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mef2c is activated directly by myogenic basic helix-loop-helix proteins during skeletal muscle development in vivo

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Abstract

Skeletal muscle development requires the coordinated expression of numerous transcription factors to control the specification of mesodermal progenitor cells to a muscle fate and the differentiation of those committed myoblasts into functional, contractile muscle. Two families of transcription factors play key roles in these processes. The myogenic basic helix-loop-helix (bHLH) proteins, MyoD and Myf5, are required for myoblast specification, while two members of the same family, myogenin and MRF4, play key roles in myoblast differentiation in vivo. All four members of the myogenic bHLH family are sufficient to dominantly induce myogenesis when introduced into a variety of non-muscle cells in culture, however this function requires the activity of a second family of transcriptional regulators, the myocyte enhancer factor 2 (MEF2) family. MEF2 factors are essential for muscle differentiation, and previous studies have shown that MyoD and MEF2 family members function combinatorially to activate transcription and myogenesis. Consistent with these observations, the majority of skeletal muscle genes require both MyoD and MEF2 family members to activate their transcription. A possible exception to this combinatorial model for activation is suggested by the observation that myogenic bHLH factors may be able to independently activate the expression of MEF2. This raises the question as to how *mef2* gene transcription is induced by MyoD factors without cooperative activation by MEF2. During skeletal muscle development, *mef2c* is the first member of the MEF2 family to be expressed. In this study, we have investigated the regulation of a skeletal muscle-specific enhancer from the mouse *mef2c* gene using a transgenic approach. We show that *mef2c* is a direct transcriptional target of the MyoD family in vivo via an essential E box in the skeletal muscle enhancer of *mef2c*, and we show that *mef2c* is not a direct target for autoregulation by MEF2.

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1. Introduction

Skeletal muscle development involves the specification of mesodermal progenitors to a muscle fate, followed by the differentiation of those myoblasts into functional, contractile myotubes. Both of these steps are controlled by a tightly regulated transcriptional program that involves two key transcription factor families: the MyoD family of basic helix-loop-helix (bHLH) proteins and the myocyte enhancer factor 2 (MEF2) family of MADS box factors. Members of the MyoD family are essential for both specification and differentiation of skeletal muscle. This family of muscle regulatory factors (MRFs) comprises four

members: Myf5, MyoD, MRF4, and myogenin (Olson, 1990; Weintraub, 1993). Genetic studies in mice have shown that the regulators MyoD and Myf5 are involved primarily in myoblast specification while myogenin and MRF4 play central roles in muscle differentiation (Arnold and Braun, 1996; Megeney and Rudnicki, 1995; Weintraub, 1993). Each of the MRFs is sufficient to dominantly induce myogenesis when introduced into a variety of non-muscle cells in culture (Olson, 1990; Weintraub, 1993), but the ability to convert cells into skeletal muscle requires the function of the MEF2 family (Black and Olson, 1998; Ornatsky et al., 1997). Like members of the *myoD* gene family, members of the *mef2* family are among the earliest markers of the skeletal muscle lineage (Edmondson et al., 1994). However, in contrast to the MRFs, members of the MEF2 family are not sufficient to induce skeletal

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myogenesis (Black and Olson, 1998; Molkentin et al., 1995; Ornatsky et al., 1997), and *mef2* transcripts are expressed outside the skeletal muscle lineage.

During development, *mef2* genes are expressed in cardiac, smooth, and skeletal muscles, as well as in a restricted set of other tissues (Edmondson et al., 1994; Leifer et al., 1993, 1994; Lyons et al., 1995). There are four *mef2* genes in vertebrates and a single *mef2* gene, *D-mef2*, in *Drosophila* (Black and Olson, 1998). Despite the early expression of *mef2* in myoblasts, MEF2 activity is not required for muscle specification since inactivation of the single *mef2* gene in *Drosophila* results in a complete loss of muscle differentiation with no apparent defect in myoblast specification (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995). Likewise, inactivation of the *mef2c* gene in mice results in embryonic lethality due to cardiac and vascular defects that included incomplete expression of downstream contractile protein genes without defects in cardiac myoblast specification (Bi et al., 1999; Lin et al., 1997, 1998). The requirement for MEF2 proteins in muscle differentiation in vertebrates is supported further by the observation that expression of a dominant-negative form of MEF2A in myoblasts blocked differentiation (Ornatsky et al., 1997).

Myogenic bHLH proteins heterodimerize with ubiquitously expressed E proteins and bind DNA at a consensus sequence known as an E box (CANNTG), found in the promoters of most skeletal muscle-specific genes (Buskin and Hauschka, 1989; Wright et al., 1991). MEF2 proteins bind as homo- and heterodimers to the consensus nucleotide sequence YTA(A/T)₄TAR, also found in the promoter region of nearly every known muscle-specific gene (Black and Olson, 1998). Most downstream skeletal muscle-specific genes require the combinatorial activities of members of the MEF2 and MyoD families to activate their transcription (Arnold and Winter, 1998; Black and Olson, 1998; Molkentin and Olson, 1996; Yun and Wold, 1996). Myogenic bHLH and MEF2 transcription factors physically interact as part of a combinatorial complex that results in synergistic activation of transcription and myogenesis (Arnold and Winter, 1998; Black and Olson, 1998; Molkentin and Olson, 1996; Yun and Wold, 1996). Members of both of these families of transcription factors are capable of binding directly to DNA to activate transcription, although the binding of only one factor to DNA may be sufficient to recruit the other to the DNA via protein–protein interactions (Black et al., 1998; Molkentin et al., 1995). However, the cooperative activation of transcription by these two classes of regulators probably occurs most efficiently in vivo when both are bound to DNA. Cooperative activation is facilitated by the close proximity and coordinated positioning of the binding sites for MyoD and MEF2 on the same face of the DNA in most muscle-specific promoters (Fickett, 1996). In this model for muscle gene activation, the binding of each

factor to its DNA binding site would help to recruit and stabilize the binding of the other factor. Synergistic protein–protein interactions then could occur while both factors were bound to DNA (Black et al., 1998).

The transcriptional control of the muscle-specific transcription factors themselves is more complex than the regulation of the broad array of downstream muscle genes. Myogenic bHLH and MEF2 transcription factors activate and repress each others' transcription in a complex network that probably serves to tightly regulate and fine tune the myogenic program (Arnold and Winter, 1998; Bergstrom et al., 2002; Cserjesi and Olson, 1991; Olson and Klein, 1994; Wong et al., 1994). This increased complexity in regulation is illustrated by the regulation of *MRF4* transcription. *MRF4* requires upstream myogenic bHLH proteins to activate its expression through an essential E box in its promoter, yet *MRF4* cannot activate its own expression (Black et al., 1995; Naidu et al., 1995; Pin et al., 1997). In addition, *MRF4* expression requires MEF2 activity (Black et al., 1995; Naidu et al., 1995). The regulation of *myogenin* transcription is also controlled by myogenic bHLH proteins and MEF2 (Cheng et al., 1992, 1993; Edmondson et al., 1992; Yee and Rigby, 1993), but expression of *myogenin* also requires the function of the Six family of homeodomain proteins via an essential MEF3 element in its promoter (Spitz et al., 1998). In addition to requiring MEF2 activity for its own expression, myogenin activates the expression of MEF2 independently of other skeletal muscle gene products, further suggesting that a reinforcing transcriptional loop functions during vertebrate skeletal myogenesis (Cserjesi and Olson, 1991; Ridgeway et al., 2000).

