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Lineage-based Primary Muscle Fiber Type Diversification Independent of MEF2 and NFAT in Chick Embryos

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Abstract

Differences in primary avian skeletal muscle fiber types are based on myoblast cell lineages and independent of innervation. To understand the basis for this mode of myogenesis, embryonic myoblasts specifically committed to the formation of either fast or fast/slow muscle fiber types were isolated, characterized, and examined for their capacities to transcriptionally regulate the slow myosin heavy chain 2 (MyHC2) gene. Myogenic basic helix-loop-helix protein binding sites within the slow MyHC2 promoter were mutated and did not direct fast versus fast/slow muscle fiber type development. Using promoter analyses coupled with overexpression studies and transcriptional sensors, the roles of Nuclear Factor of Activated T cells (NFATc1), and MEF2A in regulation of the slow MyHC2 gene were determined. MEF2A activated the slow MyHC2 promoter in both fast and fast/slow primary muscle fibers. In contrast, NFATc1 repressed promoter activity. These results do not support the roles of MEF2 and NFAT as direct regulators of primary muscle fiber type differences. Rather, the results reflect intrinsic differences in the modes of regulation of the slow MyHC2 gene in primary muscle fiber types.

Keywords

myogenesis; avian; embryonic; myoblast; fiber type; lineage; promoter

Introduction

Vertebrate skeletal muscle is composed of muscle fibers with differing contractile and metabolic characteristics. Muscle fibers types are often categorized as fast or slow based on their contraction and relaxation speeds as well as expression of myosin heavy chain (MyHC) isoform genes that correlate with the fiber's contractile properties (Reiser et al. 1985; 1988). The vast majority of fibers of chicken skeletal muscles are classified as fast or fast/slow. This classification is based on whether an individual fiber expresses a slow MyHC gene in addition to a fast MyHC gene. Nearly all chicken muscle fibers express at least one fast MyHC isoform gene throughout development and in the adult (Bandman et al. 1982; Page et al. 1992). Some chicken muscle fibers also express slow MyHC1 and/or slow MyHC2 genes. For example, the chicken pectoralis major is composed of exclusively fast muscle fibers without expression of slow MyHC1 or slow MyHC2 genes (except the rostral red strip). Conversely, all the muscle fibers of the chicken medial adductor express the slow MyHC2 gene in addition to a fast MyHC isoform gene.

Myogenic precursor cells appear at multiple times during development. Primary muscle fibers form by differentiation and fusion of primary or embryonic myoblasts. These fibers

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form before the ingression of functional innervation into muscle forming regions. In the developing chick, primary muscle fiber formation in the limbs begins at approximately embryonic day (ED) 4 and continues until approximately ED8 (Stockdale 1992). Fiber type analysis of primary muscle fibers using monoclonal antibodies that recognize fast MyHCs and slow MyHC2 demonstrated fiber type diversity at the earliest stages of primary muscle fiber formation. Muscle fibers were identified that expressed fast MyHC genes exclusively or both fast MyHC and slow MyHC2 genes (Crow and Stockdale 1986a,b). This diversity within the primary muscle fiber population persists throughout primary myogenesis (Page et al. 1992). Following primary myogenesis, fetal or secondary myogenesis ensues. In chicken development, fetal muscle fiber formation occurs from ED8-ED18 (Stockdale 1992). Similar to primary muscle fiber type formation, both fast and fast/slow muscle fibers appear during secondary myogenesis as well.

Although the final phenotypic diversity of muscle fibers generated during primary and secondary myogenesis may appear to be the same, the cellular mechanisms that generate this fiber type diversity during primary and secondary myogenesis are quite different. Regulation of the diversity of primary muscle fiber types in chicken development is independent of functional innervation. Functionally denervated primary muscle fibers continued to express either fast MyHC genes exclusively or both fast MyHC genes and the slow MyHC2 gene in patterns reflective of untreated embryos (Crow and Stockdale 1986a,b). Studies using direct neural ligation also showed persistence of diverse primary fiber types in denervated muscles (Butler et al. 1982; McLennan 1983). In conceptual agreement with the innervations-independent diversity of primary muscle fiber types in vivo, diversity of muscle fibers formed from embryonic myoblasts in vitro was also demonstrated. Avian primary embryonic myoblasts in vitro form muscle fibers that express fast MyHC genes exclusively or both fast MyHC genes and the slow MyHC2 gene (Miller and Stockdale 1986a,b). The lineage commitment of individual clonal myoblasts to form muscle fibers of specific types is stable in vivo (DiMario et al. 1993). In contrast to primary muscle fiber formation, secondary muscle fibers require functional innervation for the acquisition of diversity among fiber types (Wigmore and Evans 2002). For secondary muscle fibers in vitro, expression of slow muscle fiber type specific genes is also dependent on innervation or direct electrical stimulation (Kubis et al. 2002; Crew et al. 2010).

