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Developmental Biology 294 (2006) 541 – 553
DEVELOPMENTAL BIOLOGY
www.elsevier.com/locate/ydbio

MyoD, Myf5, and the calcineurin pathway activate the developmental myosin heavy chain genes

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Received for publication 28 July 2005; revised 5 December 2005; accepted 24 February 2006
Available online 3 April 2006

Abstract

Myogenesis is accompanied by the activation of two developmental myosin heavy chains (MyHCs), embryonic and perinatal, followed by a dramatic decrease in their expression during early postnatal life. The pathways that control the transcription of these genes have not been previously determined. In this study, we identified *cis*-acting elements and *trans*-acting factors that regulate the expression of these two developmental MyHCs in the mouse. Between 800 and 1000 bp of proximal promoter sequence is sufficient to drive muscle-specific expression in cell culture. Further, these same regions contain sequences that confer downregulation in postnatal life in vivo. For the embryonic MyHC gene, the region between −791 bp and −626 bp contains the majority of activating elements. In the proximal promoter regions of both genes, we identified two E-box elements that work in conjunction to activate transcription, but only the embryonic MyHC E-boxes bind a complex containing MyoD. In addition, our results reveal activation by calcineurin that is transduced only partially by its conventional downstream effectors, MEF2 and NFAT. Some common features are shared between the promoters of these two genes; however, the mechanisms of their regulation appear distinct.

Keywords: Developmental myosin; Skeletal muscle; Myogenic regulatory factors; Calcineurin; Promoter

Introduction

The myosin proteins responsible for muscle contraction in mammals constitute a class of at least eight isoforms, each encoded by its own gene (Weiss and Leinwand, 1996). Six of these myosin heavy chain (MyHC) genes are expressed predominantly in skeletal muscle and are organized in a conserved cluster on chromosome 17 in humans and 11 in mice (Weiss et al., 1999). The MyHC isoforms each have distinct biochemical characteristics, and the physiological properties of a muscle are based in part on which isoforms are predominantly expressed (Pette and Staron, 1997). Temporal and spatial regulation of each of the different skeletal MyHCs can therefore lead to functional differences in muscles.

Each MyHC gene exhibits a pattern of expression according to muscle type and developmental time point. One slow isoform (I) and three fast isoforms (IIA, IID, and IIB) are expressed in a mosaic pattern in skeletal muscle throughout adult life. In contrast, two MyHC isoforms are only expressed during development and in regenerating adult muscle fibers. Embryonic MyHC (MyHC-emb) and perinatal MyHC (MyHC-peri) are expressed sequentially during development with MyHC-emb transcript first detected at 9.5 days postcoitum (dpc) and MyHC-peri transcript at 10.5 dpc in the mouse (Lyons et al., 1990). Peak expression of MyHC-emb occurs at around 15 dpc, while the MyHC-peri transcript reaches its peak about 5 days after birth (Allen and Leinwand, 2001; Lu et al., 1999; Lyons et al., 1990). Expression of both developmental myosins is then downregulated until they account for less than 1% of total MyHC protein at 20 days postnatal (Allen and Leinwand, 2001; Lu et al., 1999). The developmental MyHCs are not detected in normal adult muscles (Allen and Leinwand, 2001; Lu et al., 1999) but are re-expressed transiently in muscles regenerating after injury or disease (d'Albis et al., 1988).

The MyHC genes are transcriptionally regulated (Cox and Buckingham, 1992), and several key pathways have been implicated in the transcriptional control of muscle-specific genes including some of the MyHCs. The myogenic regulatory factor (MRF) family of transcription factors and the calcineurin

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doi:10.1016/j.ydbio.2006.02.049

pathway, including the downstream effectors nuclear factor of activated T cells (NFAT) and myocyte enhancer factor 2 (MEF2), are two groups of molecules that provide good candidates for the regulation of the developmental MyHCs.

MRFs are a family of basic helix–loop–helix (bHLH) proteins that induce the transcription of many muscle-specific genes. Four members of the MRF family have been identified to date: MyoD, myogenin, Myf5, and MRF4. Members of this family induce expression of not only sarcomeric proteins but also upstream regulatory proteins and can thus cause the myogenic conversion of many non-muscle cells when ectopically expressed (Choi et al., 1990; Davis et al., 1990; Tapscott et al., 1988). Myf5, MyoD, and myogenin transcripts are first detected between 8 dpc and 10.5 dpc and therefore could influence developmental MyHC expression (Ott et al., 1991; Sassoon et al., 1989). Like all bHLH proteins, the MRFs recognize the E-box motif, which has a core sequence of CANNTG (Lassar et al., 1989). E-boxes found in the promoter regions and enhancers of many muscle-specific genes including myosin light chain 1/3 (Ceccarelli et al., 1999; Wentworth et al., 1991), MyHC-IIB (Wheeler et al., 1999), and muscle creatine kinase (Lassar et al., 1989; Weintraub et al., 1990) are critical for expression.

The Ca^{2+} induced calcineurin pathway is another candidate for the regulation of the developmental myosins, given its apparent role in myocyte differentiation and muscle regeneration (Abbott et al., 1998; Friday et al., 2000; Sakuma et al., 2003). Calcineurin (Cn) is a phosphatase that dephosphorylates NFAT, allowing it to translocate into the nucleus where it cooperates with other factors to induce transcription of target genes (Chin et al., 1998; Timmerman et al., 1996). In muscle, MEF2, which is also activated by calcineurin (Wu et al., 2001), has been shown to act cooperatively with NFAT to regulate activity of the myoglobin promoter and the troponin I (slow form) enhancer (Chin et al., 1998; Wu et al., 2000). Cyclosporine A, a specific inhibitor of calcineurin, also inhibits the differentiation of muscle cells in culture, indicating a role in the regulation of early muscle genes (Abbott et al., 1998).

We have previously shown that MRFs and the calcineurin pathway can differentially influence activity of the three adult fast MyHC promoters (Allen et al., 2001). MRFs activate the promoter of IIB, the fastest adult isoform, while constitutively active calcineurin preferentially activates the promoter of IIA, the slowest of the three isoforms. From evidence in chick embryos (Breckler and Winters, 1988; Kalamkarova et al., 1988), the developmental isoforms are considered to be slower in terms of ATPase activity than the adult isoforms and, therefore, may have regulatory input similar to the slower adult isoforms. However, novel mechanisms likely exist for transcriptional activation and inactivation of the developmental MyHCs since they have a unique temporal expression.

Transcriptional control of the developmental MyHCs has not been studied thoroughly. The activities of various lengths of rat and human MyHC-emb promoter sequences have been assessed, but specific *cis*-elements and their *trans*-acting factors have, for the most part, not been determined (Bouvagnet et al., 1987; Konig et al., 2002). One study found a positive *cis*-element in the rat MyHC-emb promoter that binds an undescribed protein complex found in C2 myoblasts and myotubes, as well as HeLa cells (Yu and Nadal-Ginard, 1989). No studies have characterized the perinatal MyHC promoter region or the factors that act on it.

In the present study, we sought to identify the *cis*-elements and *trans*-factors involved in the transcriptional regulation of the two developmental MyHCs. We isolated a portion of the proximal upstream promoter sequence for both genes that was sufficient to direct muscle-specific expression of a reporter gene in cell culture. These same sequences also contained elements responsible for decreased expression during postnatal life in vivo. Both of these promoters were responsive to MyoD and calcineurin overexpression in muscle cells. Deletion analysis identified a different region in each promoter that was essential for expression and contained three E-boxes. Mutational analysis revealed that the two proximal E-boxes in each sequence were necessary for full activity, while binding studies showed that only the MyHC-emb proximal E-boxes were able to bind MyoD.

