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Postnatal suppression of myomesin, muscle creatine kinase and the M-line in rat extraocular muscle

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Summary

The M-line and its associated creatine kinase (CK) M-isoform (CK-M) are ubiquitous features of skeletal and cardiac muscle. The M-line maintains myosin myofilaments in register, links the contractile apparatus to the cytoskeleton for external force transfer and localizes CK-based energy storage and transfer to the site of highest ATP demand. We establish here that the muscle group responsible for movements of the eye, extraocular muscle (EOM), is divergent from other striated muscles in lacking both an M-line and its associated CK-M. Although an M-line forms during myogenesis, both *in vivo* and *in vitro*, it is actively repressed after birth. Transcripts of the major M-line structural proteins, myomesin 1 and myomesin 2, follow the same pattern of postnatal downregulation, while the embryonic heart-specific EH-myomesin 1 transcript is expressed early and retained in adult eye muscle. By immunocytochemistry, myomesin protein is absent from adult EOM sarcomeres. M-line suppression does not occur in organotypic co-culture with oculomotor motoneurons, suggesting that the mechanism for suppression may lie in muscle group-specific activation or workload patterns experienced only *in vivo*. The M-line is, however, still lost in dark-reared rats, despite the developmental delay this paradigm produces in the visuomotor system and EOMs. EOM was low in all CK isoform transcripts except for the sarcomeric mitochondrial (*Ckmt2*) isoform. Total CK enzyme activity of EOM was one-third that of hindlimb muscle. These findings are singularly unique among fast-twitch skeletal muscles. Since EOM exhibits isoform diversity for other sarcomeric proteins, the M-line/CK-M divergence probably represents a key physiological adaptation for the unique energetics and functional demands placed on this muscle group in voluntary and reflexive eye movements.

Key words: extraocular muscle, M-line, myomesin, creatine kinase, myogenesis, rat, *Rattus norvegicus*.

Introduction

The sarcomere is the basic structural and functional unit of striated musculature. Within each sarcomere, two lattices of thin (actin) myofilaments, maintained in register by transversely oriented Z-lines, interdigitate with a central lattice of thick (myosin) myofilaments. The active sliding of the actin lattices along and towards the center of the myosin lattice reduces sarcomere length and is the fundamental event in muscle contraction. The incremental length changes at each longitudinally linked sarcomere are additive in producing the larger translations underlying movement. To ensure the lateral and longitudinal transfer of developed force, the striated muscle contractile apparatus is tightly integrated with the periphery *via* a myofilament–cytoskeleton–sarcolemma–extracellular matrix network. Z-lines anchor the actin lattices to a highly ordered cytoskeleton, while M-lines interconnect each myosin filament with the six adjacent myosin filaments and with the cytoskeletal protein titin (Furst et al., 1999; Luther and Squire, 1978; Obermann et al., 1997). On this basis, it was assumed that M-lines were functionally analogous to Z-lines in facilitating force transfer and in maintaining the longitudinal and lateral register of the myosin lattice (Clark et al., 2002).

Myomesin 1 (*Myom1*) and myomesin 2 (*Myom2*) represent the principal structural components of the M-line. *Myom1* exhibits tissue- and developmental-stage-specific alternative splicing; two isoforms have been identified in rodents (myomesin 1, or S-myomesin, and EH-myomesin 1) and four have been identified in chickens (S-myomesin 1 and H-myomesin 1, with EH splice variants of each). The EH and H isoforms are thought to be specific to embryonic and adult cardiac muscle, respectively (Agarkova et al., 2000; Bantle et al., 1996; Steiner et al., 1999). Only one myomesin 2 (M-protein) isoform has been described. In addition to acting as structural components in adult striated muscle, the myomesin 1 and M-line may play an important organizational role in

developing sarcomeres (Ehler et al., 1999; Grove et al., 1985).

The M-line also serves as scaffolding for localization of homodimers of the muscle creatine kinase (CK-M; Turner et al., 1973), which functions to cleave phosphocreatine and maintain physiologically adequate muscle ATP:ADP ratios. CK-M may also contribute to the cross-bridges at M-lines. A portion of total muscle CK becomes localized to the M-line in the early postnatal period (Carlsson et al., 1982), supporting renewal of ATP immediately at the site of energy utilization by myofibrillar ATPase. Striated muscle also expresses sarcomeric mitochondrial CK (sCK), localized to the mitochondria, and there is compelling evidence that CK isoforms can, at least in part, functionally compensate for one another (Roman et al., 1997). CK isoform heterogeneity is, however, tightly coupled to the organization of energy production and utilization pathways and thus may be tailored to the operational mode of a given muscle type (Ventura-Clapier et al., 1998).

We have previously shown that extraocular muscle (EOM) is fundamentally different from other skeletal muscles (Cheng and Porter, 2002; Porter and Baker, 1996; Porter et al., 2001a). Here, we investigated the temporal expression patterns of the M-line and of myomesin and CK isoforms in rat EOMs in comparison with those in other striated muscles. Convergent evidence establishes a novel developmental downregulation of adult myomesin and CK-M transcripts in EOM in coordination with a postnatal repression of the M-line. Absence of both an M-line and CK-M suggests that EOM differs from prototypic skeletal muscle in both sarcomeric structure and myofiber energetics. Finally, using organotypic co-culture we established that the developmental stage-specific M-line suppression might represent an early adaptation to activity patterns and/or load in postnatal eye muscle.

Materials and methods

Sequencing of rat myomesin

Total RNA was isolated from embryonic day 18 (E18) heart (22 embryos pooled) and from postnatal day 45 (P45) hindlimb muscles (gastrocnemius and soleus; eight animals pooled) of Sprague-Dawley rats (*Rattus norvegicus* L.; Harlan, Indianapolis, IN, USA) using Trizol (Invitrogen, Carlsbad, CA, USA), according to the manufacturers directions. 1 μg of total RNA was reverse transcribed using oligo dT primer (Invitrogen), and RT product was diluted 1:10 and used for PCR. PCR conditions were as follows: denaturation at 94°C for 5 min, followed by 30 s each at 94°C, 58°C and 72°C for 33 cycles, and then incubation at 72°C for 5 min. Myomesin 1 isoform (myomesin 1 and EH-myomesin 1) primers were derived from mouse *Myom1* (GenBank accession number AJ012072) (Fig.·1). Primers (5′ to 3′) were: pr1 (forward), GGCAAAATCATCCCAAGTAG; pr2 (reverse), ATAATAGCCTGTAATCTCTGC; pr3 (forward), CAGATGTGTGGCCTCAACTGA; pr4 (reverse), TCGGATTGACTTTGCTCCT. A myomesin 1 fragment was amplified from P45 hindlimb using primers pr1 and pr2. EH-myomesin 1 was amplified from E18 heart using both the pr1–pr4 and pr3–pr2 pairs. Rat myomesin 2 primers were derived from mouse *Myom2* (GenBank REFSEQ NM_008664). Primers were: pr5 (forward), TGTGGCGGGAACAAACAT; pr6 (reverse), ACCTTCCACCGTTAAGATCCTC. A myomesin 2 fragment was amplified from P45 hindlimb using pr5 and pr6. PCR products were purified using the QIAquick PCR purification kit (QIAGEN, Inc., Valencia, CA, USA) and then were sequenced with a PRISM 377 automated sequencer (Applied Biosystems, Inc., Foster City, CA, USA). Nucleotide sequences for rat myomesin 1, EH-myomesin 1 and myomesin 2 have been deposited in the NCBI GenBank database under accession numbers AY177415, AY177416 and AY177417, respectively. Nucleotide and deduced protein sequence alignments used ClustalW (slow/accurate, IUB) and ClustalW (slow/accurate, Gonnet), respectively, implemented in the MegAlign module of Lasergene (DNASTAR, Inc., Madison, WI, USA). Myomesin 1 secondary structure (i.e. α-helix, β-sheet, turn, coil, hydrophilic, amphipathic and flexible region content) was predicted using algorithms in the Protean module of Lasergene.