Slow tonic motor neuron activity increased calcineurin-mediated dephosphorylation of NFAT and subsequent nuclear import (Dunn et al. 2001). Chronic low frequency electrical stimulation, mimicking slow muscle neural input, of rabbit and chicken myotubes in vitro caused nuclear import of NFATc1 (Kubis et al. 2002; Crew et al. 2010). In vivo studies in which NFATc1-GFP was detected in the cytoplasmic compartment of the fast tibialis anterior (TA) muscle and in the nuclear compartment of the slow soleus muscle as well as the stimulated TA muscle supported the activity-dependent mechanism of NFAT nuclear localization (Tothova et al. 2006). The downstream effect of NFAT nuclear localization is increased NFAT-mediated transcriptional activity. Because NFAT is localized to the nucleus by calcium transients generated by slow muscle neural activity, multiple slow fiber specific genes are activated by NFAT. Such stimulation patterns increase NFAT occupancy of specific NFAT binding sites within slow fiber specific promoters and increase expression of genes indicative of the slow fiber phenotype such as myoglobin, slow troponin I, and slow MyHC (Wu et al. 2000; Chin et al. 1998; Jiang et al. 2004). Moreover, nuclear localization of NFAT was required for transition of fast to slow muscle fiber type gene expression (Kubis et al. 2002; Jiang et al. 2004; McCullagh et al. 2004). More recently, nuclear localization of individual NFAT isoforms (NFATc1-c4) due to particular stimulation patterns in specific muscle fiber types have demonstrated increased complexity of NFAT regulation in slow muscle fibers (Calabria et al. 2009).

MEF2 belongs to the MADS family of transcription factors (Shore and Sharrocks 1995), and four isoforms – MEF2A, B, C, and D – have been identified in vertebrates. MEF2 proteins are expressed in a wide variety of developing tissues and cell types including neurons, neural crest cells, chondrocytes, lymphocytes, and in smooth, cardiac, and skeletal muscle (reviewed by Potthoff and Olson 2007). In skeletal muscle development, MEF2C is the first MEF2 isoform expressed followed shortly thereafter by MEF2A (Edmondson et al. 1994). MEF2 proteins bind to the consensus DNA sequence YTA(A/T)₄TAR in transcriptional regulatory regions (Gossett et al. 1989; Andrés et al. 1995; Fickett 1996). MEF2 proteins regulate the expression of numerous structural genes within skeletal muscle cells. They also positively regulate expression of the myogenic basic helix-loop-helix (bHLH) transcription factors and thereby contribute to a positive feed-forward mechanism of myogenic differentiation (Edmondson et al. 1992; Tapscott 2005). Thus MEF2 proteins in skeletal muscle development augment the myogenic differentiation program through stabilization and enhancement of myogenic gene expression.

MEF2 proteins often activate gene transcription by cooperative interactions with other transcriptional regulators. For example, MEF2 proteins can synergistically activate muscle specific genes by interaction with myogenic bHLH factors and other promoter-bound transcription factors (Molkentin et al. 1995; Funk and Wright 1992; Naidu et al. 1995; Esser et al. 1999; Calvo et al. 1999, 2001; Allen et al. 2005). MEF2 transcription factor activity can be repressed by interaction with class II histone deacetylases (HDACs) (Miska et al. 1999). Since HDACs themselves are regulated by phosphorylation via nuclear calcium/calmodulin-dependent kinase, MEF2 activity is susceptible to modulation by calcium signaling within muscle fibers (Wu et al. 2000; Liu et al. 2005). Several reports have also indicated that calcineurin-dependent activity can lead to dephosphorylation of MEF2 in addition to NFAT, and thereby regulate MEF2 transcription factor activity (Wu et al. 2001; Dunn et al. 2001).

Since NFAT and MEF2 transcription factors are regulated by calcium signaling which is initiated by motor neuron activity or direct electrical stimulation, the roles of these factors in muscle fiber type specific gene regulation have been investigated in either myogenic cell lines or secondary muscle fibers, both of which exhibit innervation or stimulation dependence for expression of slow muscle fiber type specific genes. Both NFAT and MEF2-mediated transcription are increased by chronic low frequency stimulation (Wu et al. 2000, 2001; Liu et al. 2005). In secondary chicken muscle fibers formed from fetal myoblasts, we found that innervation and direct electrical stimulation increased NFAT and MEF2-mediated transcription as well as slow MyHC2 promoter activity. NFAT and MEF2 binding site sequences within the slow MyHC2 transcriptional regulatory region were required for innervation-induced, fiber type specific activation of the slow MyHC2 promoter in these secondary muscle fibers (Jiang et al. 2004). In contrast, it is clear that innervation is not required for the development of fast/slow muscle fibers and expression of the slow MyHC2 gene (Crow and Stockdale 1986a; DiMario and Funk 1999). The roles of NFAT and MEF2 transcription factors in the development of these fast/slow muscle fibers are unknown. This report presents the initial characterization of the mechanism of transcriptional regulation of the slow MyHC2 gene in primary skeletal muscle fibers that express the slow MyHC2 gene independently of innervation or electrical stimulation.

Materials and Methods

Isolation of Myoblasts and Cell Culture

The limb buds of ED4 quail embryos were removed, placed in Hank's Balanced Salt Solution, and passed through a 200ul pipette tip. Tissue was then incubated in 0.125% trypsin (Invitrogen) for 1 hour at 37° C with frequent agitation. Cells were plated at a

density of 100–300 cells per plate into collagen-coated 100mm plates. Cell culture medium consisted of 10% horse serum (Hyclone), 5% chick embryo extract, supplemented with 1.32mM CaCl₂, 2mM glutamine, and 1X antibiotic/antimycotic (Invitrogen) in Ham's F-10 basal medium (Sigma) mixed in equal volume with the same medium conditioned by incubation for 2 days in cultures of differentiated ED13 chicken myotubes. The ED13 cells were prepared as previously described (O'Neill and Stockdale 1972). The final medium consisted of 50% fresh medium and 50% conditioned medium based on previous studies of embryonic avian myoblasts (Bonner and Hauschka 1974; Miller and Stockdale 1986a). Clearly separated colonies of cells were trypsinized and replated into collagen-coated plates for cell population expansion.