Methods

Plasmids and DNA probes

All luciferase reporter constructs were made by replacing the cytomegalovirus (CMV) promoter of the VR1255 vector (Vical, San Diego, CA) with a segment of the mouse MyHC-emb or MyHC-peri promoter using the MscI-SacII endonuclease restriction sites. All MyHC-emb and MyHC-peri promoter sequences contained 13 bp or 9 bp, respectively, downstream of the experimentally determined major transcriptional start site for that gene (B.K. Dennehey and K.S. Krauter, personal communication). The MyHC-emb promoter contains a TATA box sequence (TATAAAAG) that begins 29 bases upstream of the major start site, and the first T in that sequence has therefore been designated base −29. Full-length and truncated constructs contained MyHC-emb sequences from −791 to +14 (EmbWT), −626 to +14 (Emb600), or −389 to +14 (Emb360). A MyHC-emb minimal promoter containing only the TATA box and 34 bp of downstream sequence (−29 to +14) was also constructed. The distal + min construct was created by placing the sequence from −791 to −627 directly upstream of the minimal promoter. To accomplish this, the sequence from −626 to −30 was removed by inverse PCR and replaced with a SpeI restriction site. Mutant constructs were made from the full length EmbWT plasmid using PCR-mediated point mutagenesis. The E-box starting at −758 bp was mutated from CATCTG to CATCCA (EE1), the E-box starting at −684 bp was mutated from CAGCTG to CAGCAT (EE2), and the E-box starting at −632 bp was mutated from CAGCTG to GCGCTG (EE3). Additional E-boxes in the MyHC-emb promoter sequence were mutated to create a construct with no E-boxes in the promoter region (EnoE). In addition to mutations in E-boxes 1–3, the following mutations of E-boxes at the indicated positions were also included in the EnoE construct: CAACTG to GCACTG starting at −466, CAACTG to GGACTG starting at −367, CAAGTG to CAAGAC starting at −346, CAGCTG to GTGCTG starting at −330, and CAGTTG to GCGTTG starting at −191. The MyHC-peri promoter also contains a TATA box sequence (TATATAAG) that begins 29 bases upstream of the major start site, and the first T in that sequence has therefore been designated base −29. Full-length and truncated constructs contained MyHC-peri sequences from −983 to +9 (PeriWT), −599 to +9 (Peri600), or −356 to +9 (Peri360). Mutant constructs were made from the PeriWT plasmid using a PCR-mediated point mutagenesis method. The E-box starting at −351 was mutated from CATCTG to ATTCTG (PE1), the E-box starting at −122 was mutated from CAAATG to CAAAGC (PE2), the E-box starting at −84 was mutated from CAAGTG to ATAGTG (PE3), and the MEF2 site located at −189 was mutated from TTATTTATAG to TTACGTATAG (PMEF2mut).

A thymidine kinase-renilla luciferase vector (pRL-TK; Promega) was used as an internal transfection control. The RSV-MyoD expression vector was a gift from Harold Weintraub, the CMV-Myf5 expression vector a gift from Stephen

Konieczny, and the β-gal, MEF2C, constitutively nuclear NFAT, and constitutively active calcineurin expression vectors as well as the NFAT sensor, GFP-SPRIEIT, and GFP-SPAIAIA plasmids were all kindly provided by Eric Olson. Plasmid DNA was purified using the QIAfilter Plasmid Midi Kit (QIAGEN) for use in cell culture experiments or purified by cesium chloride gradient for in vivo injections.

Duplex probes for mobility shift assays were made from oligonucleotides ordered from Integrated DNA Technologies, Inc. and had the following sense sequences (E-box bolded, mutated bases underlined):

EE1WT	5′ GAA ATG ACA G**CA TCT G**TT AGG CGA TAC C 3′
EE1mut	5′ GAA ATG ACA G**CA TC**C **A**TT AGG CGA TAC C 3′
EE2WT	5′ CCT ATC TAG GCT GG**C AGC TG**G CCT TTC CTA 3′
EE2mut	5′ CCT ATC TAG GCT GG**C AGC A**T**G** CCT TTC CTA 3′
EE3WT	5′ GGC CCC TAA G**CA GCT G**TC ACT GTG AA 3′
EE3mut	5′ GGC CCC TAA G**GC GCT G**TC ACT GTG AA 3′
PE1WT	5′ GAG TTG CTG GG**C ATC TG**A TCT TTG GCA 3′
PE1mut	5′ GAG TTG CTG GG**A T**T**C TG**A TCT TTG GCA 3′
PE2WT	5′ CCG AAG ATG C**CA AAT G**TT GCT ACA AA 3′
PE2mut	5′ CCG AAG ATG C**CA AAG C**TT GCT ACA AA 3′
PE3WT	5′ GGT TCC TGT TGC **CAA GTG** TTT GGC CCT 3′
PE3mut	5′ GGT TCC TGT TGC **ATA GTG** TTT GGC CCT 3′

Cell culture and transfections

C_2C_{12} and L cells were obtained from the American Type Culture Collection. Mouse C_2C_{12} myoblasts were grown on 0.5% gelatin coated dishes in DMEM (Gibco-BRL, cat #12100-061) supplemented with 1.5 g/l sodium bicarbonate and 20% FBS (Hyclone). All transfections were carried out on 24-well plates with cells that were 70–80% confluent. Cells were transfected as described previously (Allen et al., 2001). When the cells became confluent (12–24 h after transfection), cells were placed in differentiation medium (DMEM plus 1.5 g/l sodium bicarbonate and 1% horse serum (Gibco-BRL)). Myoblasts were harvested at confluence and myotubes and L cells were harvested 3 days after transfection. A dual luciferase assay system (Promega) was used to measure firefly and renilla luciferase values. Briefly, cells were rinsed in phosphate-buffered saline and then lysed in 1× Passive Lysis Buffer (Promega). 10 μl of the cell lysate was used to assay for both firefly (reporter constructs) and renilla (internal control) luciferase activity using a standard luminometer. Normalized values for reporter activities were calculated as the ratio between firefly luciferase and renilla luciferase activities in all transient transfection experiments with the exception of the NFAT inhibitor experiments where only the firefly luciferase value is shown.

Electrophoretic mobility shift assays

C_2C_{12} nuclear extracts were prepared essentially as described in Dignam et al. (1983). Complimentary oligos were annealed to create a 3′ overhang that was filled and labeled by incorporation of two α^{32}P-dCTP nucleotides using Klenow enzyme. Probes were incubated for 30 min at room temperature with 9 μg of C_2C_{12} nuclear extract and 1 μg poly dI–dC (Amersham) in 1× binding buffer (10 mM Tris pH 7.5, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1.25% Ficoll). Samples were loaded onto a 4% polyacrylamide gel and run in 0.5× TBE buffer. In competition or supershift assays, a 50× excess of unlabeled probe or 0.5 μg antibody was added to the reaction mixture. Antibodies against myogenin (M-225), MRF4 (Myf6 242), and Myf5 (c-20) were obtained from Santa Cruz Biotechnology. The NCL-MyoD1 antibody was obtained from Novocastra.

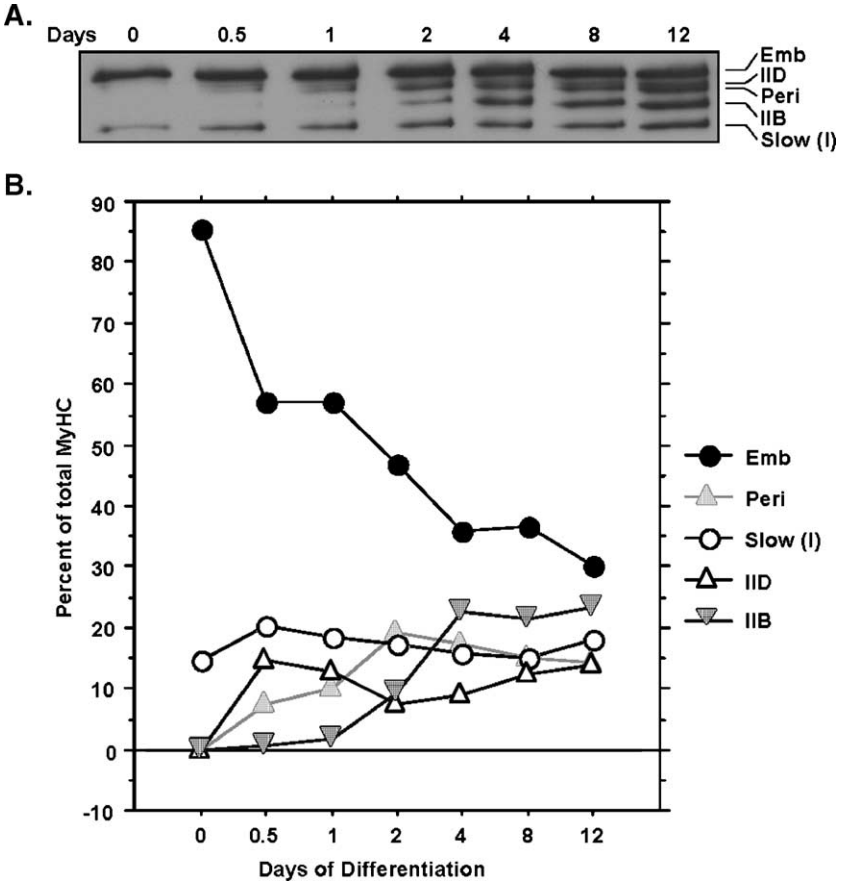

Fig. 1. Endogenous MyHC protein expression timecourse. (A) Western blot of all sarcomeric MyHCs over the course of C_2C_{12} differentiation. Protein collected from C_2C_{12} cells during differentiation was first separated by high-resolution gel electrophoresis. The band corresponding to IIA is masked by the Embryonic band, however, IIA has previously been shown to be very low in these cells (Allen and Leinwand, 2001; Allen et al., 2001) and, therefore, is making a negligible contribution to the top band. (B) Relative levels of the MyHC isoforms as a percentage of the total MyHC present in each sample.

