Myosin Heavy Chain Expression in Mouse Extraocular Muscle: More Complex Than Expected

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PURPOSE. To characterize the expression patterns of myosin heavy chain (MyHC) isoforms in mouse extraocular muscles (EOMs) during postnatal development.

METHODS. MyHC isoform expression in mouse EOMs from postnatal day (P)0 to 3 months was evaluated by quantitative polymerase chain reaction (qPCR) and immunohistochemistry. The longitudinal and cross-sectional distribution of each MyHC isoform and coexpression of certain isoforms in single muscle fibers was determined by single, double, and triple immunohistochemistry.

RESULTS. MyHC isoform expression in postnatal EOMs followed the developmental rules observed in other skeletal muscles; however, important exceptions were found. First, developmental isoforms were retained in the orbital layer of the adult EOMs. Second, expression of emb-MyHC, neo-MyHC, and 2A-MyHC was restricted to the orbital layer and that of 2B-MyHC to the global layer. Third, although slow-MyHC and 2B-MyHC did not exhibit obvious longitudinal variations, emb-MyHC, neo-MyHC, and 2A-MyHC were more abundant distally and were excluded from the innervational zone, whereas com-MyHC complemented their expression and was more abundant in the mid-belly region in both the orbital and global layers. Fourth, coexpression of MyHC isoforms in single global layer fibers was rare, but it was common among the orbital layer fibers.

CONCLUSIONS. MyHC isoforms have complex expression patterns, exhibiting not only longitudinal and cross-sectional variation of each isoform, but also of coexpression in single fibers. The highly heterogeneous MyHC expression reflects the complex contractile profiles of EOMs, which in turn are a function of the requirements of eye movements, which range from extremely fast saccades to sustained position, each with a need for precise coordination of each eye. (*Invest Ophthalmol Vis Sci.* 2010;51:6355–6363) DOI:10.1167/iovs.10-5937

The primary function of a skeletal muscle is to generate force for movement. The ocular motor system specifies that extraocular muscles (EOMs) behave in a fashion fundamentally different from that of other skeletal muscles.¹ The EOMs contract at high speeds and are constantly active, which necessitates that they be highly fatigue resistant. The degree of contractile force must also be modulated to precisely coordinate the movements of both eyes to allow clear vision. Con-

tractile properties of a skeletal muscle, such as shortening velocity and force generation, are largely determined by the composition of myosin heavy chain (MyHC) isoforms.^{2,3} Precise characterization of MyHC expression is a fundamental requirement for understanding EOM contractile properties, and by extension, the manipulation of MyHC expression may have therapeutic implications for disorders of ocular motility.

A striking feature of EOM is its expression of a diversity of MyHC isoforms. In addition to the isoforms typically observed in mammalian skeletal muscle—*Myh* 2 (fast, 2A), *Myh* 4 (fast, 2B), *Myh* 1 (fast, 2x), and *Myh* 7 (type 1, slow)—mature EOMs express the two developmental isoforms *Myh* 3 (embryonic) and *Myh* 8 (neonatal), as well as the cardiac isoform *Myh* 6 (α -cardiac) and the EOM-specific isoform *Myh* 13.^{4–6} In marked contrast to other skeletal muscles, individual EOM fibers demonstrate variation in MyHC expression along their lengths and mixed expression of MyHC isoforms within a single fiber. MyHC expression also varies between the orbital and global regions and as a function of innervation.⁶

EOM MyHC expression has been systematically studied in humans,⁷ the rabbit,⁸ the rat,^{9–12} and the dog,¹³ but not in the mouse. The lack of detailed evaluation of murine MyHC expression limits the exploitation of the numerous transgenic models that have been produced to study cellular and molecular mechanisms of MyHC expression. A clear picture of the temporal and spatial distribution of MyHC isoforms is necessary to evaluate regulatory mechanisms and manipulate MyHC expression for therapeutic benefit. In this study, we examined the composition and developmental transition of MyHC isoforms in mice, with real-time PCR and immunohistochemistry at postnatal day (P)0, P21, and 3 months of age. We examined the longitudinal as well as cross-sectional expression patterns of each MyHC isoform, with the use of a panel of antibodies specific for MyHC isoforms. We further detail the coexpression of MyHC isoforms in single EOM fibers.

METHODS

Animals

C57BL/6J mice were bred in the animal facility of Saint Louis University. The day the pups were born was designated postnatal day (P)0. The animals were maintained in accordance with National Institutes of Health (NIH) Guidelines for Animal Care. All experiments were approved by Institutional Animal Use and Care Committees at Saint Louis University and were conducted in accordance with the principles and procedures established by the NIH and the Association for Assessment and Accreditation of Laboratory Animal Care and in the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

RNA Extraction, Reverse Transcription, and Quantitative Real-Time PCR

C57BL/6J mice were euthanatized by CO₂ asphyxiation at P0, P21, and 3 months of age, and all four rectus muscles were dissected. The EOMs were immediately frozen in liquid nitrogen, and total RNA was ex-

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Supported by National Institutes of Health Grant R01 EY-015306 (HJK).

Submitted for publication May 24, 2010; revised June 21, 2010; accepted June 22, 2010.