Immunocytochemistry and Cell Fusion Indices

Differentiated myotubes were immunostained for fast MyHC(s) and slow MyHC2 with monoclonal antibodies F59 and S58, respectively, as previously described (Crow and Stockdale 1986a; Crew et al. 2010). To determine fusion indices, nuclei were stained with 1.2uM 4',6-diamidino-2-phenylindole (DAPI) in phosphate buffered saline (PBS) for 10 minutes following immunostaining. Four to six random fields of cells were analyzed by counting total nuclei and nuclei contained within myotubes immunostained with the F59 monoclonal antibody which identified all differentiated myotubes. At least 1000 nuclei were counted for each cell population. Indices were calculated as the number of nuclei within myotubes relative to the total number of nuclei.

DNA Constructs and Mutagenesis

Chicken slow MyHC2 genomic DNA containing exon 1, intron A, part of exon 2, and 1358bp of DNA upstream from exon 1 comprises a 2279bp DNA fragment. This DNA was cloned into the pGL3Basic luciferase reporter vector (Promega) yielding 2279SM2Luc (Jiang et al. 2004). The 6150SM2Luc DNA construct was generated by addition of the 3871bp SacI genomic DNA fragment immediately upstream of the 5' end of 2279SM2 DNA. The DNA sequence and site-directed mutagenesis of candidate E-box, NFAT, and MEF2 binding sites within the proximal slow MyHC2 promoter region have been previously described (Jiang et al. 2004). The NFATc1-GFP expression construct (kindly provided by G. Crabtree) contains cDNA encoding constitutively nuclear NFATc1 coupled to green fluorescent protein (GFP). The MEF2AFLAG expression construct contains cDNA encoding human MEF2A (kindly provided by E. Olson) linked to a FLAG epitope tag.

DNA Transfection and Promoter Assays

Slow MyHC2 promoter-reporter DNA constructs were transfected into myogenic cells using Lipofectamine 2000 (Invitrogen). Slow MyHC2 promoter-firefly luciferase DNA (4ug) was combined with 2ug pRL-SV40 (Promega), which encodes Renilla luciferase, in Opti-MEM medium. Cells in 35mm plates were transfected for 3 hours at 37° C in a 5% CO₂ humidified incubator. Transfection medium was then replaced with cell culture medium. Five days following transfection, luciferase activities were measured using the Dual-Glo Luciferase Assay (Promega). MEF2 and NFAT sensor transcriptional sensors (kindly provided by E. Olson) contain multimerized MEF2 and NFAT binding sites, respectively, that drive transcription of the luciferase gene.

Chromatin Immunoprecipitation

Immunoprecipitation of slow MyHC2 chromatin was conducted as previously described (Mitchell and DiMario 2010) with the following modifications. Chromatin was isolated from ten 100mm plates of myogenic cells which had been allowed to fully differentiate for 4 days. Chromatin was immunoprecipitated by addition of 4ug MEF2A antibody (Sigma),

incubation overnight at 4° C, followed by addition of 50ul Protein A agarose (Millipore). Addition of 4ug antibody directed against acetylcholine receptor (AChR; Santa Cruz) to an equal amount of chromatin served as a negative control for specificity of immunoprecipitation. Additional controls included lack of antibody addition to chromatin, amplification of input chromatin (prior to addition of antibody), and amplification of 6150SM2Luc plasmid DNA. Precipitated DNA was PCR amplified using the following primer pair; forward primer: 5'-GAGGCAGAAAGGAAAGCTCTCTCAGT-3'; reverse primer: 5'-AGAAATTCCCATGTGTCATGTCCA-3'.

Results

Isolation and characterization of distinct embryonic myoblast types

Cells from ED4 quail limb buds were isolated as described in *Methods* and plated at clonal density. After several days of incubation, well-separated colonies of cells were trypsinized from the plates and re-seeded for expansion of the cell population. Portions of each expanded cell population were cultured for 4–7 days to allow for differentiation of cell populations that were myogenic. Cells were immunostained for fast MyHCs and slow MyHC2 with monoclonal antibodies F59 and S58, respectively (Crow and Stockdale 1986a). Examples of myogenic cell populations that displayed myogenic potential and differentiated into muscle fibers are shown in Figure 1A. Muscle fibers were observed that expressed either fast MyHCs exclusively or both fast MyHCs and slow MyHC2. Embryonic myoblasts that formed exclusively fast MyHC-expressing muscle fibers continued to do so after multiple (15–20) serial passages (not shown). Fast myogenic clones were also immunostained for slow MyHC2 each day for one week until well-formed muscle fibers were present that expressed fast MyHC genes. At no time during differentiation of these muscle fibers were detectable levels of the slow MyHC2 gene observed, indicating that the slow MyHC2 gene was not transiently expressed during differentiation of fast muscle fibers (not shown). The commitment of fast/slow myoblast clones to form muscle fibers that expressed both fast MyHCs and slow MyHC2 was also stable through multiple serial passages. Of all the myogenic clones isolated and characterized to date, approximately 64% differentiate into exclusively fast MyHC-expressing muscle fibers and approximately 36% of the clones differentiate into muscle fibers that express both fast MyHCs and slow MyHC2 (Figure 1B). To determine whether the differentiation capacity varied between myoblast clones that generated fast versus fast/slow muscle fibers, the fusion indices of all clones were determined (Figure 1C). Average fusion indices for fast versus fast/slow myoblast clones were not significantly different, indicating that the differentiation capacity of these two types of myoblasts is the same.

