Published in final edited form as:

Biochim Biophys Acta. 2016 November ; 1860(11 Pt A): 2355–2362. doi:10.1016/j.bbagen.2016.07.003.

C/EBP α Represses Slow Myosin Heavy Chain 2 Gene Expression in Developing Avian Myotubes

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Abstract

Background—The CCAAT/enhancer binding proteins (C/EBP) comprise a family of transcription factors that regulate many cellular processes. Little is known of their function during embryonic and fetal myogenesis. Slow myosin heavy chain 2 (MyHC2) is a marker of the slow avian skeletal muscle fiber type, and slow MyHC2 gene regulation involves molecular pathways that lead to muscle fiber type diversification.

Methods—The biological effects of C/EBP α and C/EBP β expression were analyzed by use of a general C/EBP activity reporter and by slow MyHC2 promoter-reporter constructs transfected into specific myogenic cell lineages. The effects of C/EBP α and C/EBP β expression were also analyzed by immunocytochemical detection of slow MyHC2. C/EBP α interaction with the slow MyHC2 promoter was assessed by electromobility shift assays.

Results—C/EBP α and C/EBP β are present in embryonic fast and fast/slow avian myogenic lineages. Overexpression of C/EBP α cDNA repressed slow MyHC2 promoter activity in embryonic myotubes and in both electrically stimulated fetal myotubes. Deletion analysis of the slow MyHC2 promoter-luciferase reporter demonstrated that the transcriptional repression mediated by C/EBP α occurs within the first 222bp upstream from exon 1 of the slow MyHC2 gene. Electromobility shift assays determined that C/EBP α can bind to a non-canonical C/EBP site within the slow MyHC2 gene, and mutation of this site reduced transcriptional repression of the slow MyHC2 gene.

Conclusion—C/EBP α , but not C/EBP β , represses slow MyHC2 promoter activity via a non-canonical C/EBP binding element.

General Significance—Members of the C/EBP family of transcription factors differentially regulate genes indicative of distinct muscle fiber types.

Keywords

transcription; muscle; promoter; fiber type; avian; lineage

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Introduction

Skeletal muscle is a diverse tissue type with significant variation in contractile and metabolic properties among different muscles. It is also a dynamic tissue that alters its contractile and metabolic properties in response to changes in environmental stimuli such as exercise or disuse. The complexity of muscle properties and its capacity for adaptation are reflected in the diversity and adaptive capabilities of the individual muscle fibers that comprise the tissue. This complexity and diversity allows for many different methods of classifying skeletal muscle fiber types based on metabolism (i.e. glycolytic and oxidative), histochemical staining, or contractile speed (i.e. fast twitch and slow twitch). The classification of fiber type by contractile speed is determined by the composition of myosin heavy chain (MyHC) isoforms present within the fiber. In general, mammalian fiber types are slow type 1 fibers or fast type 2 fibers. However, muscle fiber type in mammalian systems is complex with individual muscle fibers often containing mixtures of type 1 and 2A, or type 2A and 2X, or type 2X and 2B MyHC isoforms [1]. In contrast, all chicken skeletal muscle fibers express a fast MyHC gene exclusively or express both a fast MyHC gene and a slow MyHC gene. Expression of the slow MyHC2 gene in chicken muscle fibers reflects the fully differentiated state of avian slow muscle fibers, and therefore its expression is a useful marker for fast versus slow muscle fiber type [2].

Vertebrate myogenesis occurs in two phases, yielding primary and secondary muscle fibers from separate, but closely related, myogenic precursors during embryonic and fetal development, respectively [3]. Embryonic myoblasts and fetal myoblasts have differing morphologies, nutritional requirements, and transcriptional signatures [4, 5]. In avian skeletal muscle the embryonic phase begins at embryonic day (ED) 3 and lasts until ED8. This phase produces primary muscle fibers which set up the initial architecture of the developing musculature. Fetal myogenesis begins at ED8 and persists until hatching. Secondary muscle fibers are formed at this time and constitute the majority of muscle mass postnatally [6].

The mechanisms that regulate the development and maintenance of fiber type differ between these two phases of myogenesis. Embryonic myoblasts differentiate into fast and fast/slow muscle fiber types independent of any extrinsic signal such as innervation [7]. The commitment of embryonic myoblasts to differentiate into distinct fast or fast/slow muscle fiber types, based on slow MyHC2 gene expression, is stable after multiple serial passaging of the myoblasts and after transplantation into heterologous muscle locations [8,9]. However, fetal myoblasts differentiate into muscle fibers that will only express the slow MyHC2 gene upon chronic electrostimulation or innervation [10,11]. Therefore, the molecular mechanisms that control slow MyHC2 gene expression and muscle fiber type are both fiber type and temporally specific.

Transcriptional regulation of the slow MyHC2 promoter is fiber type specific and temporally regulated. Fetal fiber type specific activation of the slow MyHC2 promoter is regulated by a 1350bp DNA sequence that contains three candidate E-box binding sites, two potential nuclear factor of activated T-cells (NFAT) binding sites and one potential myocyte enhancer factor-2 (MEF2) binding site. This is in contrast to embryonic muscle fibers in which an

additional 3871bp of DNA upstream of the proximal fetal-specific promoter is required for fiber type specific expression of the slow MyHC2 gene. Moreover, regulation of the proximal promoter region itself differs between embryonic and fetal muscle fibers. The MEF2 site, proximal E-box, and both NFAT binding sites transcriptionally activate the slow MyHC2 promoter in fast/slow fetal muscle fibers upon innervation [12]. In embryonic fast/slow muscle fibers, the MEF2 site is the only site within the proximal promoter that activates slow MyHC2 gene transcription [9].