Gel electrophoresis and Western blotting

Protein was collected from C_2C_{12} cells just before transition to differentiation media (day 0) and at specified times after the transition to differentiation media. Cells were scraped into myosin extraction buffer (Butler-Browne and Whalen, 1984) and incubated on ice for 1 h. Cell debris was removed by centrifugation, and total protein concentration was determined using the Bradford Assay (Bio-Rad). For each sample, 5 μg of total protein was loaded onto an 8% acrylamide gel according to the protocol by Talmadge and Roy (1993) with 2-mercaptoethanol added to the upper running buffer at a final concentration of 10 mM (Fritz et al., 1989). The high-resolution gel was run at 90 V for 30 h at 4°C. Proteins from the gel were then blotted onto Hybond-P PVDF membrane (Amersham Biosciences) in a mini-blot apparatus at 30 V for 16 h at 4°C. All sarcomeric myosins were visualized using the F59 antibody (Miller et al., 1989), goat anti-mouse secondary antibody conjugated to horseradish peroxidase (Bio-Rad), and the Western Lightning Chemiluminescence Reagent (Perkin Elmer Life Sciences). Bands were identified based on previous reports of MyHC migration order (Agbulut et al., 2003; Allen and Leinwand, 2001; Talmadge and Roy, 1993) and quantified using ImageQuant software (Molecular Dynamics).

In vivo injection of plasmids

Wild-type and double mutant plasmid constructs were injected into the hindlimbs of postnatal mouse pups at 1 dpn. Adult mice (2 months old) were injected directly in the tibialis anterior (TA) muscle. A pRL-CMV (Promega) plasmid was used as a control for uptake efficiency. The sterile 167 mM NaCl injection solution contained the experimental plasmid at 1 μg/μl and pRL-CMV plasmid at 42 ng/μl. Newborns were anesthetized by chilling on ice while adults were anesthetized by halothane inhalation. 25 μl of sterile injection solution plus plasmid was injected into the hindlimb of pups, and 30 μl was injected directly into the TA muscle of adults. Four days postinjection, anesthetized mice were sacrificed by cervical dislocation (adults) or decapitation (pups). Muscles of the entire hindlimb (pups) or the TA (adults) were dissected from mice and homogenized in 500 μl of buffer containing 25 mM Glygly pH 7.8, 15 mM $MgSO_4$, 4 mM EGTA pH 8.0, and 1 mM dithiothreitol. Samples were clarified by centrifugation and used in the dual luciferase assay as described above. All procedures involving live mice were performed in accordance with institutional guidelines.

Results

Endogenous MyHC protein levels in C_2C_{12} cells

To determine relative expression levels of the MyHCs during C_2C_{12} cell differentiation, Western blots were performed on total protein from cells at specific times after the addition of differentiation media (Fig. 1). Embryonic MyHC and slow (type I) MyHC appear at time 0, before differentiation media are added, indicating that the myocytes had begun to differentiate on their own upon reaching confluence. Perinatal, IIB, and IID MyHCs appear 12 h after the addition of differentiation media and generally increase over the course of differentiation. MyHC-emb remains the dominant isoform throughout the timecourse and ranges from 7- to 2-fold more abundant than MyHC-peri as differentiation progresses.

Proximal promoter sequences are sufficient for muscle-specific expression

Previous studies of the three adult fast MyHC proximal promoters indicated that 1 kb or less was sufficient to confer muscle-specific expression that mimicked endogenous relative levels of protein (Allen et al., 2001). To identify promoter sequences with similar properties for the mouse developmental MyHCs, the sequence from −791 to +14 of MyHC-emb or the sequence from −983 to +9 of MyHC-peri driving the expression of luciferase (constructs EmbWT and PeriWT, respectively) was tested for activity in myogenic C_2C_{12} cells and non-muscle L cells. The activities of the EmbWT and PeriWT promoters were significantly higher in differentiated myotubes than in proliferating myoblasts (Fig. 2A). The activity of EmbWT was significantly higher than PeriWT in both myoblasts and myotubes which is consistent with the relative levels of endogenous protein in these cells (Fig. 1). In contrast, activity for both promoters was extremely low in L cells (Fig. 2B). Taken together, these results indicate that these promoter sequences contain the regulatory elements sufficient for muscle-specific expression.

Promoters respond to MyoD, Myf5, and calcineurin

To identify signaling pathways controlling transcription of MyHC-emb and MyHC-peri, the EmbWT and PeriWT constructs were co-transfected into C_2C_{12} cells along with plasmids overexpressing a number of factors known to regulate muscle-specific genes (Fig. 3A). Of those tested, only MyoD and constitutively active calcineurin (caCn) significantly increased the activity of both promoters. EmbWT activity was induced 5.5- and 3.4-fold while PeriWT activity was induced 26.9- and 11.8-fold by MyoD and caCn, respectively. The greater fold increase in activation of PeriWT by both of these co-transfectants relative to the fold increase of EmbWT activity is likely due to the higher basal activity of EmbWT in these cells (Fig. 2A). Myf5 also increased EmbWT activity significantly by

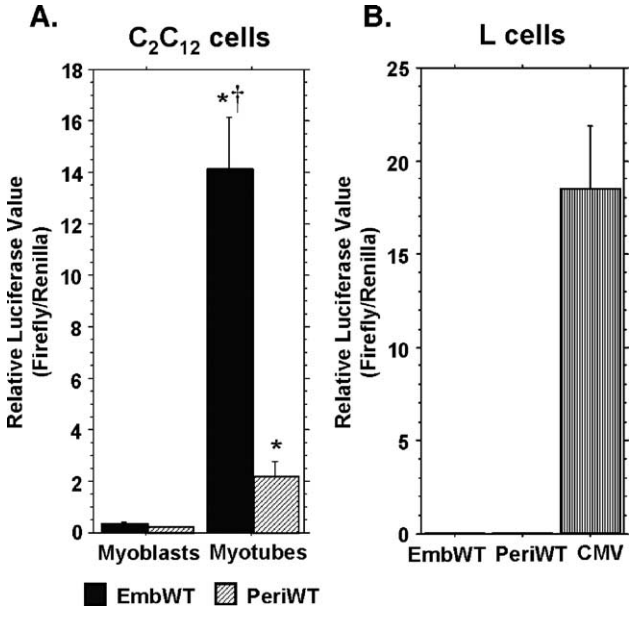

Fig. 2. Activity of MyHC-emb and MyHC-peri proximal promoters in muscle and non-muscle cells. EmbWT and PeriWT plasmids were transfected into C_2C_{12} cells (A) and non-muscle L cells (B) along with pRL-TK as a transfection control. Firefly luciferase values from each sample were first normalized to renilla luciferase activity and results are expressed as the mean ± SE. *Significantly different from myoblasts ($P < 0.0001$); †significantly different from perinatal ($P < 0.0001$).