Disclosure: Y. Zhou, None; D. Liu, None; H.J. Kaminski, None
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tracted (TRIzol reagent; Invitrogen, Carlsbad, CA). Reverse transcription was performed according to the manufacturer's instructions (Superscript First-Strand Synthesis System; Invitrogen). Quantitative real-time PCR was performed with MyHC isoform-specific primers (Table 1)¹⁴ and SYBR green PCR core reagent (Applied Biosystems Inc. [ABI], Foster City, CA) in 24-μL reaction volumes, with a sequence-detection system (Prism model 7500; ABI). GAPDH was used as an internal loading control. Relative transcript abundance was normalized to the amount of GAPDH and quantitated by the $2^{-\Delta\Delta CT}$ method.¹⁵ The percentage of a specific MyHC isoform transcript was calculated as the ratio of relative transcript abundance of this specific MHC isoform to the relative transcript abundance of all eight MHC isoforms combined. Data represent the mean of triplicate measurements and are reported as the mean $\pm$ SE.

Antibodies

The sources and dilutions of antibodies against MyHC isoforms were as follows: mouse anti-embryonic MyHC (1:20; emb-MyHC, IgG; F1.652), mouse anti-neonatal MyHC (1:5; neo-MyHC, IgM; N1.551), mouse anti-EOM-specific MyHC (1:20; eom-MyHC, IgM; 4A6), mouse anti-MyHC-2X (no dilution; 2X-MyHC, IgM; 6H1), mouse anti-MyHC 2A/2X (1:5; 2A/2X-MyHC, IgG; A4.74), and mouse anti-all MyHCs except 2X (1:10; BF-35, IgG) were obtained from the Developmental Studies Hybridoma Bank (DSHB; developed under the auspices of National Institute of Child Health and Human Development and maintained by the Department of Biology, University of Iowa, Iowa City, IA). Antibody 7A10 (against dystrophin protein, 1:30 dilution) from DSHB was used to identify myotube boundaries. Mouse anti-developmental MyHC (IgG, 1:50; Vector Laboratories, Burlingame, CA) had a staining pattern in mouse EOMs identical with that of mouse anti-emb-MyHC (F1.652) from DSHB, consistent with reports in the rabbit.^{8,16} Mouse anti-skeletal MyHC, pan-fast (1:500; fast-MyHC, IgG, My32) was obtained from Sigma-Aldrich (St. Louis, MO), and mouse anti-slow muscle MyHC (1:400, slow-MyHC, IgG) was from Chemicon (Temecula, CA). Cell culture supernatants of hybridomas were obtained from ATCC (Manassas, VA) for mouse anti-MyHC-2A (2A-MyHC, IgG; SC-71), mouse anti-MyHC-2B (2B-MyHC, IgM; BF-F3) and mouse anti- α -cardiac MyHC (IgG, BA-G5). The secondary antibodies, AlexaFluor 350, AlexaFluor 488, and AlexaFluor 594 goat anti-mouse IgG or IgM (Molecular Probes, Carlsbad, CA) were used at a 1:500 dilution.

Tissue Preparation and Immunohistochemistry

Eyes with all four rectus muscles attached were dissected from the C57BL/6J mice after euthanatization at P0, P1, P2, P3, P4, P5, P7, P14, P21, and 8 weeks of age. After dissection, the EOMs were mounted on cork with 8% tragacanth (Sigma-Aldrich, St. Louis, MO) and immediately frozen in liquid N₂-cooled 2-methylbutane and stored at -80°C until use. Ten-micrometer serial sections were collected and designated as follows: proximal sections (in the posterior aspect of the orbit, close to annulus of Zinn), mid-belly sections (innervation zone), and distal sections (near the myotendinous junction, just before the muscle attaches to the globe). The sections were air-dried for 30 minutes and rinsed with phosphate-buffered saline (pH7.4; PBS) before

they were blocked with 3% normal goat serum for at least 1 hour. The sections were incubated in diluted primary antibody for 1 hour at room temperature and washed with PBS before application of a secondary antibody. For double and triple immunostaining of mouse antibodies on mouse tissue, the slides were incubated for 1 hour at room temperature with blocking reagent (MOM; Vector Laboratories), according to the manufacturer's instruction, before the next primary antibody was applied. The reagent serves to block endogenous as well as previously applied exogenous mouse IgG antibodies. After staining, the sections were examined with a fluorescence microscope (Olympus America Inc., Center Valley, PA), and images were captured with a digital camera (Spot; Diagnostic Instruments, Sterling Heights, MI) and software (Spot Advanced; Diagnostic Instruments) before processing with image-management software (Photoshop; Adobe Systems, San Jose, CA).

Statistical Analysis

Data were analyzed and tested for statistical significance ($P < 0.05$) with ANOVA and paired t -tests.