To more fully understand this complex molecular network and to define more precisely how MEF2 factors fit into the inter- and cross-regulation among skeletal muscle-specific transcription factors, we analyzed the transcriptional regulation of the mouse *mef2c* gene in skeletal muscle. Of the four vertebrate *mef2* genes, *mef2c* is the earliest to be expressed in skeletal muscle (Edmondson et al., 1994). *mef2c* expression in the mouse is detectable by 9.0 dpc in the myotomal compartment within rostral somites, and its expression expands throughout all of skeletal muscle as development progresses (Edmondson et al., 1994). We have isolated an enhancer region from the mouse *mef2c* gene that is sufficient to direct expression to skeletal muscle during murine development. Furthermore, we have defined *cis*-acting sequences within the skeletal muscle enhancer of *mef2c* that are essential for expression in vivo. Among the *cis*-acting elements in the skeletal muscle enhancer of *mef2c* is an E box that is bound by myogenic bHLH proteins with extremely high affinity and is absolutely required for expression in vivo. The data presented in this study support a direct link between myogenic specification and differentiation by demonstrating that myogenic bHLH proteins directly activate the expression of *mef2c* in skeletal muscle in vivo.

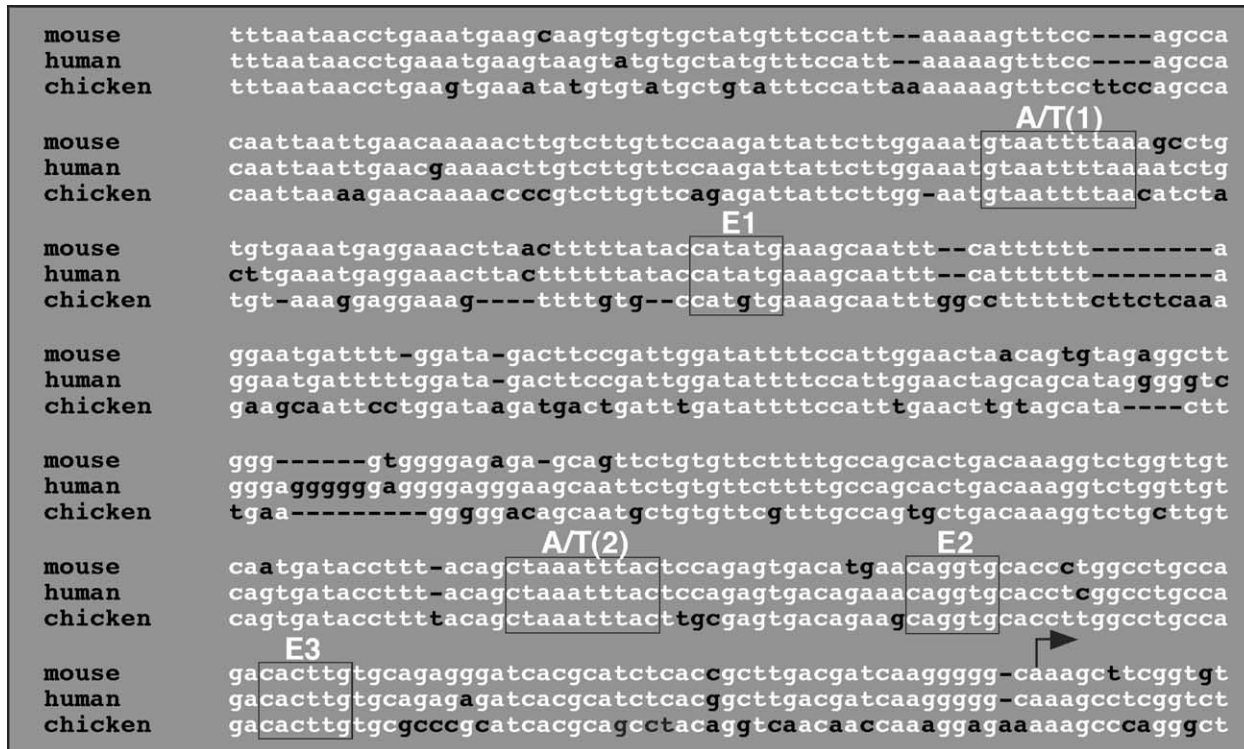


Fig. 1. An evolutionarily conserved skeletal muscle enhancer resides upstream of exon 1 in mouse *mef2c* (genbank accession #AY321453). Sequence alignment of the mouse, human, and chicken *mef2c* loci reveals an evolutionarily conserved 429 bp region upstream of the first transcribed exon. Nucleotides indicated in white have been conserved; those indicated in black are divergent. Boxes denote the evolutionarily conserved, candidate *cis*-acting sequences examined in this study. The arrow indicates the transcriptional start site obtained from genbank (accession #AK009139).

2. Results

2.1. Identification and isolation of a skeletal muscle enhancer from mouse *mef2c*

To begin to define the transcriptional enhancers controlling *mef2c* expression, we introduced the *lacZ* gene into a bacterial artificial chromosome (BAC) encompassing the *mef2c* locus by in vivo recombination in bacteria (Yang et al., 1997), and we used this recombinant BAC to generate transgenic mice. This 120 kb BAC was sufficient to direct expression of *lacZ* in the complete expression pattern of endogenous *mef2c* (data not shown) suggesting that all of the *cis*-acting elements required for *mef2c* expression resided within the BAC sequence. To assist us in defining sequence elements that might be important for controlling *mef2c* expression in vivo, we analyzed the genomic sequences of human, mouse, and chicken *mef2c*. Our sequence comparison identified eight regions of significant evolutionary conservation that were upstream of the first translated exon but did not represent known exons. One of these regions of cross-species homology was present immediately upstream of the first untranslated exon of mouse *mef2c*, which was 71 kb upstream of the first translated exon. This region comprised 429 bp of conserved sequence and contained three conserved candidate E boxes

(Buskin and Hauschka, 1989; Wright et al., 1991), and two conserved AT-rich sequences that we thought might be able to function as MEF2 sites (Andres et al., 1995; Dodou et al., 1995) (Fig. 1). The presence of these candidate motifs suggested that this region might represent a skeletal muscle enhancer for *mef2c*. To determine if this region of the *mef2c* gene could function as a skeletal muscle-specific enhancer in vivo, we cloned a 954 bp fragment encompassing this evolutionarily conserved region into the transgenic reporter plasmid HSP68-*lacZ* (Kothary et al., 1989), and we tested the ability of this fragment to direct expression in transgenic mice. This fragment of *mef2c* was sufficient to direct skeletal muscle specific expression throughout mouse development (Fig. 2). This enhancer fragment directed *lacZ* expression beginning at 9.0 dpc in rostral somites, consistent with the first myotomal expression of endogenous *mef2c* (Edmondson et al., 1994), and expression became more robust by 9.5 dpc (Fig. 2A). Expression was apparent in skeletal muscle throughout mouse development. Robust β -galactosidase activity was observed in the myotomal compartment within the somites at 11.5 dpc (Fig. 2B), and could be seen in all skeletal muscles by 13.5 dpc (Fig. 2C). No expression of β -galactosidase was observed outside of the skeletal muscle lineage, indicating that this fragment of the *mef2c* gene represented a skeletal muscle specific enhancer only and did not contain elements sufficient to

