A New Myocyte-Specific Enhancer-Binding Factor That Recognizes a Conserved Element Associated with Multiple Muscle-Specific Genes

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Exposure of skeletal myoblasts to growth factor-deficient medium results in transcriptional activation of muscle-specific genes, including the muscle creatine kinase gene (mck). Tissue specificity, developmental regulation, and high-level expression of mck are conferred primarily by a muscle-specific enhancer located between base pairs (bp) −1350 and −1048 relative to the transcription initiation site (E. A. Sternberg, G. Spizz, W. M. Perry, D. Vizard, T. Weil, and E. N. Olson, Mol. Cell. Biol. 8:2896–2909, 1988). To begin to define the regulatory mechanisms that mediate the selective activation of the mck enhancer in differentiating muscle cells, we have further delimited the boundaries of this enhancer and analyzed its interactions with nuclear factors from a variety of myogenic and nonmyogenic cell types. Deletion mutagenesis showed that the region between 1,204 and 1,095 bp upstream of mck functions as a weak muscle-specific enhancer that is dependent on an adjacent enhancer element for strong activity. This adjacent activating element does not enhance activator activity in single copy but acts as a strong enhancer when multimerized. Gel retardation assays combined with DNase I footprinting and diethyl pyrocarbonate interference showed that a nuclear factor from differentiated C2 myotubes and BC3H1 myocytes recognizes a conserved A+T-rich sequence within the peripheral activating region. This myocyte-specific enhancer-binding factor, designated MEF-2, was undetectable in nuclear extracts from C2 or BC3H1 myoblasts or several nonmyogenic cell lines. MEF-2 was first detectable within 2 h after exposure of myoblasts to mitogen-deficient medium and increased in abundance for 24 to 48 h thereafter. The appearance of MEF-2 required ongoing protein synthesis and was prevented by fibroblast growth factor and type β transforming growth factor, which block the induction of muscle-specific genes. A myoblast-specific factor that is down regulated within 4 h after removal of growth factors was also found to bind to the MEF-2 recognition site. A 10-bp sequence, which was shown by DNase I footprinting and diethyl pyrocarbonate interference to interact directly with MEF-2, was identified within the rat and human mck enhancers, the rat myosin light-chain (mlc)-1/3 enhancer, and the chicken cardiac mlc-2A promoter. Oligomers corresponding to the region of the mlc-1/3 enhancer, which encompasses this conserved sequence, bound MEF-2 and competed for its binding to the mck enhancer. These results thus provide evidence for a novel myocyte-specific enhancer-binding factor, MEF-2, that is expressed early in the differentiation program and is suppressed by specific polypeptide growth factors. The ability of MEF-2 to recognize conserved activating elements associated with multiple muscle-specific genes suggests that this factor may participate in the coordinate regulation of genes during myogenesis.

Development requires precisely coordinated control of transcription. Differentiation of skeletal myoblasts to myotubes represents a well-defined system for exploring the molecular mechanisms that underlie the selective activation of genes during development. Expression of muscle-specific genes requires conversion of multipotential stem cells to the myogenic lineage and subsequent activation of the muscle differentiation program. Recent studies have provided evidence for a family of interacting factors that can direct the establishment of the myogenic lineage (7, 11, 14, 42, 55, 62). These of these factors, MyoD1, myogenin, and Myf-5, share a domain of homology with a segment of c-myc and are each sufficient to convert fibroblasts to myoblasts (7, 11, 14, 55, 62). The actions of these myogenic regulatory factors are suppressed by growth factors and certain oncogenes that activate intracellular growth factor cascades (10, 19, 28, 29, 31, 38, 45, 50–52).

The tissue specificity and developmental regulation of many muscle-specific genes suggest that trans-acting factors may control expression of these genes through interaction with shared DNA sequences. cis-Acting sequences that confer muscle-specific regulation on marker genes have been identified by expression in tissue culture cells and transgenic mice (1–5, 13, 21, 22, 24, 26, 27, 30, 33, 35, 40, 41, 48, 49, 52, 53, 61, 65). Shared sequence motifs have also been noted among cis-acting regulatory regions from many muscle-specific genes (9, 13, 26, 33, 35, 53). However, no universal muscle-specific sequences have been identified, nor has it been established whether muscle-specific gene expression is dependent on transcription factors present only in muscle or specific combinations of ubiquitous factors.

We have selected the muscle creatine kinase gene (mck) as a representative muscle-specific gene and have begun to explore the mechanisms that confer muscle specificity, developmental regulation, and high-level expression on this gene. The region mainly responsible for mck induction during myogenesis has been shown to lie between 1,350 and 1,048 base pairs (bp) upstream of mck (14, 24, 27, 52, 53). This element functions as a classical enhancer and directs

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muscle-specific expression from the mck promoter or heterologous promoters. A weaker enhancer has also been located within intron 1 of mck (53; S. Haushka, personal communication). Both mck enhancers have recently been shown to interact with a myocyte-specific nuclear factor, referred to as MEF-1 (9).

