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The *Mef2c* gene is a direct transcriptional target of myogenic bHLH and MEF2 proteins during skeletal muscle development

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SUMMARY

Members of the MEF2 family of transcription factors are upregulated during skeletal muscle differentiation and cooperate with the MyoD family of myogenic basic helix-loop-helix (bHLH) transcription factors to control the expression of muscle-specific genes. To determine the mechanisms that regulate MEF2 gene expression during skeletal muscle development, we analyzed the mouse *Mef2c* gene for *cis*-regulatory elements that direct expression in the skeletal muscle lineage in vivo. We describe a skeletal muscle-specific control region for *Mef2c* that is sufficient to direct *lacZ* reporter gene expression in a pattern that recapitulates that of the endogenous *Mef2c* gene in skeletal muscle during pre- and postnatal development. This control region is a direct target for the binding of myogenic bHLH and MEF2 proteins. Mutagenesis of the *Mef2c* control region shows that a binding site for myogenic bHLH proteins is essential for expression at all stages of skeletal muscle development, whereas an adjacent MEF2 binding site is required for maintenance but not for initiation of *Mef2c* transcription. Our findings reveal the existence of a regulatory circuit between these two classes of transcription factors that induces, amplifies and maintains their expression during skeletal muscle development.

Key words: Skeletal muscle, MEF2C, Mouse, bHLH, MEF2, Myogenesis

INTRODUCTION

The formation of skeletal muscle during embryogenesis involves a multi-step developmental program in which mesodermal progenitors become committed to a skeletal muscle fate and then propagate and migrate to specific destinations before differentiating to form myofibers (Hauschka, 1994). Skeletal muscle development is controlled by the MyoD and myocyte enhancer factor 2 (MEF2) families of transcription factors, which interact to establish a unique transcriptional code for activation of skeletal muscle-specific genes (Molkentin and Olson, 1996). Members of the MyoD family – MyoD, myogenin, Myf5 and MRF4 – are expressed exclusively in the skeletal muscle lineage and can each activate the complete muscle differentiation program in transfected fibroblasts (Davis et al., 1987; Braun et al., 1989; Edmondson and Olson, 1989; Rhodes and Konieczny, 1989). During embryogenesis, *MyoD* and *Myf5* are expressed in distinct early populations of skeletal muscle precursor cells in the somites (Ott et al., 1991; Smith et al., 1994). Mice that lack either *MyoD* or *Myf5* are able to form skeletal muscle (Rudnicki et al., 1992; Braun et al., 1992), whereas mice lacking both genes fail to form any trace of the skeletal muscle lineage (Rudnicki et al., 1993), consistent with the notion that these genes play overlapping roles in myoblast specification. Myogenin is expressed as myoblasts enter the differentiation pathway and is required for muscle differentiation (Hasty et al., 1993; Nabeshima et al., 1993). *Mrf4* is expressed transiently during early myogenesis and in differentiated muscle fibers (Hinterberger et al., 1991); its functions appear to overlap with those of *MyoD* in the muscle differentiation pathway (Rawls et al., 1998).

Members of the MyoD family share homology in a basic helix-loop-helix (bHLH) region that mediates dimerization and binding to the E-box consensus sequence (CANNTG), which is found in the control regions of many muscle-specific genes (Olson and Klein, 1994). The bHLH region also associates with MEF2 transcription factors, which lack myogenic activity alone, but potentiate the muscle-inducing activity of myogenic bHLH proteins and are required for the activation of muscle differentiation genes (Molkentin et al., 1995). The four vertebrate MEF2 factors – MEF2A, MEF2B, MEF2C and MEF2D – belong to the MADS (MCM1, agamous, deficiens, SRF) family of transcription factors (Black and Olson, 1998). The MADS domain mediates dimerization, association with myogenic bHLH proteins, and binding to an A/T-rich DNA sequence associated with muscle-specific genes (Gossett et al., 1989; Pollock and Treisman, 1991).

In contrast to the skeletal muscle specificity of myogenic bHLH factors, MEF2 factors are expressed in skeletal, cardiac

and smooth muscle cells, as well as in neurons (Breitbart et al., 1993; Chambers et al., 1992; Yu et al., 1992; Leifer et al., 1993; Martin et al., 1993, Martin et al., 1994; Lyons et al., 1995; Edmondson et al., 1994; Ticho et al., 1996), and at lower levels in several other cell types. During embryogenesis, *Mef2c* is expressed at the onset of differentiation of the cardiac and skeletal muscle lineages and is followed by expression of the other MEF2 genes (Edmondson et al., 1994). Mice that lack *Mef2c* die at about E9.5 from cardiovascular defects, precluding analysis of the role of *Mef2c* in skeletal muscle development in vivo (Lin et al., 1997). Mice homozygous for mutations in *Mef2a* or *Mef2b* are viable, whereas mice lacking *Mef2d* die prior to gastrulation (our unpublished results). Loss-of-function mutations of the single *Mef2* gene in *Drosophila* result in a block to differentiation of all muscle cell types (Lilly et al., 1995; Bour et al., 1995; Ranganayakulu et al., 1995), indicating that MEF2 genes are required for muscle differentiation.

In addition to activating subordinate muscle structural genes, myogenic bHLH and MEF2 factors auto- and crossregulate the expression of one another. Forced expression of myogenic bHLH genes in non-muscle cell types is sufficient to activate expression of the endogenous myogenic bHLH genes and to upregulate expression of MEF2 genes (Lassar et al., 1991; Cserjesi and Olson, 1991). MEF2 alone cannot induce expression of myogenic bHLH genes (Molkentin et al., 1995), but the promoters of the myogenin and *Mrf4* genes contain binding sites for myogenic bHLH and MEF2 factors that control transcription during myoblast differentiation in vivo and in vitro (Cheng et al., 1993; Yee and Rigby, 1993; Black et al., 1995; Naidu et al., 1995). Binding of MEF2 to these sites provides a mechanism for amplifying the expression of these regulatory genes and stabilizing the muscle phenotype. Whether myogenic bHLH proteins act directly on MEF2 genes to upregulate their expression, or whether they induce MEF2 expression through an indirect mechanism is unknown because regulatory elements for vertebrate MEF2 genes have not been identified.

To further understand the mechanisms that regulate MEF2 expression during myogenesis, we sought to identify *cis*-regulatory elements responsible for transcription of the mouse *Mef2c* gene during skeletal muscle development. We describe a novel skeletal muscle-specific control region upstream of the *Mef2c* gene that is sufficient to direct the expression of a *lacZ* transgene in a spatiotemporal expression pattern that mimics that of the endogenous gene during mouse development. Mutational analysis of this control region shows that it is a direct target for myogenic bHLH and MEF2 factors in vivo, revealing a transcriptional circuit through which these transcription factors induce, amplify and maintain *Mef2c* expression during muscle development.

MATERIALS AND METHODS

5′-RACE

5′-RACE (rapid amplification of cDNA ends) cloning was performed as described previously (Wang et al., 1999). Briefly, total RNA was isolated from adult mouse skeletal muscle using Trizol reagent (LifeTech). 5 μg of RNA was used for first-strand cDNA synthesis with random hexamers. A PCR-based RACE procedure was carried out using the Marathon cDNA amplification kit (Clontech) according to the manufacturer's instructions. *Mef2c* gene-specific primers were as follows: 2C-RACE1, 5′-GTGTTTCTTCTCTCTCTCGTCCCTG-3′; and 2C-RACE2, 5′-GCACAGCTCAGTTCCCAAATCCCTG-3′.