The C/EBP family of transcription factors consists of six members -C/EBP α , C/EBP β , C/EBP γ , C/EBP δ , C/EBP ϵ and C/EBP ζ . These factors interact with the DNA consensus sequence TKNNGNAAK via a basic leucine zipper motif located near the carboxy terminus [13,14]. Transactivation and regulatory domains exist near the amino terminus with the exception of C/EBP γ and C/EBP ζ which contain only the leucine zipper region. C/EBP proteins are involved in many different cellular functions such as cell cycle regulation, cellular metabolism, and cell fate determination [14]. C/EBP $\alpha^{-/-}$, C/EBP $\beta^{-/-}$, and C/EBP $\gamma^{-/-}$ mice have no gross skeletal muscle defects, but C/EBP $\alpha^{-/-}$ mice die shortly after birth due to impaired energy homeostasis [15]. In L6 myotubes C/EBP β and C/EBP γ are upregulated by the glucocorticoid dexamethasone [16]. Exogenous expression of C/EBP β increases Pax7 gene expression, and the expression of C/EBP β in muscle satellite cells is reduced upon activation and differentiation into muscle fibers [17].

A direct connection between C/EBP isoforms and regulation of skeletal muscle fiber type has not been investigated, but there is circumstantial evidence that the C/EBP family may be involved in regulating fiber type within skeletal muscle. C/EBP γ gene expression is reduced in mouse soleus versus quadriceps muscle, suggesting that it regulates metabolic differences in muscle fiber type [18]. Denervation of the gastrocnemius muscle and zero-gravity unloading of the extensor digitorum longus (EDL) muscle which elicit fiber type transitions also increased C/EBP α gene expression [19,20]. Additionally, botulinum toxin type A (BTX) injection increased C/EBP α gene expression and downregulated slow MyHC gene expression in the supraspinatus muscle [21]. In this current study we investigate whether members of the C/EBP family regulate slow MyHC2 gene expression during embryonic and fetal avian muscle development.

Materials and Methods

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RT-PCR reactions were performed using the Access RT-PCR kit (Promega) according to manufacturer's instructions. Briefly, 1 μ g of total RNA was added to the RT-PCR reaction containing 10 μ l 5 $\times$ master mix, 1mM MgCl₂, 400 μ M dNTPs, 1 μ l AMV reverse transcriptase, 1 μ l Tfl polymerase, 125ng of each oligonucleotide primer, and nuclease free H₂O to a total of 50 μ l. Thermocycling conditions for the reaction were as follows; initial reverse transcription at 45°C for 45 minutes, and 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 58°C for 50 seconds, 68°C for 50 seconds. The oligonucleotides used for RT-PCR were C/EBP α F 5'-GTGCTTCATGGAGCAAGCCAA-3', C/EBP α R 5'-TGTCGATGGAGTGCTCGTTCT-3', C/EBP β F 5'-AACATCGCTGTGCGCAAGAGC-3', C/EBP β R 5'-ATGAAACCCCCAACGAAACCG-3', and RLP0F 5'-

GTGGGCTTCGTGTTACCAAGG-3', RLP0R 5'-ATGATGGAGTGTGGCACCGAGG-3' [22].

Cloning and Mutagenesis

C/EBP α cDNA was cloned into the pCMVFLAG vector (Stratagene) using reverse transcription polymerase chain reaction, RT-PCR, of RNA derived from clonal embryonic myoblasts [9]. The oligonucleotide primers used for cloning were C/EBP α F 5'-ATCGGTGAATTCATGGAGCAAGCCAACTTCTAC-3', C/EBP α R 5'-TTAATCCTCGAGCCCTCGCCTTTCTCCTTACA-3' [23] C/EBP β F 5'-ATCGGTGAATTCCTTCATGCAACGCCTGGTG-3', C/EBP β R 5'-TTATATCTCGAGGCAGCGGGGCGAGGAA-3'. RT-PCR conditions are described above. Cloning was confirmed by DNA sequencing.

Mutations were created using site-specific oligonucleotide primers with the Phusion high fidelity PCR kit (ThermoFisher). PCR reactions included 100ng of wild type slow MyHC2 promoter DNA, 2.5mM dNTPs, 125ng of each oligonucleotide primer, 10 μ l 5 $\times$ High Fidelity Phusion buffer, 1 μ l Phusion polymerase, and nuclease free H₂O to a total of 50 μ l. After thermal cycling, 1 μ l of DpnI restriction enzyme (Promega) was added to the reaction buffer and incubated at 37°C for 1 hour. PCR product was transformed into *E. coli* and allowed to grow on LB agar plates with ampicillin overnight. Single colonies were picked, and plasmids were purified using the Wizard SV miniprep kit (Promega). The generation of mutations was confirmed by DNA sequencing. The mutation primers used were: Cm3F 5'-AGCACCAATGGAGCTGTGTG**AGTGCAGTGT**ATGGGAATTTTGACATATC-3' Cm3R 5'-GATATGTCAAAAATTCCCAT**ACACTGCACT**CACACAGCTCCATTGGTGCT-3' Cm4F 5'-AGCACCAATGGAGCTGTGTGGACATGACACCG**TTTCCGGG**TTGACATATC-3' Cm4R 5'-GATATGTCAAC**CCGGAAAC**GGTGTTCATGTCCACACAGCTCCATTGGTGCT-3' Site-directed mutations are indicated in bold.