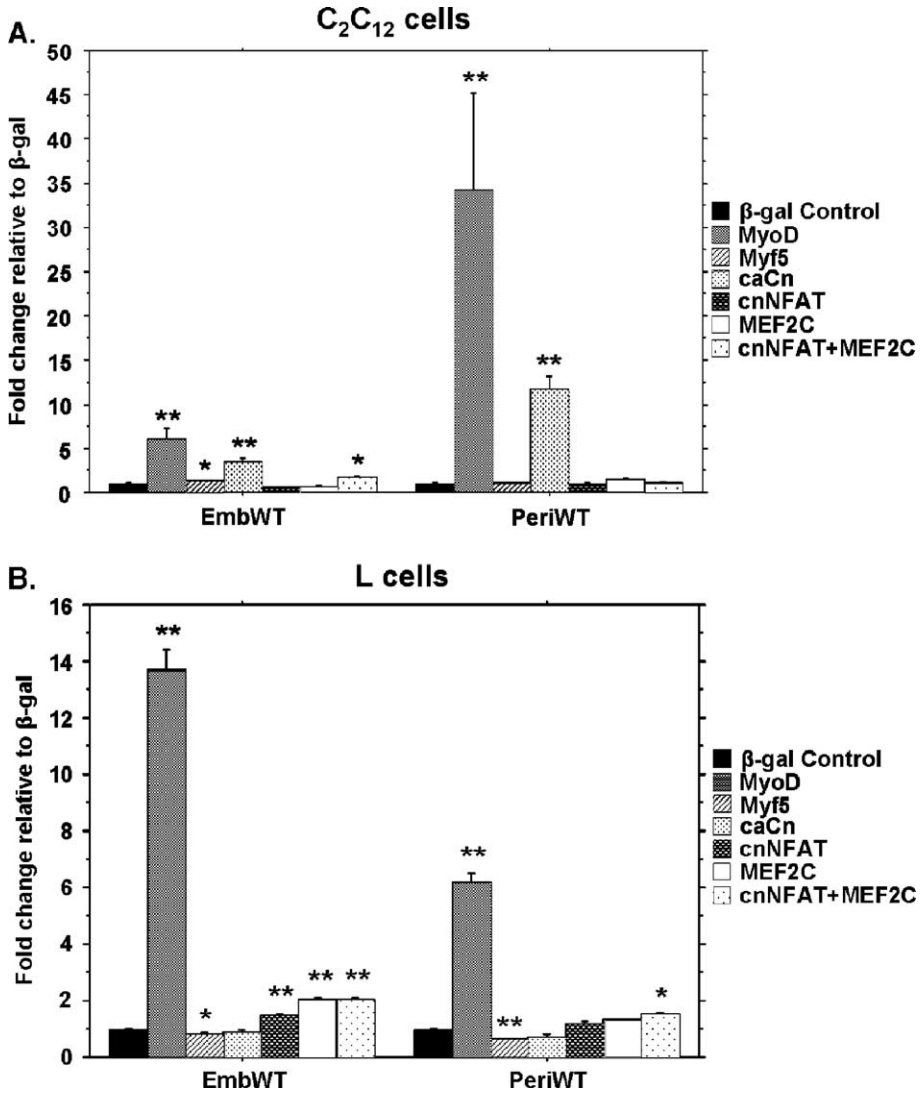

Fig. 3. Responsiveness of promoters to overexpression of known muscle regulatory proteins in cell culture. EmbWT and PeriWT were co-transfected individually with overexpression plasmids for MyoD, Myf5, constitutively active calcineurin (caCn), constitutively nuclear NFAT (cnNFAT), MEF2C, or cnNFAT and MEF2C in C_2C_{12} cells (A) and in L cells (B). Results are expressed as fold change in activation compared to the β-galactosidase overexpression control. *Significantly different from β-gal overexpression ($P < 0.05$); **significantly different from β-gal overexpression ($P < 0.0001$).

1.3-fold. Surprisingly, even though caCn overexpression increased the promoter activities of EmbWT and PeriWT, two downstream targets of calcineurin, NFAT and MEF2, had no effect on promoter activity when individually co-transfected with either EmbWT or PeriWT. Since NFAT and MEF2 have been shown to work cooperatively to activate transcription (Wu et al., 2000), we co-transfected both NFAT and MEF2 expression plasmids together with the EmbWT or PeriWT constructs. This resulted in a modest, but significant, activation of EmbWT (1.8-fold) but not of PeriWT (Fig. 3A).

All of the factors tested by overexpression are present at moderate to high levels in differentiating C_2C_{12} cells (Dedieu et al., 2002; Delling et al., 2000; Miller, 1990; Ornatsky and McDermott, 1996). Since endogenous levels of these factors may preclude any additional activation of the promoters, co-transfections were also carried out in non-muscle L cells to specifically assess the promoters' responsiveness to these regulatory factors (Fig. 3B). MyoD increased MyHC-emb and MyHC-peri promoter activity by 14- and 6-fold respectively in L cells while caCn had no effect, suggesting that calcineurin requires other muscle-specific factors to activate these promoters. Myf5 activated expression of the pRL-TK control plasmid. For this reason, when the data were normalized, it appeared that Myf5 actually inhibited the activity of the promoters (Fig. 3B). However, when the results are not normalized, this effect is no longer seen, and it appears that Myf5 does not significantly influence promoter activity. Overexpression of cnNFAT or MEF2 increased EmbWT activity by 1.5-fold and 2-fold, respectively. Overexpression of both factors simultaneously increased EmbWT activity by 2-fold and PeriWT by 1.5-fold.

Conserved motifs in the proximal promoter regions

Putative transcription factor binding sites were identified in the promoter sequences using the MATInspector program with the TRANSFAC database (Quandt et al., 1995; Wingender et al.,

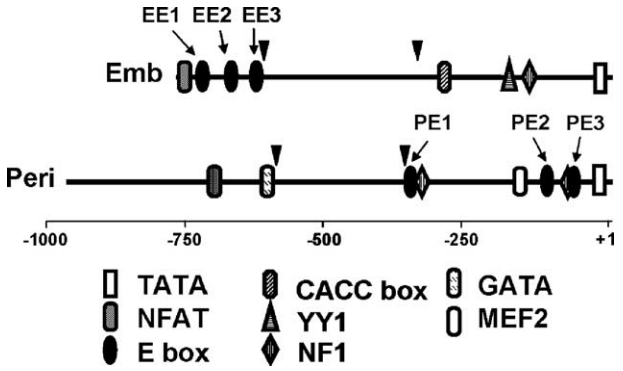

Fig. 4. Conserved putative transcription factor binding motifs. MATInspector was used with the TRANSFAC database to identify putative binding motifs. Motifs conserved in human, rat, and mouse MyHC-emb sequences or human, pig, and mouse MyHC-peri sequences are shown. Scale in base pairs. Arrowheads indicate the boundaries of truncations tested in Fig. 5. E-boxes are labeled.

1996). Putative binding sites that are conserved in human, rat, and mouse (MyHC-emb) or human, pig, and mouse (MyHC-peri) are shown in Fig. 4. Three E-boxes and one NFAT binding site are positioned in the distal region of MyHC-emb, from −791 to −627. Three conserved E-boxes and one MEF2 binding site are located in the proximal region of the MyHC-peri promoter. Other conserved binding motifs are also shown (Fig. 4).

Promoter truncations reveal important regulatory sequences

In order to determine which regions of the promoter sequences have specific regulatory functions, a series of deletion constructs in which the promoter sequence was truncated at the 5′ end were tested in C_2C_{12} cells. Deletion of bases −791 to −627 (Emb600) resulted in a 90% decrease in activity compared to EmbWT, and additional deletion of bases −791 to −340 (Emb360) did not have any further significant effect (Fig. 5A). Thus, sequences between −791 and −627 appear to be necessary for the activity of this promoter. However, this segment is not sufficient to drive full expression when placed upstream of a minimal promoter containing only the MyHC-emb TATA-box and downstream bases to +14 (distal + min, Fig. 5A) indicating that some of the intervening promoter sequences may be necessary to work in conjunction with the upstream segment. These findings are consistent with a recent study of the MyHC-emb promoter that revealed, through Bayesian analysis, two regions of likely conservation between human and mouse within the proximal 1 kb of this promoter that correspond to the sequences distal to −600 bp and proximal to −300 bp (Konig et al., 2002). Konig and colleagues also showed that the distal sequences of the human MyHC-emb promoter must work with the specific proximal sequences of the promoter, in agreement with the results presented here (Fig. 5A).

In contrast, truncation of the perinatal MyHC promoter by deleting bases −983 to −600 (Peri600) or −983 to −357 (Peri360) had no significant effect on promoter activity (Fig. 5B). This suggests that the major elements responsible for activity of the perinatal MyHC promoter are located in the proximal 356 bp. Bayesian analysis of the MyHC-peri promoter showed likely conservation between human and mouse only in the proximal region of the promoter that corresponds to our Peri360 promoter region (Konig et al., 2002).