RESULTS

MyHC Isoform Gene Expression Patterns during Postnatal Development

MyHC isoform gene transcript levels were evaluated at P0, P21, and 3 months (Table 2). At P0, the predominant isoforms were the embryonic *Myb3* (85.2%) and neonatal *Myb8* (11.4%), comprising approximately 97% of total MyHC transcripts. Levels of these two isoforms declined over 3 months, to less than 9% of total MyHC transcripts. In contrast, the transcript percentage of fast MyHC isoforms *Myb1*, *Myb2*, and *Myb4* rose from barely detectable levels at P0 to 16.8%, 4%, and 63%, respectively, at P21 and maintained these levels at 3 months. The percentage of the EOM-specific isoform *Myb13* also increased from very low level at P0 to close to 8% at P21, but decreased slightly to 4% at 3 months ($P < 0.001$). The *Myb6* and *Myb7* transcripts were less than 1% of the total during all stages of postnatal development.

MyHC Isoform Expression during Postnatal Development

BA-G5, an antibody against α -cardiac myosin that recognizes both orbital and global multiply innervated fibers (MIFs) in the rabbit¹⁶ and some orbital fibers in the rat (YZ, HJK, unpublished data, 2010), failed to immunostain any fibers in mouse EOM or heart. Thus, the expression pattern of α -cardiac myosin could not be evaluated.

Emb-MyHC, Neo-MyHC, and Slow-MyHC. At P0, myofibers in global layers were organized in clusters, with the center fiber being the largest and surrounded by five to eight smaller myofibers (Figs. 1A–D and insets). This pattern of myofiber organization was similar to the rosette arrays observed in rat

TABLE 1. Primers for qPCR

Gene	Forward	Reverse
<i>Myb1</i>	5'GAGGGACAGTTTCATCGATAGCAA 3'	5'GGGCCAACTTGTCTCTCTCAT 3'
<i>Myb2</i>	5'AGGCGGCTGAGGAGCACGTA 3'	5'GCGGCACAAGCAGCGTTGG 3'
<i>Myb3</i>	5'CTTCACCTCTAGCCGGATGGT 3'	5'AATTGTCAGGAGCCACGAAAT 3'
<i>Myb4</i>	5'CACCTGGACGATGCTCTCAGA- 3'	5'GCTCTTGCTCGGCCACTCT 3'
<i>Myb6</i>	5'CCAACACCAACCTGTCCAAGT 3'	5'AGAGGTTATTCCTCGTCTGCAT 3'
<i>Myb7</i>	5'CTCAAGCTGCTCAGCAATCTATT 3'	5'GGAGCGCAAGTTTGTCTAAGT 3'
<i>Myb8</i>	5'CAGGAGCAGGAATGATGCTCTGAG 3'	5'AGTTCCTCAAACCTTCAGCAGCCAA 3'
<i>Myb13</i>	5'GAAGCTCCTGAACTCCATCG 3'	5'GGTCAACAGCTTCTCGTCTC 3'
<i>GAPDH</i>	5'GTATGACTCCACTCAGGCAAA 3'	5'GGTCTCGTCTCTGGAAGATG 3'

TABLE 2. Percentage Composition of MyHC Isoform Transcripts in Mouse EOMs during Postnatal Development by qPCR

	Genes	P0	P21	3 mo
Developmental isoforms	<i>Myh3</i> (emb-MyHC)	85.2 ± 4.2	7.9 ± 0.3*	8.3 ± 0.3
	<i>Myh8</i> (neo-MyHC)	11.4 ± 0.8	0.25 ± 0.01*	0.27 ± 0.02
Typical Fast isoforms	<i>Myh1</i> (2X-MyHC)	1.5 ± 0.1	16.8 ± 1.0*	17.0 ± 0.9
	<i>Myh2</i> (2A-MyHC)	1.3 ± 0.01	4.0 ± 0.13*	4.0 ± 0.06
	<i>Myh4</i> (2B-MyHC)	0.08 ± 0.01	63.0 ± 0.8*	65.8 ± 1.5
	<i>Myh6</i> (α-cardiac MyHC)	0.17 ± 0.01	0.06 ± 0.01*	0.13 ± 0.001†
Other isoforms	<i>Myh7</i> (slow-MyHC)	0.42 ± 0.09	0.24 ± 0.01	0.60 ± 0.05†
	<i>Myh13</i> (eom-MyHC)	0.006 ± 0.001	7.9 ± 0.07*	4.0 ± 0.08†

Data represent the mean percentage ± SD of each MyHC isoform after normalization to GAPDH; *n* = 3.
t-Test, P21 vs. P0: **P* < 0.001.
t-Test, 3 months vs. P21: †*P* < 0.001.

EOMs.⁹ At P0, mouse EOMs expressed emb-MyHC, neo-MyHC, and slow-MyHC (Fig. 1). All myofibers in both global and orbital layers contained emb-MyHC, with intense staining of the central large fibers and weak expression in the surrounding smaller fibers (Fig. 1A and inset). The large fibers also expressed slow-MyHC (Fig. 1D and inset) and continue to express slow-MyHC over time (Figs. 1D, 1H, 1L, 1P). All fibers except the large fibers expressed neo-MyHC (Fig. 1B) at P0. The