In this study, we have delimited the core of the mck 5′ enhancer to the region between bp −1204 and −1095 relative to the transcription initiation site and have shown that maximal activity of this region requires an adjacent activating element which is conserved among multiple muscle-specific genes. This upstream activating element interacts with a myocyte-specific enhancer-binding factor, designated MEF-2, that is upregulated rapidly after exposure of myoblasts to growth-factor-deficient medium. A minor factor that is expressed in myoblasts and is down regulated before the appearance of MEF-2 was also identified and shown to bind to the MEF-2 binding site. The recognition sequence for MEF-2 is distinct from that for MEF-1 and does not correspond to the binding site of any previously described enhancer-binding factor. In addition to interacting with the mck enhancer, competition experiments demonstrated that MEF-2 interacts with the myosin light-chain (mlc)-1/3 enhancer, which is regulated in parallel with mck during myogenesis. Potential binding sites for MEF-2 were also identified within the human and rat mck enhancers and the chicken cardiac mlc-2A promoter. Although the MEF-2 binding site lacks enhancer activity in single copy, it exhibits strong enhancer activity in multimers. On the basis of its selective expression in differentiated myocytes and its ability to recognize a conserved activating element associated with the mck and mlc-1/3 enhancers, we believe that MEF-2 may contribute to the activation of gene sets during myogenesis.

**MATERIALS AND METHODS**

**Cell culture.** The C2 (63) and BC2H1 (46) mouse muscle cell lines were cultured as described previously (37, 38). Undifferentiated cultures were maintained at subconfluent densities in Dulbecco minimal essential medium (DMEM) with 20% fetal bovine serum (FBS). Differentiation of C2 myoblasts was initiated by transferring undifferentiated cultures at 80% confluency to DMEM containing 10% horse serum (fusion-promoting medium). Differentiation of BC2H1 cells was initiated by exposing myoblasts to DMEM with 0.5% FBS. NIH 3T3 and 10T1/2 cells were grown in DMEM with 20% FBS. For inhibition of protein synthesis, cycloheximide was added to cultures to a concentration of 10 μg/ml. At this concentration, [35S]methionine incorporation into protein was inhibited greater than 90% within 2 min (data not shown). Basic fibroblast growth factor (FGF) (Collaborative Research, Inc.) and type β transforming growth factor (TGF-β) (R and D Products) were added to cultures at concentrations of 20 and 5 ng/ml, respectively.

**Construction of mck-cat expression vectors.** To test for enhancer activity, DNA fragments from the mck upstream region were inserted into the unique BamHI site of the vector pCK246CAT (53). This vector contains the mck promoter region (mck −246 to +1) linked immediately upstream of cat (18). The 246-bp region functions as a weak promoter and contains a canonical TATA box, a CArG motif (35), and three 17-bp imperfect repeats that resemble sequences found upstream of several muscle-specific genes (26, 53). Restriction fragments used to generate mck-cat chimeric genes were as follows: pCKCAtE4, PvuII-BamHI; pCKCAtE5, PvuII-AvaI; pCKCAtE6, AvaI-BamHI; pCKCAtE11, AvaI-NcoI; and pCKCAtE12, NcoI-BamHI. The locations of these restriction sites within the mck enhancer are shown in Fig. 1. When necessary, protruding 5′ termini were filled with deoxynucleotide triphosphates, using the large fragment of DNA polymerase I (Klenow), and BamHI linkers were ligated to the ends (32). After digestion with BamHI, fragments were ligated to the vector. Multimers of the MEF-2 binding site were cloned into pCK246CAT, using synthetic oligomers corresponding to the mck sequence shown in Fig. 6 with BamHI sites on each end. The identities of inserted sequences were confirmed by DNA sequencing.

**Transfection by calcium phosphate precipitation and CAT assays.** Cells were plated on 10-cm-diameter dishes at a density of 5 × 10⁵ cells per dish in DMEM with 20% FBS. Approximately 24 h later, cultures were refed with 4 ml of fresh growth medium for 4 h before transfection. Calcium phosphate precipitates were prepared by a modification of the method of Graham and Van der Eb (20), and transfections were performed as described by Sternberg et al. (53). A 10-μg sample of each plasmid was used for the transfections.

For analysis of chloramphenicol acetyltransferase (CAT) activity, cells were harvested at different stages of differentiation, and extracts were prepared as described previously (53). CAT activity in cell lysates was determined as described previously, (18), and the extent of conversion of substrate to products was quantitated by excising labeled spots from thin-layer plates and scintillation counting. CAT activity recovered from transfected cultures was linear with respect to the concentration of plasmid DNA used in the transfection. To prevent expression of transfected CAT genes in undifferentiated myoblasts, it was important to maintain the cultures at subconfluent densities and to change the growth medium at least every 48 h. In cultures that showed significant cell-cell contact, we observed that CAT genes under control of the mck enhancer began to be
Prepared by 5024 GOSSETT

Preparation of nuclear extracts. Nuclear extracts were prepared by the method of Dignam et al. (12). Briefly, 5 to 10 15-cm-diameter plates of cells were rinsed in phosphate-buffered saline (150 mM NaCl, 10 mM sodium phosphate [pH 7.2]) at 4°C, and cells were harvested by scraping in buffer A (10 mM N-2-hydroxyethylpiperazine-N'2-ethanesulfonic acid [HEPES, pH 7.9], 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM dithiothreitol [DTT]). Cells were homogenized in a Dounce homogenizer and centrifuged at 3,000 rpm in a Sorvall GLC centrifuge. Nuclear pellets were resuspended in buffer A, rehomogenized, and centrifuged as described above. The nuclear pellet was then resuspended, placed in extraction buffer (20 mM HEPES [pH 7.9], 25% glycerol, 0.55 M NaCl, 1.5 mM MgCl₂, 0.2 mM EDTA, 2 μg of antipain per ml, 1 μg of leupeptin per ml, 10 μg of benzamidine per ml, 1 μg of chymostatin per ml, 1 μg of pepstatin per ml, 1 μl of aprotinin per ml, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 mM DTT), and homogenized. Extracts were then centrifuged in a microfuge at high speed for 30 min, the supernatant was dialyzed against binding buffer (40 mM KCl, 15 mM HEPES [pH 7.9], 1 mM EDTA, 0.5 mM DTT, 2% phenylmethylsulfonyl fluoride, 0.5 mM DTT, 20% glycerol) for 4 h. Samples of extracts were stored at −80°C until use.