Quantitative real-time RT-PCR (qPCR) of myomesin and CK isoforms

Total RNA was isolated from rat cardiac (E18, P0, P14, P28 and P45), hindlimb (E18, P0, P7, P14, P21, P28 and P45) and extraocular (P0, P7, P14, P28 and P45) muscle using Trizol (GibcoBRL, Rockville, MD), following the manufacturer's instructions. Reverse transcription was carried out using Superscript II RNase H- reverse transcriptase (GibcoBRL), with oligo$(dT)_{18}$ primer. The same primers used for sequencing were used to amplify myomesin 1 and EH-myomesin 1 (pr1–pr2), EH-myomesin 1 alone (pr3–pr4) and myomesin 2 (pr5–pr6). Primers were also designed for the known CK isoforms: CK-M (GenBank REFSEQ NM_012530; forward, GAGATCTTCAAGAAGGCTGGTCA; reverse, GAGATGTCGAACACGGCG; 227-bp product), sCK (GenBank accession number X59736; forward, TTTCCAACATAGATCGGATCG; reverse, AGACTTCCTGTGTCTGTCATACCA; 211-bp product), CK-B (GenBank REFSEQ NM_012529; forward, TGGCCTCACTCAGATTGAAA; reverse, GAACTTCTCGTGCTTTCCCAG; 160-bp product) and ubiquitous CK (GenBank accession number X59737; forward, GCGGATGTCTTTGACATCTCTAAT; reverse, TAGGACAGGGATTGAGAGGCA; 267-bp product). qPCR used the Roche LightCycler (Mannheim, Germany) with the LightCycler-FastStart DNA Master SYBR Green I kit, following the manufacturer's standard conditions (40 cycles; 5 s at 95°C, 5 s at 58°C, 15 s at 95°C). Human genomic DNA and β-globin primers were used to generate an external standard curve for each reaction.

EOM and oculomotor motoneuron co-culture

Organotypic explant co-culture of P0 EOM with E16 midbrain slices containing oculomotor motoneurons was

performed as described previously (Porter and Hauser, 1993). Briefly, motoneuron-containing midbrain slices were cultured for 2 days to allow for neurite outgrowth. Newborn EOMs were dissected, minced and added in close proximity to established midbrain explants. Cultures were grown on rat-tail collagen-coated plastic cover slips in 22 mm wells incubated at 34°C, in 5% CO_2/95% air and high humidity. Medium contained 18.6% donor horse serum, 59.1% alpha-modified Eagle's minimal essential medium, 292 μg ml^{-1} glutamine, 9 mg ml^{-1} glucose, 1 mmol l^{-1} sodium pyruvate, 100 μg ml^{-1} transferrin, 4 ng ml^{-1} selenium, 16.1 μg ml^{-1} putrescene and 10 μg ml^{-1} gentamycin. Cultures were fed every 3–4 days.

Reduction of EOM activity by dark rearing

Timed-pregnant Sprague-Dawley (Harlan) rats were housed in total darkness. This paradigm disrupts visual and oculomotor system development and compromises EOM maturation (Brueckner and Porter, 1998). Animal maintenance (cage changing/feeding) was conducted making brief use of a low intensity lamp with a red filter (Kodak 1A safelight filter). Rat rod photoreceptors are barely sensitive to the extreme end of the spectrum, so a dark-adapted eye may be exposed to fairly high luminance levels of deep red light without loss of adaptation (Davson, 1990). After birth, rats were kept in the dark for 14–56 days. Control rats were raised under a standard 12 h:12 h light:dark cycle.

Ultrastructural analysis of EOM

Rat EOM sarcomere morphology was assessed by electron microscopy between E17 and P56. Embryos isolated from timed-pregnant rats were fixed by immersion in 1% paraformaldehyde and 1.25% glutaraldehyde in 0.1 mol l^{-1} phosphate buffer. Postnatal animals were perfused with saline followed by the same fixative. Tissues were postfixed in osmium tetroxide, stained *en bloc* in uranyl acetate, dehydrated in graded methanols and propylene oxide, and embedded in epoxy resin. Ultrathin sections (~90 nm) were stained with uranyl acetate and lead citrate and examined with an electron microscope. Organotypic explant cultures were fixed with 4% glutaraldehyde and then similarly processed and evaluated.

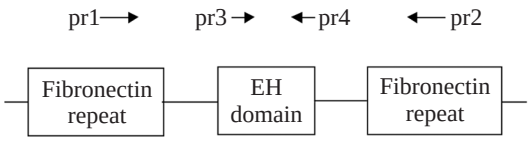

Fig. 1. Schematic representation of myomesin sequencing strategy. Myomesin 1 cDNAs were amplified and sequenced from E18 cardiac and P45 hindlimb muscle. The approximate position of the four primers used for sequencing (pr1 to pr4) is indicated. *Myom1* is present in two splice variants, with and without the EH (embryonic heart) domain. Primers pr1 and pr2 yielded a 393-bp fragment from hindlimb muscle that lacked the EH domain. The alternatively spliced EH domain is shown here relative to two of the conserved fibronectin III repeats (My6 and My7 domains shown) that dominate the central portion of myomesin 1. Primers pr1 and pr4 yielded a 542-bp fragment, and primers pr3 and pr2 yielded a 389-bp fragment, from E18 heart muscle. Collectively, sequencing of these fragments gave full-length coverage to the cDNA of the EH domain. The assembled EH-myomesin 1 sequence was then used in a BLASTn search of GenBank.

Myomesin immunocytochemistry

Myomesin was localized using a monoclonal antibody raised against skelemin, an alternatively spliced isoform of myomesin 1 (Reddy et al., 1998, 2001).

Total CK activity assay

Total CK activity was measured for hindlimb and EOM between P7 and P45 using the hexokinase/glucose-6-phosphate dehydrogenase-coupled enzyme system, which yields reduced NADH proportional to total CK activity (Sigma Chemical Co., St Louis, MO, USA) (Watchko et al., 1996).

Results

Mammalian myomesin sequences are highly conserved

Since no GenBank entries existed for rat *Myom1*, EH-*Myom1* or *Myom2*, we first amplified and sequenced rat cardiac and skeletal muscle cDNAs. Starting from E18 heart, RT-PCR using primers complementary to the mouse cDNA sequence amplified a 542-bp fragment (pr1 and pr4) and a 389-bp fragment (pr3 and pr2), each of which was predicted to contain overlapping portions of the EH-myomesin 1 domain (see Fig. 1). Following sequencing of both fragments, sequences were aligned to yield a single sequence that included all of EH. The assembled EH-myomesin 1 sequence was then used in a BLASTn search of NCBI GenBank (release 131.0). This approach identified homologous entries for chicken (AF185572 and U58204), mouse (NM_010867) and human (AF185573 and NM_003803). Sequence alignments using ClustalW showed substantial nucleotide identity for rat EH-myomesin 1 with the mouse (90.6%), human (83.8%) and chicken (71.0%) isoforms.

The deduced EH-myomesin 1 amino acid sequence was aligned with mouse, human and chicken using ClustalW, identifying homologies of 86.9%, 78.5% and 57.8%, respectively (Fig. 2). For the rat EH motif, 79 residues were identical, 13 residues represented conservative replacements (differing by <6 distance units) and five residues represented non-conservative changes (differing by ≥6 distance units, as determined from PAM 250; DNASTAR, Inc.) when compared with the consensus sequence.