FIGURE 1. Expression pattern of emb-MyHC, neo-MyHC, and slow-MyHC in the rectus muscle during postnatal development. Immunostaining for emb-MyHC (green, A, E, I, M), neo-MyHC (red, B, F, J, N), and slow-MyHC (blue, D, H, L, P) at P0 (A, B, C, D), P7 (E, F, G, H), P14 (I, J, K, L), and P21 (M, N, O, P). (C, G, K, O) Merged images of emb-MyHC and neo-MyHCs; (D, H, L, P) merged images of emb-MyHC and slow-MyHC at the same time point. Emb-MyHC was present in all fibers at P0 and then was progressively lost, first from the global secondary fibers, then from the global primary fibers, and then from the orbital-global boundary region by P21. Slow-MyHC was coexpressed in primary fibers with emb-MyHC until P21, when emb-MyHC expression disappeared from the global primary fibers. Neo-MyHC was expressed in every secondary fiber until P7 and then was progressively lost from the global layers and the outer orbital layers. (A–D, insets) Enlargements of the regions in rectangles showing global layer primary fibers (emb-MyHC⁺, slow-MyHC⁺, and neo-MyHC⁺) and the surrounding secondary fibers (emb-MyHC⁺, neo-MyHC⁺, and slow-MyHC[−]). All images were taken of mid-belly sections. Orientation: orbital layer (top left); global layer (bottom right). Scale bar, 100 μm.

relatively weak emb-MyHC and strong neo-MyHC immunoreactivity probably reflected an intermediate state of the smaller fibers, with neo-MyHC replacing emb-MyHC.

Over the next 3 weeks, the expression of emb-MyHC decreased (Figs. 1A, 1E, 1I, 1M), first disappearing from the smaller fibers of the global layer and then from the orbital layer. By P7, emb-MyHC was expressed in most fibers of the outer orbital layer but was restricted to the slow-MyHC-positive large fibers in the inner orbital layers and the global layer. The expression of emb-MyHC became weaker in global large fibers at P14 and by P21 assumed a distinct outer orbital layer location and was no longer detected in the global layers. In contrast, a reduction in neo-MyHC was not noticeable until P14 (Fig. 1J), at which time neo-MyHC was prominent in the orbital layer and weak in the global layer. By P21, neo-MyHC was expressed only in the inner orbital layer, which is the location in the adult (Fig. 1N). The longitudinal and cross-sectional distributions of both embryonic and neonatal MyHC at P21 were similar to those in the adult.

2A-MyHC, 2B-MyHC, 2X-MyHC, and Eom-MyHC. We were unable to identify 2X-MyHC fibers with antibody 6H1 (against 2X-MyHC of rabbit). Antibody A4.74 (against 2A/2X-MyHC of human) demonstrated the same staining pattern as SC-71, which is 2A-MyHC specific. Therefore, A4.74 appeared to identify 2A-MyHC-expressing fibers (data not shown). We used a negative staining approach to determine 2X-MyHC expression with antibody BF-35, which recognized all myofibers except pure 2X fibers. We deduced that fibers that were not immunoreactive with BF-35 were 2X-MyHC-containing fibers. No pure 2X-MyHC fibers were detected at P0, P14, or P21 (data not shown). Therefore, 2X-MyHC fibers or pure 2X-MyHC fibers appear not to be present in EOMs until adulthood.

Eom-MyHC expression preceded that of the other adult fast MyHC (2A-MyHC, 2X-MyHC, and 2B-MyHC). At P5, weak immunoreactivity was observed in the mid-belly region (data not shown), became stronger over time (Figs. 2A–C), and gradually extended across the length of the muscle. No 2A-MyHC (Fig. 2D) or 2B-MyHC (Fig. 2G) positive fibers were found at P7. By P14, 2A-MyHC (Fig. 2E) and 2B-MyHC (Fig. 2H) positive fibers were readily identified, and by P21, the longitudinal and cross-

sectional expression patterns of eom-MyHC (Fig. 2C), 2A-MyHC (Fig. 2F), and 2B-MyHC (Fig. 2I) were nearly the same as at 3 months.

Longitudinal and Cross-Sectional Distribution of MyHC Expression in Adult EOMs

2A-MyHC, 2B-MyHC, and Slow-MyHC. In adult EOMs, 2A-MyHC expression patterns varied greatly along the length of the muscle and in the cross sections. A dramatic degree of variation in expression of emb-MyHC, neo-MyHC, 2X-MyHC and eom-MyHC was also observed (described later). In the proximal region, 2A-MyHC was found mostly in the orbital layer, with rare positive fibers in the global layer (Fig. 3A). Toward the mid-belly region, 2A-MyHC-positive fibers were completely restricted to the orbital layer (Fig. 3B) and were nearly absent in the innervational region (Fig. 3C). However, 2A-MyHC fibers became evident in the orbital layer distally (Fig. 3D).

2B-MyHC and slow-MyHC did not vary along the length of the muscle. 2B-MyHC was expressed in the largest diameter myofibers of the global region along the length of the muscle (Fig. 3). In contrast, slow-MyHC-positive fibers were scattered throughout the global and orbital layers with no longitudinal variation (Fig. 3). In general, slow-MyHC-positive fibers in the orbital layer were relatively smaller and seemed to be less intensely immunoreactive than those in the global layer. 2A-MyHC, 2B-MyHC, and slow-MyHC isoforms were never found to be jointly expressed in a single myofiber.