Gel retardation assays. A 5-μg sample of nuclear extract from each of various cell lines was incubated with 2.0 μl of 10× binding buffer (final concentrations, 40 mM KCl, 15 mM HEPES [pH 7.9], 1 mM EDTA, 0.5 mM DTT, and 5% glycerol), 4 μg of poly(dl-dC), and various concentrations of competitor fragments in a final volume of 18 μl for 10 min (15). Then 2 μl of enhancer fragments (typically 10,000 cpm/2 ng), which were end labeled with [32P]dATP and [32P]dCTP, was added, and the reaction mixture was incubated at room temperature for an additional 20 min. In the absence of poly(dl-dC), the labeled DNA remained at the top of the gel because of interaction with nonspecific DNA-binding proteins in the nuclear extracts (data not shown). For gel retardation assays using synthetic oligomers, the oligomers were cloned into the polylinker of pUC19 by using BamHI linkers and were isolated as restriction fragments before use. The oligomer corresponding to the MEF-2 binding site within the mlc-1/3 enhancer was provided by N. Rosenthal (Boston University) and contained the sequence GATCCCTACATCTTTTTAAAAATTACTTTCTAAAG cloned into the BamHI site of pUC19.

DNase I footprinting. Single-end-labeled probes were prepared from the region between −1095 and −1048 upstream of mck, which had been cloned into the BamHI site of pUC19 by using BamHI linkers. The fragment was end labeled with the Klenow fragment of DNA polymerase I at the EcoRI site (coding strand) or at the HindIII site (non-coding strand). After labeling, DNA was precipitated with ethanol and digested with HindIII or EcoRI, respectively. DNAs were then electrophoresed on a 5% polyacrylamide gel, and labeled fragments were excised from the gel and electroeluted.

The low abundance of enhancer-binding factors within the nuclear extracts prevented direct analysis of factor binding by DNase I footprinting (17). Therefore, we performed indirect footprinting by treating DNA-protein complexes with 10 μg of DNase I per ml for 1 min at room temperature, followed by separation of free and bound DNA on a native acrylamide gel. The region of the gel containing the myotube-specific DNA-protein complex and the free probe were then excised and electrophoresed on an 8% acrylamide sequencing gel. A Maxam-Gilbert A+G reaction (34) was performed with each end-labeled DNA fragment and electrophoresed on the sequencing gel beside its respective DNase I reaction to determine the exact sequences that were protected.

DEPC interference. Determination of the precise nucleotides contacted by MEF-2 was performed by diethyl pyrocarbonate (DEPC) interference as described by Sturm et al. (54). Briefly, fragment 12 (−1095 to −1048) of the mck enhancer was single end labeled as described above. The labeled fragment was denatured, treated with DEPC, reannealed, and used in gel retardation assays as described above. The myocyte-specific band and the free DNA were then excised and electroeluted. They were then treated with piperidine at 90°C for 30 min, extracted with butanol, and separated on a sequencing gel as described above.

RESULTS

Identification of mck enhancer-binding factors by the gel retardation assay. We and others have reported previously that the mck enhancer is contained within the region between bp −1350 and −1048 5′ of the mck transcription initiation site (14, 24, 27, 52, 53). As a first step toward identification of nuclear factors that interact with the mck enhancer region, we divided the 302-bp enhancer into two parts by digestion with AvaI and analyzed factor interactions within each part by using gel retardation assays. The locations of the two AvaI fragments in addition to other DNA fragments that were used in gel retardation assays are shown in Fig. 1.

Incubation of nuclear extracts from C2 myoblasts and myotubes with the region between the AvaI and BamHI sites at bp −1204 and −1048, respectively (designated fragment 6), revealed multiple DNA-protein complexes with reduced mobility after electrophoresis on non-denaturing polyacrylamide gels (Fig. 2A). The pattern of mobility-shifted species observed with the proximal half of the enhancer region was similar in extracts from myoblasts and myotubes with the exception of a DNA-protein complex that was generated only by extracts from differentiated myotubes (designated m in Fig. 2). The distal half of the enhancer (−1350 to −1205) also bound several factors from myoblast and myotube extracts; however, none appeared to be myocyte specific (data not shown).

The specificities of factors binding to the enhancer were assessed by competition experiments using unlabeled DNA fragments as competitors. Unlabeled enhancer fragment 1 competed effectively for the formation of the myotube-specific complex, as well as the complexes that were common to both extracts, whereas nonspecific competitors, such as a fragment of equivalent size from pSVOCAT, had no effect on formation of these complexes (Fig. 2B).

Our initial interest was to define the binding site for the myocyte-specific enhancer-binding factor observed with the fragment 6 enhancer region probe. Preliminary competition experiments indicated that this factor recognized a sequence in the 3′ portion of the enhancer region (data not shown). Indeed, by using the downstream peripheral region between −1094 and −1048 (designated fragment 12) as a labeled probe, this factor was readily apparent in extracts from C2 myotubes (Fig. 3). The factor responsible for the formation of this myotube-specific complex will be referred to as myocyte-specific enhancer-binding factor-2, or MEF-2. The binding site for MEF-2 lies within a region of the mck enhancer different from the binding site for MEF-1 described by Buskin and Hauschka (9).