An aliquot of the first PCR products was also used for subsequent nested PCR. Amplified cDNAs were gel-purified and subcloned into the pGEM-T-Easy vector (Promega) and sequenced. Multiple overlapping clones were isolated through this approach.

Genomic library screening, DNA cloning, mapping and sequencing

A mouse genomic library (Stratagene) was screened using a cDNA fragment obtained from 5′-RACE as a probe. Three positive clones were isolated and sub-cloned into the pBlueScript vector (Stratagene). Restriction mapping and DNA sequencing were performed as described previously (Lin et al., 1998).

Generation and analysis of transgenic mice

Different fragments of the *Mef2c* 5-flanking region were subcloned into the hsp68-*lacZ* vector (Kothary et al., 1989) to make reporter constructs (detailed in Fig. 2). Transgenes were prepared and injected into the male pronuclei of fertilized oocytes from B6C3F1 mice as described previously (Cheng et al., 1992). Injected oocytes were then transplanted into ICR pseudopregnant females and embryos were harvested at the desired embryonic or neonatal time points indicated in the text and figure legends.

lacZ transgene expression was detected as previously described (Cheng et al., 1992). Briefly, embryos were dissected out of sacrificed mothers. Yolk sacs were removed for *lacZ* PCR genotyping. The amnion was also removed and embryos were fixed at 4°C in 2% paraformaldehyde and 0.2% glutaraldehyde in phosphate-buffered saline (PBS) for variable amounts of time, depending on the size of the embryo. Fixed embryos were washed in PBS at 4°C for 30 minutes. Embryos were stained overnight at room temperature in 1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$ and 5 mM $K_4Fe(CN)_6$ in PBS.

Mice carrying *lacZ* transgenes were identified by PCR analysis. Genomic DNA isolated from tail biopsies or embryonic yolk sacs was digested in lysis buffer (10 mM Tris (pH 8.0), 25 mM EDTA, 100 mM NaCl, 1% SDS, 0.2 mg/ml Proteinase K) at 55°C overnight, followed by removal of protein by phenol/chloroform extraction and ethanol precipitation. A typical PCR reaction contained genomic DNA template, 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.16 mM of each dNTP, 1 μM of each primer and 2.5U *Taq* polymerase (Promega) in a 25 μl total volume. A typical temperature profile included 32 cycles of DNA strand melting at 95°C for 30 seconds, primer annealing at 55°C for 30 seconds and polymerization at 72°C for 30 seconds. *lacZ* genotype analysis was performed by PCR on genomic DNA using the following primers: 5′-CAAACTGGCAGATGCACGGTTAC-3′ and 5′-CAGT-ACAGCGCGGCTGAAATC-3′, producing a 450 bp *lacZ*-specific product.

The preparation, sectioning and staining of embryos was performed using standard procedures. Briefly, β-galactosidase-stained embryos were post-fixed in 4% paraformaldehyde in PBS overnight at 4°C and embedded in paraffin after a stepwise dehydration with progressively higher concentrations of ethanol and two changes of xylene. Embryos were sectioned in increments of 5-7 μm. Sections were counterstained with nuclear Fast Red and cover-slipped with cytoseal or permount.

Gel mobility shift assays

Proteins for electrophoretic mobility shift assays (EMSA) were produced using the TNT T3/T7 Coupled Reticulocyte Lysate System (Promega). The DNA templates used for in vitro transcription of mouse MyoD, E12 and Myc-tagged MEF2C were: EMSV-MyoD, pCITE-E12 and pcDNA3.1-MEF2C-Myc, respectively. pEMSV-MyoD consists of a full length MyoD cDNA subcloned into the

expression vector pEMSVscribe (Harland and Weintraub, 1985). pCITE-E12 contains the E12 cDNA inserted into the pCITE expression vector (Novagen). pcDNA3.1-MEF2C-Myc contains the full-length mouse MEF2C cDNA with a C-terminal *Myc* epitope tag (McKinsey et al., 2000a; McKinsey et al., 2000b) subcloned into the pcDNA3.1 expression vector (Invitrogen). To ensure that proteins were appropriately translated, parallel transcription-translation reactions were performed in the presence of [^{35}S]methionine, separated by 10% SDS-PAGE and visualized using autoradiography.

The sequences of the sense strands of the oligonucleotides used as probes in the gel mobility shift assays were as follows: MEF2 site, 5′-ACCTTTACAGCTAAATTTACTCCAGAGTG-3′; and E-box, 5′-GAGTGACATGAACAGGTGCACCCTGGCCT-3′.

Gel mobility shift assays were also performed with oligonucleotides corresponding to the high-affinity right E-box (Chakraborty et al., 1991) and MEF2 sites (Cserjesi et al., 1994) from the MCK enhancer as a positive control. The oligonucleotides were generated with four extra nucleotides, GAGG, at their 5′ ends. Oligonucleotides were annealed at a concentration of 100 ng/μl and were end-labeled with [α-^{32}P]dCTP using the Klenow fragment of DNA polymerase I. All binding reactions were performed using 2 μg poly(dI:dC) and 100,000 cpm of probe in a total volume of 20 μl of binding buffer (400 mM KCl, 150 mM Hepes (pH 7.9), 10 mM EDTA, 5 mM DTT, 50% glycerol). Where indicated, unlabelled annealed oligonucleotide was used as competitor. Reactions were incubated at room temperature for 15-20 minutes. Where indicated, mouse monoclonal anti-Myc (Santa Cruz) and anti-MyoD (Pharmingen) antibodies were added to the reactions at concentrations of 45 and 33 ng/μl, respectively, and incubated for an additional 10-15 minutes. Binding reactions were analyzed by electrophoresis on 4% (MyoD binding) and 5% (MEF2C binding) non-denaturing polyacrylamide gels in 0.5× TBE. Gels were dried and binding was visualized by autoradiography.

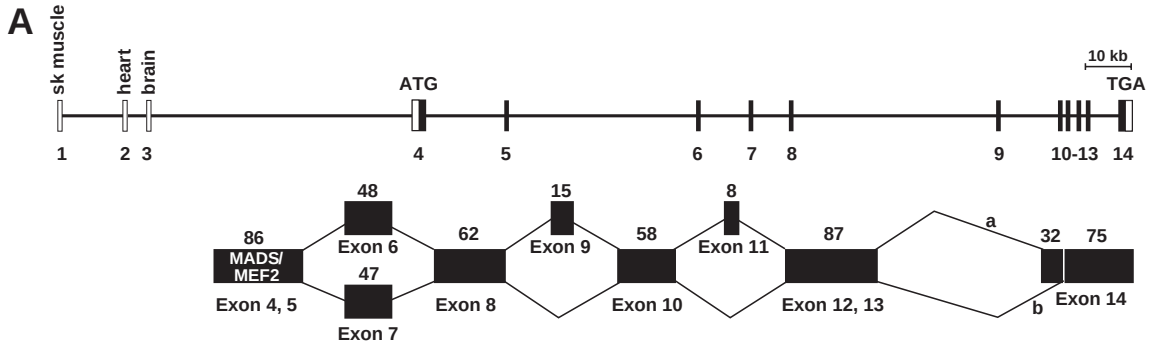
Site-directed mutagenesis

Mutagenesis of the MEF2 site and E-box was achieved using the overlap extension method, previously described (Horton and Pease, 1991). The DNA template used for mutagenesis and cloned into pBlueScript (Stratagene) included the nucleotide region from –512 bp to +41 bp. Mutant PCR products were then sub-cloned into the pGEM-T-Easy Vector (Promega). Mutated fragments were then excised using endogenous flanking restriction enzyme sites and cloned into the appropriate transgenic expression vector. All mutations were confirmed by DNA sequencing.