Emb-MyHC, Neo-MyHC, and Eom-MyHC. In adult EOMs, immunostaining patterns of antibodies to 2A-MyHC (SC-71) and neo-MyHC (N1.551) were identical (see Figs. 6A–C). However, these two antibodies did not recognize the same myosin isoform. At P0 and P7, N1.551 detected immunoreactive fibers, which would be predicted to contain neo-MyHC (Figs. 1B, 1F), whereas no positive fibers were detected with SC-71 at these early ages. Therefore, we concluded that the neo-MyHC isoform is always coexpressed with the 2A-MyHC isoform in adult EOMs.

Expression patterns of the emb-MyHC and eom-MyHC isoforms exhibited longitudinal and cross-sectional variations.

FIGURE 2. Expression pattern of fast MyHCs (eom-MyHC, 2A-MyHC, and 2B-MyHC) in rectus muscle during postnatal development. Immunostaining for eom-MyHC (A–C), 2A-MyHC (D–F), and 2B-MyHC (G–I) at P7 (A, D, G), P14 (B, E, H), and P21 (C, F, I). The expression of eom-MyHC was first detected in rectus muscle in the mid-belly region at ~P5. 2A-MyHC and 2B-MyHC were not detected until P10. The staining observed in (D) and (G) is extracellular. All three fast MyHCs exhibited adult expression patterns by P21. All images were taken from mid-belly sections. Orientation: orbital layer (top left); global layer (bottom right). Scale bar, 100 μ m.

FIGURE 3. Longitudinal and cross-sectional distributions of 2A-MyHC, 2B-MyHC, and slow-MyHC expression in adult rectus muscle. Immunostaining for 2A-MyHC (red), 2B-MyHC (green), and slow-MyHC (blue) on cross sections of rectus muscle from the proximal (A), proximal-mid (B), mid-belly (C), and distal (D) regions. 2A-MyHC expression was mostly in the orbital layer at both the proximal and distal ends and was excluded from the mid-belly. 2B-MyHC was expressed in the large myofibers only in the global region throughout the length of the muscle, and no longitudinal variation was observed. Slow-MyHC was scattered throughout the orbital and global layers with no longitudinal variation. Note that 2A-MyHC, 2B-MyHC, and slow-MyHC are not coexpressed in single myofibers. Images were taken from sections of the same rectus muscle at different regions. (C, arrows) An adjacent muscle. Orientation: orbital layer (top left); global layer (bottom right). Scale bar, 100 μ m.

Throughout the length of the muscle, emb-MyHC was restricted to the orbital layer (Figs. 4A–D) and was excluded from the innervational zone, where no or rare immunoreactive fibers were identified (Fig. 4C). Simultaneous immunostaining with antibodies against the emb-MyHC and 2A-MyHC isoforms demonstrates that emb-MyHC was located in the C-shaped outer orbital layer, whereas 2A-MyHC was restricted to the inner orbital layer.

Longitudinal eom-MyHC isoform expression was complementary to the distribution of emb-MyHC, neo-MyHC, and 2A-MyHC (Figs. 4E–G). At the very tip of the proximal region, no eom-MyHC-positive fibers were detected (data not shown). Eom-MyHC-positive fibers appeared first in the orbital layer and then extended into the global layer as the mid-belly region was approached, and within this central region, eom-MyHC was detected in most fibers of both the orbital and global layers, with the exception of some core global fibers, where 2B-MyHC-positive fibers were found (Fig. 4G, also see Fig. 6). In the transition from the mid-belly to the distal end, the number of positive fibers decreases, and eom-MyHC was detected only in the global layer in the most distal region (Fig. 4H).

2X-MyHC. Because of the limitation imposed by the BF-35 antibody, we were able to study only the distribution of pure 2X-MyHC fibers (identified as fibers unstained by BF-35). Pure 2X-MyHC fibers exhibited longitudinal variation in a fashion similar to that of emb-MyHC, neo-MyHC, and 2A-MyHC fibers, in that they were present in the proximal and distal regions but were excluded from the mid-belly (Fig. 5). However, although emb-MyHC, neo-MyHC, and 2A-MyHC fibers were mainly distributed in the orbital layer, pure 2X-MyHC fibers were present in the global layer; only a few negative fibers were observed in the orbital layer at the two ends of the muscle.

Coexpression of MyHC Isoforms in Single Muscle Fibers in Adult EOMs

Global Layer. Of the seven MyHC isoforms evaluated, only slow-MyHC, 2B-MyHC, 2X-MyHC, and eom-MyHC were found in the global layer. Since pure 2X-MyHC fibers were detected only by negative staining with BF-35, possible coexpression of 2X-MyHC with other isoforms could not be evaluated. Slow MyHC expression, which was scattered in fibers of both layers, never colocalized with the 2B-MyHC (Fig. 3) or eom-MyHC (Figs. 6B, 6D). Because antibodies against 2B-MyHC (BF-F3) and eom-MyHC (4A6) were both of the IgM type, we were not able to perform double immunostaining with complete blocking between these two antibodies. However, slow-MyHC fibers were used as landmarks to compare 2B-MyHC and eom-MyHC myofibers in serial sections. As shown in Figure 6, 2B-MyHC and eom-MyHC were expressed in a complementary fashion. 2B-MyHC was primarily expressed in the core of the global layer (also see Fig. 3), whereas eom-MyHC was predominantly found in the orbital layer with the exception of the mid-belly region where eom-MyHC extended to the global