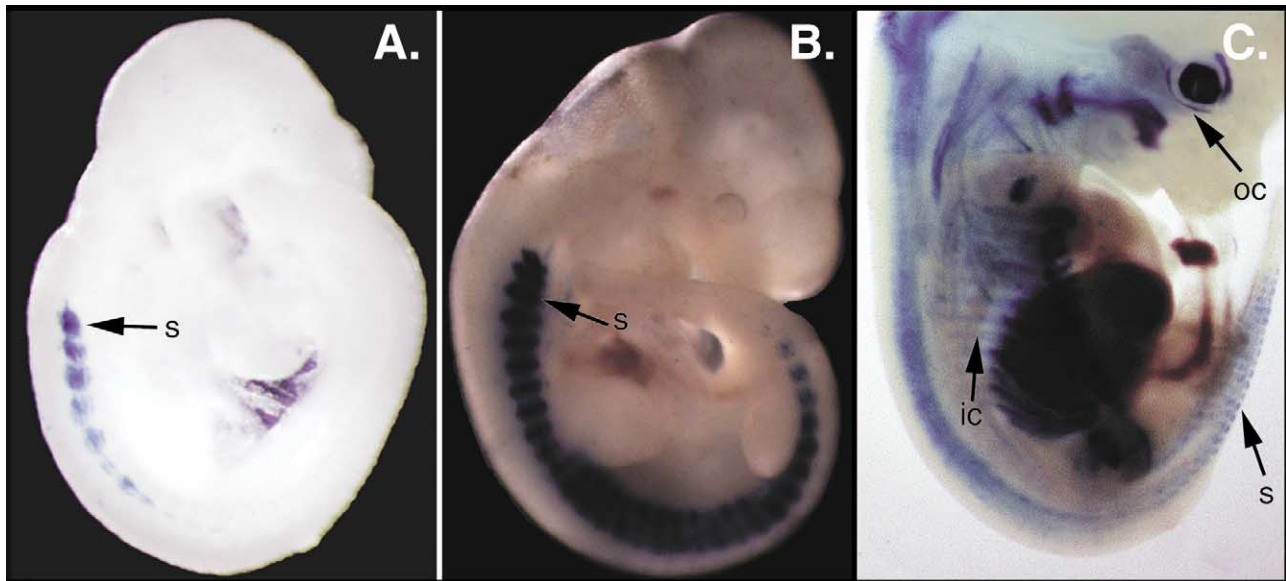


Fig. 2. Developmental expression directed by the mouse *mef2c* skeletal muscle enhancer. A 954 bp fragment of the mouse *mef2c* gene that contains the evolutionarily conserved sequences shown in Fig. 1 was cloned into the *lacZ* reporter plasmid HSP68-*lacZ*. This fragment was sufficient to direct expression of the *lacZ* reporter to skeletal muscle in vivo. Representative transgenic embryos are shown at (A) 9.5 dpc (B) 11.5 dpc, and (C) 13.5 dpc. Expression was evident in skeletal muscle throughout development beginning at 9.0 dpc. Arrows denote selected sites of expression. s, somites (myotome); ic, intercostal muscles; oc, ocular muscles.

direct expression to other regions where endogenous *mef2c* is expressed.

2.2. Analysis of cis-acting elements in the *mef2c* skeletal muscle enhancer in vitro

As a first step toward defining the *cis*-acting elements responsible for the function of this skeletal muscle enhancer, we analyzed the evolutionarily conserved candidate *cis*-acting elements for transcription factor binding by electrophoretic mobility shift assay (EMSA). We tested each of the three evolutionarily-conserved E boxes present in the enhancer (Fig. 1) for the ability to bind myogenic bHLH protein heterodimers. No binding was observed for either the E1 or the E3 E box, and neither of these E boxes was able to compete for the binding of myogenic bHLH heterodimers to the *bona fide* Right E box from the *muscle creatine kinase* (MCK) enhancer (data not shown). By contrast, myogenic bHLH heterodimers robustly bound to the E2 E box (Fig. 3). Binding to the E2 E box was specifically competed by an excess of unlabeled MCK Right E box or by unlabeled E2 E box from the *mef2c* gene (Fig. 3, lanes 9 and 11, respectively), but not by a 100 fold excess of either of the unlabeled mutant probes (Fig. 3, lanes 10 and 12). Likewise, an excess of unlabeled E2 E box probe specifically inhibited binding of myogenic bHLH heterodimer to the MCK Right E box (Fig. 3, lane 5). In this experiment, the MCK Right E box and *mef2c* E2 E box were labeled with identical specific activities and an equal

amount of labeled probe was included in each EMSA analysis. Under these conditions, the *mef2c* E2 E box represented a significantly stronger binding site for myogenic bHLH protein heterodimers than did the canonical MCK Right E box (Amacher et al., 1993), suggesting that this binding site in *mef2c* represents one of the strongest direct targets for the MyoD family identified to date.

Two A/T-rich elements, resembling MEF2 sites, are also present in the conserved region of the *mef2c* skeletal muscle enhancer (Fig. 1). While neither of these sites, A/T(1) or A/T(2), represent canonical binding sites for MEF2 (Andres et al., 1995; Dodou et al., 1995), we tested whether these elements could bind MEF2 in vitro by EMSA (Fig. 4). Fig. 4A shows that the A/T(1) element was unable to bind MEF2 (Fig. 4A, lane 8). By contrast, the *bona fide* MEF2 site from the *myogenin* enhancer (Edmondson et al., 1992; Yee and Rigby, 1993) was bound by MEF2 with high affinity in the same experiment (Fig. 4A, lane 2). The binding of MEF2 to the *myogenin* MEF2 site was efficiently competed by an excess of unlabeled *myogenin* MEF2 probe (Fig. 4A, lane 3), but was not competed by a 100 fold excess of the *mef2c* A/T(1) element (Fig. 4A, lane 5). The failure of the A/T(1) element from *mef2c* to compete for MEF2 binding even when present in 100 fold excess (Fig. 4A, lane 5), combined with the observation that the *mef2c* A/T(1) element is not bound by MEF2 protein, demonstrates that the A/T(1) element is not a *bona fide* MEF2 site.

Similarly, we tested the A/T(2) element for the ability to bind to MEF2 in vitro. The results shown in Fig. 4B