Sequences of both the partial rat EH-*Myom1* cDNA obtained here and the complete mouse *Myom1* cDNA (GenBank REFSEQ NM_010867) were used as templates for a BLASTn search (http://www.ncbi.nlm.nih.gov/genome/seq/RnBlast.html) of rat genomic data at Rat Genome Database (http://rgd.mcw.edu). The search identified draft sequence *Rattus norvegicus* clone CH230-98N18 (GenBank accession number AC103176.4). Since mouse and rat are highly conserved at the nucleotide level, we then used the mouse cDNA as reference to assemble rat EH-*Myom1*. Rat *Myom1* coding sequence extends over 130 kb of genomic sequence, including 40 exons and 39 introns. Actual coding

sequence was ~5 kb, encoding 1666 amino acids. Using ClustalW, nucleotide/protein identity of the full-length rat EH-*Myom1* with mouse and human was 92.8%/94.5% and 83.6%/85.4%, respectively.

By contrast, P45 hindlimb muscle mRNA amplified with pr1–pr2 yielded a smaller, 393-bp fragment. Sequencing this fragment showed that it lacked the EH-myomesin 1 domain, consistent with prior observations that the alternatively spliced EH domain is absent from rodent skeletal muscle (Agarkova et al., 2000). Nucleotide/deduced amino acid alignments performed using ClustalW showed this rat *Myom1* fragment to have highest homology with mouse (92.7% nucleotide/95.2% amino acid), followed by human (87.9%/92.7%) and chicken (76.1%/84.7%).

Pr5 and pr6 amplified a 376-bp fragment of *Myom2* from P45 hindlimb muscle. After sequencing, an NCBI BLASTn search with the rat *Myom2* sequence identified homologous sequences for chicken (D11474), mouse (XM_125012) and human (NM_003970) myomesin 2. Nucleotide/deduced amino acid sequence alignments performed using ClustalW showed this *Myom2* fragment to have highest homology with mouse (94.9% nucleotide/99.2% amino acid), followed by human (81.6%/92.4%) and chicken (68.4%/81.4%).

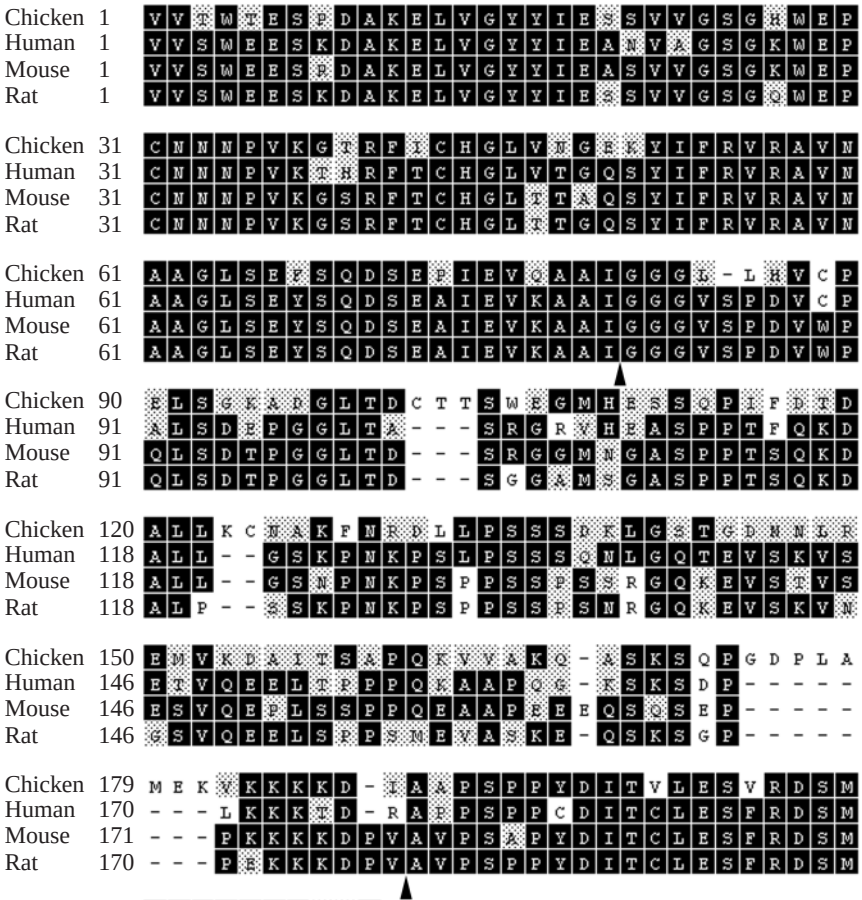

Fig. 2. The deduced amino acid sequences of rat, mouse, human and chicken EH-*Myom1* exhibit significant divergence. ClustalW was used to align the deduced amino acid sequence of the EH domain and surrounding residues for rat with homologous sequences deduced from chicken (GenBank accession numbers AF185572 and U58204), human (GenBank accession numbers AF185573 and NM_003803) and mouse (GenBank accession number NM_010867) nucleotide sequences. The start and end of the rat EH domain are indicated by arrowheads. Residues differing from consensus by <6 distance units are shown in dotted boxes, while those differing by ≥6 distance units (non-conservative substitutions) are shown in white boxes.

Deduced EH-myomesin 1 domain secondary structure is highly conserved in mammals

Agarkova et al. (2000) characterized EH-myomesin 1 in chicken and mouse embryonic heart and suggested that it may confer flexibility to an otherwise rigid molecule that is principally comprised of fibronectin III and immunoglobulin-like domains. Using algorithms in Protean (DNASTAR, Inc.), we generated and compared secondary structure predictions for chicken, rat, mouse and human EH motifs and for representative *Myom1* immunoglobulin-like (My2) and fibronectin repeat (My4) domains. Fig. 3 shows predicted secondary structure, distribution of charged domains and a backbone chain flexibility index for rat and chicken. Comparisons of the rat EH domain (Fig. 3A) with those of mouse and human (data not shown) indicate similarly low α-helical and β-sheet domain content and high turn and coil content. By contrast, the secondary structure of the chicken EH domain includes higher α-helical and lower turn/coil content than rat, yielding a less flexible structure (Fig. 3B). However, relative to the deduced secondary structure of immunoglobulin-like (My2 shown in Fig. 3C) and fibronectin (My4 shown in Fig. 3D) repeats that comprise most of myomesin 1, backbone chain flexibility of the EH domain is high for all four species.

Extraocular myomesin 1 and myomesin 2 transcripts are downregulated from birth, while EH-myomesin 1 is upregulated

To determine if myomesin transcripts exhibit developmental stage specificity, we performed qPCR analysis of rat cardiac muscle, skeletal muscle and EOM. Data showed both developmental age and muscle class specificity in myomesin transcript expression. At E18, nearly equivalent levels of *Myom1* were detected for heart and hindlimb (heart was 1.3-fold greater than leg), and this relationship was maintained as levels roughly paralleled one another throughout development (Fig. 4A). EOM *Myom1* mRNA levels were ~10-fold less than heart and hindlimb at birth and reached

