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7. The system is composed of a cooled charge-coupled device (CCD) camera (TE/CCD512BKS, Princeton Instruments, Trenton, NJ), a large custom-built turntable that holds 12 plates, and a computer (Macintosh LCII, Apple Computer) with custom software that controls the turntable and data acquisition. The turntable was programmed to make a complete circuit in 45 min, which subjected each plate to a cycle of 36 min of white light [46 $\mu E\ m^{-2}\ s^{-1}$ (E is the energy of 1 mol of photons)] and 9 min of darkness at 30°C (these were standard assay conditions). As brief, repetitive dark interruptions of continuous light do not cause major interference to circadian clocks (*5*, *15*), we assumed these conditions were equivalent to continuous light (LL). Photosynthesis during the light period maintained the metabolism of the cells. The bioluminescence image was captured by the CCD camera during the middle 3 min of the 9-min dark interval. The image was initially scanned with a border-following algorithm. On average, 500 to 1000 colonies could be recognized on each plate by this algorithm. After this initial scan, bioluminescence signals from each region were integrated and stored. The parameters of the rhythms (period, phase, and amplitude) were computed by regression procedures similar to those used for the rhythms of eukaryotic algae (*15*).
8. AMC149 cells were cultured in BG-11 medium (*16*) which was bubbled with air in LL (46 $\mu E\ m^{-2}\ s^{-1}$). At the middle of the exponential growth phase [optical density at 730 nm ($OD_{730}$) = 0.5], 5 ml of culture was spun down, and cells were resuspended in a mixture of 0.5 ml of BG-11 medium and 0.5 ml of 30 mM potassium phosphate buffer (pH 7). After adding EMS (400 mM; Sigma) and incubating for 30 to 40 min at 37°C, 10 ml of 5% sodium thiosulphate solution was added to inactivate the EMS. Cells were washed twice with BG-11 medium and incubated under dim LL (23 $\mu E\ m^{-2}\ s^{-1}$) to allow the surviving cells to grow. About 0.2 to 1% of the cells survived this treatment. When cultures reached stationary growth phase, they were subcultured by a 1/1000 dilution into fresh medium.
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12. To be sure that the mutant phenotypes were not a result of mutagenesis of cis elements of the reporter gene, we removed the P*psbAI-luxAB* reporter in several mutants and then introduced it again. This was achieved by transformation of the mutant strains with a plasmid that carries a kanamycin resistance gene in the neutral site region of the *Synechococcus* chromosome. AMC149 and the original mutant strains carry a spectinomycin resistance gene and the P*psbAI-luxAB* reporter at this locus (*5*). Transformation and selection for kanamycin resistance resulted in recombinants that had replaced the spectinomycin resistance gene and the P*psbAI-luxAB* reporter with the kanamycin resistance gene. These strains, purged of the reporter, were transformed again with pAM977, the plasmid used to generate AMC149; selection for spectinomycin resistance yielded clones which again carried the P*psbAI-luxAB* reporter.
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17. Cells were inoculated into BG-11 medium and cultured with air-bubbling under LL (46 $\mu E\ m^{-2}\ s^{-1}$) at 30°C. Cell growth was monitored by measuring $OD_{730}$ of small volumes withdrawn from the culture. Under these conditions, the cultures grew in an exponential fashion to a density of $OD_{730}$ = 0.8 (about $10^9$ cells per milliliter). The doubling time of the cells was calculated from the slope of the growth curve.
18. We thank N. Lebedeva and C. A. Strayer for technical assistance with the reporter gene replacement experiment and N. Yamaguchi for assembling the turntable system. This research was supported by grants from Science and Culture, the Ministry of Education (05670079, 06670093, and 06807029), United States–Japan Cooperative Program (BSAR382), the Shimadzu Foundation (Kyoto), and the Yamada Foundation (Osaka) to T.K. and M.I.; the National Institute of Mental Health (MH43836) and NSF (MCB-9219880 and INT-9218744) to C.H.J.; and NSF (DMB-8958089 and MCB-9311352) and NIH (GM37040) to S.S.G.