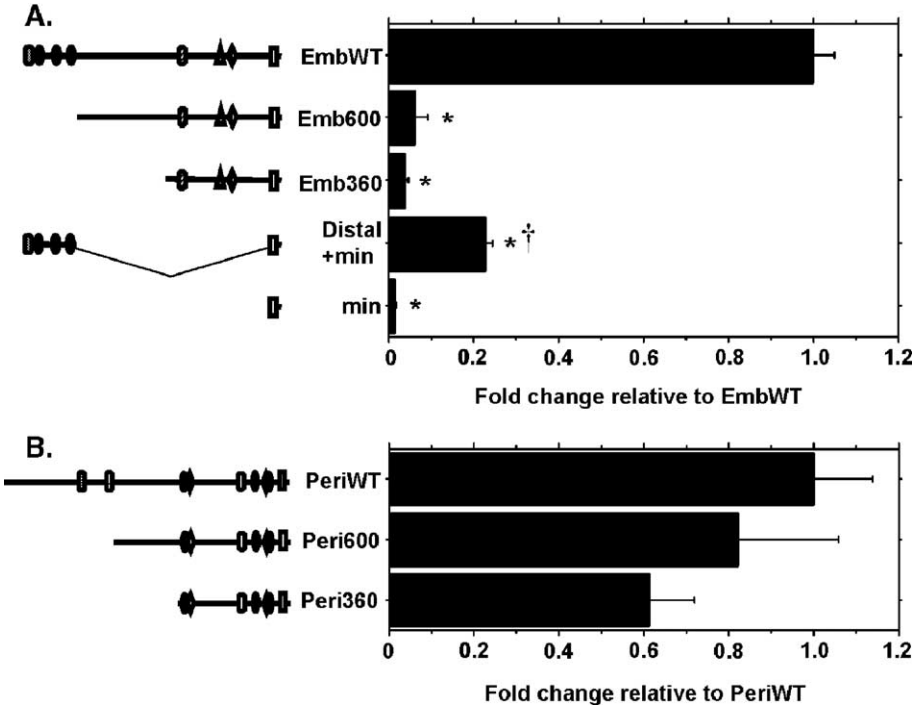

Fig. 5. Activity of truncated promoter constructs in C_2C_{12} cells. (A) Fold change in normalized activities (firefly/renilla) of truncated MyHC-emb promoter constructs compared to EmbWT. (B) Fold change in normalized activities (firefly/renilla) of truncated MyHC-peri promoter constructs compared to PeriWT. *Significantly different from WT ($P < 0.0001$); †significantly different from min ($P < 0.0001$).

NFAT and possibly MEF2 mediate caCn activation of PeriWT but not EmbWT

To understand the role of NFAT in the caCn activation of these promoters, we used an inhibitor of NFAT activation in C_2C_{12} cells. A peptide matching the portion of the NFAT protein that binds to calcineurin (SPRIEIT) was expressed from a plasmid as a fusion protein attached to the amino terminus of green fluorescent protein (Aramburu et al., 1998). The control plasmid containing a mutant peptide that does not interact with calcineurin (SPAIAIA) was also used in these experiments. As before, caCn caused an increase in EmbWT and PeriWT activity compared to the β-gal control plasmid and co-transfection of caCn and SPAIAIA gave similar activation of these two promoters (Figs. 6A and B). Co-transfection of caCn along with the NFAT inhibitor, SPRIEIT, caused a decrease in the activation level of PeriWT but not EmbWT. An NFAT sensor expression construct containing three tandem NFAT binding sites upstream of the luciferase coding sequence was used as a control for caCn activation and SPRIEIT inhibition and showed the expected responses (Fig. 6C).

The importance of a putative MEF2 binding site in the MyHC-peri promoter was also tested in C_2C_{12} cells. The conserved sequence located in the proximal region of the promoter was mutated to abolish MEF2 binding. Activity of the mutant promoter sequence was significantly decreased by over 60% relative to the wild-type promoter (Fig. 6D).

E-boxes are essential for expression

Each of the putative E-boxes (numbered 1–3 for each promoter; Fig. 4) was individually mutated within the context of the full EmbWT and PeriWT promoter sequences and tested in cell culture. For each mutant construct, a pair of point mutations was designed to change either the invariant CA or TG bases of the E-box core sequence CANNTG. Mutation of E-box 2 or E-box 3 resulted in a significant decrease in activity by 40–65% compared to wild type for both promoters (Figs. 7A and B). Simultaneous mutation of E-boxes 2 and 3 resulted in a further decrease in activity to about 10% of the wild-type activity (Figs. 7A and B). Mutation of E-box 1 in these promoters resulted in a slight but significant increase in activity, suggesting that this E-box may bind a negative regulator (Figs. 7A and B).

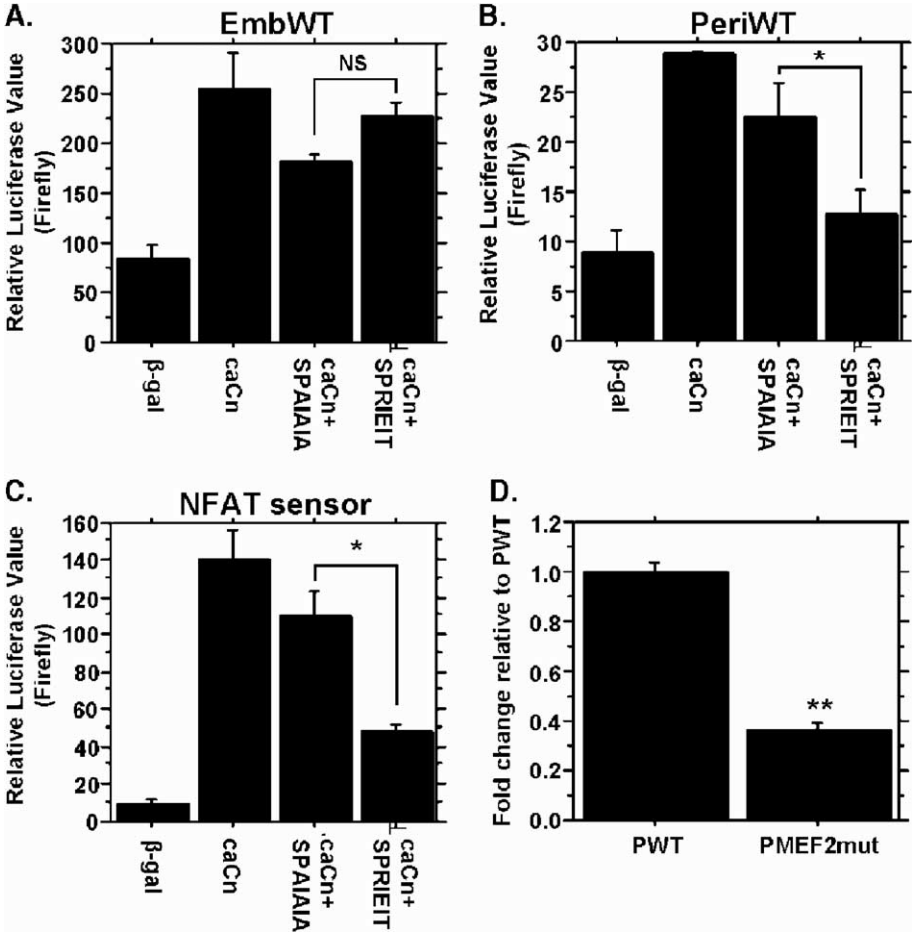

Fig. 6. NFAT and MEF2 contribution to promoter activation. The role of NFAT in caCn activation of EmbWT (A) and PeriWT (B) is shown using a specific inhibitor of NFAT activation (SPRIEIT) and a control analog that has no inhibitory activity (SPAIAIA). Panel C shows the activity of a control NFAT sensor plasmid under the same conditions. (D) Activity of a MyHC-peri construct with the conserved MEF2 site mutated is compared to PeriWT. NS, not significantly different; *$P < 0.05$; **$P = 0.0001$.