RESULTS

Cloning of the *Mef2c* 5′-UTR and determination of *Mef2c* genomic structure

The 5′-untranslated sequences of the *Mef2c* gene have not been previously defined. Therefore, as a first step toward identifying regulatory regions responsible for *Mef2c* transcription in skeletal muscle, we performed 5′-RACE on mouse skeletal muscle mRNA using primers from the cDNA sequence



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ATCACGCATCTCACCGCTTGACGATCAAGGGGGCAAAGCTTC
GGTGTTCATAGAAAAGGAGAGGAGGCGAGCGCAGCCCAAACT
GGGGGGTTTCTCTTCAAAGCCAGCTGGTCTGGCTTTATTCTA
CAGGAATTTTTTTACCTGTCAGAGTTTGGACAACAAAGCCCT
CAGCAGGTGCTGACGGGAACAACTTCTGGAGAAGCAGAAAGG
CACTGGTGCCAACAAAAGCATTGAAAACTGTGAACGTAACCT
CTGGAGAGATGAAGTGAAGCGTGGAAGGCCAATGGCTGTGGC
AGTGAAGAAGTGCAGAGGGAACGAATGCAGGGATTTGGGAAC
TGAGCTGTGCGCGTGCTGAAGAAGGAAACGTGTTTGAAGGAA
ACAGGAAAGAGAAAGAAAAGGAAGGAAAAAATACATAATTTC
AGGGACGAGAGAGAGAAGAAACACGGGGACTATG
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Fig. 1. Schematic diagram of the mouse *Mef2c* gene. (A) The genomic structure of the mouse *Mef2c* gene is shown. Open boxes represent UTR. Closed boxes represent translated sequence. Skeletal muscle specific-transcripts contain exon 1 spliced directly to exon 4. Exons 2 and 3 are specific to heart and brain, respectively. The translational start site for *Mef2c* is located in exon 4 (ATG) and the stop codon (TGA) is located in exon 14. The contributions of exons 4-14 to the translated MEF2C protein are detailed in the black boxes below the genomic structure. Numbers above the boxes indicate the number of amino acids in each exon. The MADS/MEF2 domain is encoded by exons 4 and 5. Exons 6, 7, 9, 11 and 14 are used alternatively (see text). Exons 14a and 14b are generated by alternate splice acceptor sites that maintain the same open reading frame. However exon 14a is longer than exon 14b by 32 amino acids. (B) The skeletal muscle-specific 5′-untranslated sequence of *Mef2c* from the beginning of exon 1 to the translational start site (boxed) is given. The junction between exons 1 and 4 is located in the region between the arrowheads.

immediately 5′ of the translation initiation codon. Several overlapping cDNA clones containing extended 5′-untranslated sequence were obtained. One of the 5′-RACE clones was then used to screen a mouse genomic library, resulting in three overlapping genomic clones.

The structure of the mouse *Mef2c* gene was characterized by genomic DNA sequencing and restriction mapping, as well as comparison of human and mouse genomic DNA sequences from several databases. The deduced structure of the mouse *Mef2c* gene is shown in Fig. 1A. The protein-coding region of the gene comprises 11 exons (exons 4-14) distributed over approximately 200 kb of genomic DNA. As reported previously, several exons are contained in all *Mef2c* transcripts, while others are used alternatively (Martin et al., 1993; McDermott et al., 1993). Based on RT-PCR analysis of RNA from different tissue sources and on the presence of exon sequences in expressed sequence tags, exons 6 and 7 appear to be mutually exclusive, with exon 6 being used in transcripts from heart and brain, and exon 7 being specific to skeletal muscle. Exons 9 and 14b are also detected in heart and brain transcripts, but not in skeletal muscle transcripts; and exon 11 is specific to brain. The AUG codon is contained in a 193 bp exon (exon 4) that, in skeletal muscle transcripts, is spliced to an approximately 250 bp exon (exon 1), located approximately 80 kb upstream. The sequence of the 5′-UTR from skeletal muscle *Mef2c* transcripts are shown in Fig. 1B.

We also performed 5′-RACE with RNA from mouse heart and brain and identified two additional 5′ exons that appear to be preferentially used in those tissues (Fig. 1A). We did not identify additional 5′ exon sequence in RACE products from these tissues, suggesting that different promoters are used in