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# Activation of the Myogenic Lineage by MEF2A, a Factor That Induces and Cooperates with MyoD

Sunjay Kaushal,* Jay W. Schneider,† Bernardo Nadal-Ginard,‡ Vijak Mahdavi‡

**Muscle enhancer factor–2A (MEF2A), a member of the MADS family, induced myogenic development when ectopically expressed in clones of nonmuscle cells of human clones, a function previously limited to the muscle basic helix-loop-helix (bHLH) proteins. During myogenesis, MEF2A and bHLH proteins cooperatively activate skeletal muscle genes and physically interact through the MADS domain of MEF2A and the three myogenic amino acids of the muscle bHLH proteins. Thus, skeletal myogenesis is mediated by two distinct families of mutually inducible and interactive muscle transcription factors, either of which can initiate the developmental cascade.**

When ectopically expressed, the family of muscle bHLH transcription factors—MyoD, myogenin, MRF4, and myf5—independently activates the cascade of myogenic development (*1*). The myogenic activity of muscle bHLH factors was mapped to three amino acid residues located within the basic junction domain, which is predicted to reside within the major groove during DNA binding (*2*, *3*). In addition to binding DNA as heterodimers with bHLH E-proteins, the muscle bHLH proteins associate with several non-HLH proteins, including c-Jun, the retinoblastoma protein (Rb), and p107 (*4*–*6*). Despite this functional diversity, gene transfer and gene knockout studies have shown that certain features of the genetic regulation of myogenesis cannot be attributed to the bHLH proteins (*1*, *7*).

The muscle enhancer factor–2 or related to serum response factor (MEF2-RSRF) family, a second class of muscle transcription factors that belong to the MADS superfamily (MCM1, agamous, deficiens, and human serum response factor), also plays an important role in myogenic differentiation. Four distinct genes encoding the MEF2 family (*MEF2A*, *B*, *C*, and *D*) have been cloned in mammals, two in *Xenopus* (*SL1* and *SL2*), and a single gene in *Drosophila* (*D-MEF2*) (*8*, *9*). Whereas the muscle bHLH factors are expressed only in skeletal muscle cells, the MEF2 factors are predominantly, though not exclusively, expressed in skeletal, cardiac, and smooth muscle and in neuronal cells (*8*, *9*). Like the

Department of Cardiology, Children's Hospital, Harvard Medical School, Boston, MA 02115, USA.

*Present address: CVRC, Massachusetts General Hospital-East, Charlestown, MA 02129, USA.
†Present address: Cardiology Division, Department of Internal Medicine, Brigham and Women's Hospital, Harvard Medical School, Boston, MA 02115, USA.
‡The two senior authors (B.N.-G. and V.M.) can be reached at their private address: 340 Beacon Street, Boston, MA 02116, USA.

**Table 1.** Comparison of the efficiency of myogenic conversion after stable transfection of MyoD, myogenin, or MEF2A in mouse fibroblasts. 10T1/2, 3T3-L1, and 3T3-C2 fibroblasts were cotransfected as described (Fig. 2) and immunostained with the MHC mAb MF-20 and the ABC-Vectastain kit (Vector Laboratories). Myogenic conversion represents the number of myogenic colonies divided by the total number of *neo*$^r$ colonies. Control, pMSV.