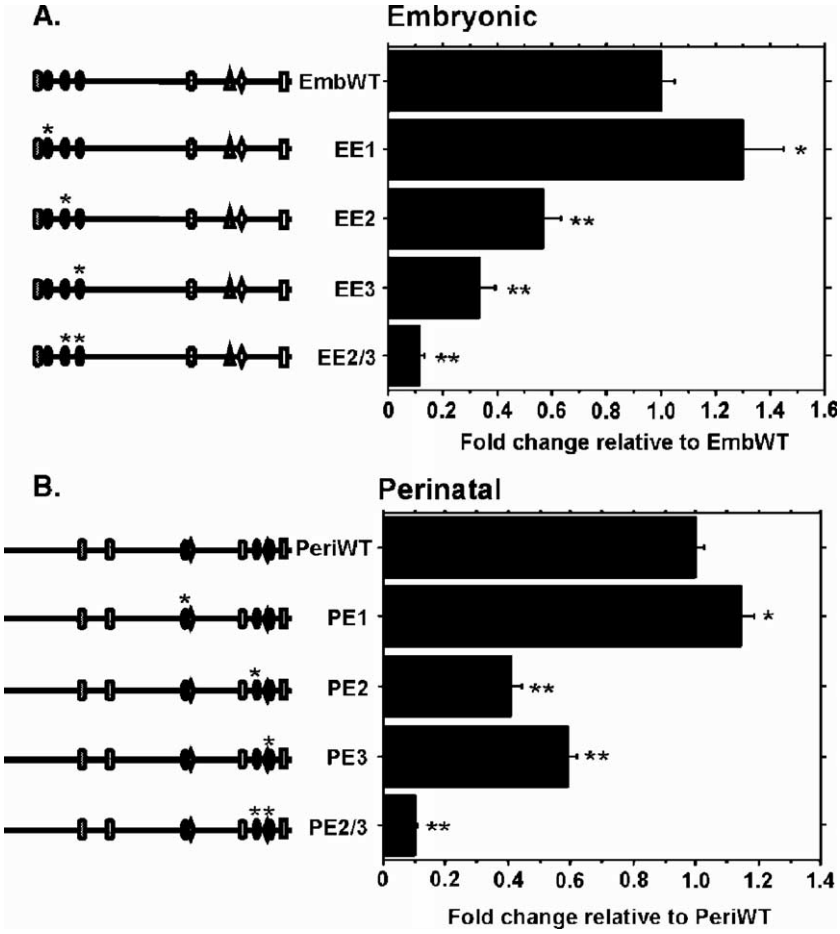

Fig. 7. Relative activity of wild-type and mutant promoters. (A) Fold change in promoter activity indicated by normalized luciferase activity for MyHC-emb E-box mutant constructs compared to EmbWT. (B) Fold change in promoter activity indicated by normalized luciferase activity for MyHC-peri E-box mutant constructs compared to PeriWT. *Significantly different from WT ($P < 0.05$); **significantly different from WT ($P < 0.0001$).

E-box mutant constructs were also transfected into L cells along with a plasmid overexpressing MyoD to assess the role of the binding sites in MyoD activation of these promoters (Fig. 8). EmbWT and PeriWT were activated by MyoD in this experiment by 28-fold and 6.5-fold, respectively. The embryonic double mutant (EE2/3) and triple mutant (EE1/2/3) showed an approximately 55% decrease in activation by MyoD compared to EmbWT (Fig. 8A). Five additional non-conserved E-boxes in the mouse MyHC-emb promoter were also mutated, and the construct containing no intact E-boxes was tested (EnoE). This construct had activity 75% lower than EmbWT (Fig. 8A). The only mutant MyHC-peri construct showing a significant loss of MyoD activation was PE1/2/3 which contained no intact E-boxes. PE1/2/3 was a modest 31% less active than PeriWT (Fig. 8B). The original luciferase expression plasmid containing the CMV promoter was activated almost 2-fold by MyoD in L cells (Fig. 8).

A subset of E-boxes form complexes with myocyte nuclear proteins

Duplex probes containing wild-type or mutant E-boxes from the MyHC-emb and MyHC-peri promoters were used in mobility shift assays to determine the protein binding capability of each sequence. The wild-type probes for MyHC-emb E-boxes 2 and 3 (EE2 WT and EE3 WT) both formed two specific complexes with components of C_2C_{12} nuclear extract (Figs. 9C and D). The corresponding mutant probes were unable to form these complexes indicating that an intact E-box is necessary for the binding of these complexes to the DNA (data not shown). One or both of the complexes were supershifted by a MyoD-specific antibody (Figs. 9C and D, lane Ab). The wild-type probe for MyHC-emb E-box 1 (EE1 WT) also formed similar complexes that were supershifted with MyoD antibody (Fig. 9A). While the faster mobility complex was not able to form with the mutant probe, the slower mobility protein complexes did show some binding ability and, therefore, are E-box independent (Fig. 9B).

Despite decreased MyHC-peri promoter activity in response to mutation of the proximal E-boxes (Fig. 7B), these sequences do not appear to bind any of the MRFs. PE1 WT probe formed many complexes with components of the C_2C_{12} nuclear extract (Fig. 9E); however, only the faster migrating complexes were specific since they were not formed in the presence of an excess of specific competitor (lane SC) and were also not present with the mutant probe (data not shown). Two specific complexes

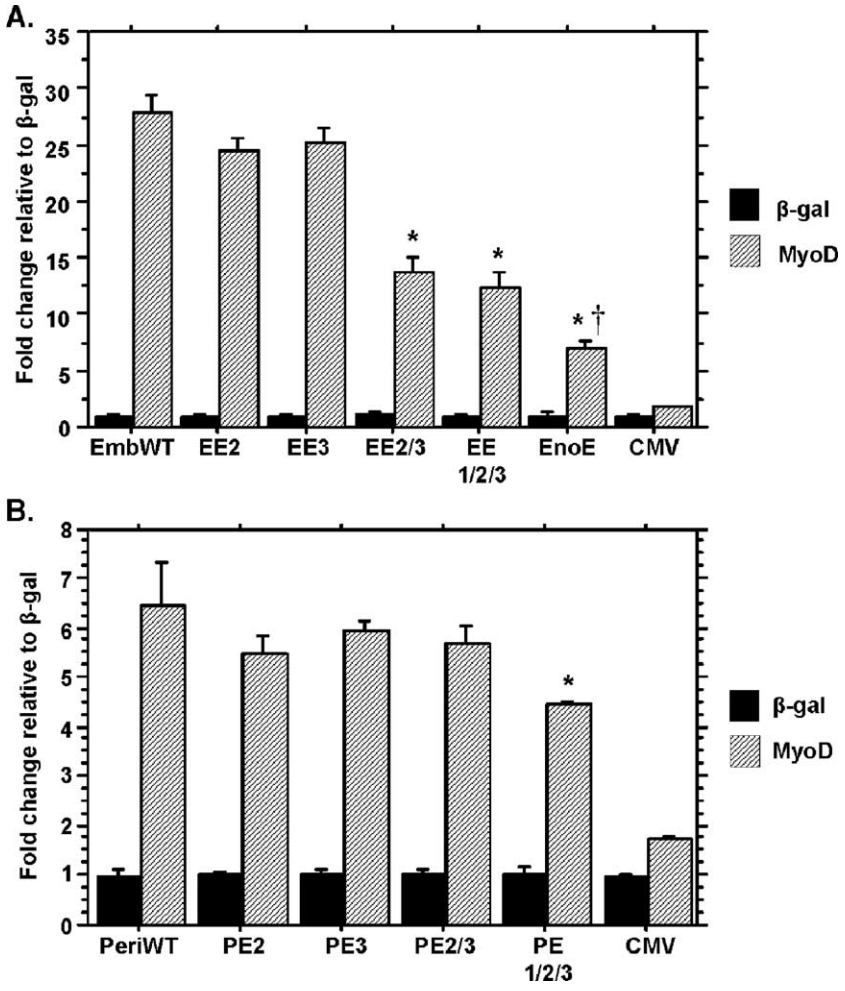

Fig. 8. Response of mutants to MyoD overexpression. Wild-type and E-box mutant constructs were co-transfected with a plasmid overexpressing MyoD or β-gal into L cells. The induction of each promoter is shown relative to the promoter activity in the presence of β-galactosidase overexpression. Panel A shows embryonic MyHC constructs. Panel B shows perinatal MyHC constructs. *Significantly different from WT ($P < 0.005$); †significantly different from EE2/3 and EE1/2/3 ($P < 0.005$).

were formed with the PE2 WT but not the mutant probe (Fig. 9F and data not shown). The PE3 WT probe formed three complexes that were inhibited by a specific competitor, but the complexes also formed with the PE3 mut probe indicating that the complexes do not require the E-box sequence but rather were binding to flanking sequences in the probe (Figs. 9G and H). None of the complexes that contained either the PE1 WT probe or the PE2 WT probe were supershifted by antibodies specific for MyoD, Myf5, myogenin, or MRF4 (data not shown). The PE1 WT probe also contained a putative NF1 binding site, but an antibody against NF1 also failed to supershift any of the complexes (data not shown).

