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Muscle fiber type specific activation of the slow myosin heavy chain 2 promoter by a non-canonical E-box

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ABSTRACT

Different mechanisms control skeletal muscle fiber type gene expression at specific times in vertebrate development. Embryonic myogenesis leading to formation of primary muscle fibers in avian species is largely directed by myoblast cell commitment to the formation of diverse fiber types. In contrast, development of different secondary fiber types during fetal myogenesis is partly determined by neural influences. In both primary and secondary chicken muscle fibers, differential expression of the slow myosin heavy chain 2 (MyHC2) gene distinguishes fast from fast/slow muscle fibers. This study focused on the transcriptional regulation of the slow MyHC2 gene in primary myotubes formed from distinct fast/slow and fast myogenic cell lineages. Promoter deletion analyses identified a discrete 86 bp promoter segment that conferred fiber type, lineage-specific gene expression in fast/slow versus fast myoblast derived primary myotubes. Sequence analysis and promoter activity assays determined that this segment contains two functional cis-regulatory elements. One element is a non-canonical E-box, and electromobility shift assays demonstrated that both cis-elements interacted with the E-protein, E47. The results indicate that primary muscle fiber type specific expression of the slow MyHC2 gene is controlled by a novel mechanism involving a transcriptional complex that includes E47 at a non-canonical E-box.

1. Introduction

Skeletal muscles are comprised of muscle fibers with different patterns of gene expression that contribute to variation in contractile dynamics and metabolic characteristics. In general, muscle fiber types are categorized as fast or slow, depending on the expression of contractile protein genes, such as myosin heavy chain (MyHC) genes, that significantly determine muscle fiber function in the adult and during development [1,2]. Species differences exist with respect to muscle fiber type nomenclature, reflecting differences in MyHC gene expression. Whereas mammalian species typically have fast type IIa, IIb, and IIx and slow type I muscle fibers, avian skeletal muscle is comprised of fast and fast/slow fibers. The distinction between fast and fast/slow fibers in avian species is based principally upon regulated expression of the slow MyHC2 gene in fast/slow versus fast muscle fibers [3].

The development of these different muscle fiber types occurs across several stages of myogenesis [4]. The embryonic stage of myogenesis is characterized by the proliferation of embryonic myoblasts and their subsequent differentiation into primary muscle fibers of both epaxial and hypaxial musculature. In chicken development, embryonic myogenesis ceases at approximately embryonic day 8 (ED8). Fetal myogenesis then ensues. This latter stage of myogenesis is characterized by expansive proliferation of fetal myoblasts and their differentiation into secondary muscle fibers that constitute the majority of skeletal muscle mass. Finally, adult myogenesis occurs through satellite cell activation, proliferation, and differentiation in response to muscle degeneration or injury.

The diversity of fast and fast/slow muscle fiber types is evident at each stage of avian myogenesis. Primary muscle fibers, formed from embryonic myoblasts, are either fast or fast/slow from the time of their initial formation, both in vitro and in vivo [5,6]. The embryonic myoblasts that form fast and fast/slow primary muscle fibers are distinct in their lineal commitment to the formation of specific fiber types. Clonal populations of embryonic myoblasts consistently and stably formed either fast or fast/slow primary muscle fibers in vitro and in vivo [7,8]. This myoblast type-specific formation of different muscle fiber types in independent of

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functional innervation [9]. The intrinsic commitment of embryonic myoblasts to the formation of distinct muscle fiber types is a dominant feature of embryonic myogenesis.

In contrast to primary myogenesis, differentiation of distinct secondary muscle fiber types from fetal myoblasts is dependent on both cell lineage commitment and innervation [10]. Denervation and cross-reinnervation of developing and adult muscles typically leads to muscle fiber type transitions [11,12]. However, in both mammals and birds, not all muscle fiber types respond to altered innervation equally with fibers retaining diversity in MyHC gene expression. Some muscles in both mammals and birds are refractory to innervation-dependent muscle fiber type transitions [13]. For example, fetal avian myotubes in vitro do not express the slow MyHC2 gene. Innervation of these myotubes induced slow MyHC2 gene expression. However, the innervation-induced fiber type transition was also dependent on the origin of the myoblasts that formed the myotubes. Myoblasts from the fetal medial adductor muscle, a fast/slow muscle, formed myotubes that expressed the slow MyHC2 gene in response to innervation or electrical stimulation. Myoblasts from the exclusively fast fetal pectoralis muscle formed myotubes that did not express the slow MyHC2 gene when innervated or electrically stimulated at low, tonic stimulation frequencies [14,15].

Reflecting the cellular differences in the regulation of muscle fiber type between embryonic and fetal stages of myogenesis and between fiber types themselves, the mechanisms that control slow MyHC2 gene transcription differ at embryonic and fetal stages of myogenesis and within different fiber types. The proximal 1358 bp of DNA upstream from exon 1 of the slow MyHC2 gene control gene expression in fetal fast and fast/slow muscle fibers [16]. Within this transcriptional promoter region, functional E-boxes, a Myocyte Enhancer Factor 2 (MEF2) binding site, and a Nuclear Factor of Activated T Cells (NFAT) binding site regulate slow MyHC2 gene transcription in fast/slow medial adductor muscle fibers. NFAT-mediated transcriptional activation was found to be dependent on innervation. In contrast, slow MyHC2 gene expression in embryonic fast/slow muscle fibers was independent of innervation and NFAT activity [17]. The proximal 1358 bp of the slow MyHC2 promoter was not sufficient to direct fiber type specific transcription in embryonic fast/slow versus fast muscle fibers.