| Muscle regulatory gene | Myogenic colonies (*n*) | Total colonies (*n*) | Myogenic conversion (%) |
|---|---|---|---|
| | *10T1/2 cells* | | |
| Control | 0 | 50 | 0 |
| MyoD | 17 | 65 | 26 |
| Myogenin | 10 | 35 | 28 |
| MEF2A | 35 | 250 | 14 |
| | *3T3-L1 cells* | | |
| Control | 0 | 56 | 0 |
| MyoD | 8 | 20 | 40 |
| Myogenin | 3 | 20 | 10 |
| MEF2A | 5 | 35 | 14 |
| | *3T3-C2 cells* | | |
| Control | 0 | 54 | 0 |
| MyoD | 5 | 15 | 33 |
| Myogenin | 7 | 19 | 37 |
| MEF2A | 3 | 35 | 8 |

muscle bHLH protein family, the four MEF2 genes (A through D) are sequentially expressed during the development of muscle cells in embryos and in cell culture (*8–10*). Whereas MEF2D accumulates in mouse C2C12 myoblasts, MEF2A, B, and C can be detected only in differentiated myotubes. The MEF2 family gains additional complexity from tissue-specific splicing of exons in muscle and neural cells (*8*, *9*).

Ectopic expression of myogenin or MyoD induces MEF2 activity in many nonmuscle cells, which suggests that the genes encoding MEF2 are downstream of the muscle bHLH genes in the hierarchy of myogenic regulators (*4*, *11*). However, both the mouse myogenin and *Xenopus* MyoDa genes have essential MEF2 DNA-binding sites in their control regions, which suggests that the MEF2 proteins are important regulators of muscle bHLH factor gene expression (*12*). During *Drosophila* development, mRNA that encodes D-MEF2 appears at the late cellular blastoderm stage preceding the appearance of mRNA for *nautilus*, the homolog of MyoD (*9*). Thus, at least in *Drosophila*, MEF2 is an upstream regulator of myogenic development, although direct induction of the endogenous muscle bHLH gene by *Drosophila* MEF2 has not been established.

Functional MEF2 DNA-binding sites are essential for the high expression levels of many striated muscle genes (*11*, *13*). In these muscle-specific genes, MEF2 DNA-binding sites are often juxtaposed to one or more muscle bHLH factor DNA-binding sites (E-boxes). This configuration of cis elements, which has been conserved through millions of years of muscle gene evolution, suggests an important and intimate relation between these two families of regulatory factors. Here, we investigated the functional and biochemical relationship of the MEF2A and muscle bHLH factors in the regulatory hierarchy of skeletal myogenesis.

The muscle bHLH proteins can initiate the program of skeletal muscle differentiation when forcibly expressed in nonmuscle cells (*1*). To test if MEF2A, which is a strong transactivator of muscle genes (*8*), can also convert nonmuscle cells into skeletal muscle cells, we selected two cell lines with little or no endogenous MEF2A protein for gain-of-function experiments (*8*). 10T1/2 embryonic mouse fibroblasts and CV-1 monkey kidney cells were permanently transfected with expression plasmids for human MEF2A under the control of the Moloney sarcoma virus long terminal repeat and the neomycin resistance gene (*neo*$^r$). Transfectant colonies were selected in neomycin, tested for ectopic MEF2A expression, and examined for myogenic differentiation after 5 days in differentiation medium. Whereas colonies transfected with the *neo*$^r$ plasmid alone exhibited no phenotypic changes, a large percentage of 10T1/2 colonies cotransfected with human MEF2A differentiated into multinucleated myotubes, whose phenotype was confirmed by double-immunostaining for human MEF2A and sarcomeric myosin heavy chain (MHC) (Fig. 1). Transfection of MEF2A in CV-1 cells, which are blocked in the myogenic differentiation pathway downstream of the muscle bHLH factors (*1*), induced the expression of myogenin but not of other muscle lineage markers. Therefore, like the muscle bHLH proteins, ectopic expression of MEF2A triggered skeletal muscle differentiation in 10T1/2 but not in CV-1 cells.