FIGURE 4. Longitudinal and cross-sectional distribution of 2A-MyHC (neo-MyHC), emb-MyHC, and eom-MyHC expression in adult rectus muscle. Immunostaining for 2A-MyHC (red, serves as a landmark), emb-MyHC (green, A–D), and eom-MyHC (green, E–H) on cross-sections of rectus muscle from the proximal (A, E), proximal-mid (B, F), mid-belly (C, G), and distal (D, H) regions. (E–H) Adjacent sections of (A–D), respectively. Similar to 2A-MyHC, emb-MyHC expression was restricted to the orbital layer of both the distal and proximal regions, tapering off in the mid-belly region. On the other hand, eom-MyHC was located predominantly in the orbital layer at the proximal and proximal-mid regions, in both the orbital and global layers in the mid-belly region, and predominantly in the global layer in the distal region. (A–D, arrows) 2A-MyHC (neo-MyHC) and emb-MyHC double-positive myofibers; (E–H, dashed arrows) 2A-MyHC (neo-MyHC) and eom-MyHC double-positive myofibers. Orientation: orbital layer (top left); global layer (bottom right). Scale bar, 100 μ m.

FIGURE 5. Longitudinal and cross-sectional distribution of 2X-MyHC expression in adult rectus muscle. Cross sections of EOM from the proximal (A), mid-belly (B), and distal (C) regions were double-immunostained with antibody BF-35, which recognizes all but pure 2X-MyHC myofibers and with antibody 7A10 (against dystrophin), to identify all myotube boundaries. Pure 2X-MyHC fibers were observed in the global layers in the proximal and distal regions but not in that of the mid-belly region. Orientation: orbital layer (*top left*) global layer (*bottom right*). Arrows: pure 2X-MyHC myofibers that were identified by the absence of immunostaining. Scale bar, 100 μ m.

layer (Figs. 3, 6). Coexpression of the two isoforms was restricted to a small fraction of myofibers in the orbital-global boundary area (Fig. 6).

Orbital Layer. The orbital layer expressed at least five MyHC isoforms: emb-MyHC, neo-MyHC, 2A-MyHC, slow-MyHC, and eom-MyHC. As shown in Figures 3 and 6, slow-MyHC was excluded from 2A-MyHC, 2B-MyHC, and eom-MyHC-positive fibers. Slow-MyHC also was not detected in neo-MyHC-positive fibers (Figs. 7P-R), which was expected, as 2A-MyHC and neo-MyHC-positive fibers were identical (Figs. 7A-C). The only MyHC isoform that coexisted with slow-MyHC was the emb-MyHC isoform (Figs. 7D-F) in a small number of orbital fibers.

2A-MyHC was never detected in the same fibers as 2B-MyHC or slow-MyHC-positive (Fig. 3), but colocalized with neo-MyHC-positive fibers (Figs. 7A-C). Some of the 2A-MyHC-

positive fibers expressed emb-MyHC (Figs. 4A-D, arrows) or eom-MyHC (Figs. 4E-G, arrows).

In addition to being coexpressed in some of the 2A-MyHC and neo-MyHC-positive fibers (Figs. 4E-H, arrows), eom-MyHC was detected in some emb-MyHC-positive fibers (Figs. 7J-L).

Except for 2B-MyHC, which was located only in the global layer (Figs. 7M-O), emb-MyHC was coexpressed to some extent with the other four MyHC isoforms (slow-MyHC, neo-MyHC, 2A-MyHC, and eom-MyHC) that were present in the orbital layer (Figs. 7D-L). Some emb-MyHC-positive fibers were also positive for slow-MyHC (Figs. 7D-F), 2A-MyHC (Figs. 4A-D), eom-MyHC (Figs. 7J-L), or neo-MyHC (Figs. 7G-D). The MyHC isoform coexpression patterns are summarized in Table 3.

DISCUSSION

In our detailed analysis of gene and protein expression, MyHC isoform expression was found to be highly complex, similar to observations of other species.^{8,9,11,13} MyHC isoform expression had similarities to that in typical skeletal muscle in the decreasing expression of developmental isoforms and increasing of fast isoforms after birth, as well as the restriction of expression of slow-MyHC, 2A-MyHC, and 2B-MyHC isoforms to single muscle fibers. However, MyHC expression in the mouse EOM was divergent from that in other muscles and EOMs of other species in several ways. First, the two developmental isoforms were retained in the orbital layer of adult EOMs. Second, although slow-MyHC and 2B-MyHC did not exhibit obvious longitudinal variations, emb-MyHC, neo-MyHC, and 2A-MyHC were more abundant at both the distal and proximal ends and were excluded from the innervational zone. On the other hand, eom-MyHC complemented their expression patterns and was more abundant in the mid-belly region and in only a few fibers distally. Furthermore, pure 2X-MyHC fibers were present in the global layer distally but were absent from the innervation zone. Third, except for slow-MyHC, which appeared to be scattered throughout both the global and or-

FIGURE 6. Complementary and overlapping expression of 2B-MyHC and eom-MyHC in the adult rectus muscle. Expression of 2B-MyHC (A, C, *green*) was restricted to large fibers in the global layer, whereas that of eom-MyHC (B, D, *green*) was distributed in both the orbital and global layers. With slow-MyHC-positive fibers (*blue*) as landmarks for comparison, some double-positive fibers (*white arrows*) were observed in the orbital and global layer boundary area. *Red arrows*: 2B-MyHC-only-positive fibers; *white arrowheads*: eom-MyHC-only-positive fibers. (A, B) Proximal mid-belly region; (C, D) mid-belly to distal region. Orientation: orbital layer (*top left*) global layer (*bottom right*). Scale bar, 100 μ m.