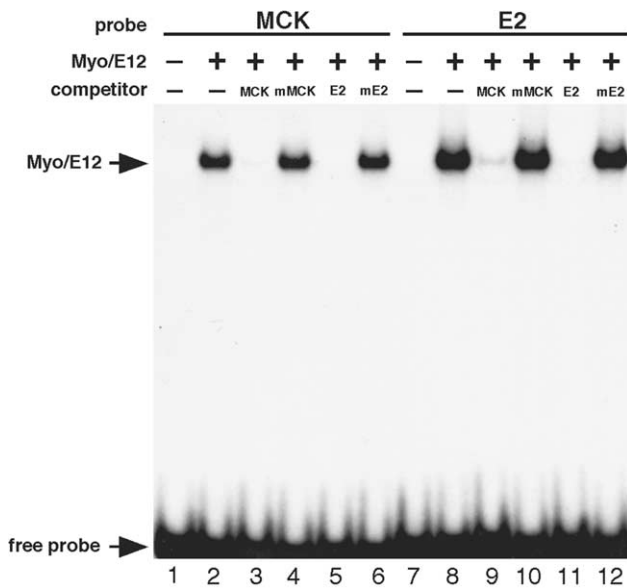


Fig. 3. The E2 E box in the *mef2c* skeletal muscle enhancer is bound efficiently by myogenic bHLH/E12 heterodimers. Myogenin and E12 were cotranscribed and cotranslated in vitro and incubated with a radiolabeled E2 E box from the *mef2c* skeletal muscle enhancer (lanes 7–12). A radiolabeled MCK Right E box was used for comparison and for competition with the E2 E box (lanes 1–6). Myo/E12 heterodimers retarded the mobility of the E2 E box (lane 8) indicating that myogenic bHLH heterodimers bind to the E2 E box. This binding was specific as it was competed by excess unlabeled MCK Right E box (lane 9) and by excess unlabeled E2 E box (lane 11) but not by unlabeled mutant E boxes (lanes 10 and 12). The E2 E box also efficiently competed for Myo/E12 binding to the MCK Right E box (lane 5), but the mutant version of the E2 E box was completely incapable of competing for binding to the MCK Right E box (lane 6). For Myo/E12, plus and minus signs represent the presence or absence of in vitro cotranslated Myo/E12. In samples where an in vitro translated protein was absent (minus sign), an equal amount of unprogrammed lysate was included. An equal amount of radiolabeled probe was included in each sample and the MCK and E2 probes were labeled with nearly identical specific activity.

demonstrate that the A/T(2) element was also incapable of MEF2 binding (Fig. 4B, lane 8). Again, we compared the binding of MEF2 to the *mef2c* A/T(2) element with binding to the canonical *myogenin* MEF2 site in the same experiment. MEF2 efficiently retarded the mobility of the *myogenin* MEF2 site (Fig. 4B, lane 2), and this binding complex was specific since it was efficiently competed by an excess of unlabeled *myogenin* MEF2 site probe (Fig. 4B, lane 3). The *mef2c* A/T(2) element was unable to compete for MEF2 binding to the *bona fide* MEF2 site from the *myogenin* gene, even when present in a 100 fold excess (Fig. 4B, lane 5). We tested the ability of three different MEF2 isoforms, MEF2A, MEF2C, and MEF2D, to bind to the A/T(2) element from the *mef2c* skeletal muscle enhancer, and none showed any binding in EMSA analyses (data not shown). These results, like those for the A/T(1) element, demonstrate that MEF2 cannot bind to the *mef2c* A/T(2) element and support the notion that neither of these elements represent high affinity MEF2 binding sites.

2.3. Trans-activation of the *mef2c* skeletal muscle enhancer by *MyoD* but not by *MEF2*

To test whether the *mef2c* skeletal muscle enhancer might be a target for autoregulation by MEF2 in vivo, in spite of a lack of MEF2 binding in vitro, we examined the ability of MEF2 to *trans*-activate the wild-type 954 bp *mef2c-lacZ* reporter in 10T1/2 cells (Fig. 5A). The *mef2c* skeletal muscle enhancer was efficiently *trans*-activated by cotransfected *MyoD* plasmid (Fig. 5A, lane 5), but the enhancer was not *trans*-activated by cotransfected MEF2 expression plasmid (Fig. 5A, lane 3). By contrast, the MEF2 expression plasmid significantly activated a cotransfected *myogenin-lacZ* reporter (Fig. 5A, lane 4). Neither the *mef2c* nor the *myogenin* reporters were significantly activated by cotransfection of the empty parental expression plasmid (Fig. 5A, lanes 1 and 2, respectively). The observation that *MyoD* efficiently activated expression of the *mef2c-lacZ* reporter in 10T1/2 cells by approximately twenty fold (Fig. 5A, lane 5) prompted us to investigate which *cis*-acting elements within the *mef2c* skeletal muscle enhancer were important for activation by *MyoD* (Fig. 5B). Consistent with the results of the gel shift analysis shown in Fig. 3, the ability of *MyoD* to *trans*-activate the reporter was dependent on the E2 E box (Fig. 5B, lane 4). Surprisingly, mutation of the A/T(1) and A/T(2) sites also impacted the ability of *MyoD* to *trans*-activate the reporter (Fig. 5B, lanes 6 and 8, respectively). Mutation of the A/T(1) site had a modest effect on *MyoD trans*-activation (Fig. 5B, compare lanes 2 and 6), while mutation of the A/T(2) site had a more dramatic effect on *MyoD trans*-activation (Fig. 5B, compare lanes 2 and 8).

The results presented in Fig. 5B show that *MyoD* requires the two A/T-rich elements in the *mef2c* skeletal muscle enhancer for efficient *trans*-activation. These data suggest that one or more cofactors for *MyoD* might bind to the A/T-rich elements in the *mef2c* skeletal muscle enhancer to positively affect *MyoD* function. This notion raised the possibility that the binding of MEF2 to these sites might be facilitated by binding of myogenic bHLH proteins to neighboring E boxes. While we considered this model to be unlikely, we believed that it might be possible for MEF2 to bind to the A/T(2) element if *MyoD* was already bound to the E2 E box, based on the position of those sites relative to each other (Fickett, 1996).

We tested the idea that cooperative binding might occur at the E2 E box and the A/T(2) element by EMSA analysis using an oligonucleotide probe encompassing both sites and the extended flanking sequences surrounding both elements (Fig. 6). Myogenic bHLH/E12 heterodimers efficiently bound to this long oligonucleotide probe, E2-A/T(2), and retarded its mobility (Fig. 6, lane 5). The binding of Myo/E12 to this probe encompassing both the A/T(2) and E2 sites was specific since binding was competed by the MCK Right E box (Fig. 6, lane 6) and by the *mef2c* E2 E box (Fig. 6, lane 7). Consistent with the results presented in