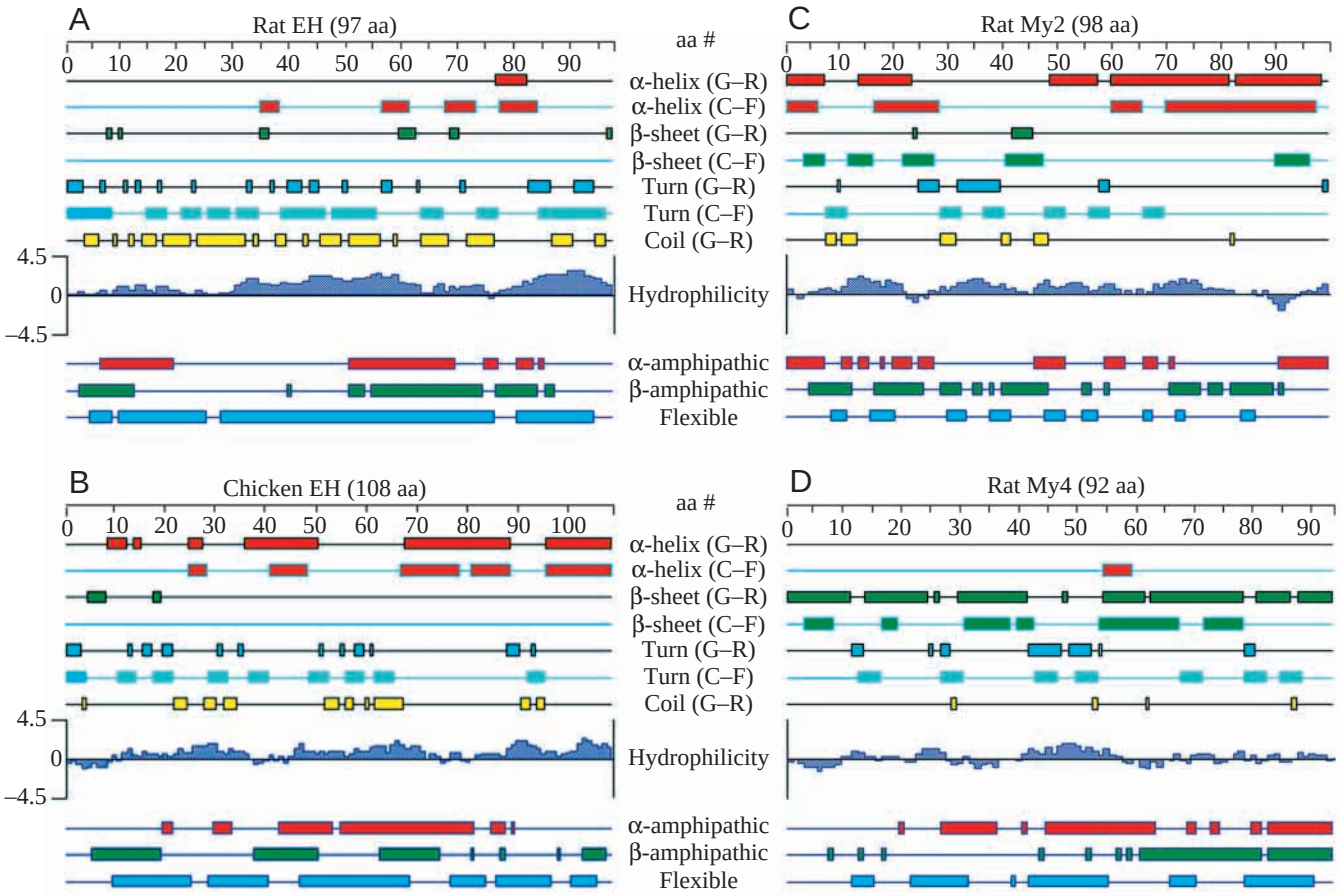

Fig. 3. Deduced secondary structure for myomesin 1 domains in rat and chicken. From the deduced amino acid sequences, secondary structure features were determined for the rat (A) and chicken (B) EH domains, the My2 immunoglobulin-like repeat domain (C) and the My4 fibronectin III repeat domain (D). My2 and My4 were deduced from a full-length rat *Myom1* cDNA assembled from GenBank AC103176.4 using full-length mouse sequence as a template. Algorithms used for secondary structure predictions were from Lasergene software (DNASTAR, Inc.) and included Garnier–Robson (G–R) and Chou–Fasman (C–F) for α-helix, β-sheet, turn and coil content, Eisenberg for α- and β-amphipathic regions, Kyte–Doolittle for the hydrophilicity plot, and Karpus–Shultz to identify flexible regions. The secondary structure pattern for rat EH was highly conserved in mouse and human (data not shown), while the chicken EH region had substantially higher α-helical content and lower turn and coil content, rendering it with a lower flexibility index. The remainder of rat myomesin 1 is comprised of seven immunoglobulin-like repeats (My2, My3, My9, My10, My11, My12 and My13), with My2 shown as representative (C), and five fibronectin repeats (My4–My8), with My4 shown as representative (D). Immunoglobulin and fibronectin repeats are principally comprised of α-helical and β-sheet structure, respectively.

a peak roughly equivalent to the other muscle classes at P7. EOM *Myom1* then declined to a level ~160-fold less than hindlimb and ~200-fold less than cardiac muscle by P45. EH-*Myom1* mRNA was initially highest in heart and lowest in EOM (Fig. 4B). Cardiac muscle showed a small decline between E18 and P45, while there was a substantial drop in EH transcripts in hindlimb muscle. EOM EH-*Myom1* showed the opposite pattern, with an ~10-fold increase between P0 and P45.

Myom2 transcript levels were closest among the three muscle classes in early development (at P0, hindlimb was 3.9-fold greater than heart and 10-fold greater than EOM; Fig. 4C) and diverged thereafter. Hindlimb and cardiac muscle showed increases that correlated with age, peaking by P28 and then slightly declining by P45. At P45, cardiac and hindlimb transcript levels were nearly equivalent (heart 1.4-fold greater than leg), and both were higher (40- to 53-fold) than EOM.

Structural M-lines formed in developing EOM are subsequently suppressed

Since expression levels of the major structural constituents of the M-line, myomesin 1 and myomesin 2, decline during postnatal eye muscle development, we assessed the presence of an M-line in this muscle group by electron microscopy. M-lines were detected at the center of sarcomeres of all primary myotubes at the earliest stage examined, E17 (Fig. 5A). M-lines were present from the earliest stages of myofibril

assembly, forming in incomplete sarcomeres as myofibrillogenesis occurs in the perinuclear region. Both primary and secondary myotubes exhibited M-lines at P0, but these were noted less regularly by P7 and were absent from P14 (Fig. 6A) and P56 (Fig. 6C) EOM. Cellular localization by immunocytochemistry established that myomesin 1 protein was absent from adult EOM (Fig. 7).

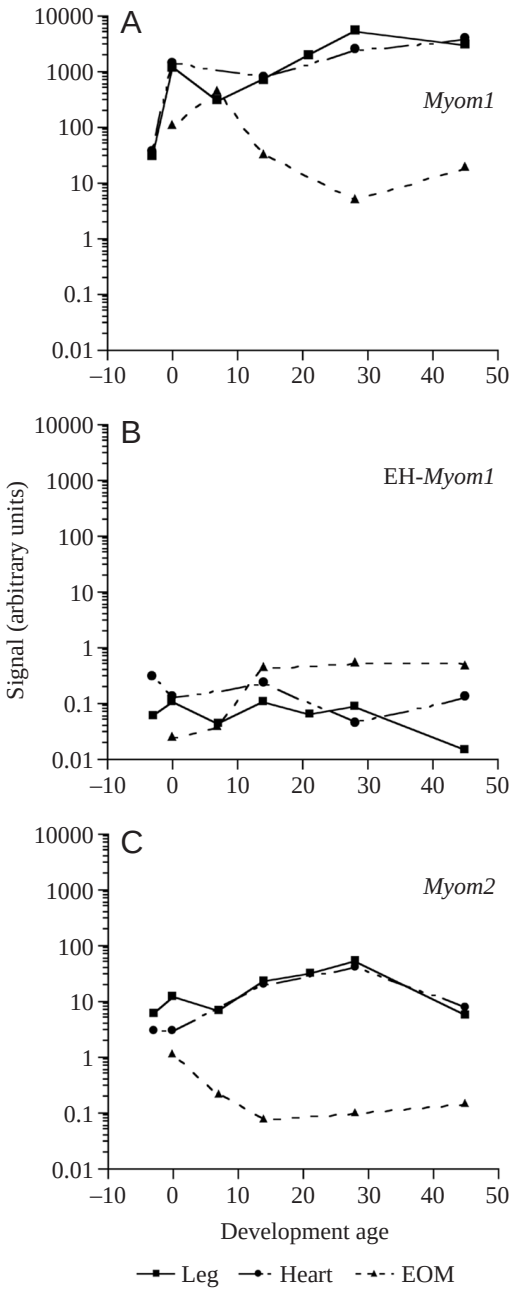

Fig. 4. Myomesin isoforms are differentially regulated in muscle classes during development. qPCR analysis of hindlimb, cardiac and extraocular muscle (EOM) was performed with the Roche LightCycler and SYBR green reagent. Different primer sets were used to evaluate expression levels of (A) myomesin 1, (B) EH-myomesin 1 and (C) myomesin 2 in the three muscle groups between the ages of E18 and P45. Data establish differential regulation patterns for each transcript and muscle group during development.