The basic helix-loop-helix (bHLH) family of myogenic regulatory factors (MRFs) is required throughout myogenesis. In general, MyoD and Myf5 direct myogenic specification, and myogenin and MRF4 control myogenic differentiation [18–20]. Each of the MRFs bind to E boxes (CANNTG) in transcriptional control regions as heterodimers with Class I bHLH E proteins, including the E2A gene products, E12 and E47, HEB, daughterless, and E2-2 [21–23]. Studies in vitro and in vivo have indicated that the myogenic cell specific MRFs avidly heterodimerize with the more ubiquitously expressed E proteins and that MRF homodimerization was weak in the presence of the E box consensus binding site [22,24]. Binding site specificities of MRF:E protein heterodimers are also partly determined by nucleotide sequence within the E box consensus sequence. For example, MyoD and myogenin preferentially recognize the E box sequence CAGCTG [25]. Sequences flanking the consensus CANNTG binding site also contribute to MRF:E protein binding specificities [24]. Furthermore, interaction of specific MRFs with non-canonical E box sequences in the myogenin promoter has indicated greater diversity in MRF:E protein binding potential in the regulation of myogenesis [26].

In the present study, we investigated the transcriptional regulation of the slow MyHC2 gene in embryonic fast/slow versus fast myotubes formed from clonally derived embryonic myoblasts. Analysis of the slow MyHC2 promoter revealed that a specific region of 86 bp in length controlled promoter activity in fast/slow versus fast myotubes. Furthermore, results indicate that a non-canonical E box sequence within this promoter segment regulates promoter activity and interacts with the E protein E47.

2. Materials and methods

Cell Culture, DNA Transfection, and Promoter Activity Assays. Embryonic myoblasts were derived from ED4 limbs and cultured as previously described [8,17]. After 24 h, myoblasts were transfected with promoter-reporter DNA constructs using Lipofectamine 2000 (Invitrogen). The 6150SM2Luc DNA construct was previously described [17]. Transfections consisted of 3ug slow MyHC2 promoter-luciferase or promoterless-luciferase (pGL3Basic; Promega) DNA constructs plus 2ug pRLSV40 DNA (Promega). Cells were transfected for 5 h in medium without antibiotics at 37 °C. Luciferase activities were measured 5 days after transfection. Firefly luciferase activities from slow MyHC2 promoter activities were normalized relative to constitutive expression of the Renilla luciferase gene from pRLSV40. Student's t-test was applied to determine statistical differences among mean luciferase activities. P values ≤ 0.05 were considered statistically significant.

2.1. Mutagenesis

Site-directed mutations were introduced into slow MyHC2 promoter-reporter DNA constructs by PCR amplification of template DNA using complementary mutagenic primers. Each reaction consisted of 200 ng template DNA, 5ul 10X Pfu polymerase buffer, 100 ng of each PCR primer, 2.5 mM of each dNTP, 1ul PfuUltra High Fidelity DNA polymerase (Stratagene) in a total volume of 50ul. After amplification, 1ul DpnI was added and incubated with the reaction for 1 h at 37 °C before bacterial transformation. DNA sequencing verified the generation of each mutation.

2.2. Electromobility shift assays

Nuclear cell extracts from differentiated cultures of fast and fast/slow myotubes were prepared as previously described [27]. Protein concentrations were determined using the Bio-Rad Protein Assay Kit. The electromobility shift assay (EMSA) protocol was performed as previously described [28]. For EMSA supershift assays, 4ug E47 antibody (Santa Cruz) was added to the reaction prior to addition of the labeled probe and incubated on ice for 30 min. A 5% non-denaturing polyacrylamide gel, run at 200 V for 90 min, resolved the protein-DNA complexes. The gel was dried and exposed to X-ray film at −80 °C overnight before development.

3. Results

3.1. Localization of positive transcriptional control region

Previous studies determined that the 1358 bp of slow MyHC2 DNA sequence positioned upstream from exon 1 was sufficient to direct slow MyHC2 promoter activity in innervated and stimulated secondary myotubes derived from fetal myoblasts [15,16]. However, it was insufficient to direct transcription in a fiber type specific manner in primary myotubes formed from embryonic myoblasts [17]. To determine the location of slow MyHC2 promoter regions that regulate gene expression in fast/slow versus fast primary myotubes, clonal populations of myoblasts, stably committed to the formation of fast/slow and fast myotubes [8,17], were transfected with a series of DNA constructs containing increasing lengths of slow MyHC2 promoter sequence coupled to the luciferase reporter gene. Transfected myoblasts were allowed to differentiate for 5 days, and slow MyHC2 promoter activities were measured and

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