The ability of MEF2A to induce myogenic differentiation in these cells was further documented by protein and RNA blot of permanently transfected cells (Fig. 2). Constitutively expressed human MEF2A activated the full program of skeletal myogenesis in 10T1/2 cells, as documented by the induction of myogenin, MyoD, MHC, and muscle-specific α-actin mRNA (Fig. 2A). In contrast to 10T1/2 cells, CV-1 cells responded to permanent transfection of human MEF2A with the induction of myogenin but not of the other muscle lineage markers. Equivalent amounts of human MEF2A polypeptide were detected by protein blot in permanently transfected 10T1/2 and CV-1 cells (Fig. 2B).

The efficiency of myogenic conversion induced by MEF2A was compared with that induced by MyoD and myogenin in three mouse fibroblast cell lines known to be highly responsive to the muscle bHLH proteins (*1*) (Table 1). After stable cotransfection and induction of differentiation, 10T1/2, 3T3-L1, and 3T3-C2 transfectant colonies were examined for myogenic differentiation

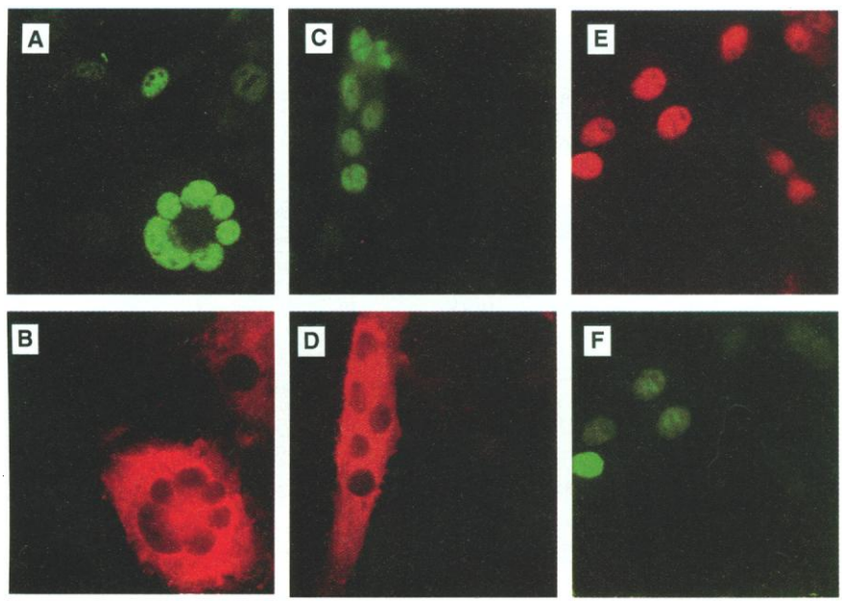


**Fig. 1.** Differential induction of skeletal muscle differentiation by stably transfected human MEF2A in 10T1/2 and CV-1 cells. Cells were cotransfected as described (*8*) with 10 μg of pMSV-MEF2A (containing the muscle-specific axon SEEEELEL) and 0.1 μg of pSV-neo, selected for 2 weeks in Dulbecco's modified essential medium (DMEM) with 20% fetal calf serum and G418 (500 μg/ml), induced to differentiate in DMEM with 2% horse serum, and prepared for double immunostaining as described (*8*). (**A**) MEF2A-transfected 10T1/2 cells immunostained with MEF2A antiserum (fluorescein, green). (**B**) Same field as in (A), immunostained with MHC mAb (rhodamine, red). (**C**) MEF2A-transfected 10T1/2 cells from a different experiment immunostained with MEF2A antiserum (fluorescein). (**D**) Same field as in (C), immunostained with MHC mAb (rhodamine). (**E**) MEF2A-transfected CV-1 cells immunostained with MEF2A antiserum (rhodamine). (**F**) Same field as in (E), immunostained with myogenin mAb (fluorescein).