In some experiments, such as the one presented in Fig. 3,
FIG. 2. Detection of nuclear factors from C2 cells that interact with the mck 5' enhancer by the gel retardation assay. (A) End-labeled fragment 6 (−1204 to −1048) was incubated with 5 µg of nuclear extract from C2 myoblasts and myotubes, and DNA-protein complexes were resolved on a native 5% polyacrylamide gel. The myocyte-specific complex (m) was present only in myotubes. (B) Competition of factors from myotube nuclear extract, using unlabeled fragment 6 or a fragment of equivalent size from pSV0CAT as the competitor.

The myocyte-specific complex appeared as a doublet. Since the lower band of the doublet was often undetectable and when present was generally a minor species, it may represent the binding of a proteolytic fragment of MEF-2. Alternatively, the doublet may be attributable to multiple forms of the same protein. Nevertheless, the factors giving rise to each band of the doublet showed identical footprints over this region (see below).

Extracts from C2 myoblasts also contained a minor species that disappeared during differentiation. This myoblast-specific factor, designated MBF-1, was down regulated during differentiation (indicated MBF-1 in Fig. 3). Both MEF-2 and MBF-1 bound specifically to the enhancer, as demonstrated by the ability of unlabeled enhancer fragment 6, but not pUC19, to block formation of the corresponding DNA-protein complexes. Neither MEF-2 nor MBF-1 was detected in nuclear extracts from proliferating or quiescent 3T3 fibroblasts or C3H10T1/2 cells (see below). An additional shifted species that could not be eliminated by competition with homologous or heterologous DNA fragments was also observed near the center of the gels. Since the factor responsible for the generation of this species is not developmentally regulated and seems to lack sequence specificity, we have not focused on it further.

Regulation of MEF-2 expression in BC3H1 cells. To determine whether MEF-2 was expressed during differentiation of other muscle cell types and to begin to examine its regulation, we investigated whether this enhancer-binding factor was expressed in BC3H1 cells. Unlike C2 cells, which form myotubes and differentiate irreversibly in serum-deficient medium, BC3H1 cells lack the ability to form myotubes or to terminally differentiate (25, 28, 29, 37, 38, 46, 50, 51). BC3H1 cells also differ from C2 cells in that they do not express MyoD1 at detectable levels (11, 14). MEF-2 was undetectable in nuclear extracts from undifferentiated BC3H1 cells in growth medium (Fig. 4). After exposure to differentiation medium, MEF-2 began to appear within 2 h and reached a maximal level 24 to 48 h thereafter. These kinetics for MEF-2 expression precede the induction of mck mRNA by several hours (50). MBF-1 was down regulated rapidly after a stimulus to differentiate and was undetectable after 4 h in mitogen-deficient medium. Comparison of the pattern of mobility-shifted species generated with extracts from C2 and BC3H1 cells suggests that these two muscle cell lines express an indistinguishable set of factors which interact with this region of the mck enhancer (Fig. 4).

In mitogen-deficient medium containing FGF or TGF-β, BC3H1 cells exit the cell cycle but do not differentiate (25,

FIG. 3. Binding of MEF-2 to the 3’ portion of the mck enhancer. Fragment 12 (−1094 to −1048) was labeled and used as a probe in gel retardation assays with 5 µg of extract from C2 myoblasts or myotubes, as indicated. Sequence specificity of factor binding was determined by using fragment 6 or Hpal-cut pUC19 as the competitor. Positions of a myocyte-specific complex (MEF-2) and a myoblast-specific complex (MBF-1) are indicated.
Thus, polypeptide growth factors, which are coupled to the cessation of proliferation or were associated more directly with the onset of differentiation. MEF-2 was not expressed in the presence of FGF or TGF-β, but MEF-1 continued to be expressed in cultures that were prevented from differentiating by these growth factors (Fig. 4). Thus, MEF-2 and MEF-1 can be regulated by single polypeptide growth factors, and their altered expression represents an early event associated with activation of the differentiation program.

Certain enhancer-binding factors have been shown to be converted to an active form through posttranslational mechanisms (43, 47). To determine whether the appearance of MEF-2 upon removal of growth factors reflected conversion of a preexisting factor to a form that could bind DNA, we investigated whether MEF-2 was expressed in the presence of cycloheximide. The major myocyte-specific species failed to appear after exposure of BC3H1 myoblasts to mitogen-deficient medium containing cycloheximide (Fig. 4). However, a faint smear in the region of the faster-migrating myocyte-specific species was detectable after exposure to mitogen-deficient medium for 30 min in the presence and absence of cycloheximide. These data can be interpreted to indicate that MEF-2 is synthesized de novo upon removal of growth factors. Alternatively, a possible precursor for MEF-2 may have a short half-life and be rapidly degraded in the presence of cycloheximide. MEF-1 expression was down regulated in mitogen-deficient medium containing cycloheximide. The decline in expression of MBF-1, therefore, does not appear to require new protein synthesis and may be a primary response to the removal of growth factors.

Identification of the binding site for MEF-2 by DNase I footprinting and DEPC interference. The recognition sequence for MEF-2 was determined initially by indirect DNase I footprinting in which myotube nuclear extracts were incubated with end-labeled enhancer fragments, and DNA-protein complexes were treated with DNase I before separation on a native polyacrylamide gel. The complex containing MEF-2 was then isolated from the gel and analyzed on a sequencing gel. This technique revealed significant protection from DNase I digestion on both strands over an A+T-rich sequence within the 3′ region of the enhancer (Fig. 5A). The region protected by MEF-2 extended from −1063 to −1082 on the noncoding strand, with the region between −1075 and −1082 showing only partial protection. A hypersensitive site was observed at −1062 on the noncoding strand. The coding strand showed a more extensive footprint, with partial protection toward the 5′ region of the binding site. Because of the low level of expression of MBF-1, we were unable to determine the precise footprint for this factor. However, experiments shown below demonstrate that MBF-1 binds to a synthetic oligomer corresponding to the MEF-2 binding site.