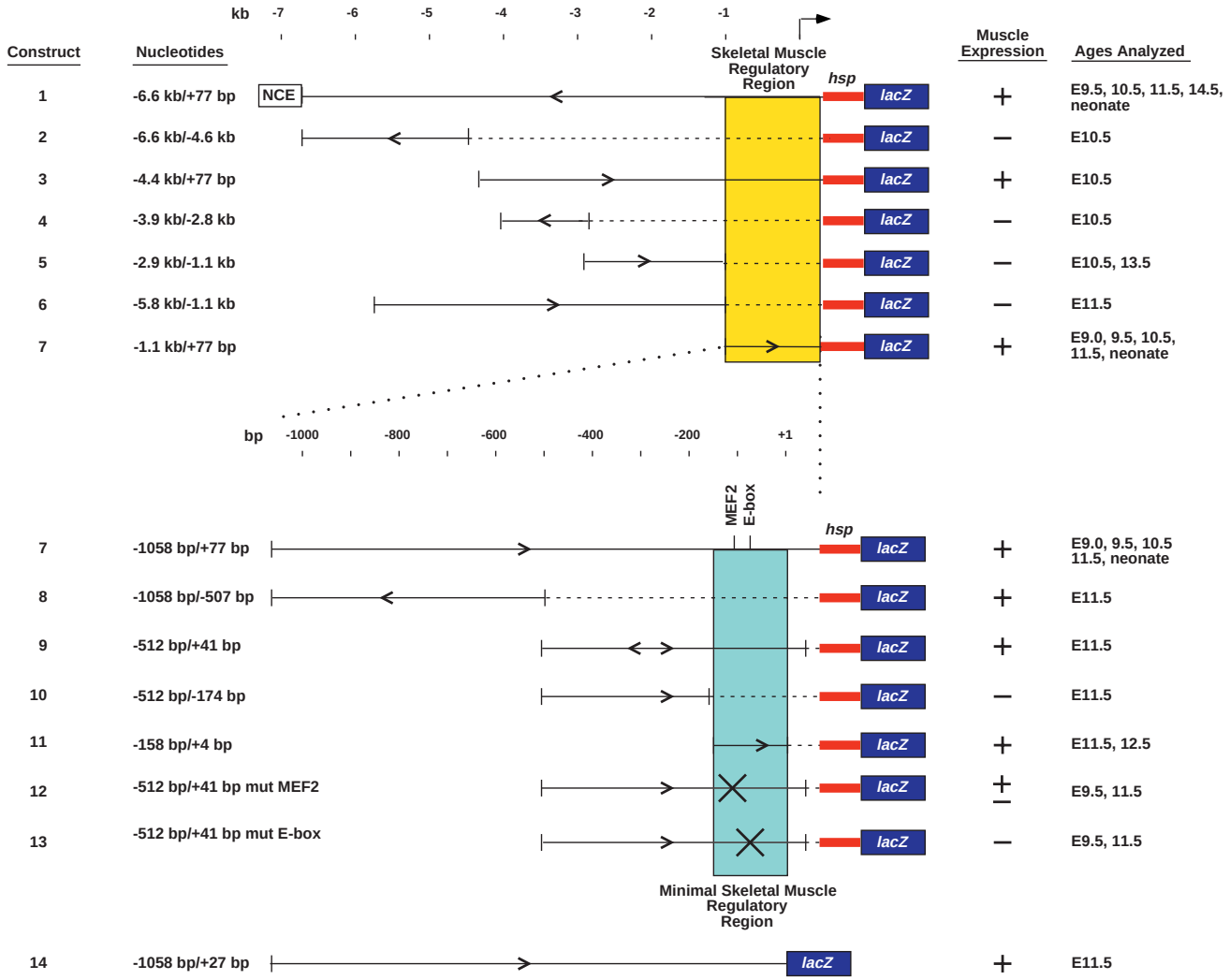

Fig. 2. Transgenes used to identify the *Mef2c* skeletal muscle control region. Regions of *Mef2c 5′* flanking DNA used in *lacZ* transgenes are shown. (Top) The region containing endogenous skeletal-muscle specific promoter activity is indicated in yellow. The position of the *Hand2* neural crest enhancer (NCE), which was used as an internal control for *lacZ* expression with construct 1, is shown. (Bottom) +1 indicates the transcriptional start site for skeletal muscle-specific transcripts. The minimal skeletal muscle regulatory region is indicated in pale blue. The location of the MEF2 binding site at –64 bp and the E-box at –38 bp are indicated in the enlarged construct 7. ×, mutation; >, sense orientation; <, antisense orientation; < >, sense or antisense orientation. A minimum of two transgenic F_0 embryos were analyzed for each construct. Multiple independent stable transgenic lines were also generated with constructs 7 and 11; they showed the same expression patterns seen in F_0 transgenic mice. Broken lines indicate deleted regions.

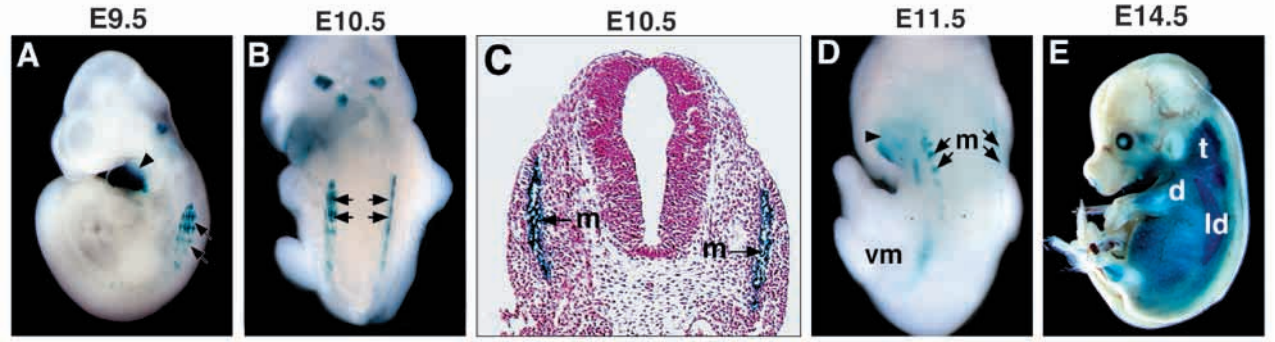

Fig. 3. β-galactosidase staining of mouse embryos harboring the *Mef2c-lacZ* transgene. F_0 embryos harboring construct 1, containing the region from –6.6 kb to +77 bp (see Fig. 2), were stained for *lacZ* expression. A neural crest enhancer (NCE) was also included in this construct as a positive control for *lacZ* activity. (A) An E9.5 transgenic embryo shows *lacZ* expression in rostral somites and weaker activity in more caudal somites. (B) Dorsal view of an E10.5 embryo shows expression in rostral and caudal somites. (C) Transverse section of an E10.5 embryo at the level of thoracic somites demonstrates *lacZ* staining in the myotome. (D) Dorsal view of an E11.5 embryo shows staining in somites and ventral myoblasts (vm). (E) At E14.5, transgene expression is evident throughout much of the embryonic musculature. Also evident is pharyngeal arch expression driven by the NCE (A,D), which is indicated by arrowheads. Arrows, somites; d, deltoid; ld, latissimus dorsi; m, myotome; t, trapezius; vm, ventral myoblasts.

skeletal muscle, brain and heart to generate the distinct 5′-untranslated regions of the transcripts from these tissues.

Identification of the *Mef2c* skeletal muscle regulatory region

To search for the regulatory region responsible for skeletal muscle expression of *Mef2c*, we fused a series of 5′ genomic fragments to the *hsp68* basal promoter upstream of a *lacZ* reporter gene and tested for expression in F_0 transgenic mouse embryos (Fig. 2). We initially included the *hsp68* basal promoter in the transgene constructs because this promoter is extremely sensitive and permissive to heterologous enhancers (Lien et al., 1999; McFadden et al., 2000) and it therefore enabled us to search for potential *Mef2c* regulatory elements without knowing the precise location of the promoter. As shown in Fig. 3, the 6.6 kb region immediately 5′ of the skeletal muscle-specific exon 1 was sufficient to direct strong skeletal muscle-specific expression in vivo (construct 1). As a positive control for *lacZ* transgene expression, a well-defined neural crest enhancer (NCE) of the mouse *dHAND* (*Hand2* – Mouse Genome Informatics) gene, which has been shown to be active specifically in the branchial arches of developing embryos (Charité et al., 2001), was fused upstream of this 6.6 kb genomic sequence. The NCE gave rise to an expression pattern in the branchial arches and their derivatives (Fig. 3), as predicted. Because this enhancer is active in cell types in which *Mef2c* is not expressed, we were able to use it initially to rapidly survey numerous genomic fragments for the *Mef2c* skeletal muscle enhancer by identifying embryos that contained *lacZ* transgenes integrated into regions of chromatin permissive to gene expression. Once we had identified the region of genomic DNA with skeletal muscle regulatory activity, we no longer included the NCE in subsequent transgenes. Expression from construct 1 was localized to the somite myotomes at E9.5-11.5 (Fig. 3A-D). At E14.5, this construct was highly active in differentiated skeletal muscle fibers throughout the body (Fig. 3E). No expression in other cell types, including cardiac and smooth muscle, was detected at any developmental stage examined.