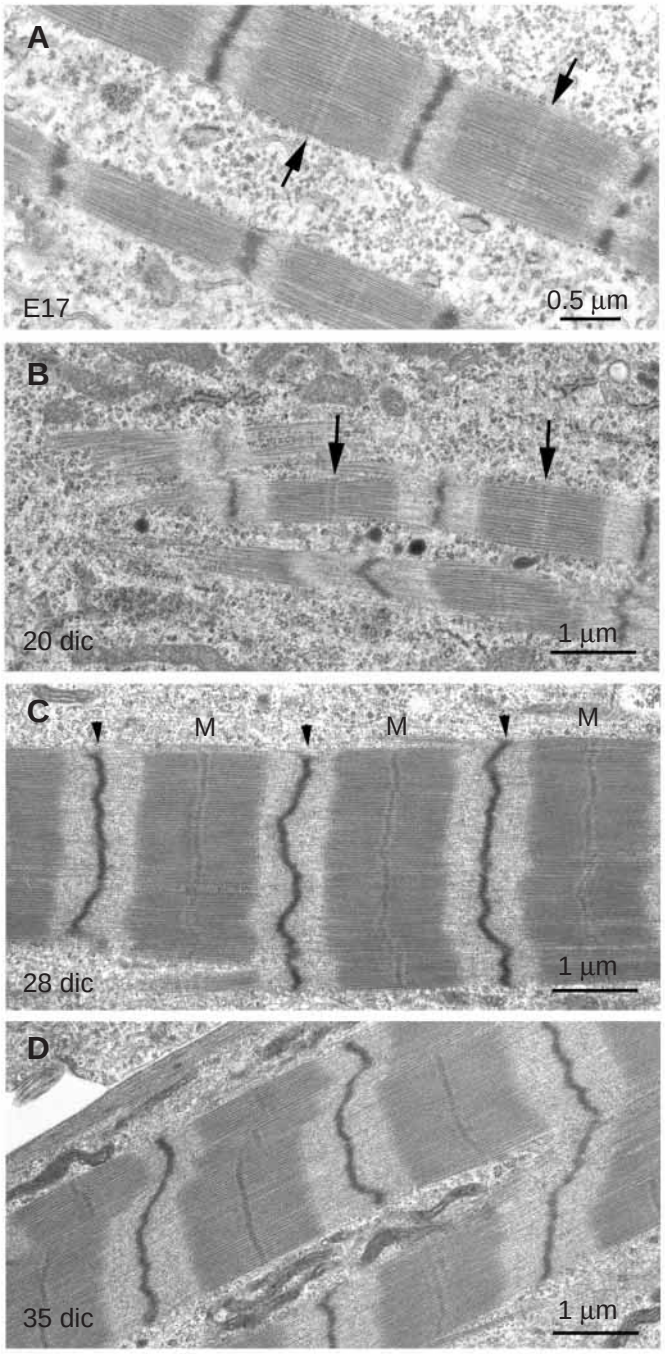

Fig. 5. M-lines are expressed early during *in vivo* development and continuously in organotypic culture of extraocular muscle (EOM). The presence or absence of a structural M-line was evaluated in EOMs of rats between the ages of E17 and P45. A representative electron photomicrograph illustrating the presence of an M-line in E17 eye muscle (A) is shown here. M-line morphology was also assessed in EOM grown in organotypic co-culture with oculomotor motoneurons after 20 days (B), 28 days (C) and 35 days (D) *in vitro*. M-lines were present in newborn and prenatal EOM and in organotypic co-cultures at all stages. M-lines are indicated by arrows or by the letter M; arrowheads denote Z-lines.

M-lines are not suppressed in EOM organotypic nerve–muscle co-cultures

To begin to assess how the M-line is regulated in developing EOM, we characterized M-line appearance in organotypic nerve–muscle co-cultures. Explant cultures mimic the developmental environment, including innervation by appropriate motoneuron pools, but lack the postnatal activity patterns from sensory inputs into higher order oculomotor system structures and the functional load represented by the globe. M-line appearance during myogenesis in organotypic co-cultures was identical to that *in vivo* (compare Fig. 5A and Fig. 5B). As actin and myosin filaments were assembled, M-lines were present in the first sarcomeres formed adjacent to myonuclei (Fig. 5B). Moreover, distinct M-lines were still detected in myofibers maturing after 28–35 days in culture (Fig. 5C,D). By these stages, ultrastructural traits of the two major extraocular myofiber types, singly and multiply innervated, had emerged and distinctive M-lines were present in both types.

EOM M-line suppression is not prevented by altered visuomotor development

If animals are deprived of vision during a postnatal critical period, ocular dominance columns do not properly develop in primary visual cortex (V1) and there are additional upstream and downstream consequences. Oculomotor motoneuron output is altered and the EOMs undergo critical period-dependent changes (Brueckner and Porter, 1998). Since M-lines are actively suppressed in postnatal EOMs but retained in organotypic co-cultures, we tested whether the dark-rearing-mediated alteration of oculomotor output would influence M-line expression. Rats reared in darkness from birth were evaluated at P14, P28 and P56 for the presence or absence of M-lines. As in age-matched control rats, an M-line was absent from extraocular myofibers of P14–P56 dark-reared rats (Fig. 6B,D). We also evaluated myomesin transcript levels and found no differences between P45 dark-reared and normally reared rats (data not shown).

CK isoform expression patterns exhibit muscle class specificity

In addition to its role as a structural component of the sarcomere, the M-line is a binding site for CK-M homodimers, which, in turn, are responsible for phosphocreatine metabolism at the site of ATP utilization in muscle contraction. Absence of an M-line in adult eye muscle probably has profound consequences for myofiber energetics that may require adaptations at the level of phosphocreatine metabolism. We determined muscle class- and developmental age-specific expression patterns for all known CK isoforms by qPCR.