It is important to note that expression of the slow MyHC2 gene in muscle fibers derived from fast/slow myoblast clones occurs in the absence of innervation or other distinguishing factors extrinsic to the muscle fibers. The generation of fast and fast/slow muscle fiber types in the absence of innervation or direct electrical stimulation is unique to the myoblast populations isolated from embryonic stages of primary muscle development. In contrast, fetal myoblasts isolated from ED13 avian limbs undergoing secondary myogenesis form muscle fibers that express only fast MyHC genes in the absence of innervation or direct electrical stimulation (DiMario and Stockdale 1997; Jiang et al. 2004; Crew et al. 2010). In total, these results indicate that regulation of the slow MyHC2 gene in diverse muscle fiber types formed from distinct embryonic myoblast clones is intrinsic to the myoblast lineages present at embryonic stages of primary muscle formation.

Regulation of the proximal promoter region of the slow MyHC2 gene in embryonic muscle fibers

To investigate the myoblast lineage-based control of fast and fast/slow muscle fiber formation, analysis of the regulation of expression of the slow MyHC2 gene, which is differentially expressed in the muscle fiber types, was conducted. Figure 2 shows the sequence of the proximal region of the slow MyHC2 promoter. Within this sequence, candidate binding sites for known transcription factors are underlined. The proximal promoter contains three candidate E-box sites for binding the myogenic regulatory factors MyoD, Myogenin, Myf5, and MRF4. The sequence also contains two potential binding sites for NFAT and a single candidate MEF2 binding site. This proximal sequence is contained within the 1358bp of upstream sequence in the wild type promoter-reporter DNA construct 2279SM2Luc. This construct also contains the first exon, first intron, and part of the second exon of the slow MyHC2 gene (Jiang et al. 2004).

Previous analysis of the regulation of this promoter region was conducted in secondary muscle fibers, formed from fetal ED13 myoblasts (Jiang et al. 2004). The promoter region contained within the 1358bp upstream region directed innervation-dependent transcription of the slow MyHC2 gene. Activation of the promoter in fetal secondary muscle fibers was mediated by the transcription factors NFATc1 and MEF2A. Forced expression of nuclear-localized NFATc1, coupled with innervation, changed muscle fiber type from fast to fast/slow, by expression of the slow MyHC2 gene. The effects of NFATc1 and MEF2A on slow MyHC2 gene expression and secondary muscle fiber type occurred via the MEF2 and NFAT binding sites located within the proximal promoter.

Since fast/slow primary embryonic muscle fibers expressed the slow MyHC2 gene without innervation or stimulation, the candidate transcription factor binding sites were tested for their ability to regulate slow MyHC2 promoter activity in primary muscle fibers. Each of the candidate sites was mutated, and the mutant promoters were linked to the firefly luciferase reporter gene. Transcriptional activities of the mutant promoters in fast and fast/slow myoblast-derived muscle fibers were compared to the activity of the wild type 2279SM2Luc construct (Figure 3). Mutation of the candidate E-box sites yielded variable results depending on the site and on the muscle fiber type. Individual mutation of the first and third E-box sites (Ebox1 and Ebox3) did not reduce slow MyHC2 promoter activity in either fiber type. Rather, mutation of the first E-box site (Ebox1) caused modest increases of 38% and 55% in slow MyHC2 promoter activities in fast and fast/slow muscle fibers, respectively, relative to the wild type promoter. Mutation of Ebox3 did not cause a significant change of promoter activity in fast muscle fibers, and it caused an 80% increase of relative activity in fast/slow muscle fibers. Mutation of the two candidate NFAT binding sites and the MEF2 binding site yielded similar results in both fast and fast/slow muscle fibers. Slow MyHC2 promoter activity was increased between 1.6–1.9 fold by mutation of the first NFAT binding site (NFAT1) in both fast and fast/slow muscle fibers. Mutation of the second NFAT binding site (NFAT2) caused a very modest decline (22%) of relative promoter activity in fast muscle fibers and no effect on promoter activity in fast/slow muscle fibers. Mutation of the candidate MEF2 binding site caused a similar reduction of 38% and 45% of relative slow MyHC2 promoter activity in fast and fast/slow muscle fibers, respectively.

MEF2 transcription factors have been implicated in the regulation of numerous muscle-specific genes, and the results above suggest that the MEF2 site activates the slow MyHC2 promoter. To determine whether MEF2 interacts with the slow MyHC2 promoter, MEF2 occupancy of the MEF2 binding site was tested by chromatin immunoprecipitation (Figure 4). Using an antibody directed against MEF2A, chromatin from muscle fibers derived from a fast/slow myoblast clone was specifically immunoprecipitated. Amplification of precipitated DNA using primers that flanked the MEF2 binding site yielded the expected

PCR product of 233bp. These results demonstrate that MEF2A occupies the candidate MEF2 binding site of the slow MyHC2 proximal promoter.