Cell Culture and Transfection

Cells were cultured as previously described [9]. Clonally derived embryonic myoblasts were plated on collagen coated plates. Cell culture medium contained a 1:1 formulation of fresh medium (FM) and conditioned medium (CM), comprised of 10% horse serum, 5% chick embryo extract, 2mM glutamine, 1.32mM CaCl₂, 1 $\times$ antibiotic/mycotic in F-10 medium. Fetal myoblasts were cultured in FM only. Medium was replaced every other day. Myogenic cells in 35mm dishes were transfected with 2 μ g of slow MyHC2 promoter-luciferase reporter constructs using Lipofectamine 2000 (Invitrogen). In some experiments embryonic myoblasts were also transfected with 2 μ g of pCMVC/EBP α . For analysis of native C/EBP transcription factor activity, embryonic myoblasts were transfected with 2 μ g of C/EBP cis-Reporting system (Agilent Technologies). Slow MyHC2 promoter constructs and the C/EBP cis-reporter DNA were co-transfected with 0.3 μ g of the SV40 Renilla luciferase expression construct (Promega) to control for variations in transfection efficiencies. Transfection of the empty CMV vector served as a transfection control. Cells were allowed to differentiate for 3 days after transfection, and luciferase activities were determined using a Dual-Glo

Luciferase assay according to the manufacturer's instructions (Promega). Fetal myotubes were electrostimulated as previously described [10], and then luciferase activities were determined.

Electromobility Shift Assay

Double-stranded oligonucleotide probes were end labeled using T4 kinase (Promega) and ^{32}P -ATP. Samples were purified using Sephadex G-50 spin columns (Roche). A 20 μl reaction containing 40mM KCl, 15mM HEPES pH 7.9, 1mM EDTA, 0.5mM DTT, 5mM MgCl_2 , 5% glycerol, 6 μg bovine serum albumin (BSA), 2 μg poly dI-dC and 10 μg of cell extract was incubated at room temperature for 20 minutes. Some reactions included 1 μg of anti-FLAG or anti- β -galactosidase antibody. Other binding reactions contained 50 fold molar excess of competitor oligonucleotide probe. Radiolabeled oligonucleotide probe (100,000cpm) was added to the reaction and incubated at room temperature for an additional 20 minutes. Protein-DNA complexes were resolved by electrophoresis in a 5% non-denaturing polyacrylamide gel in 0.5 $\times$ Tris-Borate EDTA buffer at 160 volts for 90 minutes. Gels were dried and exposed to X-ray film.

Immunocytochemistry

Embryonic myogenic cultures were washed three times with phosphate buffered saline (PBS). Cells were then fixed with methanol for 5 minutes and washed 3 more times with PBS. Blocking buffer consisting of 5% horse serum and 2% BSA in PBS was added and incubated for one hour at room temperature. Cells were then incubated with a FLAG antibody (Sigma), α -actin antibody (Sigma), and the slow MyHC2 monoclonal antibody, S58 [4], diluted 1:4000, 1:750, and 1:10, respectively, in blocking buffer for 1 hour at room temperature. Cells were then washed 3 times with PBS. Detection of the primary antibodies occurred using anti-mouse IgG FITC, biotinylated anti-mouse IgM, and anti-mouse IgA TRITC secondary antibodies (Vector Labs) diluted 1:200 in blocking buffer. Cells were washed three times with PBS. Pacific Blue-Tyramide, diluted to 0.5 μl /1ml PBS with 0.01% H_2O_2 , was incubated with the cells for 30 minutes at room temperature to recognize the biotinylated IgM. Cells were washed as before, and coverslips were applied. Immunofluorescence was quantitated using Nikon NIS Elements software. Background fluorescence was established in a region devoid of cells. Subsequent regions of interest for fluorescence measurements were made encompassing myotubes that were α -actin positive. Myotubes were scored as positive if the region of interest had slow MyHC2 fluorescence that was 25% higher than background fluorescence. The myotubes were scored negative if the slow MyHC2 fluorescence was lower than 25% above background fluorescence.

Statistics

All individual experiments were done independently. The mean was calculated using the results from each independent experiment (N). These were also used to calculate the standard error of the mean (SEM). Student's T-test was used to determine statistical significance. Any p-values ≤ 0.05 were considered to be statistically significant.

Results

C/EBP α and C/EBP β in Fast and Fast/Slow Embryonic Myogenic Cells

Microarray analysis of fast and fast/slow embryonic myogenic clones previously indicated that C/EBP family members may be involved in the regulation of differentiation of fast and fast/slow embryonic myoblasts into distinct muscle fiber types [24]. RT-PCR analysis was performed to determine whether genes encoding C/EBP α and C/EBP β are expressed in both fast and fast/slow embryonic myoblasts and myotubes (Figure 1A). Both C/EBP α and C/EBP β transcripts were detected in each cell type. A CEBP-mediated transcription reporter plasmid containing multiple C/EBP binding sites that regulate transcription of the luciferase reporter gene was used to assess C/EBP transcription factor activity in fast and fast/slow embryonic myotubes (Figure 1B). No significant difference in C/EBP transcription factor activity was detected using the non-specific transcriptional reporter.

C/EBP α Represses Slow MyHC2 Promoter Activity

Although the non-fiber type specific C/EBP transcriptional sensor did not demonstrate differential activity in fast and fast/slow embryonic myotubes, the slow MyHC2 promoter which is differentially active in fast/slow myotubes was used to determine the effects of C/EBP transcription factor activity. A slow MyHC2 promoter-luciferase reporter construct containing 6150bp of slow MyHC2 DNA sequence [9] was co-transfected along with C/EBP α or C/EBP β expression plasmids into fast/slow myoblasts. The myoblasts were allowed to differentiate into myotubes, and luciferase activities were measured (Figure 2). C/EBP β did not significantly alter slow MyHC2 promoter activity in fast/slow myotubes compared to activity in myotubes co-transfected with the empty control CMV expression plasmid. However, slow MyHC2 promoter activity was significantly reduced in fast/slow myotubes by expression of C/EBP α . Slow MyHC2 promoter activity was repressed 3.5 fold compared to activity in control transfections. These results indicate that slow MyHC2 promoter activity in embryonic myotubes can be regulated by a C/EBP transcription factor(s). Furthermore, its activity can be repressed by C/EBP α , and not by C/EBP β .