Prenatal CK-M (*Ckm*) transcript levels were similar for cardiac and skeletal muscle (heart 1.6-fold greater than leg), remained close at birth (leg 2.0-fold greater than heart) but then diverged thereafter as hindlimb levels continued to rise while cardiac transcripts leveled off (Fig. 8A). By P45, skeletal muscle *Ckm* mRNA content was 80-fold greater than that of

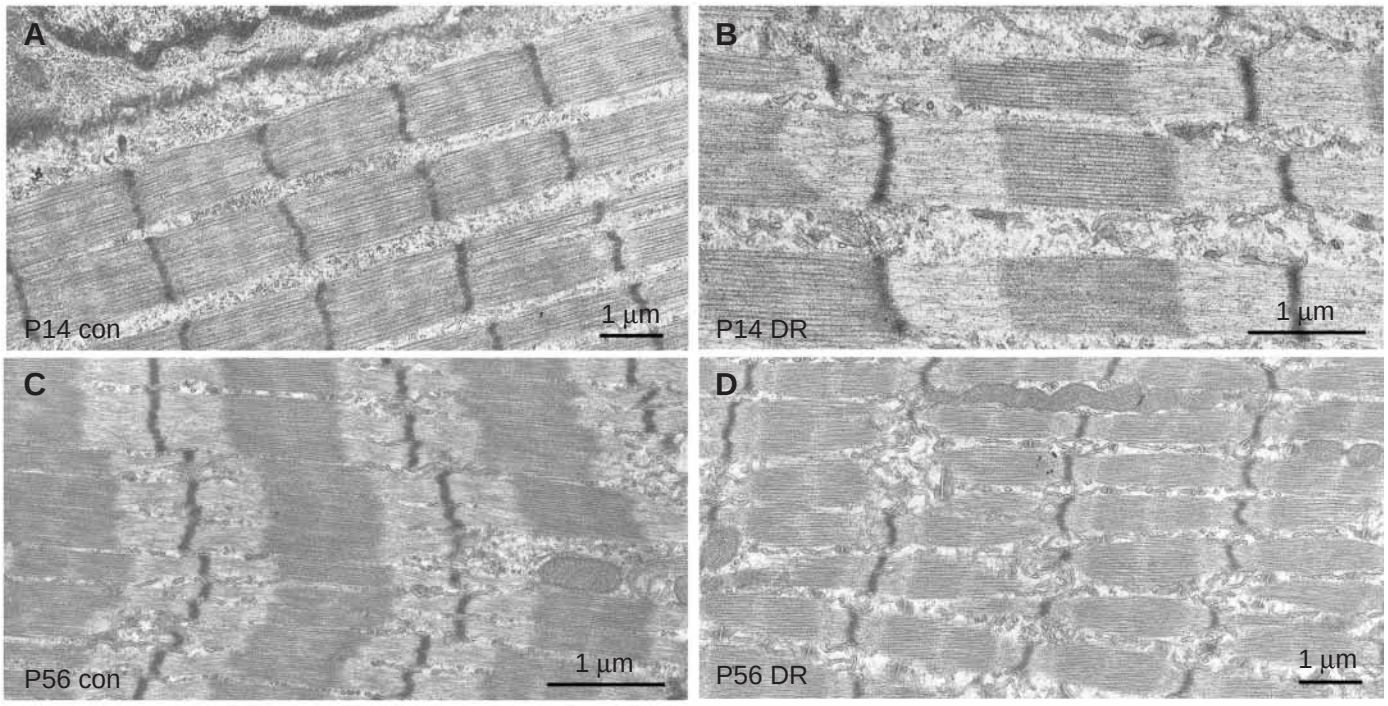

Fig. 6. Extraocular muscle (EOM) M-line expression is not preserved in dark-reared rats. Dark rearing is known to alter eye movements and to delay development of EOM-specific traits. We raised newborn rats either in complete darkness (DR) or in a 12 h:12 h light:dark cycle (con). M-line morphology was evaluated by electron microscopy. At P14 (A) and P56 (C), EOMs of normally reared rats lack M-lines. M-lines were not preserved in rats dark reared for 14 days (B) or 56 days (D).

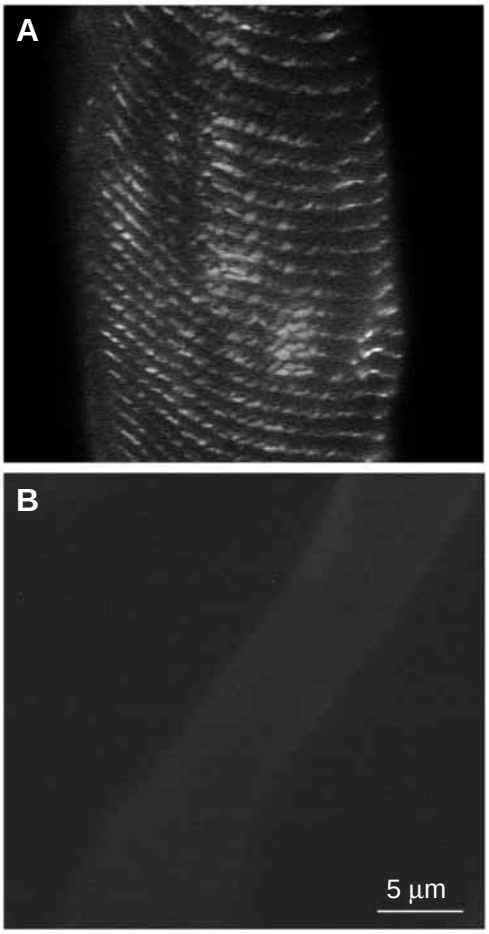

Fig. 7. Immunocytochemical localization of myomesin 1 in adult skeletal muscle (A) and absence from adult extraocular muscle (EOM) (B), consistent with low *Myom1* transcript levels in EOM.

heart. EOM *Ckm* transcripts were, however, 90-fold greater than those of hindlimb muscle at birth and increased only marginally during the developmental stages studied here. In P45 rats, EOM *Ckm* transcript levels were negligible, as qPCR showed that hindlimb and cardiac muscle were 90-fold and 10-fold higher, respectively, than eye muscle.

By contrast, muscle class-specific developmental patterns of the sCK (*Ckmt2*) transcript were nearly reversed from those of *Ckm* (Fig. 8B). Extraocular and hindlimb muscles expressed nearly equal levels of *Ckmt2* at birth, when both were ~4.5-fold greater than those in heart. From P0 onwards, both EOM and cardiac muscles showed substantial increases in *Ckmt2* mRNA, while hindlimb muscle exhibited only a modest increase (4.2-fold between P0 and P45). In adult rats, EOM *Ckmt2* transcript levels were 432-fold greater than those in cardiac muscle, and heart levels were 1.4-fold greater than hindlimb muscle levels.

Expression of the brain-type CK-B isoform (*Ckb*) was modest in the three muscle classes (Fig. 8C). Transcript levels declined from birth for cardiac and hindlimb muscles, while EOM showed only a modest increase (1.8-fold) between P0 and P45. Likewise, the ubiquitous mitochondrial CK (*Ckmt1*), the predominant type in smooth muscle, was expressed at low levels in all muscle classes at the earliest stages and declined during development (Fig. 8D).

Assuming equal amplification efficiency by the four primer pairs used in qPCR (primers were designed to have very close T_ms, and data were normalized using a β-globin standard control reaction), the relative abundance of CK transcripts for the three muscle classes was compared and yielded: EOM, *Ckmt2*⩾*Ckm*>*Ckb*⩾*Ckmt1*; hindlimb, *Ckm*⩾*Ckmt2*⩾*Ckb*>*Ckmt1*; cardiac, *Ckm*>*Ckmt2*>*Ckb*⩾*Ckmt1* (where ⩾ denotes a difference of more than one order of magnitude).

Total CK activity is low in EOM

To determine the functional impact of differential regulation of CK isoform transcripts in EOM, we conducted a total CK activity assay for hindlimb and EOM at P7, P14, P21 and P45. Data show a postnatal increase in CK activity for both muscle groups, but EOM CK activity is less than that of hindlimb muscle at all developmental stages except for P7 (P<0.01, N=3; Fig. 9). In the adult (P45) rat, EOM total CK activity was approximately one-third that of the hindlimb (EOM, 503.0±128.0 μmol min^{-1} mg^{-1} protein; leg, 1532.3±143.1 μmol min^{-1} mg^{-1} protein; P<0.001).