Effect of NFAT and MEF2 transcription factor activities on embryonic muscle fiber type

Since NFAT and MEF2 regulate slow muscle fiber type specific genes, including the slow MyHC2 gene in fetal secondary myotubes (Jiang et al. 2004), the ability of NFAT and MEF2 to regulate muscle fiber type development in fast versus fast/slow embryonic primary muscle fibers was investigated. To determine whether increased levels of NFAT or MEF2 transcription factor was sufficient to drive endogenous slow MyHC2 gene expression and fiber type transition in fast primary muscle fibers, genes encoding NFATc1 and MEF2A were overexpressed in fast primary muscle fibers. The NFATc1 expression construct contains cDNA encoding constitutively nuclear-localized NFATc1 linked to the green fluorescent protein (GFP) (Beals et al. 1997). The MEF2A expression construct contains the full length MEF2A cDNA linked to a FLAG epitope sequence.

Fast myoblasts were transfected with each expression construct individually and then allowed to differentiate into muscle fibers. After 3–5 days, muscle fibers formed from transfected myoblasts were identified by GFP fluorescence (NFATc1) or by immunocytochemical detection of the FLAG epitope (MEF2A) (Figure 5). Muscle fibers were also immunostained for fast MyHCs and slow MyHC2. Muscle fibers formed from fast myoblasts and overexpressing NFATc1 or MEF2A immunostained for fast MyHCs and not slow MyHC2. Therefore, neither NFATc1 nor MEF2A were sufficient to induce expression of the endogenous slow MyHC2 gene and conversion of fast embryonic primary muscle fibers to a fast/slow phenotype.

NFAT-mediated transcriptional activities in fast and fast/slow embryonic muscle fibers were compared by use of an NFAT transcriptional sensor in the different muscle fiber types (Figure 6). Fast and fast/slow myoblasts were transfected with a DNA expression construct consisting of multimerized NFAT DNA binding sites that control transcription of the luciferase gene. We have used this construct before to investigate NFAT-mediated transcription in fetal secondary muscle fibers and found sensor activity to be significantly greater in muscle fibers expressing the slow MyHC2 gene (Jiang et al. 2004). However, in embryonic primary muscle fibers, fast muscle fibers yielded nearly 10 times ($p < 0.0001$) the relative level of NFAT-mediated transcription compared to fast/slow muscle fibers. To determine the responsiveness of the NFAT transcriptional sensor in the muscle fiber types, constitutively active NFATc1-GFP was co-transfected along with the NFAT sensor. In fast muscle fibers, expression of NFATc1-GFP increased relative NFAT sensor activity by 11.4 times ($p < 0.0001$). In fast/slow muscle fibers, relative NFAT sensor activity increased more than 40 times ($p < 0.0001$) by expression of NFATc1-GFP. Yet, relative NFAT sensor activity was significantly lower (2.8 times; $p < 0.0001$) in fast/slow muscle fibers expressing NFATc1-GFP compared to fast muscle fibers expressing the same transgene. These results indicate that NFAT-mediated transcription in the embryonic fast/slow muscle fiber type does not activate slow muscle fiber type specific genes.

Figure 3 presented results of slow MyHC2 promoter activity with mutated proximal NFAT binding sites. To determine whether NFAT could activate slow MyHC2 gene expression via other regions of the slow MyHC2 promoter, or indirectly via other transcriptional control mechanisms, an additional 3.8kb of slow MyHC upstream promoter sequence was included in 6150SM2Luc. Slow MyHC2 promoter activities were measured in fast and fast/slow embryonic muscle fibers transfected with 2279SM2Luc and 6150SM2Luc with and without the constitutively nuclear NFATc1-GFP expression construct (Figure 7). Relative 2279SM2Luc activity was not significantly different ($p = 0.18$) in fast muscle fibers with and without transfected NFATc1-GFP. However, relative 2279SM2Luc activity was

significantly reduced ($p < 0.0001$) by 61% in fast/slow muscle fibers expressing the NFAT transgene. Similar results were obtained in the analysis of the effects of NFATc1-GFP expression on 6150SM2Luc activities in fast and fast/slow muscle fibers. Relative 6150SM2Luc activities were not significantly different ($p = 0.31$) between fast muscle fibers with and without expressed NFATc1-GFP. However, promoter activity was significantly reduced ($p < 0.0001$) by 56% in fast/slow muscle fibers expressing the NFATc1-GFP transgene. These results further indicate that NFAT transcription factor activity does not activate the slow MyHC2 gene in fast/slow embryonic muscle fibers. Indeed, the results indicate that NFAT represses slow MyHC2 promoter activity in fast/slow muscle fibers.

To determine whether levels of MEF2 mediated transcription are different between fast and fast/slow embryonic muscle fibers, cells were transfected with a MEF2 transcriptional sensor containing multimerized DNA binding sites that regulate transcription of the luciferase gene. MEF2 sensor activities were compared between fast and fast/slow muscle fibers with and without the co-transfected MEF2A expression construct (Figure 8). Fast and fast/slow embryonic muscle fibers without the MEF2A expression construct yielded similar ($p = 0.63$) MEF2 sensor activities. Co-transfection of the MEF2A expression construct increased MEF2 sensor activities in both fast and fast/slow muscle fibers. The relative increases were not significantly different ($p = 0.24$). Therefore, overall MEF2 mediated transcription is similar in both fast and fast/slow embryonic muscle fibers.