FIGURE 7. Coexpression of MyHC isoforms in single myofibers of adult rectus muscle. Double immunostaining with combinations of MyHC antibodies showed that 2A-MyHC and neo-MyHC (A–C) were colocalized; slow-MyHC and emb-MyHC (D–F), neo-MyHC and emb-MyHC (G–I), and emb-MyHC and eom-MyHC (J–L) were also colocalized in some muscle fibers. However, emb-MyHC and 2B-MyHC (M–O) and neo-MyHC and slow-MyHC (P–R) did not coexist in any single fibers. Orientation: orbital layer (*top left*) global layer (*bottom right*). Scale bar, 100 μ m.

bital layers, and eom-MyHC, which was expressed in both layers at the mid-belly region, the other MyHCs were restricted either to the orbital layer (emb-MyHC, neo-MyHC, and 2A-MyHC) or global layer (2B-MyHC and 2X-MyHC). Fourth, complex coexpression patterns exist in mouse EOM fibers. In the global layer, MyHC isoform coexpression was rare and restricted to only a few eom-MyHC and 2B-MyHC double-positive fibers in the orbital–global boundary area. In the orbital layer, however, five of the six MyHC isoforms were found to have overlapping expression patterns with at least one other isoform (emb-MyHC and slow-MyHC, emb-MyHC and 2A-MyHC

[neo-MyHC], emb-MyHC and eom-MyHC, 2A-MyHC and neo-MyHC, and 2A-MyHC and eom-MyHC).

The complex expression patterns of MyHC isoforms in the orbital layer are most likely a reflection of functional requirements. Orbital layer fibers are nearly continuously active throughout the oculomotor range,^{17,18} participating in all types of eye movements.¹⁹ The orbital layer has been hypothesized to be involved in the slow and tonic eye movements responsible for positioning the globe and fixation in specific gaze directions.²⁰ These smooth and finely graded eye movements require fine control of muscle contraction and force

TABLE 3. Coexpression of MyHC in Adult EOMs

MyHC (Antibody)	2A	2B	EOM	Emb	Neo
s-MHC	No	No	No	Yes	No
2A	NA	No	Yes	Yes	Yes
2B		NA	Yes	No	No
EOM			NA	Slight	Yes
Emb				NA	Yes
Neo					NA

generation. The expression of multiple MyHC and combinations of MyHC isoforms in single fibers of the orbital layer, most likely provides contractile diversity, allowing the EOMs to achieve the precise muscle force generation and small force increments necessary for orbital layer function.

The expression of MyHC isoforms in EOMs of small mammals share common features but demonstrate distinct differences (summarized in Table 4). The longitudinal and cross-sectional expression patterns of slow-MyHC and 2B-MyHC appear consistent among the rabbit, rat, and mouse; both myosin isoforms are continuous along the length of EOM fibers, with no longitudinal variation.^{9,10} Developmental MyHCs are expressed in the orbital layers at the proximal and distal ends but are excluded from the endplate zone of all three species. Eom-MyHC, on the other hand, is expressed at the highest levels, spanning the endplate zone.^{9–11,16,21} There are differences in expression. First, Eom-MyHC is expressed only in the orbital layer in the rat,^{9,10} whereas in the rabbit, eom-MyHC is expressed at high levels in the orbital layer, less in the global layer endplate zone, and disappears from orbital fibers outside the endplate zone.^{16,22,23} Eom-MyHC in mice is abundantly expressed in the orbital and global layers in the endplate zone, but only in the orbital layer in the proximal region and only in the global layer in the distal region (Fig. 4). Second, emb-MyHC is expressed in most of the orbital fibers of the rat^{9,10,21} and in almost all of the outer orbital fibers, 40% to 50% of the inner orbital fibers, and 10% to 20% of the global fibers in the rabbit.^{8,16} In the mouse, it is expressed only in a subset of orbital fibers, mostly in the outer orbital layer (Fig. 4). Third, 2A-MyHC is expressed in most of the fibers in the orbital layer and in scattered, small-diameter fast fibers in the global layer in the rat, with no longitudinal variation observed,^{10,22} similar to the pattern in the rabbit.⁸ However, in the mouse, 2A-MyHC is restricted to the orbital layer toward the inner orbital layer, and its expression is nearly absent from the innervational zone (Figs. 3, 4). Fourth, in the rabbit, neo-MyHC expression is scattered across the global and inner orbital layers and has been identified in the proximal, endplate, and distal regions, with no longitudinal variation.¹⁶ However, neo-MyHC expression in the mouse was identical with 2A-MyHC and was found exclusively in the orbital layer, not in the global layer, except in the proximal end (Figs. 3, 4). Similarly, emb-MyHC, neo-MyHC, and 2A-MyHC were excluded from the endplate zone. The difference in myosin isoform expression among animals may reflect variations in eye movement requirements across species; however, correlation of MyHC to eye movement differences would be purely speculative.