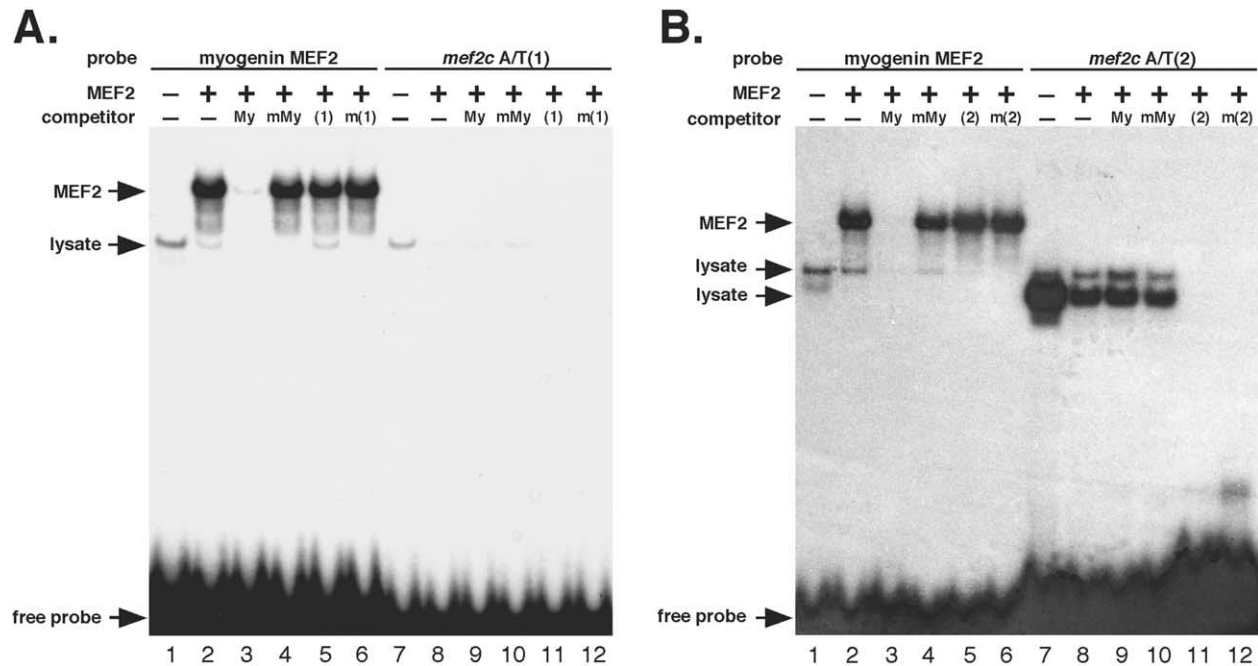


Fig. 4. MEF2 proteins do not bind to either of the A/T-rich sequences in the *mef2c* skeletal muscle enhancer. (A) MEF2 EMSA of the *myogenin* MEF2 site and the *mef2c* A/T(1) element. (B) MEF2 EMSA of the *myogenin* MEF2 site and the *mef2c* A/T(2) element. MEF2A was transcribed and translated in vitro and used in EMSA analyses with radiolabeled double-stranded oligonucleotides representing the *myogenin* MEF2 site (panels A and B, lanes 1–6), the *mef2c* A/T(1) element (panel A, lanes 7–12), or the *mef2c* A/T(2) element (panel B, lanes 7–12). MEF2 efficiently bound to the *myogenin* MEF2 site (panels A and B, lane 2), but failed to bind to either of the A/T-rich elements from the *mef2c* skeletal muscle enhancer (panels A and B, lane 8). Binding of MEF2 to the *myogenin* MEF2 site was specific since a 100 fold excess of cold competitor site (My) efficiently competed for binding (both panels, lane 3), but a 100 fold excess of a mutant *myogenin* MEF2 site (mMy) did not compete for binding (both panels, lane 4). By contrast, 100 fold excesses of unlabeled A/T(1) site (panel A, lane 5) and unlabeled A/T(2) site (panel B, lane 5) failed to compete for binding to the *myogenin* MEF2 site. Addition of mutant A/T-rich sequences, mutA/T(1) or mutA/T(2), denoted in the figure as m(1) and m(2), respectively, had no effect on competition (panels A and B, lanes 6 and 12). In both panels, lanes 1 and 7 contains unprogrammed cell lysate (lysate-derived mobility shifts are noted).

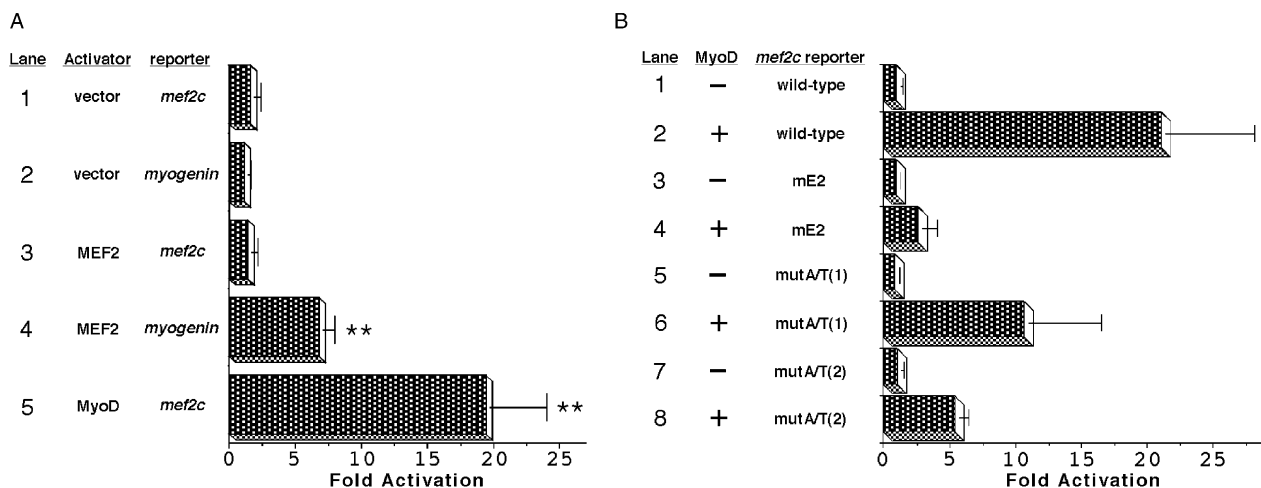


Fig. 5. *Trans*-activation of the *mef2c* skeletal muscle enhancer by MyoD but not by MEF2. (A) Either a MEF2C expression plasmid (lanes 3 and 4), MyoD expression plasmid (lane 5), or the parental expression vector (lanes 1 and 2) were cotransfected with either a *mef2c* or *myogenin-lacZ* reporter plasmid, as indicated. MEF2 failed to *trans*-activate the *mef2c* reporter (lane 3) under conditions in which it significantly activated the *myogenin* reporter (lane 4). MyoD also significantly activated the *mef2c* reporter (lane 5). Asterisks indicate significant activation, $p < 0.001$. (B) A MyoD expression plasmid (lanes 2, 4, 6 and 8) or parental expression vector without cDNA insert (lanes 1, 3, 5 and 7) was cotransfected with the indicated *mef2c* skeletal muscle enhancer fragments directing expression of a *lacZ* reporter gene. MyoD efficiently *trans*-activated the wild-type reporter (lane 2) and this activation was dependent on the E2 E box in the enhancer (lane 4). *Trans*-activation by MyoD was also partly dependent on the A/T(1) and A/T(2) sites in the enhancer (lanes 6 and 8, respectively). 5 μ g of each plasmid were transfected in each sample (10 μ g plasmid total for each sample). The data shown represent the mean values obtained in three independent transfections and analyses. Error bars represent the standard error of the mean for each of the sets of three experiments.