Although overall MEF2 mediated transcriptional activity as determined by the MEF2 sensor appeared to be similar between fast and fast/slow muscle fibers, the ability of MEF2A to specifically regulate slow MyHC2 promoter activity was tested (Figure 9). Overexpression of MEF2A in fast muscle fibers did not significantly increase ($p = 0.39$) relative 2279SM2Luc activity (Figure 9A). There was a very modest increase (15%) of relative 2279SM2Luc activity in fast/slow muscle fibers expressing MEF2A compared to fast/slow muscle fibers without exogenously expressed MEF2A ($p < 0.002$). MEF2 activation of the slow MyHC2 promoter was also tested using the 6150SM2Luc reporter construct (Figure 9B). MEF2A expression in fast muscle fibers caused a very modest (13%) increase of 6150SM2Luc relative activity ($p < 0.0001$). Overexpression of MEF2A in fast/slow muscle fibers resulted in a modest 2.1 fold increase ($p < 0.002$). In conjunction with the reduced activity of the slow MyHC2 promoter with a mutation of the MEF2 binding site in both fast and fast/slow muscle fibers (Figure 3), these latter results indicate that MEF2 is a transcriptional activator of the slow MyHC2 gene. However, this activation is not strictly muscle fiber type specific (i.e. fast versus fast/slow).

Discussion

Investigation of the roles of MEF2 and NFAT transcriptional regulators in the development of different muscle fiber types have focused on fetal stages of myogenesis. Fetal secondary muscle fibers respond to phasic, high frequency and tonic low frequency innervation or stimulation by altering patterns of gene expression indicative of the fast and slow muscle fiber type, respectively. In addition, adult muscle has been used for analysis of muscle fiber type transition in models of denervation, disuse, and hypertrophy. Very little is known regarding the roles of these potential modulators of fiber type specific gene expression in primary muscle fibers. In vivo, it is clear that innervation is not required for the development of fast/slow primary muscle fibers and expression of the slow MyHC2 gene in avian development (Crow and Stockdale 1986a; DiMario and Funk 1999). The roles of MEF2 and NFAT transcription factors in these events are virtually unknown.

To investigate the mechanism of primary muscle fiber type development, distinct myoblast clones were isolated from embryonic (ED4) quail limbs during primary myogenesis. These

clones were expanded in vitro and characterized for their differentiation into distinct muscle fiber types. Myoblast clones were isolated that differentiated into muscle fibers expressing exclusively fast MyHC genes or muscle fibers expressing fast MyHC genes and the slow MyHC2 gene. The commitment of individual clones to the differentiation of specific fast and fast/slow fiber types is stable in vitro and in vivo (Miller and Stockdale 1986a,b; DiMario et al. 1993). Formation of these different muscle fiber types – fast and fast/slow – and expression of the slow MyHC2 gene occurred in the absence of innervation or stimulation.

The slow MyHC2 promoter contains several E boxes and NFAT binding sites as well as a MEF2 binding site. In secondary muscle fibers in which slow MyHC2 promoter activity is dependent upon innervation or direct stimulation, both NFAT binding sites, the MEF2 binding site, and the most proximal E box mediated promoter activation in muscle fibers derived from myoblasts of the fetal fast/slow medial adductor muscle (Jiang et al. 2004). Mutagenesis of each of the sites reduced slow MyHC2 promoter activity. In primary muscle fibers, the MEF2 binding site also mediated slow MyHC2 promoter activation and this site interacted with MEF2A. However, in contrast to secondary muscle fibers, mutation of the first and third E box sites and mutation of the first NFAT binding site increased slow MyHC2 promoter activity in fast/slow primary muscle fibers. Furthermore, overexpression of constitutively nuclear NFATc1 was not sufficient to drive endogenous slow MyHC2 gene expression in fast primary muscle fibers. These results indicate that slow MyHC2 gene expression and promoter regulation are very different in primary versus secondary muscle fibers.

The E box sites of the slow MyHC2 promoter yielded some differences in relative promoter activities between fast and fast/slow muscle fibers with promoters containing mutations at these sites. However, mutation of the first and third E boxes increased promoter activity in fast/slow muscle fibers, suggesting a repressive function in the cells that normally express the gene. Also the second E box mutation reduced promoter activity in fast muscle fibers that do not normally express the gene. Therefore, primary muscle fiber type specific regulation of the slow MyHC2 promoter does not appear to be coordinated via the three identified E box sites.

Although mutation of the MEF2A binding site reduced slow MyHC2 promoter activity in fast/slow muscle fibers, it also decreased promoter activity to a similar relative amount in fast muscle fibers. In fact, mutation analysis of the proximal 2279bp of the slow MyHC2 promoter, which directs innervation-dependent and fiber type specific transcription in secondary muscle fibers, demonstrated no fiber type specific effects in primary muscle fibers. MEF2 activity can be controlled by calcineurin activity which is typically thought to regulate the slow muscle fiber phenotype. However, MEF2 isoforms also regulate expression of fast MyHC genes (Allen and Leinwand 2002; Allen et al. 2005; Dunn et al. 2001). Moreover, in these studies MEF2 differentially activated various fast MyHC gene promoters. MyHCIIb was preferentially activated by MEF2 compared to MyHCIIa and IId/x (Allen et al. 2005). Similarly, Dunn et al. (2001) found that although calcineurin-MEF2 signaling was present in all fiber types tested, fiber types IIb and IId/x were preferentially affected. Therefore, MEF2 is capable of activating both fast and slow muscle fiber type specific genes.