The effects of C/EBP α and C/EBP β on slow MyHC2 gene expression were also assessed in single cells. Fast/slow myoblasts were transfected with DNA constructs that directed expression of C/EBP α and C/EBP β tagged with the FLAG epitope (C/EBP α FLAG and C/EBP β FLAG). After differentiation, myotubes were immunostained with antibodies directed against the FLAG epitope, slow MyHC2, and α -actin (Figure 3A). Immunodetection of α -actin identified all differentiated myotubes. Myotubes containing nuclei immunostained with the FLAG antibody were scored for slow MyHC2 gene expression. Those myotubes with mean fluorescence 25% higher than background were scored as slow MyHC2-positive (Figure 3B). Whereas 54% of myotubes transfected with C/EBP β FLAG were slow MyHC2-positive, only 17% of myotubes transfected with C/EBP α FLAG were slow MyHC2-positive. Ensuring that C/EBP α was in fact repressing slow MyHC2 gene expression, we chose to quantitate the mean fluorescence above background of the slow MyHC2 signal. The mean slow MyHC2 fluorescence above background of the C/EBP β -positive myotubes was 47% compared to 14% above background for the C/EBP α -positive myotubes (Figure 3C). Therefore, C/EBP α gene expression reduced both the frequency of myotubes expressing the

slow MyHC2 gene and the level of slow MyHC2 gene expression in myotubes that expressed both C/EBP α and slow MyHC2 genes.

C/EBP α -mediated Repression Is Not Developmental Stage Specific

The mode of regulation of slow MyHC2 gene expression is developmental stage specific. Whereas slow MyHC2 gene expression in embryonic primary myotubes is intrinsically controlled by distal promoter elements, expression in fetal secondary myotubes is controlled by proximal promoter elements including E-box and NFAT regulatory sites [9,12]. In addition, innervation or chronic electrostimulation is required for slow MyHC2 gene expression in fetal myotubes [10,11]. To determine whether C/EBP α -mediated repression of slow MyHC2 gene expression is restricted to embryonic myotubes, C/EBP α -mediated regulation of slow MyHC2 promoter activity was assessed in fetal myotubes. Myoblasts were harvested from fetal ED13 chicken medial adductor muscle. C/EBP α and C/EBP β expression constructs were transfected into the fetal myoblasts along with the 6150bp slow MyHC2 promoter-reporter construct. Myotubes were electrically stimulated for 4 days to activate slow MyHC2 gene expression [10] after which promoter activities were determined (Figure 4). C/EBP α expression repressed slow MyHC2 promoter activity 3.26 fold in electrically stimulated fetal myotubes. C/EBP β expression did not repress slow MyHC2 promoter activity in these myotubes. Therefore, C/EBP α functions as a transcriptional repressor of slow MyHC2 promoter activity in both embryonic and fetal myotubes.

C/EBP α Interacts with the Slow MyHC2 Promoter

Previous studies have identified a number of functional and candidate transcriptional regulatory sites including multiple E-boxes, two NFAT binding sites, and a MEF2 binding site within the slow MyHC2 promoter. To determine the location within the slow MyHC2 promoter that mediates C/EBP α transcriptional repressor activity, a series of deletions of the full length 6150bp slow MyHC2 promoter-reporter DNA construct was made. The deletions resulted in promoter constructs that contained 488bp and 222bp upstream from exon 1 (Figure 5A). The full length 6150bp promoter and deletion constructs were co-transfected with the C/EBP α expression construct into fast/slow embryonic myoblasts. Promoter activities were measured in the resulting fast/slow myotubes (Figure 5B). C/EBP α expression significantly repressed activity of the 6150bp, 488bp, and 222bp slow MyHC2 promoters by 10.53 fold, 3.8 fold, and 1.9 fold, respectively. Although deletion constructs containing 488bp and 222bp of promoter sequence demonstrated less CEBP/ α -mediated repression relative to the full length 6150bp promoter, these truncated promoters were nonetheless still significantly repressed by CEPB/ α . Additional deletions of the slow MyHC2 promoter reduced promoter activity to near basal levels (data not shown).

To more narrowly define the location of the slow MyHC2 proximal promoter site that functioned in C/EBP α mediated transcriptional repression, overlapping oligonucleotide probes were designed to span the proximal 222bp of the slow MyHC2 promoter. These probes were used in electromobility shift assays with extracts from cells expressing C/EBP α FLAG (Figure 6). An antibody to the FLAG epitope was included in some of the reactions to identify the protein-DNA complex consisting of C/EBP. Probes A, B, and D did not show robust binding to form protein-DNA complexes. However, probe C that contains

slow MyHC2 promoter sequence between 66–116bp upstream from exon 1 formed a protein-DNA complex compared to other oligonucleotide probes. Furthermore, inclusion of the FLAG antibody resulted in a supershift of this protein-DNA complex containing probe C. This segment of the slow MyHC2 promoter includes one of the three previously identified E-boxes [9], but does not contain the canonical C/EBP binding site TKNNGNAAK.

Additional oligonucleotides were then made with 10bp mutations that spanned the first 40 basepairs of oligonucleotide probe C (Figure 7A). Each of these mutant probes was incubated in protein extract from cells expressing C/EBP α FLAG. These probes were also incubated with the FLAG antibody (Figure 7B–F). A protein-DNA complex formed with the wild type probe and with mutated oligonucleotide probes 1, 2, and 4. Initial overnight exposure of EMSAs of probes 2 and 4 showed no supershifted protein-DNA (data not shown), but a prolonged exposure did reveal that these complexes were supershifted by addition of the FLAG antibody. Mutated oligonucleotide probe 3 did not form a supershifted complex with the FLAG antibody. Wild type and mutated oligonucleotides of probe 3 were also used in competition assays in formation of protein-DNA complexes (Figure 7G). Mutated oligonucleotide probe 3 competed less effectively in formation of a protein-DNA complex compared to the wild type and other mutated oligonucleotide competitors. These results indicate that the mutated 10bp within the mutated oligonucleotide probe 3 are necessary for C/EBP α binding under these conditions.