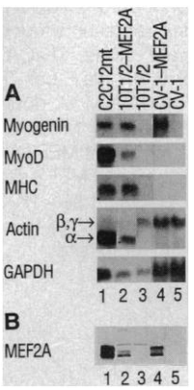


**Fig. 2.** Differential induction of muscle-specific mRNA in MEF2A-transfected 10T1/2 and CV-1 cells (*20*). (**A**) RNA blot of C2C12 myotubes (mt) (lane 1), MEF2A-transfected 10T1/2 cells (lane 2), 10T1/2 cells (lane 3), MEF2A-transfected CV-1 cells (lane 4), and CV-1 cells (lane 5) hybridized to radiolabeled cDNAs as indicated on the left. (**B**) Protein blot of extracts prepared from the same cells as in (A) detected with MEF2A antiserum (*21*). On this blot, a faint, nonspecific cross-reactive band is present in parental and MEF2A-transfected 10T1/2 cells. GAPDH, glyceraldehyde phosphate dehydrogenase.

by immunostaining for MHC. Human MEF2A induced skeletal myogenesis in these fibroblasts in a manner similar, but at lower efficiency, to that of the myogenic bHLH factors.

Although constitutive, high-level expression of MEF2A in 10T1/2 fibroblasts activated skeletal muscle–specific gene expression (Figs. 1 and 2), the endogenous MEF2A activity in cardiac muscle, smooth muscle, and neuronal cells do not. Thus, endogenous MEF2A might require a skeletal muscle–specific cofactor to efficiently induce the expression of skeletal muscle genes. Evidence from DNA binding and transfection experiments suggested that the MEF2 and the MyoD factors might stabilize the binding of each other to collinear DNA sequences and thus might cooperate in transactivation. For example, the CASTing (cyclic amplification and selection of target sequences) protocol done with C2C12 myotube extracts and the MEF2 antiserum resulted in the coselection of adjacent E-box and MEF2 DNA-binding sites (*14*). Similar results were previously obtained in a reciprocal experiment with a myogenin antiserum (*15*).

To gain insight into the potential interaction between MEF2A and muscle bHLH proteins, we performed a series of transfection experiments (Fig. 3). Constructs containing E-box (E) or MEF2 (M) DNA-binding sites from the gene, enhancers of muscle creatine kinase (MCK) or myosin light chain 1/3 (MLC1/3) fused to the thymidine kinase promoter–chloramphenicol acetyltransferase (TK-CAT) reporter gene were transiently transfected in skeletal muscle cells. Whereas MCK reporter genes containing either a single E-box or a single MEF2 binding site were inactive in C2C12 myotubes, reporters containing duplicated copies of either element (E-E or M-M) were active (Fig. 3A). Maximal reporter activity was achieved when a single E-box site was combined with a single MEF2 site (E-M). The cooperative effect of the E-M reporter was abrogated by mutation at either E-box or MEF2 site. Identical results were observed with MLC1/3 E-box and MEF2 binding sites (Fig. 3B). Furthermore, physical separation of the MLC1/3 E-box and MEF2 sites by an additional half or full turn of the double helix had no effect on the transcriptional activity of the corresponding reporter genes. These results, which extend previously reported data (*16*), document a sequence-specific cooperative activation of reporter genes bearing adjacent E-box and MEF2 DNA-binding sites in differentiated skeletal muscle cells.

Certain E-box–containing muscle genes are expressed at high levels in cardiac as well as skeletal muscle cells, even though cardiac muscle cells lack functional equivalents of the muscle bHLH factors. Cardiocytes, therefore, provide a natural control for the cooperative activation of the E-M reporter gene. When transfected in primary cardiocytes from neonatal rats, the M-M reporter gene was highly active, but the E-M reporter was silent (Fig. 3C). Thus, both MEF2A and muscle bHLH factors are required for the cooperative gene activation in skeletal muscle cells. These results also suggest that cardiac muscle cells achieve high-level muscle gene expression by a different mechanism.