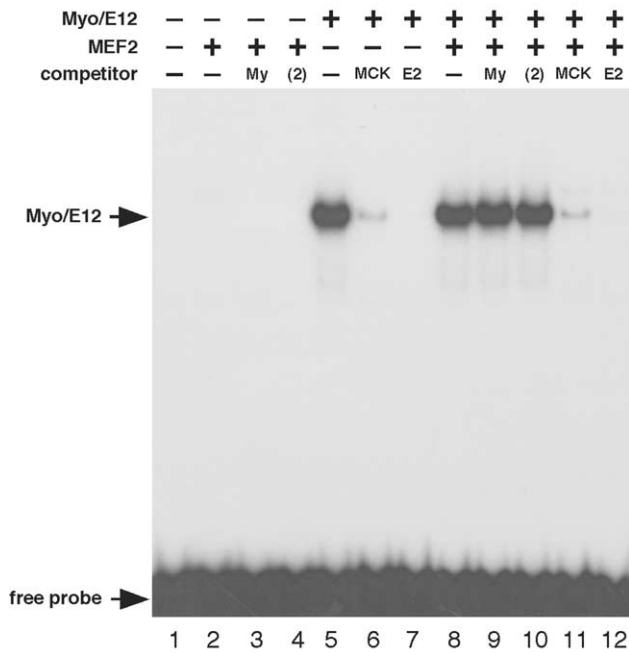


Fig. 6. MEF2 fails to bind to the A/T(2) element when myogenic bHLH heterodimers are bound at the E2 E box. A long, double-stranded oligonucleotide, E2-A/T(2), encompassing the A/T(2) element and the E2 E box from the *mef2c* skeletal enhancer was radiolabeled and used in EMSA analyses with in vitro transcribed and translated MEF2A (lanes 2–4, 8–12) and with in vitro cotranscribed and cotranslated Myogenin/E12 (lanes 5–12). Myo/E12 heterodimers efficiently bound to the E2-A/T(2) probe and retarded its mobility (lane 5). Binding of Myo/E12 to this probe was mediated by the E2 E box present in the probe since binding was efficiently competed by an excess of unlabeled MCK Right E box (lane 6) and by an excess of the *mef2c* E2 E box (lane 7). MEF2 failed to bind to the A/T(2)-element present in this long oligonucleotide probe (lane 2). The binding of Myo/E12 to the probe failed to facilitate binding of MEF2 to the site and MEF2 had no effect on the binding of Myo/E12 heterodimers (lane 8). Binding of myogenic bHLH heterodimers in the presence of MEF2 was competed by a 100 fold excess of the MCK Right E box (lane 11) and by the *mef2c* E2 E box (lane 12) but not by a 100 fold excess of either the *myogenin* MEF2 site (My) or the *mef2c* A/T(2) element, denoted as (2), (lanes 9 and 10). Lane 1 contains unprogrammed cell lysate included in the EMSA with the E2-A/T(2) probe.

Fig. 4B, MEF2 was also completely incapable of binding to this longer probe encompassing the A/T(2) element (Fig. 6, lane 2). Even in the presence of bound Myo/E12 heterodimer, MEF2 failed to bind to the E2-A/T(2) probe (Fig. 6, lane 8). These results demonstrate that a myogenic bHLH factor bound at the E2 E box cannot facilitate binding of MEF2 to the nearby A/T(2) element. This conclusion is further supported by the observation that cotransfection of MyoD and *mef2c* expression plasmids with the 954 bp *mef2c* skeletal muscle enhancer *lacZ* reporter plasmid resulted in no cooperative activation of the reporter (data not shown), demonstrating that MyoD activity on the *mef2c* enhancer is not modulated by MEF2. This observation further supports the data presented in Figs. 4–6, which demonstrate that the A/T-rich elements in the *mef2c* skeletal muscle enhancer do not represent MEF2 sites.

2.4. Analysis of cis-acting elements in the *mef2c* skeletal muscle enhancer in vivo

Based on the *trans*-activation studies presented in Fig. 5B, which showed that the A/T(1) A/T(2), and E2 sites were important for MyoD-dependent activation of *mef2c*, we tested the role of each of these sites on enhancer function in vivo. We generated transgenic mice that contained mutations in the E2 E box, A/T(1) element, or the A/T(2) element in the context of the 954 bp skeletal muscle enhancer fragment, and we determined the effect of those mutations on enhancer function at 11.5 dpc (Fig. 7). The wild-type fragment directed strong expression of *lacZ*

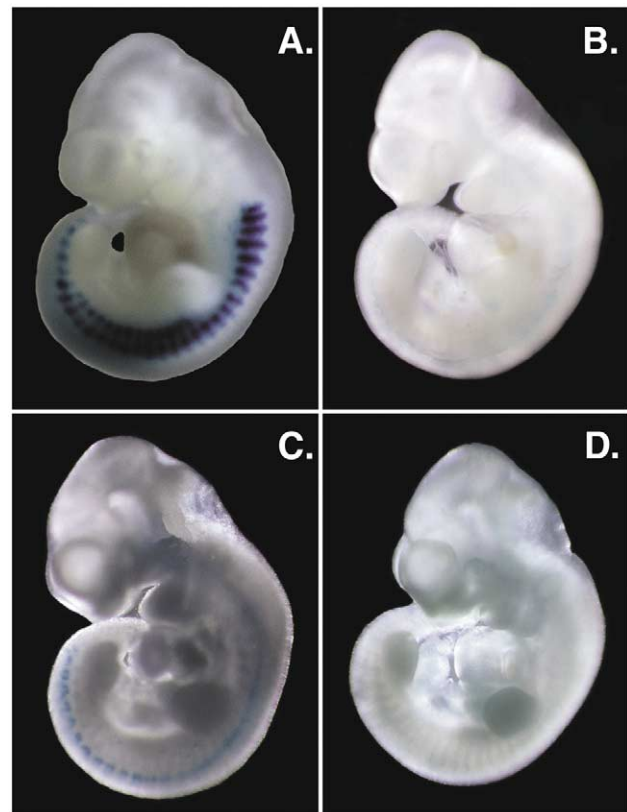


Fig. 7. Expression directed by the *mef2c* skeletal muscle enhancer in vivo is dependent on multiple evolutionarily conserved *cis*-acting elements. The wild-type *mef2c* skeletal muscle enhancer fragment (panel A), the E2 E box mutant (panel B), the A/T(1) mutant (panel C), and the A/T(2) mutant (panel D) were fused to the HSP68-*lacZ* reporter and used to generate transgenic mice. Representative transgenic embryos for the wild-type enhancer construct and each mutant at 11.5 dpc are shown. The wild-type fragment directed strong expression to the myotomal compartment of the somites (panel A). The E2 E box mutant and the A/T(2) mutant ablated expression (panels B and D, respectively). The A/T(1) mutant severely reduced expression of the reporter, but weak expression could be seen in caudal somites (panel C). For the wild-type enhancer construct, 8 independent transgenic events all resulted in skeletal muscle specific expression. For the E2 E box mutant, 5 independent transgenic founders were examined and none exhibited skeletal muscle expression of *lacZ*. For the A/T(1) and A/T(2) mutants, 7 and 5 independent transgenic founders were examined, respectively.

in the somites at this stage (Figs. 2B and 7A). By contrast, mutation of the E2 E box completely ablated activity of the enhancer (Fig. 7B). We also examined the effect of this mutation at 9.5 and 13.5 dpc, and again no X-gal staining was observed at any stage (data not shown). These results indicate that the E2 E box is essential for expression of this *mef2c* skeletal muscle enhancer throughout embryonic muscle development. Mutation of the A/T(1) element consistently disrupted enhancer activity, but weak expression in caudal somites was sometimes observed (Fig. 7C). Likewise, mutation of the A/T(2) element also ablated enhancer activity, although very weak staining could occasionally be observed in the myotomal region of the somites (Fig. 7D). The results of these mutational analyses on enhancer function in vivo are strikingly consistent with the results of the MyoD *trans*-activation studies shown in Fig. 5B. In both sets of experiments, the E2 E box was completely required for activity, the A/T(1) element had a quantitative effect on expression, and the A/T(2) site was critical for enhancer function, but less so than the E2 E box.

3. Discussion

In this study, we have shown that *mef2c* is a direct target for myogenic bHLH proteins during skeletal muscle development in vivo via a high affinity E box in the *mef2c* skeletal muscle enhancer. In addition, our results suggest that the skeletal muscle enhancer of *mef2c* requires two A/T-rich *cis*-acting sequences for activation by myogenic bHLH proteins. Previous studies have shown that MyoD family members are sufficient to activate myogenesis (Olson, 1990; Weintraub, 1993), yet myogenic bHLH factors require MEF2 function for this dominant inducing ability (Ornatsky et al., 1997). While initially this may seem paradoxical, our observations presented here suggest that MyoD family members may be able to independently activate and maintain the expression of their own essential cofactor, MEF2. Consistent with this notion, the binding of myogenic bHLH factors to the E2 E box in the *mef2c* skeletal muscle enhancer was the strongest binding we have ever observed (Fig. 3 and data not shown). This suggests that *mef2c* represents a rare example of a skeletal muscle gene which is a direct target of myogenic bHLH specification factors, yet does not require activation by MEF2.