Using the MEF2 transcriptional sensor to detect endogenous MEF2-mediated transcription and transcriptional activity following forced expression of MEF2A, no fiber type specific differences were detected. An incremental increase of 2279SM2Luc activity was detected in fast/slow muscle fibers, but not in fast muscle fibers, overexpressing MEF2A. However, a similar incremental increase of promoter activity in fast muscle fibers overexpressing

MEF2A was detected using the 6150SM2Luc promoter construct. Therefore, in the context of a longer upstream promoter construct, MEF2 did not display strict fiber type specific activation of the slow MyHC2 promoter. There was a modest increase of 6150SM2Luc activity in fast/slow muscle fibers overexpressing MEF2A relative to 2279SM2Luc activity suggesting that upstream promoter sequence enhances MEF2 activation of the promoter. Nonetheless, in the case of primary muscle fibers, MEF2A functions as an activator of the slow MyHC2 promoter in both fast and fast/slow fibers.

NFAT-mediated transcription did display primary muscle fiber type specificity. General NFAT transcription factor activity as determined by the NFAT transcriptional sensor was significantly lower in fast/slow primary muscle fibers than fast fibers. Sensor activity was also significantly lower in fast/slow fibers overexpressing constitutively nuclear NFATc1. These results indicate that NFAT activity is reduced in fast/slow primary muscle fibers. NFATc1 also repressed slow MyHC2 promoter activity. This is evident from the mutation analysis in which slow MyHC2 promoter activity in fast/slow muscle fibers was increased by mutation of the most proximal NFAT binding site. The repressor function of NFAT was also supported by reduced 2279SM2 Luc and 6150SM2Luc activity in fast/slow muscle fibers overexpressing nuclear NFATc1.

As the transcriptional functions of NFAT isoforms continue to be investigated, it is clear that NFATs confer both transcriptional activation and repression, depending on the cellular and molecular context. NFAT isoforms are well recognized for transcriptional activation of numerous genes, including the slow MyHC2 gene in fast/slow secondary muscle fibers. However, NFAT isoforms also repressed the adiponectin gene in adipocytes (Kim et al. 2006), the cyclin A2 gene in lymphocytes (Carvalho et al. 2007), the growth-arrested protein 43 (GAP-43) gene in neurons (Nguyen et al. 2009), and the osteocalcin gene in osteoblasts (Choo et al. 2009). NFATc1 repressed the fast troponin I gene via an intronic regulatory element in slow muscle fibers, thereby contributing to fiber type specificity (Rana et al. 2008). Constitutively active NFATc1 also repressed the fast MyHCIIb promoter in fast muscle fibers (McCullagh et al. 2004). In addition, several reports yielded no fiber type specific effects of the calcineurin-NFAT pathway (Swoap et al. 2000) and differential activation of the fast MyHCIIa promoter relative to the fast IId promoter by calcineurin-NFAT signaling (Allen et al. 2001). Furthermore, expression of mammalian slow MyHC during development was unaffected by calcineurin inhibition. However, slow MyHC gene expression was reduced in adults with calcineurin inhibition (Oh, et al., 2005). These varying results may be partly explained by NFAT isoform specific responses to specific stimulation patterns, yielding both activity-dependent and muscle fiber type specific changes in muscle gene expression (Calabria et al. 2009) and by inherent differences in regulation of slow MyHC gene expression in developing and adult skeletal muscle.

It is tempting to speculate that the same cis- and trans-regulatory system that controls slow MyHC2 gene expression in primary and secondary fibers may exert opposite effects in these cells. Innervation-dependent activation of the slow MyHC2 gene is mediated by MEF2 and NFAT in secondary muscle fibers derived from myoblasts of fast/slow muscle origin (Jiang et al. 2004). In primary muscle fibers, MEF2 activity controls slow MyHC2 promoter activity in a non-fiber type specific manner. NFAT displays repressive activity via slow MyHC2 promoter elements in primary muscle fibers, whereas it activates the promoter via these same sites in innervated secondary muscle fibers. Such dichotomous regulation of the same gene via the same promoter elements may be the basis for the distinct modes of slow MyHC2 gene regulation in primary versus secondary muscle fiber types – innervations-independent and innervation-dependent, respectively.

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Fig. 1. Characterization of embryonic myoblast clones. A) Fast and fast/slow differentiated muscle fiber types derived from myoblast clones. Fast and fast/slow myoblast clones were allowed to differentiate into muscle fibers and were then immunostained for fast MyHCs and slow MyHC2 with monoclonal antibodies F59 and S58, respectively. F59 was detected with a Texas Red-conjugated anti-mouse IgG, and S58 was detected with a FITC-conjugated antimouse IgA. B) Each myogenic clone was characterized for expression of fast MyHCs and slow MyHC2 and tallied. Sixty four percent of myogenic clones immunostained exclusively for fast MyHCs with F59. The remaining 34% of myogenic clones immunostained with both F59 and S58. C) Myogenic clones were allowed to differentiate into myotubes and then immunostained with F59 to identify differentiated muscle fibers. Nuclei were stained with DAPI. Graph indicates the average number of nuclei in myotubes versus the total number of nuclei in cultures of fast and fast/slow myogenic clones.