Bisection of the 6.6 kb region into fragments from –6.6 to –4.6 and from –4.4 to the first exon (constructs 2 and 3, respectively) showed that all skeletal muscle activity was localized to the 3′ DNA fragment (Fig. 4 and data not shown). Further dissection of this region localized the skeletal muscle

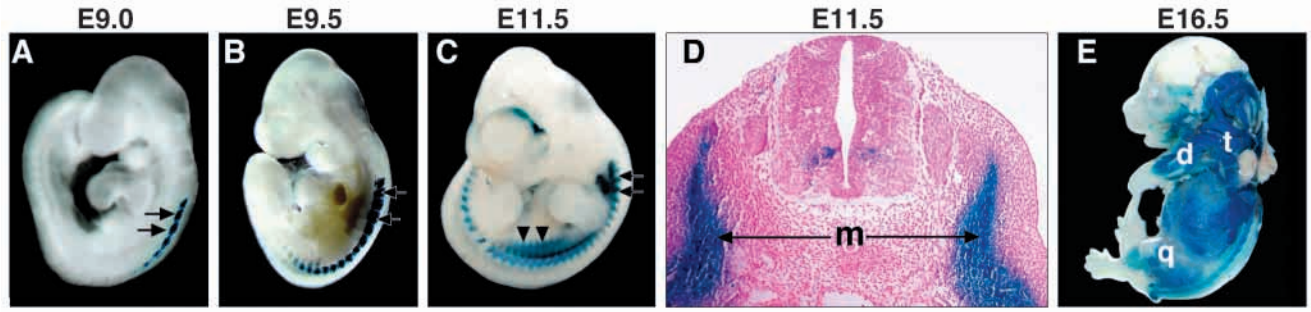

Fig. 4. Expression of construct 7 during embryogenesis. The nucleotide region from –1.1 kb to +77 bp was fused to *hsp68-lacZ* and used to create transgenic mice (see Fig. 2). (A) At E9.0, *lacZ* expression is detected in rostral somites. (B) An E9.5 embryo shows *lacZ* reporter expression from the most rostral somites, to some somites in the region of the hind limb. (C) At E11.5, transgene expression is evident throughout the somites and in ventral myoblasts. (D) Transverse section through somites at the level of the fore limb shows *lacZ* expression in the myotome. Neural tube staining is not reproducible. (E) At E16.5, expression of the transgene is seen throughout embryonic musculature, including facial, epaxial, hypaxial and limb muscle. Arrows, somites; arrowheads, ventral myoblasts; d, deltoid; m, myotome; q, quadriceps; t, trapezius.

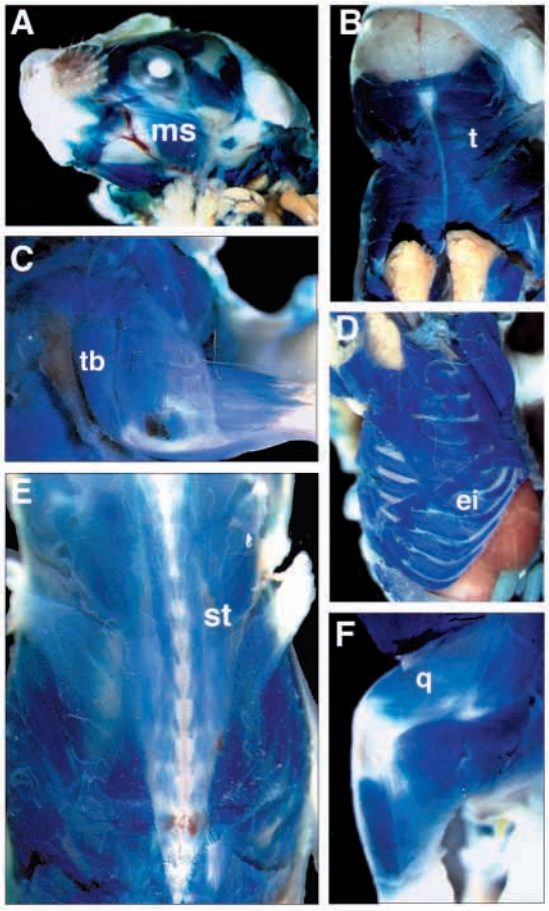

Fig. 5. Expression of construct 7 in postnatal skeletal muscle. Expression of construct 7 was analyzed in F_0 neonatal transgenic mice. High expression was observed in muscles of the (A) face, (B) neck, (C) fore limb, (D) rib cage, (E) back and (F) hindlimb. ei, external intercostals; ms, masseter; q, quadriceps; st, spinalis thoracis; t, trapezius; tb, triceps brachii.

control region to a fragment extending from –1.1 kb to exon 1 (construct 7, Fig. 4).

Because there is a precedent in other muscle genes for modularity of regulatory elements in which individual control regions direct only part of the muscle expression pattern and the complete pattern requires combinations of independent regulatory regions (Firulli and Olson, 1997), we carefully analyzed the expression pattern of construct 7 at various stages of development from E9.0 to E16.5 (Fig. 4). This construct was active in all embryonic skeletal myocytes that express *Mef2c*. The construct was also expressed at extremely high levels in all post-natal skeletal muscle fibers (Fig. 5). These results suggest that this upstream DNA region contains the *cis*-regulatory elements sufficient to direct the skeletal muscle expression pattern of *Mef2c* in vivo.

Mapping the minimal *Mef2c* skeletal muscle regulatory region

The proximal position of the *Mef2c* control region relative to exon 1 suggested that this region might function as a skeletal muscle-specific promoter, although no consensus TATA-binding sites are found in this 1.1 kb region. To test this, we

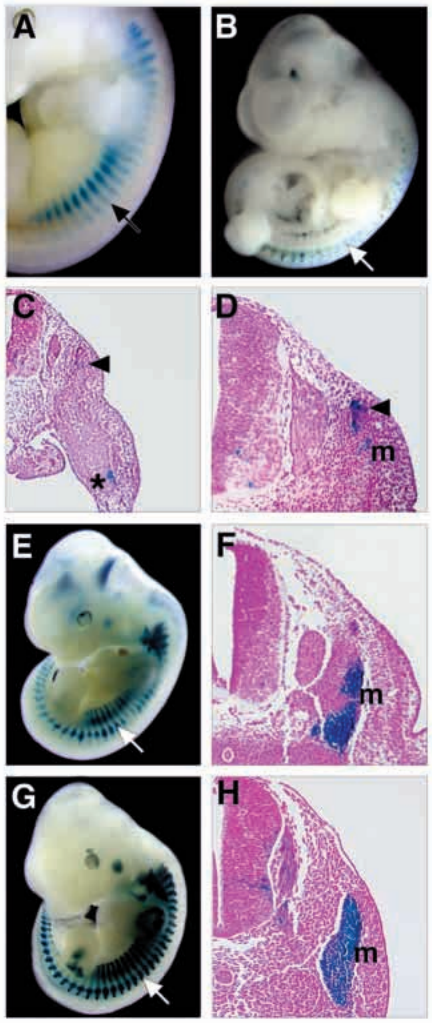

Fig. 6. Delineation of the minimal *Mef2c* skeletal muscle regulatory region. Whole-mount E11.5 embryos expressing constructs 14, 8, 9 and 11 are shown. (A) The nucleotide region from –1058 to +27 (construct 14) was fused directly upstream of the promoterless *lacZ* cassette and was used to generate F_0 transgenic embryos. At E11.5, β-gal staining is evident from rostral somites through somites at the level of the hind limb. This expression is weaker and less extensive than that of approximately the same nucleotide region fused to hsp68-*lacZ* (see Fig. 4C). (B) The nucleotide region from –1058 bp to –507 bp (construct 8) was fused to hsp68-*lacZ* and used to create F_0 transgenic mice. Small, discrete regions of β-galactosidase staining are seen in a metameric pattern throughout rostral and caudal somites. (C,D) Transverse sections at the level of forelimb somites of the embryo in B demonstrate that the β-galactosidase staining marks the extreme dorsomedial aspect of the myotome and ventrolateral myoblasts in the limb. (E) The nucleotide region from –512 bp to +41 bp (construct 9) was fused to hsp68-*lacZ* and used to create F_0 transgenic mice. Strong *lacZ* expression is evident throughout the somites and in ventral myoblasts. (F) Transverse section through the somites of an E11.5 embryo expressing construct 9. Expression is evident throughout the myotome. (G) The nucleotide region from –158 bp to +4 bp (construct 11) was fused to hsp68-*lacZ* and used to create F_0 transgenic mice. Somites and ventral myoblasts show strong *lacZ* expression. (H) Transverse section through the somites of an E11.5 embryo expressing construct 11. Section shows *lacZ* expression throughout the myotome. Arrows, somites; m, myotome; arrowheads, dorsomedial myotome; *, limb myoblasts.

fused the region from −1058 bp to +27 bp directly to a promoter-less *lacZ* transgene (construct 14). This transgene showed an expression pattern at E11.5 (Fig. 6A) that was the same as that of construct 7, although its level of expression was weaker, which we presume reflects the stronger potential activity of the *hsp68* basal promoter, which was included in construct 7. This indicated that the 1.1 kb proximal regulatory region could also function as a skeletal muscle-specific promoter for *Mef2c*.