Discussion

The M-line is a virtually ubiquitous feature of skeletal (extrafusal or intrafusal, twitch or tonic) and cardiac muscle fibers. We establish here that EOM is divergent in lacking an M-line and the associated CK-M. This finding represents a fundamental departure from a key tenant of striated muscle organization. Although we show that a distinct M-line forms during myogenesis, both *in vivo* and *in vitro*, it is repressed in the early postnatal period. *Myom1* and *Myom2* transcripts follow the M-line pattern of developmental downregulation, while upregulation of the embryonic heart-specific EH transcript occurs during eye muscle maturation. Myomesin 1 protein does not localize to sarcomeres of adult EOM. We did not, however, observe M-line suppression in EOM in organotypic culture with oculomotor motoneurons, suggesting that the mechanism for suppression may lie in muscle group-specific activation or workload patterns experienced only *in vivo*. A manipulation that compromises visuomotor system development and is known to alter EOM-specific myosin heavy chain expression (Brueckner and Porter, 1998) does not produce M-line retention in EOM.

Multiple myomesin isoforms are expressed in striated muscle, but their separate functions are not fully understood. The My1 amino-terminal domain of myomesin 1 binds the light meromyosin component of myosin, while My4–My6 bind titin (Obermann et al., 1997). Based upon its primary structure, embryonic heart-specific EH-myomesin 1 probably has similar binding capability (the EH insert lies between domains My6 and My7; Agarkova et al., 2000). However, expression of EH-*Myom1* alone does not support the formation of M-lines (Agarkova et al., 2000). We show here that EH-*Myom1*, the

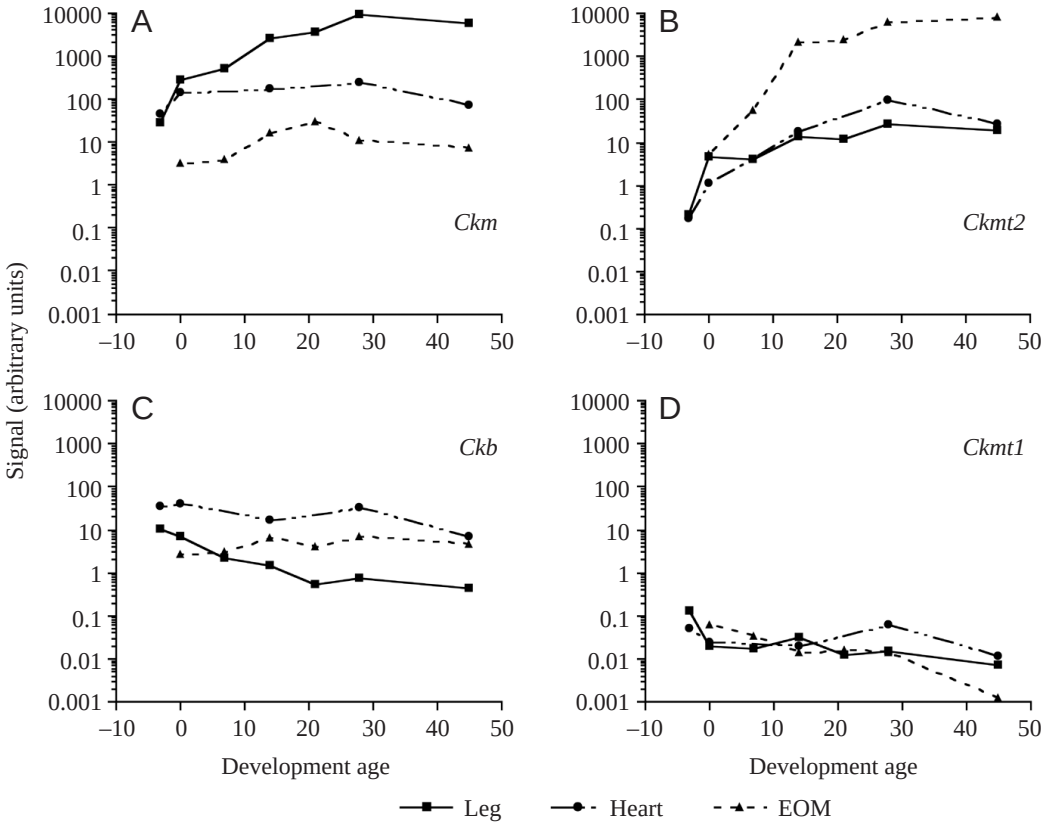

Fig. 8. Creatine kinase (CK) isoforms are differentially regulated in muscle classes during development. qPCR analysis of hindlimb, cardiac and extraocular muscle (EOM) was performed with the Roche LightCycler and SYBR green reagent. qPCR analysis of E18 to P45 muscles used primers specific for CK-M (A), sCK (B), CK-B (C) and ubiquitous mitochondrial CK (D) transcripts. These data established tissue-specific regulation patterns for each isoform during development.

major isoform in prenatal rodent and chicken hearts (Agarkova et al., 2000), is not restricted to embryonic cardiac muscle but is also expressed in adult EOM. Adult EOM is known to use several other traits of cardiac and embryonic skeletal muscle in meeting its distinct functional roles (Cheng and Porter, 2002; Jacoby et al., 1990; Khanna et al., 2003; McLoon and Wirtschafter, 1996; Porter et al., 2001a; Rushbrook et al., 1994) and then may have the phenotypic plasticity and need to express EH-myomesin. Our data further suggest that EH-*Myom1* is not restricted to embryonic cardiac muscle and EOM but rather is present in adult heart and is detected at trace levels in adult hindlimb muscle. Finally, qPCR data show that *Myom1* transcripts are present at a relatively low level and *Myom2* is absent, contributing to the absence of myomesin 1 protein and M-lines in adult EOM.

We sequenced rat *Myom1* and EH-*Myom1* fragments to validate qPCR primers, deduce EH-myomesin segment function and extract full-length *Myom1* from existing rat genomic DNA sequence data. We show that, with the exception of a divergent EH domain, *Myom1* is highly conserved in rat striated muscle. Previous studies have established that myomesin 1 is principally comprised of rigid immunoglobulin-like and fibronectin III domains (Agarkova et al., 2000), a pattern repeated in the rat. Although the greatest phylogenetic divergence in the deduced primary amino acid sequence of myomesin 1 is found in the EH domain (see Fig. 2), the deduced secondary structures of mouse, rat and human EH are still very similar and the algorithms used here predict high flexibility for the EH domain in all three species. Agarkova et al. (2000) likewise used the Karplus–Schultx algorithm to predict elasticity in the EH domain, a finding they confirmed through its circular dichroism spectrum. Chicken EH-*Myom1* is more divergent; we predict a higher α-helical component and a lower degree of flexibility. It has been

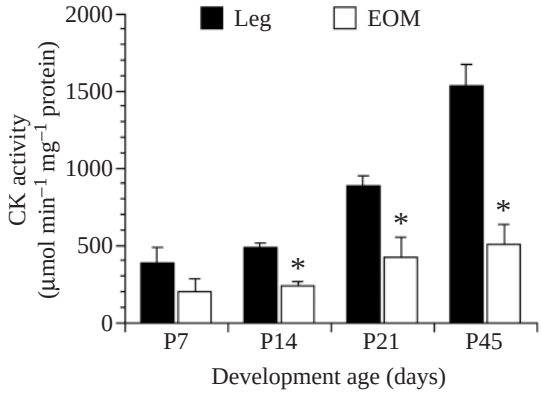

Fig. 9. Evaluation of total creatine kinase (CK) enzyme activity in hindlimb and extraocular muscle (EOM). Total CK enzyme activity was evaluated in triplicate for homogenates of hindlimb and EOM between P7 and P45. P7 activity levels were not different among the two muscle groups and both showed postnatal increases in activity. Activity levels, however, subsequently diverged, with adult hindlimb muscle attaining values 3-fold higher than those of EOM. Values are means + S.D.; * denotes $P<0.01$.

suggested that flexibility in the EH domain is important during myofibrillogenesis (Agarkova et al., 2000), but such flexibility in myosin alignment or cytoskeletal linkage may be a liability in the adult. This hypothesis is consistent with the perinatal replacement of the EH-splice variant by the more rigid myomesin 1 in heart (Agarkova et al., 2000). While myomesin may be an integral component of myofibrils even in the absence of a structurally visible M-line (Strehler et al., 1980), suggesting that EH-based myofilament linkages might be present in eye muscle, it is not yet clear what adaptive value EH-myomesin 1 may serve in a muscle that lacks the stereotypical structural connections between adjacent myosin filaments.