It has long been appreciated that the typical skeletal muscle fiber typing scheme⁶ cannot be applied to EOMs. Instead, based on location, innervational pattern, and mitochondrial content, the EOM fibers have been categorized into six types, two orbital fibers—the orbital singly innervated fibers (SIFs) and orbital MIFs—and four global fibers—red, intermediate and, white SIFs, as well as global MIFs.^{19,24} Although there is agreement that both orbital and global MIFs express slow-MyHC in the rat and the mouse,^{9,10} and α -cardiac myosin in the

rabbit,²⁵ a strict correlation of fiber type with MyHC expression is not possible. In the rat, attempts have been made to correlate the orbital SIF to emb-MyHC and eom-MyHC-expressing fibers and the four global fibers to the typical skeletal muscle fiber types.¹⁰ In the orbital layer of mouse EOMs, at least five of six myosin isoforms examined are expressed, and at least six different combinations of two myosin isoforms in single fibers are found. The expression of myosin isoforms in the global layer is complicated, with at least four isoforms (slow, EOM-specific, 2B, and 2X) present. Although 2A-MyHC is absent in the global layer, eom-MyHC is expressed in most of the orbital fibers as well as in some global fibers, some of which are also positive for 2B-MyHC. Because of technical limitations with immunostaining, we were not able to demonstrate fibers positive for four or more myosin isoforms.^{4,10,11,13,21,23,26} All evidence taken together, a strict fiber categorization scheme appears to be impossible and of limited value, given the great degree of molecular diversity of EOM fibers.

Slow-MyHC-expressing fibers are present in both the orbital and global layers in mouse EOMs and most likely are MIFs. The global MIFs in rats contract in a tonic fashion across their length and have small en grappe endplates,²⁷ and this contractile property correlates with a uniform slow-MyHC expression pattern with no coexpression of other isoforms.²¹ In contrast, the orbital MIF exhibits twitchlike contraction near the central innervational band, which has large en plaque endplates and tonic contraction at the ends of the fiber associated with en grappe endplates.²⁸ The slow-MyHC fibers of the orbital layer have varied expression of MyHC isoforms across their length.²¹ In our study in the mouse, the slow-MyHC expressing fibers of the orbital region coexpressed emb-MyHC (Figs. 7D–F), but only outside the central innervational zone, and no coexpression was found in the central region. These results suggest that specific innervation regulates MyHC expression in the MIF.

Several studies have shown a discrepancy between gene transcripts and protein levels in MyHC isoforms. Electrophoretic analysis of MyHC isoforms in rat EOMs revealed that eom-MyHC makes up 14% to 25% of total myosin^{26,29}; however, for eom-MyHC, the mRNA content estimated by competitive PCR was only 1%.¹² Transcript levels of 2A-MyHC, 2X-MyHC, 2B-MyHC, and slow-MyHC (29%, 30%, 25%, and 1%, respectively) differ from protein levels (8%, 8%, 50%, and 8%, respectively).^{12,26} In our study, mRNA levels for slow-MyHC were less than 1% at P0, significantly lower than the number of positive fibers that was approximately 20%. Eom-MyHC was

TABLE 4. Comparison of MyHC Isoform Expression in the Mouse, Rat, and Rabbit

	2A	2B	Eom	Emb	Slow
Mouse					
Orbital layer	+	–	++	+	+
Global layer	–	+	+	–	+
Longitudinal variation	Yes	No	Yes	Yes	No
Rat					
Orbital layer	+	–	++	++	+
Global layer	+	++	–	–	+
Longitudinal variation	No	No	Yes	Yes	No
Rabbit					
Orbital region	++	–	++	++	+
Global region	+	+	+	–	+
Longitudinal variation	No	No	Yes	Yes	No

++, +, and – indicate that most, a few, or none of the fibers, respectively, express the corresponding MyHC isoform.

detected in the proximal orbital, distal global, and most end-plate zone fibers, but *Myb13* transcripts were found in only 8% at P21 and in 4% at 3 months. Although the present data indicate that protein and transcript levels of eom-MyHC isoform correlate better in the mouse than in the rat,^{12,26,29} the use of qPCR could also have contributed to better correlations of protein and gene expression. The relationship of mRNA and protein expression may be influenced by RNA stability, translational efficiency, and posttranslational modification, which could enhance protein stability, all of which may contribute to a discrepancy between expression levels of MyHC gene transcript and protein levels.

In summary, MyHC isoforms have complex expression patterns in mouse EOMs, exhibiting not only longitudinal and cross-sectional variations of each isoform, but also an array of coexpression in single fibers. Detailed understanding of MyHC expression will aid understanding of the unique contractile activity of EOMs, which may then shape studies of how modification of MyHC expression can modify muscle contraction and offer a novel approach for treatment of ocular motility disorders.

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