To assess the functional significance of the CEBP binding site within probe 3, the same mutation was introduced into the full length slow MyHC2 promoter-reporter DNA construct which was then transfected along with the C/EBP α expression construct into fast/slow myoblasts as before. Luciferase assays were performed on differentiated myotubes (Figure 8). Exogenous C/EBP α repressed slow MyHC2 promoter activity under all conditions. The wild type slow MyHC2 promoter activity was repressed 7.84 fold by CEBP α , and activity of the slow MyHC2 promoter containing the mutation of the C/EBP α binding site (Cm3) was repressed 6.3 fold. This reflects a significant 20% reduction ($p = 0.01$) in CEBP α -mediated transcriptional repression due to introduction of the CEBP site mutation. The adjacent 10bp mutation contained within Cm4 (see Figure 7A) and introduced into the slow MyHC2 promoter did not significantly alter CEBP α -mediated repression of slow MyHC2 promoter activity.

Discussion

We have shown that C/EBP α and C/EBP β genes are expressed within fast and fast/slow embryonic myoblasts and myotubes. General C/EBP transcription factor activity was not significantly different between fast and fast/slow myoblasts and myotubes. However, C/EBP α and C/EBP β did demonstrate differential transcription factor activity in regulation of the slow MyHC2 promoter. C/EBP α effectively repressed the slow MyHC2 promoter in both embryonic and fetal myotubes. In contrast, C/EBP β had no detectable effect on slow MyHC2 promoter activity. This result was initially unexpected since both C/EBP α and C/EBP β bind the same consensus DNA sequence. Additionally, these results suggest that C/EBP α and C/EBP β may regulate genes such as the slow MyHC2 gene via different

transcriptional complexes, the specific composition of which may then determine C/EBP-mediated transcriptional regulation. Nevertheless, C/EBP α expression has now been demonstrated for the first time to have a direct link to the repression of a specific marker of skeletal muscle fiber type.

C/EBP α effectively repressed activity of the full length 6150bp slow MyHC2 promoter. However, it is possible that promoter activity was not completely abrogated by 3.5 fold promoter repression. Complete slow MyHC2 promoter repression may be regulated by other transcription factors in addition to C/EBP α . We have previously shown that the slow MyHC2 promoter is controlled by E-box, NFAT, and MEF2 binding sites [9]. The MEF2 binding site mediated transcriptional activation. In contrast, mutation of proximal E-box and NFAT binding sites resulted in 80% and 90% promoter activation, indicating that these sites mediate transcriptional repression and that complex transcriptional control exists via both multiple activators and repressors. Therefore, it is likely that the C/EBP α binding site proportionally controls slow MyHC2 promoter activity rather than complete abrogation.

Additional deletions that contained 488bp and 222bp upstream of the 5' end of exon 1 were also repressed by C/EBP α , but the fold repression was reduced in these constructs. The deletion of obligate activators, two E-boxes, and a MEF2 site, located within the deleted region may explain why the fold repression was reduced. In these constructs, promoter activation is reduced thereby providing less promoter activity for C/EBP-mediated transcriptional repression. Mutation of the identified C/EBP α binding site, which abrogated C/EBP α binding, resulted in a significant 20% reduction in C/EBP α -mediated repression of the slow MyHC2 promoter. It should be noted that the 6150bp slow MyHC2 promoter sequence contains 9 C/EBP consensus binding sites. Interaction of C/EBP α with one or more of these sites would likely contribute to transcriptional repression of the full length slow MyHC2 promoter in an additive or multiplicative manner. Therefore, the difference in transcriptional repression between the wild type and mutated full length slow MyHC2 promoter is likely due to the existence and function of additional C/EBP α binding sites.

We have shown that C/EBP α can bind to the proximal slow MyHC2 promoter and repress its activity. However, the DNA sequence to which C/EBP α binds does not contain a consensus C/EBP binding site. Analysis of the 50bp DNA segment that binds C/EBP α by Transcription Factor Affinity Prediction (TRAP) indicates that this segment contains a possible AP-1 binding site at the location where the electromobility shift assays demonstrated interaction with C/EBP α . The apparent bind of C/EBP α to a candidate AP-1 binding site can be explained if C/EBP α binds to the slow MyHC2 promoter as a heterodimer with AP-1. Previous studies have shown that C/EBP α and AP-1 can interact as heterodimers that bind to the sequence TGACGCAA rather than the consensus TKNNGNAAK sequence for C/EBP α or the AP-1 homodimer binding sequences TGACGTCA and TGACTIONA [25]. This hybrid site is contained within over 300 genes in the human and murine genomes and adds an additional potential mode of regulation for both C/EBP α and AP-1 [26,27].

Another explanation for the C/EBP α -mediated transcriptional control is that the mechanism of action for C/EBP α does not always require that C/EBP α be directly bound to the

promoter. C/EBP α can stabilize p21 to produce an anti-proliferative effect on preadipocytes [28]. Mutation of sites within the basic region of C/EBP α that are highly conserved but do not directly contact the promoter can inhibit E2F-mediated transcriptional repression and prevent adipogenesis in NIH3T3 fibroblasts [29]. Although there is not a canonical E2F binding site within the slow MyHC2 promoter segment used in this study, it would not be unusual to find that C/EBP α utilizes a similar mechanism of transcriptional repression within a protein complex that may include transcription factors other than members of the E2F family.