DNase I footprinting suggests the boundaries of the region protected by a DNA-binding factor but does not indicate the specific nucleotides that contact the factor. To determine the nucleotides that interact directly with MEF-2, we used the DEPC interference assay. In this assay, purines are modified by DEPC, resulting in disruption of factor interactions with the DNA. Adenines interact preferentially with DEPC, giving rise to a sequencing ladder with A’s more intense than G’s. Because of the A+T-rich nature of the MEF-2 binding region, we chose DEPC interference over methylation interference, which allows detection only of G’s in a binding site. Figure 5B shows the results of DEPC interference assays with the coding and noncoding strands of the mck enhancer in the region of the MEF-2 binding site. On the coding strand, modification of six adenosines in the center of the binding site interfered completely with MEF-2 binding. On the noncoding strand, a single adenine was detected which, when modified by DEPC, interfered with MEF-2 binding. The results obtained from DNase I protection and DEPC interference are summarized in Fig. 5C.

Binding of MEF-2 to oligomers corresponding to sequences within the mck and mlc-1/3 enhancers. Comparison of the mouse MEF-2 binding site with regulatory sequences associated with other cellular and viral genes revealed exact homology between the core of the binding site and a 10-bp A+T-rich element within the human and rat mck enhancers (24, 56), the rat mlc-1/3 enhancer (13), and the chicken mlc-2A promoter (1). The A+T-rich core, which is identical in all of these genes, is flanked by pyrimidines on each end. The alignment of these sequences and a derived consensus for the MEF-2 binding site are shown in Fig. 6.

The ability of MEF-2 to recognize the conserved A+T-rich element was explored further by using corresponding oligomers as competitors in gel retardation assays. Initially, we examined the interaction of MEF-2 and MEF-1 with the 16-bp sequence from the mouse mck enhancer. The unlabelled oligomer corresponding to the binding site for MEF-2 (OL-mck) competed effectively for the binding of MEF-2, as well as MEF-1, when fragment 12 (−1094 to −1048) was the labeled probe (Fig. 7A). Conversely, with the oligomer as the labeled probe, nuclear extracts from C2 myoblasts and myotubes gave rise to the MBF-1- and MEF-2-dependent

![Image](http://mcb.asm.org/Downloaded from http://mcb.asm.org on May 8, 2021 by guest)
FIG. 5. Determination of the recognition site for MEF-2 by DNase I footprinting and DEPC interference. (A) Fragment 12 (−1094 to −1048) was single end labeled on either the coding or noncoding strand and used as a probe in gel retardation assays with 5 μg of nuclear extract from C2 myotubes. DNA-protein complexes were incubated with DNase I as described in the text, and reactions were then electrophoresed on a nondenaturing polyacrylamide gel. The myocyte-specific complex was visualized by autoradiography, electroeluted, and electrophoresed on a sequencing gel. Bound and Free in panels A and B refer to MEF-2-bound probe and free probe, respectively. The extent of protection from DNase I cleavage is shown by the brackets. Dashed lines indicate partial protection. (B) Fragment 12, which had been single end labeled on either the coding or noncoding strand, was partially carboxethoxylated by exposure to DEPC as described in the text. The probe was then used in a gel retardation assay with 5 μg of nuclear extract from C2 myotubes. The myocyte-specific complex was visualized by autoradiography, electroeluted, cleaved at modified residues and electrophoresed on a sequencing gel. * Modified nucleotides that interfered with MEF-2 binding. Maxam-Gilbert A+G sequencing reactions are shown for each strand. (C) Nucleotides protected by MEF-2 from DNase I cleavage; solid line indicates complete protection; dashed line indicates partial protection.* Hypersensitive nucleotide. Dots denote nucleotides that interfere with MEF-2 binding when modified by DEPC.

FIG. 6. Sequence homologies between the MEF-2 binding site in the mouse mck enhancer and regulatory elements associated with other genes. The sequence on the coding strand of the MEF-2 binding site from the mouse mck enhancer is compared with sequences associated with other muscle-specific genes. Human and rat mck sequences are from references 24 and 56. The mlc-1/3 sequence lies on the coding strand of the enhancer, which is located >24 kilobase pairs downstream of the mlc-1 promoter (13). The mlc-2 sequence lies on the noncoding strand between bp −42 and −57 (1). The indicated region of the mck enhancer was synthesized as a double-stranded oligomer (OL-mck) and used as a probe and competitor to analyze MEF-2 binding in Fig. 7.
FIG. 7. Gel retardation assays using oligomers corresponding to the binding site for MEF-2. The sequence from the mck enhancer shown in Fig. 6 was synthesized as a double-stranded oligomer and used in gel retardation assays. (A) Fragment 12 (−1094 to −1048) was end labeled and incubated with nuclear extracts from C2 myoblasts and myotubes as indicated. Specificity of factor binding was determined by competition with unlabeled fragment 12 and OL-mck. (B) OL-mck was end labeled and incubated with nuclear extracts from C2 myoblasts and myotubes as indicated. Specificity of factor binding was assessed by competition experiments as in panel A. (C) Cloned oligomers corresponding to the mck or mlc-1/3 sequences shown in Fig. 6 were labeled and used as probes in gel retardation assays with 5 μg of nuclear extract from C2 myoblasts, myotubes, and 10T1/2 cells. (D) Oligomers corresponding to the mck and mlc-1/3 sequences were labeled and used as probes in gel retardation assays with nuclear extract from C2 myotubes. The relative affinity of MEF-2 for the two sequences was determined by assaying the ability of increasing amounts of each unlabeled oligomer to compete for the formation of the myocyte-specific complex.

complexes, respectively (Fig. 7B). These complexes were eliminated in the presence of an excess of unlabeled fragment 12 or OL-mck (Fig. 7B), whereas nonspecific DNA fragments failed to compete for these complexes (data not shown).