When the proximal 1.1 kb fragment (construct 7) was cut approximately in half, each part directed a distinct pattern of *lacZ* expression within the myotome. At E11.5, the distal portion (−1058/−507, construct 8) was expressed only in the dorsomedial lip of the myotome and in ventrolateral myoblasts in the limb (Fig. 6B-D). By contrast, the proximal portion (−512/+41, construct 9) was expressed in the entire myotome (Fig. 6E,F). The expression pattern of construct 9 was indistinguishable from the expression pattern of construct 7, from which it was derived. Thus, constructs 8 and 9 identify distinct, but overlapping myogenic precursor populations.

Because construct 9 was expressed throughout the entire myotome, and was as strong as construct 7, we attempted to further localize the *cis*-regulatory elements within this fragment. Bisection of construct 7 into two fragments (−512/−174, construct 10, and −158/+4, construct 11) demonstrated that all of its transcriptional activity was contained in the 3′ fragment (Fig. 6G,H and data not shown).

Together, the above results demonstrate the existence of two independent *Mef2c* regulatory regions capable of directing transcription in the skeletal muscle lineage. The region from −158/+4, which acts as a promoter, appears to be primarily responsible for the skeletal muscle-specific expression of *Mef2c*. This region can also direct muscle-specific expression in the opposite orientation when combined with the *hsp68* promoter, which suggests that it can also act as an enhancer.

Regulation of the *Mef2c* skeletal muscle enhancer by MyoD and MEF2

The sequence of the minimal skeletal muscle regulatory region is shown in Fig. 7A. Comparison of the sequence from the mouse and human genes showed extensive cross-species homology (data not shown). Within the minimal regulatory region, we identified an E-box and a potential MEF2 site (Fig. 7A,B). To determine whether myogenic bHLH or MEF2 proteins could bind these sequences, we performed gel mobility shift assays using oligonucleotide probes and in vitro translated proteins. As shown in Fig. 7C, Myc-tagged MEF2C bound avidly to the MEF2 site-containing oligonucleotide and binding was competed by the cognate sequence, but not by a nonspecific sequence (data not shown). The identity of the MEF2C-containing complex was confirmed by its supershift with anti-Myc antibody. Similarly, the E-box-containing sequence was bound by MyoD/E12 heterodimers, which were supershifted with anti-MyoD antibody (Fig. 7D). Homodimers of MyoD or E12 showed only weak binding to this site.

The potential roles of the above transcription factor binding sites in *Mef2c* transcriptional regulation were determined by mutagenesis of each site individually within the context of the −512/+41 bp region (constructs 12 and 13 of Fig. 2). As shown in Fig. 8D-F, the E-box mutant (construct 13) was inactive at

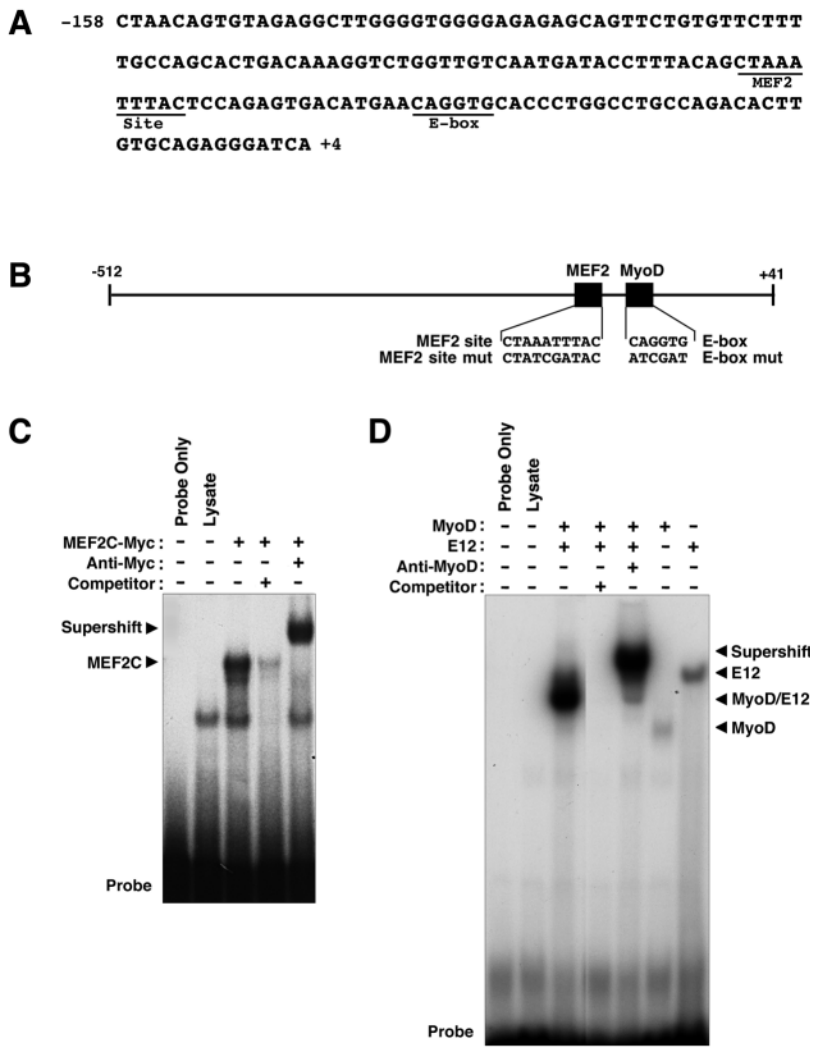

Fig. 7. Binding of MyoD and MEF2 to the *Mef2c* skeletal muscle regulatory region. (A) Sequence of the minimal skeletal muscle regulatory region is shown. The MEF2-binding site extends from nucleotides −64 to −56. The E-box includes nucleotides −38 through −33. (B) Schematic representation of the 1.1 kb *Mef2c* skeletal muscle control region. The region encompassing nucleotides −512 to +41, with respect to the transcriptional start site of *Mef2c* (see Fig. 1). The specific MEF2 site and E-box nucleotides mutated for the experiments shown in Fig. 8 are indicated beneath the wild-type sequence. α-^{32}P-labeled oligonucleotides for the (C) MEF2 site and (D) E-box of the *Mef2c* skeletal muscle regulatory region were used as probes in gel mobility shift assays with the in vitro translated (C) Myc-tagged MEF2C or (D) MyoD and E12 proteins. Gel shift reactions using unprogrammed reticulocyte lysate are included in parallel lanes. A 100-fold excess of unlabeled (C) MEF2 site or (D) E-box oligonucleotides was used as competitor. (C) Anti-Myc and (D) anti-MyoD antibodies were used for supershifts. Labeled arrows indicate the positions of the various protein-DNA complexes.