Promoter activity in vivo

One interesting aspect of developmental MyHC expression is the downregulation of mRNA and protein after birth. This aspect cannot be easily modeled in cell culture. To understand the role of E-boxes 2 and 3 of the MyHC-emb and MyHC-peri promoters in vivo, wild-type and mutant plasmid DNA constructs were injected into the hindlimb of newborn mouse pups or the tibialis anterior (TA) of 2-month-old mice. As shown in Fig. 10A, activity of both wild-type promoters was significantly higher at 5 dpn than at 2 months of age. This corresponds to the time period when expression of the endogenous genes decreases. To determine the role of E-boxes in this developmental downregulation of expression, mutants were tested at the same time points. Constructs with double mutants have approximately 20% of the activity as wild type at both ages tested (compare Figs. 10A and B), mirroring our cell culture results. Also, double mutant constructs showed a significant decrease in activity from 5 dpn to 2 months of age indicating that these E-boxes are not required for the postnatal decrease in expression (Fig. 10B).

Discussion

The pathways involved in regulating MyHC-emb and MyHC-peri transcription have not been previously examined. The results of this study implicate relatively short segments of proximal promoter sequences in conferring high activity in muscle cells to these two promoters. Further, we show that

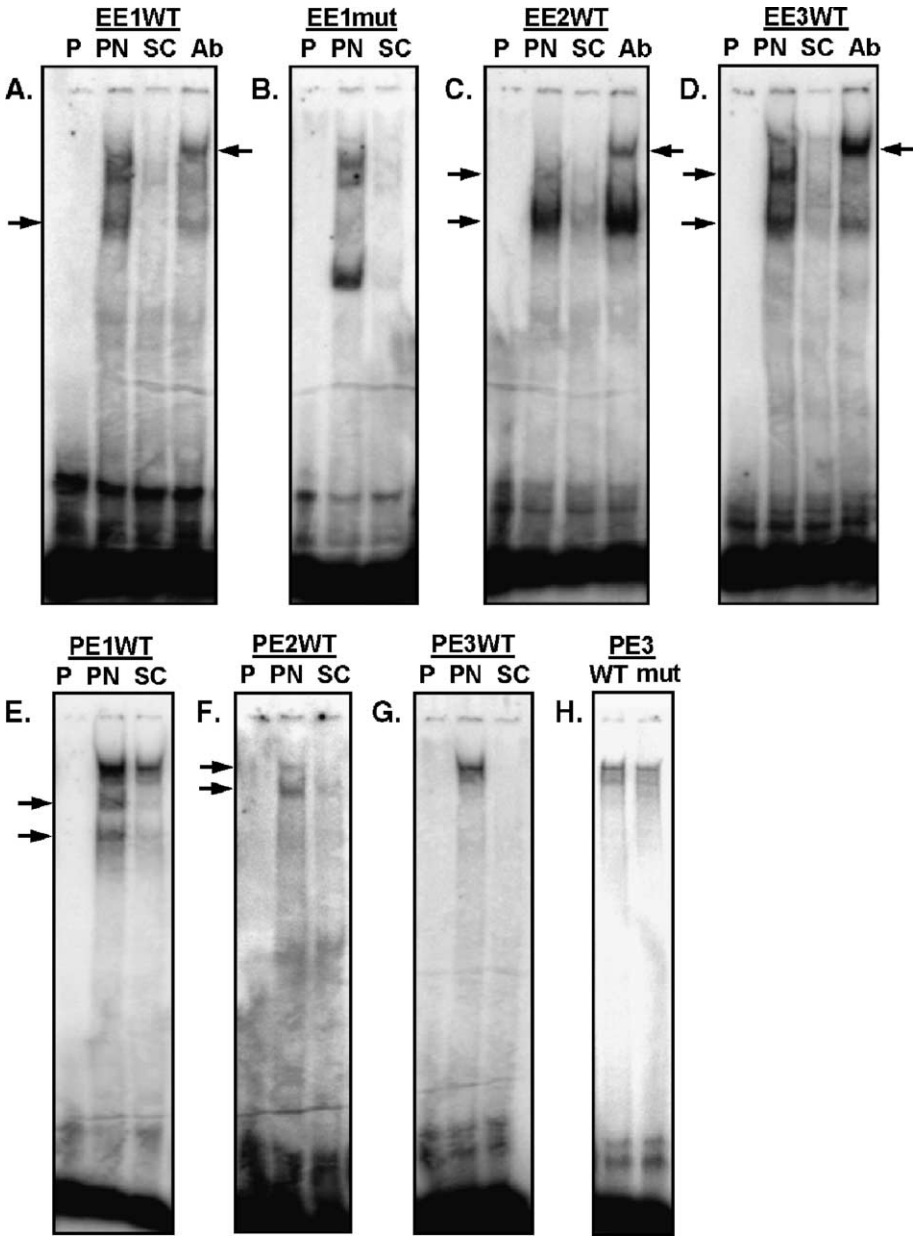

Fig. 9. Electrophoretic mobility shift assay of wild-type and mutant E-boxes. Labeled probes containing E-boxes from MyHC-emb and MyHC-peri promoters were incubated with a complex C_2C_{12} nuclear extract and run on a non-denaturing gel. (A) MyHC-emb wild-type E-box 1, (B) MyHC-emb mutant E-box 1, (C) MyHC-emb wild-type E-box 2, (D) MyHC-emb wild-type E-box 3, (E) MyHC-peri wild-type E-box 1, (F) MyHC-peri wild-type E-box 2, (G) MyHC-peri wild-type E-box 3, (H) MyHC-peri wild-type and mutant E-box 3. P, probe alone; PN, probe and C_2C_{12} nuclear extract; SC, probe, extract, and 50× specific competitor; Ab, MyoD specific antibody. Left arrows indicate bands that are both E-box dependent and competed by a specific competitor. Right arrows indicate supershifted bands.

MRFs and the calcineurin pathway play a role in their activation. While these promoters are induced largely by the same molecules, the details of their transcriptional regulation appear distinct.

In both of the developmental MyHC promoters, we have identified an important region of 200–300 bp, each containing three E-box elements (Figs. 4 and 5). Reporter constructs containing mutations in either of the two proximal E-boxes had lower activity relative to wild type, and mutations in both proximal E-boxes revealed an additive decrease of promoter activity (Figs. 7A and B). This suggests that both E-boxes are necessary for full activity of the promoter. Injection of the wild-type and double mutant constructs into hindlimb muscle in vivo indicates that these two E-boxes are also important for expression in maturing muscle fibers (Fig. 10). Because of greater variability in data from injection studies in general, the difference between the PeriWT and PE2/3 constructs was not significant at 5 dpn but shows the same trend as in cell culture. Both wild-type and mutant sequences also exhibited lower activity in adult mouse muscle than in newborn mouse muscle (Fig. 10), indicating that these E-boxes are not required for the postnatal downregulation of these genes.