To determine whether MEF-2 and MBF-1 also recognized the corresponding region of the mlc-1/3 enhancer (Fig. 6), we examined the ability of these factors to recognize a cloned oligomer corresponding to the region of the mlc-1/3 enhancer encompassing the MEF-2 recognition sequence. The mck and mlc-1/3 oligomers generated comparable myoblast-specific and myotube-specific complexes, indicating that MBF-1 and MEF-2 recognize the same sequence in the two enhancers (Fig. 7C). It was also of interest to determine whether
MBF-1 and MEF-2 were specific to muscle cells or were present in nonmuscle cells. Extracts from proliferating 10T1/2 cells did not appear to give rise to the MBF-1- or MEF-2-dependent complexes with either the mck or mlc-1/3 enhancer oligomers (Fig. 7C) or with larger fragments of the enhancer (data not shown). Minor DNA-protein complexes with different mobilities from the MBF-1/MEF-2 complexes were detectable with 10T1/2 extracts, however, after prolonged exposure to film. MBF-1 and MEF-2 also were not detected in extracts from 3T3 fibroblasts (data not shown). It should be pointed out that the sensitivity of this assay is such that we might not detect factors that bind to the MEF-2/MBF-1 binding site if they were present at levels 5- to 10-fold lower than the level of MBF-1 in myoblasts.

The relative affinity of MEF-2 for the mck and mlc-1/3 enhancers was determined by competition experiments with the two oligomers. Each oligomer was able to compete for the formation of the myocyte-specific complex when the opposite oligomer was used as the labeled probe (Fig. 7D). However, the mck oligomer was a more effective competitor than the mlc-1/3 oligomer and gave rise to a more intense myocyte-specific complex, suggesting that MEF-2 may have a higher affinity for the mck enhancer than for the mlc-1/3 enhancer.
Functional analysis of the MEF-2 binding site. To investigate whether the MEF-2 binding site was important for enhancer function, we created a series of deletions within the 302-bp upstream region (−1350 to −1048), which was shown previously to contain the enhancer (24, 27, 52, 53). Initially, each half of the 302-bp enhancer region was inserted downstream of cat, which was under transcriptional control of the mck promoter. None of the mck upstream regions tested showed significant activity in proliferating myoblasts or in 10T1/2 or 3T3 cells (Fig. 8A). However, as reported previously, the 302-bp region, contained in pCKCATe4, directed high levels of CAT expression in terminally differentiated myotubes. Although neither half of the 302-bp region retained full enhancer activity, the proximal half of this region, contained in pCKCATe6, exhibited strong enhancer activity in myotubes, whereas the distal half, contained in pCKCAT5, was inactive. These results suggest that the core of the upstream enhancer lies between bp −1204 and −1048. Sequences upstream from −1204 appear to contribute to activity of the core but alone lack activity.

The 156-bp active enhancer was subdivided further into two fragments, −1204 to −1095 and −1094 to −1048, to generate pCKCATe11 and pCKCATe12 (Fig. 8A). Analysis of these constructs in myoblasts and myotubes showed that the 109-bp region retained differentiation-specific expression but was only about 10% as active as the full enhancer. The 47-bp region, which contains the MEF-2 binding site, showed little or no activity. The MEF-2 binding region seems, therefore, to be required for maximal activity of the larger enhancer region but does not possess significant activity alone.

Since enhancers are generally composed of multiple elements that act in concert (16, 23, 39, 44), we examined whether the MEF-2 binding site might exhibit enhancer activity in multiple copies. Indeed, when present in two or three copies, the MEF-2 binding region (−1094 to −1048) showed strong enhancer activity in C2 myotubes (Fig. 8A). However, in contrast to the strict developmental regulation of the larger enhancer regions, multimers of the MEF-2 region also showed significant activity in myoblasts. In 10T1/2 and 3T3 cells, the basal level of activity of the MEF-2 region showed little, if any, increase upon multimerization.

We also tested whether the synthetic oligomers that were shown to bind MEF-2 and MBF-1 would exhibit enhancer activity when oligomerized as a tandem repeat. The mck oligomer in multiple copies directed moderate levels of CAT expression from the mck promoter (Fig. 8B). Like multimers of the larger region (−1094 to −1048) from which it was derived, the oligomers showed higher activity in myotubes than in myoblasts but were not absolutely myotube specific. Together, these results indicate that the MEF-2 binding site acts as a peripheral enhancer element, or enhanser, that functions preferentially but not exclusively in differentiated myotubes. This element has little or no activity of its own and is dependent on cooperative homotypic or heterotypic interactions with adjacent elements for activity.