In an earlier study, Wang et al. reported similar findings for the skeletal muscle enhancer of *mef2c* (Wang et al., 2001). They showed that myogenic bHLH proteins activate the expression of *mef2c* during skeletal muscle development and that expression of *mef2c* was maintained by the combinatorial activities of MEF2 and myogenic bHLH proteins (Wang et al., 2001). The work presented in that study showed the importance of the E2 E box and the A/T(2) element for expression of *mef2c* in vivo. In the studies presented here, we also show the critical role of the E2 E

box and A/T(2) site for expression in vivo (Fig. 7). The work presented here also defines an important role for an additional A/T-rich element, A/T(1) (Figs. 5B and 7C), which was not examined in the earlier study. The major difference between the two studies is in the interpretation of the role of MEF2 in the activation and maintenance of *mef2c* expression. In the earlier study, Wang and coworkers define the A/T(2) element as a MEF2 site (Wang et al., 2001). By contrast, our studies clearly show that MEF2 does not bind to either of the A/T-rich elements in the *mef2c* skeletal muscle enhancer in the same experiments where MEF2 robustly bound to a *bona fide*, control MEF2 site (Fig. 4). Furthermore, neither of the A/T-rich sequences could compete for MEF2 binding to the *bona fide* MEF2 site from the *myogenin* gene, even when present in a 100 fold excess (Fig. 4). The notion that the A/T-rich elements in the *mef2c* skeletal muscle enhancer do not represent binding sites for MEF2 is further supported by the observation that MEF2 cannot *trans*-activate the *mef2c* skeletal muscle enhancer in vivo in spite of the presence of the A/T-rich elements in the enhancer fragment (Fig. 5A). All of these observations are consistent with the fact that neither of the A/T-rich elements in the *mef2c* enhancer matches a consensus MEF2 sequence (Andres et al., 1995; Dodou et al., 1995). The work presented in this study suggests that the essential role for the A/T-rich elements in the *mef2c* skeletal muscle enhancer is due to their requirement for MyoD-dependent activation rather than MEF2 binding. We cannot explain the difference between the two studies regarding the role of MEF2 in binding and activation of the skeletal muscle enhancer of *mef2c*. However, this represents an important distinction between the two studies since the earlier study suggests a model in which MEF2 plays an essential role in autoregulation of *mef2c* (Wang et al., 2001). By contrast, our study suggests a model in which myogenic bHLH proteins are able to activate and maintain expression of *mef2c* independent of MEF2 binding to the enhancer.

The mechanism through which the A/T-rich elements in the *mef2c* enhancer may function in MyoD-dependent activation and expression of *mef2c* in vivo is intriguing. Our data show that these elements do not bind MEF2 in vitro (Figs. 4 and 6), nor are they *trans*-activated by MEF2 in cell culture (Fig. 5A). However, these elements, particularly the A/T(2) element, are important for enhancer function in vivo (Figs. 5B and 7)(Wang et al., 2001). There are several possible explanations, based on the results of the studies presented here, that might account for the requirement of the A/T(2) site for enhancer function. Our results demonstrate that MEF2 does not bind directly to the A/T(2) site (Figs. 4 and 6), and our data further show that neither cooperative binding (Fig. 6) nor cooperative activation (data not shown) of the enhancer by MEF2 and myogenic bHLH proteins occurred. We cannot completely rule out the possibility that MEF2 proteins could bind to the A/T-rich elements in the *mef2c* enhancer in vivo. However, if binding of MEF2 to these elements does occur in vivo, then the binding must

take place with extremely low affinity or represent a transient interaction, such that the binding is not detectable in vitro (Figs. 4 and 6) and is not sufficient for *trans*-activation of the enhancer in vivo (Fig. 5A). Based on the studies presented here, we favor a model in which the A/T-rich elements in the enhancer are required in vivo for the binding of regulators other than MEF2 proteins. Consistent with this possibility are the results of several earlier studies that have demonstrated that transcription factors other than MEF2 bind to A/T-rich sequences in other muscle enhancers (Beason et al., 1999; Ghatpande et al., 1999; Zhou et al., 1993; Zhu et al., 1993). If this is the case then the A/T-rich elements in the skeletal muscle enhancer of *mef2c* may represent binding sites for transcriptional coregulators of myogenic bHLH factors based on the requirement for these elements for efficient *trans*-activation by MyoD (Fig. 5B).

The results presented here suggest a model for the activation and maintenance of *mef2c* expression that is dependent on direct and indirect activation by myogenic bHLH proteins. In this model, the myogenic bHLH specification factors MyoD and Myf5 would serve to activate the expression of *mef2c*, while the myogenic bHLH differentiation factors myogenin and MRF4, would account for the maintenance of *mef2c* expression. This model provides an explanation for why myogenin is able to activate MEF2 independent of other muscle gene products (Cserjesi and Olson, 1991) and would explain the mechanism through which myogenin and *mef2c* upregulate each other's expression (Ridgeway et al., 2000). This would then serve to reinforce skeletal muscle differentiation since together these two factors cooperatively activate the cascade of downstream contractile protein expression required to make a functional myocyte.

The enhancers for several skeletal muscle transcription factors that are activated downstream of MyoD and Myf5 have now been characterized, including *myogenin*, *MRF4*, and *mef2c* (Black et al., 1995; Cheng et al., 1992, 1993; Edmondson et al., 1992; Naidu et al., 1995; Pin et al., 1997; Spitz et al., 1998; Wang et al., 2001; Yee and Rigby, 1993). While the *mef2c* skeletal muscle enhancer appears to be organized similarly to the *myogenin* and *MRF4* enhancers, it is regulated in quite a different manner. In the case of *mef2c*, myogenic bHLH proteins directly activate *mef2c* expression via an extremely high affinity E box in the enhancer, but autoregulation of *mef2c* by MEF2 does not occur in the context of the 954 bp skeletal muscle enhancer. Overall, these different enhancers create an emerging picture of myogenesis in which the same factors may be involved in the activation of transcription factor genes required for muscle differentiation. However, the complex circuitry for how these factors interact with each other and with their respective *cis*-acting elements is likely to account for the fine tuned regulation of gene expression during myogenesis.

During mouse development, *mef2c* is expressed in skeletal, cardiac, and smooth muscle, as well as in other

tissues including brain, spleen, and neural crest (Edmondson et al., 1994; Leifer et al., 1993, 1994; Lyons et al., 1995; Swanson et al., 1998). The studies presented here have defined the regulation of a single, modular enhancer that is sufficient to direct expression of *mef2c* only in skeletal muscle. The regulation of *mef2c* in skeletal muscle by myogenic specification factors via an independent enhancer element raises the question as to whether *mef2c* expression in other tissues is controlled by other independent enhancers. In this regard, we have recently identified separate enhancer fragments from the *mef2c* gene that are capable of independently directing *lacZ* transgene expression to a variety of other lineages, including embryonic heart, smooth muscle, and neural crest (ED and BB, unpublished observations). It will be interesting to determine whether these additional enhancers are also targets of tissue specific transcription factors in a manner analogous to the independent activation of the skeletal muscle enhancer by myogenic bHLH factors described here.