We tested whether the cooperative effect between the E-box and the MEF2 DNA-binding sites observed in skeletal myotubes was mediated by their corresponding transacting factors, or whether additional muscle-specific factors might be required. The MLC1/3 reporter plasmids were cotransfected with expression plasmids encoding myogenin, MEF2A, or both factors in CV-1 cells, which lack both endogenous muscle bHLH and MEF2 activities (Fig. 3D). Compared to either MEF2A or myogenin alone, the highest reporter activity was obtained when both factors were co-

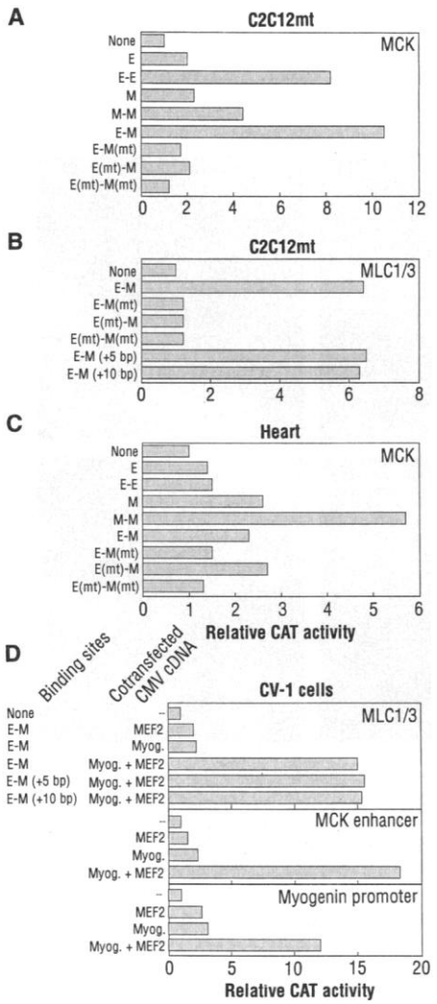


**Fig. 3.** MEF2A and muscle bHLH proteins cooperate in the transactivation of muscle reporter genes (*22*). (**A**) TK-CAT reporter gene constructs containing wild-type or mutant E-box or MEF2 DNA-binding sites from the MCK gene enhancer were transiently transfected in C2C12 myotubes. (**B**) Reporter constructs with binding site sequences from the MLC1/3 enhancer were transfected in C2C12 myotubes as in (A). (**C**) The same reporter constructs as in (A) were transfected in neonatal rat cardiocytes. (**D**) Expression plasmids for MEF2A or myogenin (Myog.) in pCMV were cotransfected with reporter constructs containing synthetic binding sites from the MLC1/3 enhancer upstream from TK-CAT as in (B) or the natural promoter-enhancer sequences from the mouse MCK (p3300MCKCAT) or myogenin (pMyo184CAT) genes in CV-1 cells. Relative CAT activity represents the experimental CAT activity divided by the activity of TK-CAT, p3300MCKCAT, or pMyo184CAT alone. Each result represents four different experiments in duplicate. None, TK-CAT; E, single E-box–TK-CAT; E-E, double E-box–TK-CAT; M, single MEF2 site–TK-CAT; M-M, double MEF2 site–TK-CAT; E-M, E-box and MEF2 site–TK-CAT; E-M(mt), E-box and mutant MEF2 site–TK-CAT; E(mt)-M, mutant E-box and MEF2 site–TK-CAT; E(mt)-M(mt) double mutant site–TK-CAT; and +5 bp or +10 bp, insertion of 5 or 10 base pairs between the E-box and MEF2 site.