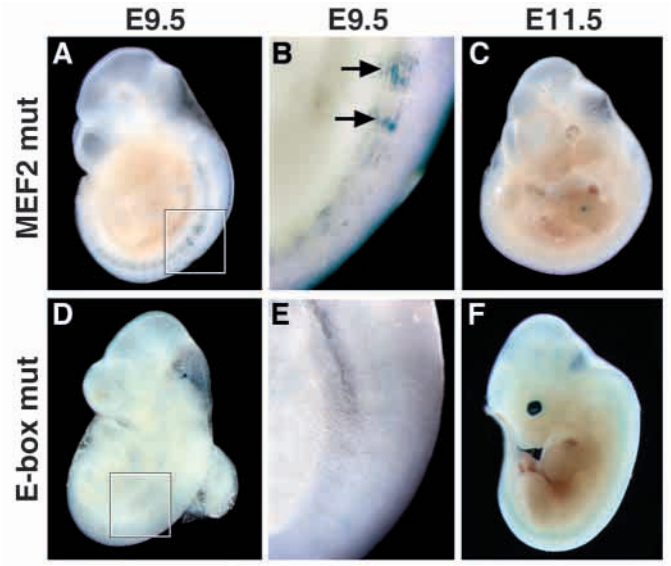

Fig. 8. Inactivation of the *Mef2c* skeletal muscle enhancer by individual mutations in the MEF2 binding site and E-box. Mutations of the (A-C) MEF2 site (MEF2 mut) and (D-F) E-box (E-box mut), shown in Fig. 7B, were introduced individually in the context of the nucleotide region from –512 bp to +41 bp fused to *hsp68-lacZ* and F_0 transgenic embryos were generated. Embryos were analyzed for β-galactosidase expression at E9.5 (A,B,D,E) and E11.5 (C,F). (B,E) Enlargements of the regions indicated by the boxes in A,D, respectively. (A,B) Only the MEF2 mut-hsp68-*lacZ* construct was weakly active in somites at E9.5. Constructs were inactive at all other time points. Arrows, somitic expression.

both E9.5 and 11.5. Nine F_0 transgenic embryos harboring this construct were analyzed. None showed expression in the skeletal muscle lineage, but several showed ectopic expression in other cell types, reflecting random integration sites of the transgene. This data indicates that the E-box is necessary for initiation, as well as maintenance, of *Mef2c* expression in vivo.

In contrast to the complete inactivity of the E-box mutant, the MEF2 site mutant (construct 12) was weakly active in the myotome at E9.5 (Fig. 8A,B). However, at E11.5 this transgene was inactive (Fig. 8C). These results suggest that the MEF2 site is not required for the initiation of *Mef2c* transcription, but is essential for the amplification and maintenance of *Mef2c* expression at later stages in vivo.

DISCUSSION

MEF2 transcription factors play a central role in the control of skeletal muscle development by enhancing the muscle-inducing activity of myogenic bHLH proteins. While much has been learned about the mechanisms whereby MEF2 proteins activate muscle-specific transcription, little is known of the mechanisms that regulate expression of MEF2 genes and no *cis*-regulatory elements that control transcription of vertebrate MEF2 genes have been previously identified. This has been a difficult problem because the 5′-UTRs of MEF2 transcripts are encoded by multiple alternative exons. For *Mef2c*, these exons are distributed over approximately 80 kb of genomic DNA. To locate the control region responsible for the expression of *Mef2c* in skeletal muscle, we used tissue-specific 5′-RACE to identify 5′-exons encoding skeletal muscle transcripts. We also took advantage of the completed human genome sequence by searching the human genome database using the tissue-specific 5′-untranslated sequences that we identified. Our results demonstrate the presence of tissue-specific alternatively spliced exons in the 5′-UTR of the *Mef2c* gene and suggest that distinct *Mef2c* transcripts are directed by different regulatory elements.

Our results are consistent with the possibility that the *Mef2c* gene is a direct target for transcriptional activation by myogenic bHLH and MEF2 proteins in the developing skeletal muscle lineage in vivo. These results confirm and extend previous findings that MEF2 DNA binding activity is upregulated by myogenic bHLH proteins (Lassar et al., 1991; Cserjesi and Olson, 1991) and reveal a direct positive feedback loop between these two classes of transcription factors. This is the first demonstration of a direct role of myogenic bHLH factors in the activation of MEF2 gene transcription in any organism.

Mef2c expression and skeletal muscle development

Skeletal muscle development is initiated in the rostral somites of the mouse embryo at E8.0 (Hauschka, 1994). Signals from the notochord induce cells from the ventral region of the somite to undergo an epithelial-to-mesenchymal transformation and migrate away from the somite, forming the sclerotome (Christ et al., 1978), which gives rise to the axial skeleton. The remaining epithelial cells of the dorsal somite give rise to the dermomyotome, which serves as the source of myogenic precursors of the trunk and limb musculature, and the myotome, from which the muscles of the back are derived (Denetclaw et al., 1997; Ordahl et al., 2001). Myf5 is the first marker of the skeletal muscle lineage and is expressed in the dorsomedial lip of the dermomyotome at E8.0 in the mouse followed soon after by the expression of myogenin, MRF4 and MyoD at E8.5, E9.5 and E10.5, (Sassoon et al., 1989; Ott et al., 1991; Hinterberger et al., 1991), respectively.

Like the myogenic bHLH transcription factors, MEF2 factors are expressed early in myogenic precursors and in developing skeletal muscle. *Mef2c* is the first member of the MEF2 family to be expressed in skeletal muscle in vivo; its expression is initially detected at E9.0 in the rostral myotomes at the onset of myocyte differentiation (Edmondson et al., 1994) and expression is maintained throughout skeletal muscle development. In the current study, we were able to detect expression of the *Mef2c-lacZ* transgene in the myotome as early as E9.0, throughout skeletal muscle development, and into adult muscle, recapitulating that of the endogenous *Mef2c* gene. The lag between the expression of Myf5/myogenin and *Mef2c* in developing somites suggests that the myogenic bHLH factors, but not MEF2C, are required for the initiation of skeletal muscle differentiation, whereas MEF2 proteins may be involved in the maintenance and/or amplification of the skeletal muscle differentiation program.

Regulation of *Mef2c* gene expression in skeletal muscle – initiation versus amplification/maintenance

Forced expression of myogenic bHLH proteins in non-muscle cells is sufficient to upregulate MEF2 expression (Lassar et al., 1991; Cserjesi and Olson, 1991). Our results suggest that this reflects the direct binding of myogenic bHLH proteins to an E-

box in the proximal promoter of the *Mef2c* gene. Our data also indicate that in the context of the *Mef2c* gene (–512 to +41) region, this E-box is essential for the initial activation of *Mef2c* transcription in the skeletal muscle lineage. An E-box mutation in the *Mef2c* promoter completely abolished *lacZ* reporter expression at E9.5. Given that the E-box mutation also abolished expression at later stages of development, binding of myogenic bHLH proteins to this site also appears to be required for the maintenance of *Mef2c* expression. As Myf5 and myogenin are expressed prior to *Mef2c*, these factors are potential initiators of *Mef2c* transcription. In mature skeletal muscle fibers, it is likely that MRF4 maintains *Mef2c* expression, as this factor is the most highly expressed in postnatal muscle.