Prior studies (Ehler et al., 1999; Eppenberger et al., 1981; Van der Ven et al., 1999; Yang et al., 2000) have shown that an M-line appears within H-zones of forming myofibrils and that myomesin 1 accumulation is coordinated with that of other myofibrillar proteins. By contrast, myomesin 2 is known to accumulate several days later and thus may not be essential for initial sarcomeric organization (Carlsson et al., 1990; Grove et al., 1985, 1987; Grove and Thornell, 1988). *In vivo* and *in vitro* data obtained for rat EOM are consistent with such a requirement for both myomesin 1 and an M-line in sarcomere formation. We show here that distinct M-lines form as myofibrillogenesis proceeds in perinuclear regions of prenatal EOM, yet the M-line is repressed within 7 days of birth. On the basis of its transient appearance, we conclude that the M-line is essential to sarcomere formation, even in this novel situation where it is not vital to postnatal muscle function.

The morphological absence of an M-line in the adult is supported by prior electron microscopic studies, one of which reported a faint M-line in only one of the six recognized EOM fiber types (Mayr, 1971). Downregulation of extraocular myomesin mRNA, as determined here by qPCR, continued well after an M-line was no longer visible. Only in skeletal slow-twitch muscle is any similar repression of an adult myomesin isoform observed. Myomesin 2 is initially present in slow-twitch (type I) myofibers but then is lost during postnatal maturation (Carlsson et al., 1990; Grove et al., 1985, 1987, 1989; Grove and Thornell, 1988). This observation cannot, however, reconcile the postnatal loss of *Myom1*, *Myom2* and a structural M-line from EOM, since 80–85% of its myofibers are fast-twitch (Porter and Baker, 1996; Porter et al., 1995; Spencer and Porter, 1988), a functional mode generally regarded as dependent upon an M-line and the associated muscle CK. Moreover, while myomesin 2 is lost from postnatal slow-twitch fibers, they still retain morphological M-lines (Thornell et al., 1987); myomesin 1 and CK-M may both contribute to the structural M-lines in the absence of myomesin 2.

Notably, M-line suppression did not occur in our organotypic oculomotor motoneuron-EOM co-cultures, even after substantial myofiber maturation during >35 days in culture. Although the normal suppression of myomesin 2 in slow-twitch muscle fibers can be blocked by neonatal denervation (Carlsson et al., 1990), the absence of M-line suppression in our co-cultures cannot be attributed to the absence of innervation *per se*. Neuromuscular junctions form in the co-culture system, myotube contractile activity becomes synchronized and the *in vitro* twitch contractions can be blocked by an acetylcholine receptor antagonist (Porter and Hauser, 1993). Thus, we suggest that the functional demands placed upon postnatal EOM require an operational mode in which the retention of a structural M-line is not adaptive. To test this notion, we altered oculomotor motoneuron activity patterns *in vivo* by raising newborn rats in complete darkness. The lack of visual experience in this paradigm delays maturation of ocular dominance columns in primary visual cortex and, in turn, alters the maturation of visuomotor control systems. In prior studies, we have shown that the postnatal emergence of the EOM-specific myosin heavy chain isoform is blocked by dark rearing (Brueckner et al., 1996; Brueckner and Porter, 1998). If the active suppression of the M-line and its molecular constituents were dependent upon the emergence of sophisticated visuomotor behavior, then we would have expected M-line retention in dark-reared rats. Instead, suppression of the M-line was not blocked in our studies, suggesting that mechanisms behind its downregulation lie in more fundamental neuromuscular interactions and eye movement behaviors that emerge immediately after birth.

The phosphocreatine shuttle serves a vital role in striated muscle, providing an efficient means to buffer ATP demands during contraction with high-energy phosphate storage and transport. Skeletal muscle transcribes both the *Ckm* and *Ckb* genes, although the CK-M isoform predominates. Muscle CK is distributed among sarcoplasmic and M-line bound compartments, the latter pool functionally coupling ATP regeneration with local hydrolysis by myofibrillar ATPase. As *Ckm* mRNA rises in parallel with the increased energy demands of postnatal skeletal and cardiac muscle, we noted here that transcript levels remain flat in EOM. Coupled with the absence of an M-line, these data support the paradoxical notion that *Ckm* does not play an important role in the predominately fast-twitch EOM group. Sarcomeric mitochondrial CK (*Ckmt2*) is also expressed in striated muscles and may substitute for *Ckm*. EOM exhibits a rapid postnatal increase in, and high adult levels of, *Ckmt2* mRNA, a finding supported by our prior serial analysis of gene expression study (Cheng and Porter, 2002). *Ckmt1* appears not to play a role in eye muscle. *Ckb* expression levels in EOM are similar to those in heart, suggesting that CK-B homodimers or CK-B/CK-M heterodimers not localized to the M-line may participate in energy metabolism in these muscles. However, regardless of isoform content, total CK enzyme activity in EOM is substantially less than that of hindlimb, a finding inconsistent with the skeletal muscle prototype. The eye muscles are, however, atypical in that they are largely fast-twitch, with most fiber types having high intermyofibrillar mitochondrial and oxidative enzyme content that confers considerable fatigue resistance. This constitutive phenotype is very much like the adapted phenotype of mice deficient in *Ckm* (de Groof et al., 2001; van Deursen et al., 1993), in which

the short burst mode of fast-twitch glycolytic fibers is replaced by speed with endurance. We suggest that reliance upon energy transfer mechanisms other than CK-M then may be a signature of muscles that combine speed, lower force and fatigue resistance.

The novel divergence of EOM from the striated muscle M-line organizational pattern, as shown here, serves to reinforce the concept that this muscle group represents a fundamentally distinct type of skeletal muscle. During eye movements, there are demands for precision, speed and fatigue resistance that are experienced by few other skeletal muscles. Consequently, EOM is phenotypically unlike other skeletal muscles across a wide range of traits, including basic fiber type classification schemes, gene expression profiles and disease susceptibility (Cheng and Porter, 2002; Fischer et al., 2002; Kaminski et al., 2002; Porter, 2002; Porter and Baker, 1996; Porter et al., 1995, 2001a,b, 1998). The absence of the M-line system is consistent with known cytoskeletal organization differences between extraocular and other skeletal musculature (Cheng and Porter, 2002; Porter et al., 2001a) and suggests that eye muscle may use novel mechanisms to transmit contractile force to the sarcolemma and tendon. We speculate that such differences in the myofilament–cytoskeleton–sarcolemma–extracellular matrix linkage may underlie the established protection of EOM in dystrophin–glycoprotein complex-based muscular dystrophies (Kaminski et al., 1992; Karpati and Carpenter, 1986; Khurana et al., 1995; Porter and Karathanasis, 1998; Porter et al., 1998, 2001b; Ragusa et al., 1996).

In summary, our data establish the absence of an M-line, low or absent expression of *Myom1*, *Myom2* and CK-M, and the presence of EH-*Myom1* in EOM. These findings represent a novel operational mode for mammalian striated muscle and further show that EOM represents an alternative paradigm for both sarcomere structure and cellular energetics among striated muscle types. Moreover, our data suggest that postnatal suppression of the M-line and CK-M is dependent upon *in vivo* maturation of activity patterns and/or load. Since EOMs exhibit diverse roles in voluntary and reflexive eye movements that are accompanied by an isoform diversity of sarcomeric proteins, M-line divergence probably represents a key physiological adaptation for the unique functional roles of this muscle group.

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