-600 GAGGTGAGTATGCTTTGGGAGCAGGATTTGGGCATTTAGTGATGGACAGA
 -550 ATCCTCCCACAATCAGTCCCTGGCAAAAACATTACATTTCACTTACCCGT
 -500 AAAGCAAAGTTGTGACTTTTGGTGCCAAATGGGAAGGGTCTGGGTGAACC
 NFAT2 *Ebox3*
 -450 AGGCAAACCCT**TTGGGGAAAGAG****CAAGTG**GGGAAGGTAGTAGGGGCCAGC
 -400 CCTGAGCCCCTTGCCCTGGTTCTCCACTCCCGGGCTGTTTGATGAGGAGC
 -350 GACTGGACCAGCGCTGAGCCAGAGGTTCTGGGTGCGCCGGCCCAGGGAGGC
 NFAT1
 -300 A**GAAAGGAAAGCT**CTCAGTGCTGCAGTCCTTGCTGGTTTGCAGAGCTGCA
 -250 CTGCCTGCAGGGACTGCAGTCCCTCTCTCTGCCTTTGTGCTCGCATGACA
 MEF2 *Ebox2*
 -200 ATACAGGA**GTAAAAATAAC**CAGGCTG**CATTTG**CAGAGAGATAAAAGCGAA
 -150 GTTTGCCTTTTGTGTCTCTCCGGGCAGTGGGACAGCAGCACCAATGGAGC
 Ebox1
 -100 TGTGTGGACATGA**CACATG**GGGAATTTCTGACATATCTTTTCCCACTCTGA
 TATA Box
 -50 CCTTGACAGACAC**AATAAA**AAGGGGCTTTGCGGCATGCACACAGCCGCTC

Fig. 2.

Sequence of the proximal promoter of the slow MyHC2 gene. Candidate transcription factor binding sites within the proximal 600bp promoter are underlined and identified. Potential binding sites include three E-box consensus sites, two NFAT sites, and a single MEF2 binding site. The non-canonical TATA box is also indicated.

Fig. 3. Relative activities of the wild type and mutated slow MyHC2 promoters in fast and fast/slow muscle fibers. Five fast and five fast/slow myoblast clones were transfected with the indicated slow MyHC2 wild type (2279SM2Luc) or mutated constructs. pGL3Basic is a promoterless luciferase gene construct. Firefly luciferase activities were normalized for variations in transfection efficiencies by co-transfection of pRL-SV40 and measurements of Renilla luciferase activities and by normalization to variances in fusion indices among the different myoblast clones. For each DNA construct tested in each myoblast clone, $n=5$. Results are shown as mean $\pm$ S.E. Statistically significant differences are labeled with: *, $p < 0.02$, and **, $p < 0.005$, using a Student's two-tailed t test.

Fig. 4.

Chromatin immunoprecipitation of the MEF2 binding site. Chromatin from fast/slow muscle fibers was prepared. Shown are the PCR products from: Lane 1, Chromatin before immunoprecipitation (input chromatin); Lane 2, Chromatin incubated with no antibody (Ab); Lane 3, Chromatin incubated with a non-specific antibody (N.S. Ab); Lane 4, Chromatin incubated with the MEF2A antibody; Lane 5, plasmid DNA containing the slow MyHC2 promoter.

Fig. 5.

Fiber type characterization of fast primary muscle fibers overexpressing NFATc1 and MEF2A. Fast myoblasts were transfected with the NFATc1GFP (A, B) or the MEF2AFLAG (C, D) expression constructs. After allowing myoblast differentiation into muscle fibers, cells were immunostained for fast MyHC (A, C) and slow MyHC2 (B, D) with antibodies F59 and S58, respectively, followed by Texas Red conjugated secondary antibodies. MEF2A expression was detected by use of a FLAG epitope antibody followed by FITC conjugated secondary antibody (C, D). NFATc1 expression was detected by GFP fluorescence.

Fig. 6. Relative activities of the NFAT transcriptional sensor in fast and fast/slow muscle fibers. Relative luciferase activities from fast (Fast) and fast/slow (Slow) muscle fibers were measured. Some muscle fibers also expressed constitutively nuclear NFATc1-GFP (+NFAT). Results are shown as mean $\pm$ S.E. All activities were significantly different from each other ($p < 0.0001$; $n=6$) using a Student's two-tailed t test.

Fig. 7.

Effect of NFATc1-GFP expression on slow MyHC2 promoter activity. Relative activities of the slow MyHC2 promoter-luciferase constructs containing (A) 2279bp and (B) 6150bp of slow MyHC2 gene sequence in fast (Fast) and fast/slow (Slow) embryonic muscle fibers with and without the transfected NFATc1-GFP transgene (+NFAT) were measured. Results are shown as mean $\pm$ S.E. Statistically significant differences ($p < 0.0001$; $n = 6$) are labeled with an asterisk and were determined using a Student's two-tailed t test.

Fig. 8. Relative activities of the MEF2 transcriptional sensor in fast and fast/slow muscle fibers. Relative luciferase activities from fast (Fast) and fast/slow (Slow) muscle fibers were measured. Some muscle fibers also expressed MEF2A (+MEF). Results are shown as mean $\pm$ S.E. (n = 6).

Fig. 9. Effect of MEF2A expression on slow MyHC2 promoter activity. Relative activities of the slow MyHC2 promoter-luciferase constructs containing (A) 2279bp and (B) 6150bp of slow MyHC2 gene sequence in fast (Fast) and fast/slow (Slow) embryonic muscle fibers with and without the transfected MEF2A transgene (+MEF2) were measured. Results are shown as mean $\pm$ S.E. Statistically significant differences are labeled with: *, $p < 0.002$, **, $p < 0.02$, ($n = 6$) using a Student's two-tailed t test.