Binding of C/EBP α to the slow MyHC2 promoter and subsequent reduction in the slow MyHC2 gene activity suggest that one of the functions of C/EBP α is to repress a fast/slow muscle fiber phenotype. However, other hypotheses may be put forth as well. For example, the repression of slow MyHC2 gene expression by C/EBP α may reflect the capacity of C/EBP α to function as a potent regulator of adipogenesis. Exogenous expression of C/EBP α alone is not sufficient to convert cell identity from myoblasts into adipocytes. However, complete cell lineage conversion of myoblasts into adipocytes is possible by expression of C/EBP α in combination with peroxisome proliferator-activated receptor (PPAR) γ [30]. Furthermore, exogenous C/EBP α gene expression is sufficient to convert fibroblasts into adipocytes. Therefore, C/EBP α gene expression may modulate expression of genes that characterize the myogenic lineage and promote expression of genes characteristic of adipocytes.

Acknowledgments

Research reported in this publication was supported by the National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under award number R01AR058043. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

References

1. DeNardi C, Ausoni S, Moretti P, Gorza L, Velleca M, Buckingham M, Schiaffino S. Type 2X-myosin heavy chain is coded by a muscle fiber type-specific and developmentally regulated gene. *J. Cell Biol.* 1993; 123:823–835. [PubMed: 8227143]
2. Kennedy JM, Kamel S, Tambone WW, Vrbova G, Zak R. The expression of myosin heavy chain isoforms in normal and hypertrophied chicken slow muscle. *J. Cell Biol.* 1986; 103:977–983. [PubMed: 3528174]
3. Hutcheson DA, Zhao J, Merrell A, Haldar M, Kardon G. Embryonic and fetal limb myogenic cells are derived from developmentally distinct progenitors and have different requirements for beta-catenin. *Genes Dev.* 2009; 23:997–1013. [PubMed: 19346403]
4. Crow MT, Stockdale FE. Myosin expression and specialization among the earliest muscle fibers of the developing avian limb. *Dev. Biol.* 1986; 113:238–254. [PubMed: 3943663]
5. Biressi S, Tagliafico E, Lamorte G, Monteverde S, Tenedini E, Roncaglia E, Ferrari S, Ferrari S, Cusella-De Angelis MG, Tajbakhsh S, Cossu G. Intrinsic phenotypic diversity of embryonic and fetal myoblasts is revealed by genome-wide gene expression analysis on purified cells. *Dev Biol.* 2007; 304:633–651. [PubMed: 17292343]
6. Stockdale FE, Miller JB. The cellular basis of myosin heavy chain isoform expression during development of avian skeletal muscles. *Dev. Biol.* 1987; 123:1–9. [PubMed: 3305110]
7. Miller JB, Stockdale FE. Developmental regulation of the multiple myogenic cell lineages of the avian embryo. *J. Cell Biol.* 1986; 103:2197–2208. [PubMed: 3782296]

8. DiMario JX, Fernyak SE, Stockdale FE. Myoblasts transferred to the limbs of embryos are committed to specific fibre fates. *Nature*. 1993; 362:165–167. [PubMed: 8383807]
9. Theobald J, DiMario JX. Lineage-based primary muscle fiber type diversification independent of MEF2 and NFAT in chick embryos. *J. Muscle Res. Cell Motil.* 2011; 31:369–381. [PubMed: 21290171]
10. Crew JR, Falzari K, DiMario JX. Muscle fiber type specific induction of slow myosin heavy chain 2 gene expression by electrical stimulation. *Exp. Cell Res.* 2010; 316:1039–1049. [PubMed: 20070941]
11. DiMario JX, Stockdale FE. Both myoblast lineage and innervation determine fiber type and are required for expression of the slow myosin heavy chain 2 gene. *Dev. Biol.* 1997; 188:167–180. [PubMed: 9245520]
12. Jiang H, Jordan T, Li J, Li H, DiMario JX. Innervation-dependent and fiber type-specific transcriptional regulation of the slow myosin heavy chain 2 promoter in avian skeletal muscle fibers. *Dev Dyn.* 2004; 231:292–302. [PubMed: 15366006]
13. Akira S, Isshiki H, Sugita T, Tanabe O, Kinoshita S, Nishio Y Y, Nakajima T, Hirano T, Kishimoto T. A nuclear factor for IL-6 expression (NF-IL6) is a member of a C/EBP family. *EMBO J.* 1990; 9:1897–1906. [PubMed: 2112087]
14. Ramji DP, Foka P. CCAAT/enhancer-binding proteins: structure, function and regulation. *Biochem. J.* 2002; 365:561–575. [PubMed: 12006103]
15. Wang ND, Finegold MJ, Bradley A, Ou CN, Abdelsayed SV, Wilde MD, Taylor LR, Wilson DR. Impaired energy homeostasis in C/EBP alpha knockout mice. *Science*. 1995; 269:1108–1112. [PubMed: 7652557]
16. Yang H, Mammen J, Wei W, Menconi M, Evenson A, Fareed M, Petkova V, Hasselgren PO. Expression and activity of C/EBPbeta and delta are upregulated by dexamethasone in skeletal muscle. *J. Cell Physiol.* 2005; 204:219–226. [PubMed: 15669083]
17. Marchildon F, Lala N, Li G, St-Louis C, Lamothe D, Keller C, Wiper-Bergeron N. CCAAT/enhancer binding protein beta is expressed in satellite cells and controls myogenesis. *Stem Cells*. 2012; 30:2619–2630. [PubMed: 23034923]
18. Campbell WG, Gordon SE, Carlson CJ, Pattison JS, Hamilton MT, Booth FW. Differential global gene expression in red and white skeletal muscle. *Am. J. Physiol. Cell Physiol.* 2001; 280:C763–C768. [PubMed: 11245591]
19. Wagatsuma A. Upregulation of gene encoding adipogenic transcriptional factors C/EBPalpha and PPARgamma2 in denervated muscle. *Exp. Physiol.* 2006; 91:747–753. [PubMed: 16675498]
20. Allen DL, Bandstra ER, Harrison BC, Thorng S, Stodieck LS, Kostenuik PJ, Morony S, Lacey DL, Hammond TG, Leinwand LL, Argraves WS, Bateman TA, Barth JL. Effects of spaceflight on murine skeletal muscle gene expression. *J. Appl. Physiol.* 2009; 106:582–595. [PubMed: 19074574]
21. Das R, Rich J, Kim HM, McAlinden A, Thomopoulos S. Effects of botulinum toxin-induced paralysis on postnatal development of the supraspinatus muscle. *J. Orthop. Res.* 2011; 29:281–288. [PubMed: 20803483]
22. Stern-Straeter J, Bonaterra GA, Hörmann K, Ralf K, Goessler UR. Identification of valid reference genes during the differentiation of human myoblasts. *BMC Mol. Biol.* 2009; 10:66. [PubMed: 19573231]
23. Liu S, Wang Y, Wang L, Wang N, Li Y, Li H. Transdifferentiation of fibroblasts into adipocyte-like cells by chicken adipogenic transcription factors. *Comp. Biochem. Physiol.* 2010; 156:502–508.
24. Weimer K, Theobald J, Campbell KS, Esser KA, DiMario JX. Genome-wide expression analysis and EMX2 gene expression in embryonic myoblasts committed to diverse skeletal muscle fiber type fates. *Dev. Dyn.* 2013; 242:1001–1020. [PubMed: 23703830]
25. Manke T, Heinig M, Vingron M. Quantifying the effect of sequence variation on regulatory interactions. *Hum. Mutat.* 2010; 31:477–483. [PubMed: 20127973]
26. Cai DH, Wang D, Keefer J, Yeaman C, Hensley K, Friedman AD. C/EBP alpha:AP-1 leucine zipper heterodimers bind novel DNA elements, activate the PU.1 promoter and direct monocyte lineage commitment more potently than C/EBP alpha homodimers or AP-1. *Oncogene*. 2008; 27:2772–2779. [PubMed: 18026136]