DISCUSSION

Enhancers are composed of multiple positive and negative elements that interact with combinations of ubiquitous and cell-type-specific transcription factors (16, 23, 39, 44). Whereas each enhancer element may lack activity alone, in concert these elements confer highly specialized patterns of expression on associated genes. The mck 5' enhancer has been shown to possess the potential to direct tissue specificity, developmental regulation, and high-level expression on the mck promoter and heterologous promoters (14, 24, 27, 52, 53). Little is known, however, of the factors that confer these properties on the mck enhancer or other muscle-specific regulatory elements.

In this study, the mck enhancer was shown to contain multiple elements that are diffusely distributed between bp −1350 and −1048 relative to the transcription initiation site. The major regulatory region of the enhancer was found to lie between 1,204 and 1,048 bp upstream of mck. Within this region, we identified an enhancer core between −1204 and −1095 that exhibits muscle specificity, in addition to a conserved activating element immediately 3' from this core that is essential for maximal enhancer activity. The sequence of this peripheral activating element is conserved among multiple muscle-specific genes and interacts with a novel myocyte-specific factor, designated MEF-2, which is rapidly up-regulated in C2 and BC3H1 myoblasts after a stimulus to differentiate. The pattern of regulation and the recognition sequence for MEF-2 suggest that this represents a new enhancer-binding factor which may be specific to the myogenic lineage. It remains to be determined whether MEF-2 is also expressed in cardiac muscle, which expresses mck at high levels.

MEF-2 expression is an early molecular marker for activation of the muscle differentiation program and precedes the expression of other muscle-specific genes, such as mck, by several hours. Expression of MEF-2 was prevented by cycloheximide, suggesting that the appearance of this DNA-binding activity may require new protein synthesis rather than posttranslational activation of a preexisting factor. A myoblast-specific enhancer-binding activity (MBF-1) was also found to bind to the recognition sequence for MEF-2. MBF-1 was rapidly down regulated after removal of mitogens from myoblasts. FGF and TGF-β, which block induction of muscle-specific genes but do not exhibit mitogenic activity for BC3H1 cells, were able to maintain MBF-1 at high levels and to prevent expression of MEF-2. The ability of these purified growth factors to modulate the expression of MEF-2 and MBF-1 in the absence of mitogens indicates that the regulation of these enhancer-binding factors is coupled to the state of differentiation and does not simply reflect whether or not the cells are proliferating.

Although MEF-2 and MBF-1 could represent alternate forms of the same factor which differ, for example, by posttranslational modification, there are several observations that argue against this possibility. First, MBF-1 appears to be a relatively minor species in myoblasts, compared with the abundance of MEF-2 in myocytes. Second, MBF-1 is down regulated completely within 4 h in mitogen-deficient medium, whereas MEF-2 continues to accumulate for at least 48 h. Finally, the dependence of MEF-2 expression on protein synthesis suggests that it does not appear as a result of posttranslational conversion of MBF-1.
enhancer region. Since enhancers often contain redundant elements, it is possible that the activating potential of the MEF-2/MBF-1 binding site is exaggerated when it is deleted from the partial 156-bp enhancer region placed 3' from cat rather than from the complete enhancer region in its normal orientation with respect to the promoter. In this regard, Horlick and Benfield have also performed deletion mutagenesis on the 5'-flanking region of the rat mck gene and found that deletions that removed the MEF-2/MBF-1 site dramatically reduced the level of expression of a linked cat gene under control of a partially deleted promoter but had little effect when the complete 5'-flanking region was present (24). Although we used a region of the MEF-2/MBF-1 binding site that showed little enhancer activity when present as a monomer, multiple copies of this region were sufficient to strongly activate the mck promoter. On the basis of the strict developmental regulation of the complete mck enhancer, we expected that MBF-1 might function as a negative regulatory factor and that the oligomerized MEF-2/MBF-1 motif would be inactive as an enhancer in myoblasts. However, an intriguing and unanticipated result was that multimers of the MEF-2/MBF-1 binding site exhibited enhancer activity in myoblasts as well as myotubes. It is unclear why MBF-1 and MEF-2 are differentially expressed during myogenesis if both factors possess the ability to act as positive regulatory factors. There are, however, several possible explanations for these observations. Enhancers are generally considered to be composed of modular arrays of elements that exhibit unique cell-type specificities, with the overall pattern of expression of the complete enhancer reflecting combinatorial interactions among the individual elements (16, 23, 39, 44). A particular enhancer motif may, in fact, exert either positive or negative transcriptional effects, depending on the cell type and the adjacent elements with which it can interact. Thus, the contribution of an individual enhancer motif to the activity of a large and complex enhancer such as the mck enhancer may not necessarily parallel its activity as a multimer removed from its normal genomic context. The results of this study are consistent with the conclusion that both MEF-2 and MBF-1 possess the potential to function as positive regulatory factors that require cooperative interactions for generation of enhancer activity. Within the context of the complete mck enhancer, this cooperativity would result from association with additional factors that bind to other nearby sites. The net effect on enhancer activity would thus be dictated by positive interactions in myotubes and negative interactions or a lack of interactions in myoblasts. Oligomerization of the MEF-2/MBF-1 motif would allow homotypic interactions between MBF-1 or MEF-2, thereby leading to enhancer activation in myoblasts or myotubes. In contrast to the moderate levels of enhancer activity of the multimerized MEF-2/MBF-1 motif in myoblasts, this element failed to exhibit significant enhancer activity in 10T1/2 or 3T3 cells. The low level of activity of this motif in these cells is most easily explained by an absence of corresponding binding factors. Indeed, gel shift assays failed to reveal MEF-2 or MBF-1 in extracts from 3T3 or 10T1/2 cells.