4. Experimental procedures

4.1. Cloning, plasmids, and mutagenesis

A 954 bp fragment of the mouse *mef2c* gene encompassing the skeletal muscle enhancer region examined in this study was cloned by PCR using the primers: 5'-agcatactcttcttagcagc-3' and 5'-gtccaaactctgactagtaaaaa-3'. The resulting product was cloned as an XbaI-SpeI fragment into the SpeI site of pBluescript SKII(+) (Stratagene) and was subcloned further into the HSP68-*lacZ* transgenic reporter plasmid (Kothary et al., 1989) to generate the plasmid 73k-HSP68-*lacZ*. This reporter was used to generate transgenic mice and for transfection analyses in tissue culture. The expression plasmids EMSV.MyoD, pCDNA1.MEF2A, and pCDNA1.MEF2C are described elsewhere (Black et al., 1995). The *myogenin-lacZ* reporter plasmid p1565myolacZ has also been described (Cheng et al., 1992). Plasmids pCITE.E12, pCITE.myogenin, and pCITE.MyoD contain the coding regions for E12, myogenin, and MyoD cloned into the translational enhancement vector pCITE-2A (Novagen). Mutations in the *mef2c* skeletal muscle enhancer fragment were generated using the PCR mutagenesis technique of gene splicing by overlap extension (Gene SOEing) (Horton, 1997) to create the following mutant sequences in the context of the 954 bp fragment: mE2, 5'-actccagagtgcacatgaattcgtgcac-3'; mutA/T(1), 5'-tcttgaaatgtaattttggcgctgt-3'; mutA/T(2), 5'-caatgatacctttacagatctattac-3'. The entire sequence of each mutant fragment was confirmed by sequencing on both strands and was subcloned into HSP68-*lacZ* (Kothary et al., 1989) for analysis in transgenic mice and in cell culture *trans*-activation analyses. The GenBank accession number for the sequence of the mouse *mef2c* skeletal muscle enhancer defined in these studies is AY321453.

4.2. Generation and analysis of transgenic mice

Transgenic reporter fragments were digested and gel purified from the plasmid backbone using standard techniques. Transgene fragments were suspended in 5 mM Tris–Cl, 0.2 mM EDTA, pH 7.4 at a concentration of 2 ng/μl and injected into the male pronuclei of fertilized oocytes from C57Bl6xCBA F1 hybrid mice as described previously (Hogan et al., 1994). Injected embryos were implanted into pseudopregnant CD-1 females, and embryos were collected at indicated time points for transient analysis or were allowed to develop to adulthood for establishment of stable transgenic lines. β-galactosidase expression from *lacZ* transgenic embryos was detected as described previously (Hogan et al., 1994). Briefly, embryos were removed from humanely sacrificed pregnant female mice. The yolk sac and amnion were removed and saved for genotype analysis. Embryos were fixed in 2% formaldehyde, 0.2% glutaraldehyde in phosphate buffered saline (PBS) at 4 °C for 30 min to 2 h, based on the size of the embryo. Embryos were rinsed twice in PBS and stained for β-galactosidase overnight in the dark at room temperature in PBS containing 5 mM K₄Fe(CN)₆, 5 mM K₃Fe(CN)₆, 2 mM MgCl₂, 1 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal). Following staining, embryos were rinsed twice in PBS and post-fixed in 4% formaldehyde overnight at 4 °C. Embryos collected at 13.5 dpc were dehydrated in ethanol and cleared for 1–3 h in a 1:1 mixture of benzyl alcohol and benzyl benzoate prior to photography for better visualization of staining under the skin.

DNA was extracted from the yolk sac and amnion of embryos or from tail biopsies from mice by digestion in tail lysis buffer (100 mM NaCl, 25 mM EDTA, 1% SDS, 10 mM Tris–Cl, 200 μg/ml proteinase K, pH 8.0) at 56 °C overnight. Digested samples were extracted once with phenol:chloroform and ethanol precipitated. DNA preparations were digested with SacI and analyzed by Southern blot using a radiolabeled *lacZ* probe.

4.3. Cell culture, transfections, and β-galactosidase assays

C3H10T1/2 (10T1/2) cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfections were performed by calcium phosphate precipitation as described elsewhere (Black et al., 1995). In each transfection, 5 μg of either *myogenin-lacZ* (Cheng et al., 1992) or *mef2c-lacZ* reporter plasmid or the indicated mutant version of the reporter were cotransfected along with either 5 μg of EMSV.MyoD expression plasmid or 5 μg of pCDNA1.MEF2C expression plasmid by mixing the DNA with 0.187 ml of 0.25 M CaCl₂ and 0.187 ml of 2 × BBS (50 mM BES, 250 mM NaCl, 1.5 mM Na₂HPO₄, pH 6.95) and adding this mixture to subconfluent monolayers of cells in 60 mm dishes. In samples where a cDNA expression plasmid was not

transfected, an equal amount of the parental expression vector was transfected. Transfected cells were harvested, and cellular extracts were prepared by sonication and were normalized as described previously (Cripps et al., 1998). Chemiluminescent β-galactosidase assays were performed using the Luminescent β-gal kit (Clontech) according to manufacturer's recommendations and relative light units were detected using a Tropic TR717 microplate luminometer (PE Applied Biosystems).

4.4. Electrophoretic mobility shift assays

DNA binding reactions were performed as described previously (Gossett et al., 1989). 2 μg of double-stranded oligonucleotides for use in binding reactions were labeled with ³²P-dCTP using Klenow to fill in overhanging 5' ends and purified on a nondenaturing polyacrylamide-TBE gel. Binding reactions were preincubated at room temperature for 10 min prior to probe addition in 1 × binding buffer (40 mM KCl, 15 mM HEPES pH 7.9, 1 mM EDTA, 0.5 mM DTT, 5% glycerol) containing 2 μg of recombinant protein (MEF2A, MEF2C, MEF2D, MyoD/E12, Myogenin/E12) or unprogrammed lysate, 1 μg of poly dI–dC, and competitor DNA (100 fold excess where indicated). Reactions were incubated an additional 20 min at room temperature after probe addition and then were electrophoresed on a 6% nondenaturing polyacrylamide gel. The MCK Right E box oligonucleotides and the mutant form of the MCK Right E box that does not bind bHLH heterodimers (mMCK) have been described (Amacher et al., 1993; Brennan and Olson, 1990). The oligonucleotides for the myogenin MEF2 site (My) and a mutant form of that site (mMy) have also been described (Yee and Rigby, 1993). The sense strand sequences of the oligonucleotides used for EMSA of the putative elements in the *mef2c* skeletal muscle enhancer were each confirmed by independent sequence analysis and were as follows: E1 E box, 5'-ggttataccatgaaagcc-3; E2 E box, 5'-gagtgacatgaacaggtgcaccctggcctg-3; E3 E box, 5'-ggcctgccagacactgtgcagagggatcacgca3; A/T(1), 5'-ggaaatgtaattttaaagccc-3; A/T(2), 5'-ggatacctttacagctaaatttactccagagtgaca-3; mE2, 5'-gagtgacatgaattcgtgcaccctggcctg-3; mutA/T(1), 5'-ggaaatgtaatttggcgc-3; mutA/T(2), 5'-ggatacctttacagatctatttactccagagtgaca-3; E2-A/T(2) double site oligo, 5'-gatacctttacagctaaatttactccagagt-gacatgaacaggtgcaccctcctg-3'.

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