27. Hong S, Skaist AM, Wheelan SJ, Friedman AD. AP-1 protein induction during monopoiesis favors C/EBP: AP-1 heterodimers over C/EBP homodimerization and stimulates FosB transcription. *J. Leukoc. Biol.* 2011; 90:643–651. [PubMed: 21543584]
28. Timchenko NA, Wilde M, Nakanishi M, Smith JR, Darlington GJ. CCAAT/enhancer-binding protein alpha (C/EBP alpha) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. *Genes Dev.* 1996; 10:804–815. [PubMed: 8846917]
29. Porse BT, Pedersen TA, Xu X, Lindberg B, Wewer UM, Friis-Hansen L, Nerlov C. E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo. *Cell.* 2001; 107:247–258. [PubMed: 11672531]
30. Hu E, Tontonoz P, Spiegelman BM. Transdifferentiation of myoblasts by the adipogenic transcription factors PPAR gamma and C/EBP alpha. *Proc. Natl. Acad. Sci.* 1995; 92:9856–9860. [PubMed: 7568232]

Highlights

- C/EBP α , but not C/EBP β represses slow MyHC2 promoter
- C/EBP α mediated promoter repression occurs embryonic and fetal myogenesis
- C/EBP α binds to a non-canonical C/EBP site in the slow MyHC2 promoter

Figure 1.

C/EBP α and C/EBP β genes are expressed in embryonic myogenic cells. A) RNA was isolated from clonal fast and fast/slow embryonic myoblasts and myotubes and analyzed by RT-PCR for the presence of RNA encoding C/EBP α and C/EBP β . Detection of RLP0 RNA served as a normalizing control. B) Relative C/EBP transcription factor activity in fast and fast/slow embryonic myotubes was determined by assay of activity of a C/EBP transcriptional sensor. Bars show mean luciferase activity $\pm$ SEM, n=6.

Figure 2.

C/EBP α represses slow MyHC2 promoter activity. The slow MyHC2 promoter-luciferase DNA construct was co-transfected along with the empty control expression plasmid (CMV), C/EBP α expression plasmid or C/EBP β expression plasmid into fast/slow embryonic myoblasts. Following myoblast differentiation, luciferase activities were measured. Bars show mean ($\pm$ SEM) fold repression of slow MyHC2 promoter activity. C/EBP α significantly repressed slow MyHC2 promoter activity (n=7; *p=0.037).

Figure 3.

Immunofluorescence analysis of slow MyHC2 gene expression in myotubes expressing C/EBP α FLAG and C/EBP β FLAG. A) Fast/slow myoblasts were transfected with C/EBP α FLAG or C/EBP β FLAG, allowed to differentiate into myotubes, and immunostained for C/EBP α or C/EBP β , slow MyHC2, and α -actin. C/EBP α and C/EBP β were detected with a FLAG epitope antibody and an FITC conjugated secondary antibody. Slow MyHC2 was detected using the S58 antibody and a Texas Red conjugated secondary antibody. An α -actin antibody and tyramide signal amplification with tyramide conjugated to Pacific Blue detected α -actin. B) Myotubes containing FLAG-positive nuclei were assessed for slow MyHC2 immunofluorescence. A significantly greater number of myotubes expressing C/EBP α FLAG did not express slow MyHC2 compared to myotubes expressing C/EBP β FLAG (* $p=0.044$). C) The mean fluorescence above background for α -actin positive myotubes that were expressing either C/EBP α or C/EBP β (* $p=0.036$). At least 15 myotubes per plate were counted with total number of 4 plates being assayed.

Figure 4.