In contrast to the requirement of the E-box for initiation of *Mef2c* transcription, a mutation of the MEF2 site did not affect the timing for initial activation of the reporter gene such that weak *lacZ* expression was detected in the somites at E9.5. However, at later stages, this MEF2 site mutant was unable to maintain *lacZ* expression and was completely inactive at E11.5. Together, these results suggest that MEF2C acts on its own promoter to amplify and maintain its expression in differentiating myoblasts and differentiated muscle. Other members of the MEF2 family also become expressed at high levels in the somite myotome after E9.5 (Edmondson et al., 1994), which could further reinforce the expression of *Mef2c*.

Our results suggest that myogenic bHLH transcription factors are required for the initial activation of *Mef2c* expression in vivo. Although MEF2 proteins are not sufficient to activate *Mef2c* transcription, they cooperate with the myogenic bHLH proteins, which provide the muscle specificity necessary to direct the expression of *Mef2c* in cells of the myogenic lineage throughout development and into adulthood. A model of how myogenic bHLH and MEF2 proteins might cooperatively regulate the expression of *Mef2c* during skeletal myogenesis is shown in Fig. 9A.

Recently, we have shown that MEF2 proteins associate with histone deacetylase5 (HDAC5) in myoblasts, resulting in repression of muscle genes controlled by MEF2 sites (McKinsey et al., 2000a). When myoblasts are triggered to differentiate, HDAC5 is exported to the cytoplasm, allowing for enhanced activity of MEF2 factors and upregulation of MEF2 target genes. As the *Mef2c* gene is itself a target for the MEF2 transcription factor, this type of derepression mechanism could also account for the upregulation of MEF2 expression during myogenesis.

Crossregulation of myogenic bHLH and MEF2 genes

The structure of the *Mef2c* skeletal muscle promoter/enhancer is remarkably similar to that of the *myogenin* and *MRF4* promoters, both of which contain MyoD- and MEF2-binding sites (Edmondson et al., 1992; Cheng et al., 1993; Yee and Rigby, 1993; Black et al., 1995; Naidu et al., 1995). Like *Mef2c*, *myogenin* and *MRF4* are upregulated during myocyte differentiation, presumably through direct transactivation by bHLH and MEF2 factors. This type of crosstalk provides a powerful mechanism for amplification of both types of regulatory genes, thereby reinforcing and stabilizing the transcriptional program for myogenesis.

In transfected cells, MyoD and MEF2 can cooperatively activate transcription through a binding site for either factor alone (Molkentin et al., 1995). By contrast, mutation of either the MEF2 or MyoD site in the *Mef2c* promoter severely diminished transcriptional activity in vivo. These findings suggest that the levels of expression of the endogenous factors are insufficient to activate transcription solely through protein-protein interactions and without binding sites for both factors. This further demonstrates the existent crossregulation between the myogenic bHLH transcription factors and the MEF2 proteins in skeletal muscle development. A model to account for the regulation of *Mef2c* transcription during myogenesis is shown in Fig. 9B.

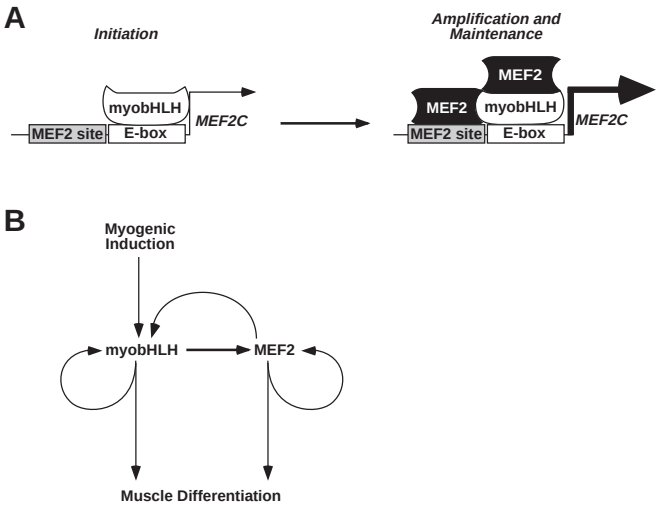

Fig. 9. A model for the regulation of *Mef2c* transcription during skeletal muscle development. (A) Regulation of *Mef2c* expression during myogenesis. The skeletal muscle regulatory region of *Mef2c* contains binding sites for myogenic bHLH and MEF2 proteins. The E-box is required for initiation and maintenance of *Mef2c* expression. By contrast, the MEF2 site is required for amplification and maintenance of *Mef2c* expression. (B) A model for the genetic network of myogenic bHLH and MEF2 factors during myogenesis.

The *Drosophila Mef2* gene is also expressed at high levels in differentiated skeletal muscle fibers (Lilly et al., 1995; Bour et al., 1995). Previously, we and others showed that transcription of *Mef2* is controlled by an array of independent enhancers that are activated in specific subsets of myocytes at distinct developmental stages (Cripps et al., 1998; Cripps et al., 1999; Nguyen and Xu, 1998). Recently, we identified an enhancer that directs *Mef2* transcription specifically in differentiated myofibers of the *Drosophila* embryo (R. Cripps and E. N. O., unpublished). This enhancer contains both a high-affinity MEF2-binding site that is essential for transcriptional activity and an E-box that is necessary for full activity. Thus, the type of positive auto- and crossregulatory loops identified in the present study seems likely to reflect an evolutionarily conserved mechanism for the control of *MEF2* gene expression in the skeletal muscle lineage.

Modular regulation of *Mef2c* transcription

A common theme that has emerged through the analysis of muscle gene transcription is the modularity of *cis*-regulatory elements, in which multiple independent regulatory regions are required to generate the complete spatiotemporal expression pattern of a gene throughout development (Firulli and Olson,

1997). Our results demonstrate the existence of two independent regulatory regions that direct *Mef2c* transcription in discrete, but overlapping, sets of embryonic skeletal muscle cells.

A distal upstream region (−1058/−507) activates transcription specifically in cells from the dorsomedial myotome and ventrolateral dermomyotome, which give rise to extreme epaxial, as well as hypaxial and limb muscles, respectively. To our knowledge, these two populations of muscle cells related and have not been previously recognized as being molecularly distinct from other mnyogenic populations. Thus, this *Mef2c* transgene has revealed unique compartments of the myogenic lineage. The specific transcription factors that activate this distal regulatory region remain to be identified.

The proximal ~500bp regulatory region of *Mef2c* acts independently of this distal enhancer and is sufficient to direct expression of *lacZ* in a pattern that appears to recapitulate the expression pattern of the endogenous *Mef2c* gene during pre- and postnatal skeletal muscle development. Although our studies do not allow us to conclude that these two regions are solely responsible for *Mef2c* transcription in the skeletal muscle lineage, we have found no other skeletal muscle regulatory elements within 24 kb of genomic DNA analyzed.

In addition to its expression in the skeletal muscle lineage, *Mef2c* is expressed in the developing heart, in specific sets of neurons in the brain, and in the spleen (Lyons et al., 1995; Martin et al., 1993; Edmondson et al., 1994). Unlike many other muscle-specific enhancers, the *Mef2c* enhancer described here is absolutely specific for the skeletal muscle lineage with no expression in the heart or other organs, suggesting the presence of completely separate regulatory mechanisms for expression in tissues other than skeletal muscle. We have not yet identified the regulatory elements that control *Mef2c* transcription in the latter cell types. However, our studies suggest that cardiac and neural transcripts for *Mef2c* contain unique 5′ exons that probably reflect alternate promoters in these tissues. The mechanisms that regulate MEF2 expression in these cell types and whether MEF2 positively autoregulates its expression in cardiac myocytes and neurons, as in skeletal myocytes, is currently under investigation.

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