The two proximal MyHC-emb E-boxes described here correspond to two E-boxes found by Konig et al. (2002) in a

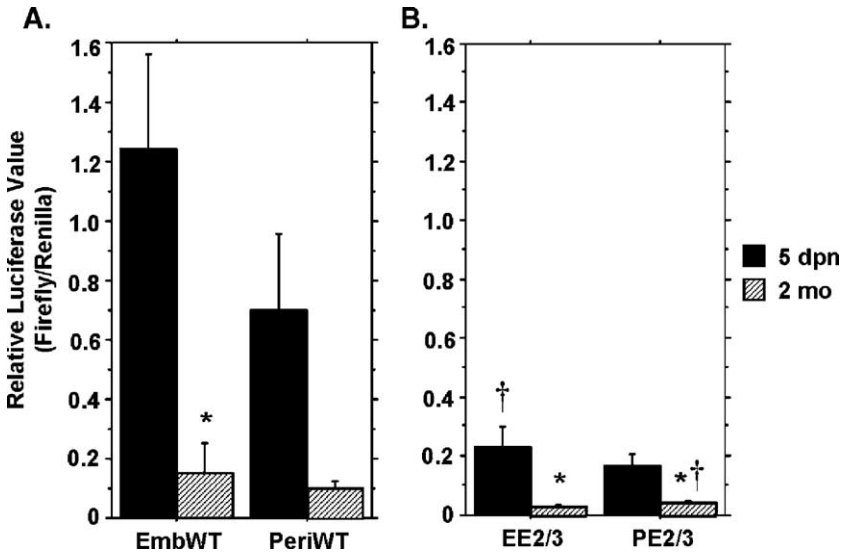

Fig. 10. Activity of wild-type and double mutant promoters in vivo. Wild-type (A) and E-box double mutant plasmid constructs (B) were injected into the hindlimb of 1 dpn mice or the tibialus anterior of 2-month-old mice and muscles were collected for luciferase assay 4 days later. Normalized (firefly/renilla) values are shown. *Significantly different from same construct at 5 dpn ($P < 0.05$); †significantly different from wild type at same time point ($P < 0.05$).

critical segment of promoter sequence and were postulated by those researchers to bind MRFs (ChemE-2 E-boxes 5 and 6). In their work, the authors describe a construct containing distal and proximal regions of the human promoter that has activity similar to their full length promoter (Konig et al., 2002). While the sequences of these constructs cannot be directly compared to our distal + min construct, the sequences that the Konig construct contains that are absent in our construct appear to include a conserved YY1 binding site and a conserved NF1 site. These or other unidentified sequences may be playing an important cooperative role in MyHC-emb transcription.

The distal E-box for both MyHC-emb and MyHC-peri also showed similar characteristics. Mutations in either resulted in slightly increased activity compared to the wild-type promoter in cell culture (Figs. 7A and B). While none of the MRFs has ever been shown to be inhibitory, an E-box in the MLC enhancer binds to a complex that has a weak negative regulatory role (Ceccarelli et al., 1999). The MLC enhancer E-box is not transactivated by MyoD, but the EE1 site is able to bind a complex that includes MyoD (Fig. 9A). Because the EE1 mut probe binds a complex that does not bind the EE1 WT probe (Figs. 9A and B), we may have created a binding site for an activator. To examine this possibility, a different mutation of embryonic E-box 1 was made (CATCTG to TCTCTG; EE1′) in the context of the full length promoter and tested in cell culture. The EE1′ construct also showed a slight increase in activity compared to EmbWT (data not shown) making this scenario unlikely. The PE1 WT probe formed two E-box-dependent, sequence specific complexes, but neither of these complexes was super-shifted with MRF antibodies (Fig. 9E and data not shown). These complexes appear to contain unidentified E-box binding proteins that suppress the transcription of the MyHC-peri gene.

Overexpression studies in cell culture highlight other similarities as well as differences between the mechanisms regulating MyHC-emb and MyHC-peri. Induction of both promoters by constitutively active calcineurin is seen in muscle cells and appears to require muscle specific factors that are not present in fibroblastic L cells since caCn overexpression in these cells had no effect on either promoter (Fig. 3). This is consistent with the finding that a muscle environment is necessary for caCn activation of other muscle genes (Chin et al., 1998). Interestingly, although caCn was able to induce the developmental MyHC promoters in muscle cells, the two best characterized of calcineurin's downstream targets do not seem to be driving this induction entirely (Fig. 3A). Overexpression of constitutively nuclear NFAT or of MEF2 caused no change in promoter activity. However, when overexpressed together, these two transcription factors were able to induce the EmbWT construct (Fig. 3A). EmbWT was induced to the same level by caCn in the presence of a peptide inhibitor of NFAT and a control peptide (Fig. 6A), indicating that calcineurin induction of this promoter is not mediated by NFAT. This, taken together with the fact that there is no apparent MEF2 binding site in the EmbWT promoter, suggests that the slight induction we see with co-transfection of cnNFAT and MEF2 is due to a secondary effect that may not have relevance to endogenous conditions. In the case of MyHC-peri, the combination of cnNFAT and MEF2 overexpression did not increase promoter activity in C_2C_{12} cells (Fig. 3A). However, when caCn is co-transfected with an NFAT inhibitor, the promoter's activity is diminished back to the basal level (Fig. 6B). The discrepancy between these results indicates that while NFAT is a necessary mediator, additional downstream factors are also required for the activation of MyHC-peri by caCn in a muscle cell environment. The conserved MEF2 binding site located in the proximal 360 bp of the promoter (Fig. 4) is also important for PeriWT activity (Fig. 6D). At this time, other calcineurin effectors that may be involved in the activation of MyHC-peri transcription are unknown.

Overexpression of MRFs in cell culture also delineated the similarities and differences between the two promoters. Myf5 and myogenin are the first MRFs to be expressed during myogenesis and are the only ones present at the time that the MyHC-emb transcript is first detected (Lyons et al., 1990; Ott et al., 1991). Therefore, one might expect Myf5 or myogenin to be involved in the activation of MyHC-emb transcription. Myf5 overexpression in C_2C_{12} cells modestly but significantly activated the EmbWT promoter by 1.3-fold (Fig. 3A), indicating a possible role in the initiation of MyHC-emb transcription. MyoD overexpression also induces activity of the MyHC-emb promoter in muscle and non-muscle cell lines (Fig. 3). The ability of MyoD to transform non-muscle cell types into muscle (Choi et al., 1990; Davis et al., 1990; Tapscott et al., 1988) dictates that the induction of the MyHC promoters in L cells may be due to either direct binding of MyoD to the promoters or indirect induction via a larger signaling network that is activated by MyoD. Because the expression of MyoD trails MyHC-emb expression by a day (Lyons et al., 1990; Ott et al., 1991), it is possible that MyoD acts as an amplifying signal. In contrast, the PeriWT promoter responded to MyoD overexpression but not to Myf5 overexpression (Fig. 3A). MyoD may be the initiating factor for MyHC-peri transcription because their onset of expression coincides (Lyons et al., 1990; Ott et al., 1991).

The abilities of MRFs to bind the MyHC-emb and MyHC-peri E-boxes were also distinct. The decreased activity of promoters containing mutations in each of the two proximal E-box mutants is consistent with the possibility of MRFs binding to the promoter to induce expression. Surprisingly, only the MyHC-emb proximal E-boxes formed complexes with MyoD (Fig. 9). The MyHC-peri E-boxes failed to bind complexes containing any of the MRFs. Because MRFs cooperate with other family members as well as other transcription factors to activate transcription, the complexes that bind the MyHC-peri E-boxes likely require a co-factor that binds a sequence that is present in the full-length promoter but is not present on the probe used for binding assays. Alternatively, an MRF could be activating at these binding sites but is not detectable using these methods. This idea is supported by the fact that binding affinity and transcriptional activation ability of MyoD are not always correlated (Huang et al., 1996).

To understand whether MyoD induction of these promoters is direct or indirect as well as to reconcile the lack of MRF binding to MyHC-peri E-boxes, we performed co-transfection experiments with E-box mutant constructs and the MyoD overexpression plasmid in L cells (Fig. 8). In these experiments, MyHC-emb E-boxes 2 and 3 show a clear role in activation by MyoD. Mutation of additional E-boxes in the MyHC-emb promoter decrease the activity further, indicating that MyoD may also bind at one or more of these other sites to activate transcription. In the case of MyHC-peri, all three of the promoter's E-boxes must be mutated to diminish activation by MyoD. Both of the developmental MyHC promoter constructs with no intact E-boxes are still activated by MyoD to a higher extent than the control plasmid with the same backbone sequence. This activation is, therefore, likely via secondary muscle-specific effects.

The control of muscle transcription is a complex web of cooperating factors from a number of pathways. The present data suggest that the promoters of MyHC-emb and MyHC-peri are controlled by members of the MRF family and the calcineurin pathway. Based on the timing of MRF expression, we believe Myf5 initiates the expression of MyHC-emb, while MyoD enhances it. MyHC-peri is also activated by MyoD, though this activation is mostly due to an indirect interaction. Calcineurin also plays a role in the upregulation of both developmental MyHCs. Some, but not all, of this regulation is mediated by NFAT and MEF2 in the case of MyHC-peri, while activation of MyHC-emb by calcineurin is not mediated by NFAT.

Acknowledgments

We thank Dr. Debra McDonough and Amy Nunnally for their technical assistance and Drs. Briana Dennehey, Hugo Olguin, and Angelika Paul for their critical reading of the manuscript. This work was supported by National Institutes of Health Grant R01 AR048817.

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