In addition to recognizing the mck enhancer, MEF-2 and MBF-1 were shown to interact with a homologous region within the mcl-1/3 enhancer. Since a similar sequence motif is also found within a region of the mcl-2 promoter that has been shown to be required for maximal expression in primary chick myoblasts (8), it is likely that MEF-2 also interacts with this sequence. We reported previously that the region of intron 1 of mck that exhibits enhancer activity contains eight tandem copies of the motif TAAAA (53). Although this motif is a truncated form of the MEF-2/MBF-1 binding site, the nucleotides that were shown by DEPC interference to participate in binding are present. It will, therefore, be interesting to determine whether MEF-2 and MBF-1 also recognize this region of the mck gene. The MEF-2 binding site does not appear to be present within regulatory regions associated with the genes for a-skeletal (3, 22) and a-cardiac (35) actin, troponin-I (65), troponin-T (33), acetylcholine receptor a (41, 61) and 8 (2) subunits, desmin (40), or myosin heavy chain (5), suggesting that these genes are regulated by distinct sets of factors.

Although nuclear factors have been shown to interact with regulatory elements associated with other muscle-specific genes, it is not known whether this occurs in differentiating myocytes. The conserved motif CC(ATC)4GG, referred to as a CarG box/C-bar (3, 35), in the 5' proximal regions of the a-skeletal and a-cardiac actin genes has been shown to interact with a ubiquitous nuclear factor that may share identity with the serum response factor which interacts with the serum response element in the promoter of the c-fos proto-oncogene (22, 36, 57). A different factor, designated MAPF2, that appears to be specific to the myogenic lineage but is not developmentally regulated, also has been shown to interact with this motif (58-60). Like the CarG box/C-bar, the MEF-2 binding site is A+T-rich. However, the two sites are clearly distinct, as are the patterns of expression of the corresponding binding factors. Competition experiments have also shown that MEF-2 does not interact with the CarG box/C-bar located between -1233 and -1224 in the mck enhancer (L. Gossett and E. Olson, unpublished results).

A factor present in nuclear extracts from C2 myoblasts, myotubes, and HeLa cells has been shown to interact with a positive regulatory element associated with the rat embryonic myosin heavy-chain gene (64). The upstream region of the chicken acetylcholine receptor a-subunit gene also has been shown to interact with several factors that are expressed in a wide range of cell types, in addition to a factor that is expressed preferentially in myotubes (41). Binding of this factor to the a-subunit upstream region can be displaced by an oligomer corresponding to a region of the simian virus 40 enhancer core. The recognition sequence for this factor does not resemble the binding site for MEF-2.

Buskin and Haushka have identified a myocyte-specific factor, MEF-1, that interacts with the region of the mck enhancer that we have defined as the core binding site for MyoD1 (1). Antibodies directed against MyoD1 cross-react with MEF-1, indicating that these factors are similar or identical (31). Identity between MEF-1 and MyoD1 is suggested further by the observation that bacterially expressed MyoD protein binds to the same sequence in the mck enhancer as does MEF-1 (31). Although MEF-1 binding activity is detectable only in myocytes, MyoD1 protein is expressed in myoblasts and myocytes (55), suggesting that MEF-1 exists in myoblasts in an inactive state that is converted into a form that can bind DNA after a stimulus to differentiate. The ability of cycloheximide to prevent the expression of MEF-2 suggests that this factor is synthesized de novo after removal of mitogens and may therefore be regulated differently from MyoD1/MEF-1. Further evidence against a relationship between MEF-2 and MyoD1/MEF-1 comes from the fact that MEF-2 is expressed in BC3H1 myocytes, which do not express MyoD1 at detectable levels (11, 14). The recognition sequences for MyoD1/MEF-1 and MEF-2 also do not share homology. We have recently identified two sites within the mck enhancer core that bind nuclear factors from differentiated BC3H1 and C2 cells (D. Kelvin, L. Gossett, T. Brennan, E. Sternberg, and E. Olson, manuscript in prepa-
ration). Since BC3H1 cells lack MEF-1/MyoD, it will be interesting to determine the relationship between these factors.

Our laboratory has reported recently that myogenin is expressed in BC3H1 and C2 cells with kinetics similar to that of MEF-2 and that myogenin will activate the expression of mck-cat genes after transient transfection into 10T1/2 fibroblasts (14). Deletion mutagenesis revealed that the region between mck –1204 and –1048 was necessary and sufficient to confer myogenin-dependent regulation on the mck promoter. Myogenin does not bind to the MEF-2 site but does bind as a heterodimer to the mck enhancer core (T. Brennan and E. Olson, manuscript in preparation).

The results of the present study demonstrate that the mck enhancer, like other cellular and viral enhancers, contains multiple regulatory elements that interact with a complex array of nuclear factors. Two of these factors, MEF-2 and MBF-1, bind to the same site and are apparently regulated in a reciprocal manner. The selective expression of MEF-2 in differentiated muscle cells, along with the presence of the MEF-2 binding site within regulatory regions of the mck, mle-1/3, and mle-2 genes, suggests that MEF-2 may participate in regulation of multiple muscle-specific genes during myogenesis. While MEF-2 appears to be a novel myocyte-specific enhancer-binding factor that plays an important role in transcriptional regulation of mck, it is clear that additional factors are required for complete regulation of mck by myogenesis. Studies to identify and characterize these factors and to further define the relationship between MEF-2 and MBF-1 are underway.

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LITERATURE CITED


MYOCYTE-SPECIFIC ENHANCER-BINDING PROTEIN

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