C/EBPα repression of slow MyHC2 promoter activity is not temporally dependent. The slow MyHC2 promoter-luciferase DNA construct was co-transfected with the empty control expression plasmid (CMV), C/EBPα expression plasmid or C/EBPβ expression plasmid into medial adductor myoblasts. Resulting myotubes were electrically stimulated for 4 days, and promoter activities were then measured. Bars represent mean promoter activities ± SEM (n=5). C/EBPα repressed slow MyHC2 promoter activity (*p=0.005).

A.

540 AAAACCATTACATTTCACTTACCCGTAAAGCAAAGTTGTGACTTTTGGTGCCAAATGGGA SM2 488
 NFAT#2 EBOX#3
 480 AGGGTCTGGGTGAACCAGGCAAACCCCTTGGGGAAAGAGGCAAGTGGGAAGGTAGTAGGGG
 420 CCAGCCCTGAGCCCCTTGCCCTGGTTCTCCACTCCTGGGCTGTTTGATGAGGAGCGACTG
 NFAT#1
 360 GACCAGCGCTGAGCCAGAGGTTCTGGGTGCGCCGGCCAGGGAGGCAGAAAGGAAAGCTCT
 300 CAGTGCTGCAGTCCTTGCTGGTTTGCAGAGCTGCACTGCCTGCAGGGACTGCAGTCCCTC
 SM2 222 MEF2 EBOX#2
 240 TCTCTGCCTTTGTGCTCGCATGACAATACAGGAGTAAAAATAACCAGGCTGCATTTGCAG
 180 AGAGATAAAAGCGAAGTTTGCCTTTTGTGTCTCTCCGGGCAGTGGGACAGCAGCACCAAT
 EBOX#1
 120 GGAGCTGTGTGGACATGACACATGGGAATTTTGTGACATATCTTTTCCCACTCTGACCTTG
 TATA BOX EXON 1
 60 ACAGACACAATAAAAAGGGGCTTTGCGGCATGCACACAGCCACTCAGTCTTATCACTAAG

B. **Figure 5.**

Deletion analysis of slow MyHC2 promoter-reporter. A) Nucleotide sequence of the proximal slow MyHC2 promoter. The 5' ends of slow MyHC2 deletion constructs and transcription factor binding sites are underlined. B) Slow MyHC2 promoter-reporter constructs with 6150bp, 488bp, or 222bp of upstream sequence were co-transfected into fast/slow embryonic myoblasts with the C/EBP α expression plasmid. Bars represent mean promoter repression $\pm$ SEM. C/EBP α expression repressed the full length 6150bp slow MyHC2 promoter, 488bp promoter (* p =0.012; n =5), and 222bp promoter (** p =0.002; n =5).

Probe A CATGACAATACAGGAGTAAAAATAACCAGGCTGCATTTGCAGAGAGATAA
TGACTGTTATGTCCACTTTTTTATTTGGTCAGCTGCAAACGTCTCTCTATT

Probe B GATAAAAGCGAAGTTTGCCTTTTGTGTCTCTCCGGGCAGTGGGACAGCAG
CTATTTTCGCTTCAAACGGAAAACACAGAGAGGCCCGTCACCCTGTCGTC

Probe C AGCACCAATGGAGCTGTGTGGACATGACACATGGGAATTTTTTGACATATC
TCGTGGTTACCTCGACACACCTGTACTGTGTACCCTTAAAACTGTATAG

Probe D ATATCTTTTCCCACTCTGACCTTGACAGACACAATAAAAAGGGGCTTTGC
TATAGAAAAGGGTGAGACTGGAAGTGTCTGTGTTATTTTTCCCCGAAACG

Ext	+	+	+	+	+	+	+	+	+	+	+	+
Probe A	+	+	+	-	-	-	-	-	-	-	-	-
Probe B	-	-	-	+	+	+	-	-	-	-	-	-
Probe C	-	-	-	-	-	-	+	+	+	-	-	-
Probe D	-	-	-	-	-	-	-	-	-	+	+	+
α-FLAG	-	+	-	-	+	-	-	+	-	-	+	-
α-βgal	-	-	+	-	-	+	-	-	+	-	-	+

Figure 6. Localization of a C/EBPα binding site. Protein extract (Ext) from fast/slow myotubes, transfected with C/EBPα, were incubated with probes A–D. Some reactions contained an anti-FLAG or an anti-β-galactosidase (βgal) antibody. Arrow indicates C/EBPα-DNA complex, and arrowhead denotes a supershifted complex.

Figure 7.

Identification of the C/EBPα binding site. A) Sequences of the oligonucleotide probes used in the electromobility shift analysis. Bold text indicates altered nucleotide sequence within each mutated oligonucleotide probe (Cm1, Cm2, Cm3, and Cm4). B–F) Protein extract (Ext) from fast/slow myotubes, transfected with the C/EBPα expression construct, was incubated with wild type probe C and mutated oligonucleotides Cm1, Cm2, Cm3, and Cm4. Some reactions contained either anti-FLAG or anti-β-galactosidase antibody. G) Formation of the protein-DNA complex between protein extract and the wild type probe C was challenged by competition for binding using unlabeled wild type probe C and the mutated oligonucleotide competitors (comp) Cm1, Cm2, Cm3, and Cm4. Arrows denote the C/EBPα-DNA complex, and arrowheads denote the supershifted complex.

Figure 8.

Functional analysis of the putative C/EBP α binding site. Mutations contained within mutated oligonucleotides Cm3 and Cm4 were introduced into the 6150bp slow MyHC2 promoter. Wild type (6150) and mutated 6150bp slow MyHC2 luciferase constructs (Cm3 and Cm4) were transfected into fast/slow embryonic myoblasts along with the CMV control vector or the C/EBP α expression construct. Transfected myoblasts were allowed to differentiate. Bars show mean fold repression of slow MyHC2 promoter activity $\pm$ SEM. The Cm3 mutation reduced transcriptional repression compared to repression of the wild type promoter (n=5, *p=0.008).