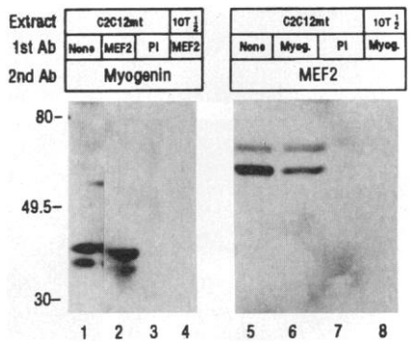


**Fig. 4.** Direct association of MEF2A and myogenin in C2C12 myotubes (*23*). Whole-cell extracts of C2C12 myotubes or 10T1/2 cells were immunoprecipitated with the first antibody (1st Ab) and immunoblotted with the second antibody (2nd Ab) as shown. Because of different amounts of input protein (25 and 100 μg, respectively), the signals obtained in the direct immunoblots (lanes 1 and 5) are not directly comparable to the co-immunoprecipitations. PI, preimmune serum for MEF2A polyclonal or a control unrelated mAb (PAb101) for the myogenin monoclonal antibody. Size markers are on the left in kilodaltons.

transfected. Although MEF2A activated the endogenous myogenin gene when permanently transfected in CV-1 cells, the level of transiently induced myogenin was apparently inadequate to induce cooperative transcriptional activation in this assay. Similar results were obtained with reporter constructs containing the native MCK enhancer and the myogenin promoter (Fig. 3D). Taken together, these results show that in the presence of their respective collinear cis elements, myogenin and MEF2A activate muscle gene transcription in a cooperative manner, which is largely independent of the precise spacing and sequence of the E-box and MEF2 DNA-binding sites.

To determine whether MEF2A and myogenin are associated in vivo, even in the absence of exogenously added DNA, we performed a series of co-immunoprecipitation experiments (Fig. 4). Equal amounts of C2C12 myotube extracts were incubated with either the MEF2A antiserum or preimmune serum, and the resulting pellets were washed, then analyzed by immunoblot with a myogenin monoclonal antibody (mAb). The MEF2A antiserum specifically co-immunoprecipitated myogenin from the C2C12 myotube extract. In the reciprocal experiment, the myogenin mAb specifically co-immunoprecipitated MEF2A from C2C12 myotubes. Neither antibody coprecipitated these factors from 10T1/2 nonmuscle cells. Although the precise stoichiometry of MEF2A and myogenin and the possible participation of other factors in this complex remain to be established, MEF2A and myogenin directly associate in C2C12 myotubes.

The domains of each polypeptide required for this interaction were mapped by in vitro protein binding experiments (Fig. 5). Full-length, in vitro–translated MEF2A efficiently bound to a fusion protein of glutathione-S-transferase (GST) and MyoD, but did not bind to a GST fusion protein containing the bHLH domain of the E-protein E2-2C (Fig. 5, A and B). Deletions of the MEF2A polypeptide had little effect on GST-MyoD binding as long as the MADS domain remained intact. An internal deletion mutant [DM(57–322)] containing only the MADS domain and a portion of the distal COOH-terminus of MEF2A retained significant binding activity. Thus, the MADS domain of MEF2A is both necessary and sufficient for binding to MyoD. The specificity of this binding was shown by the inability of MyoD to interact with a related MADS family member, serum response factor (SRF). Furthermore, even in the presence of a high concentration of ethidium bromide (an agent known to disrupt DNA-dependent protein-protein interactions), full-length MEF2A efficiently and specifically bound GST-MyoD (*17*). To confirm these results, we performed reciprocal binding experiments with in vitro–translated MyoD and GST-MEF2A (Fig. 5, C and D). Full-length MyoD, myogenin, and MRF4 efficiently bound GST-MEF2A. Among the MyoD mutants tested, only the internal deletion mutant encompassing the basic junction and first helix domains [DM(102–135)] failed to bind to GST-MEF2A. Together, these results document that binding to MEF2A is mediated by the basic and first helix domains of muscle bHLH factors.

Detailed mapping experiments have identified three amino acid residues within the basic junction domain of muscle bHLH factors that encode the myogenic activity of this protein family (*3*). To determine if these amino acids of MyoD are required for the interaction with MEF2A, we tested chimeric proteins containing different swap domains of E12 and MyoD for their ability to bind GST-MEF2A (Fig. 5, E and F). Substitution of the nonmyogenic basic or basic junction domains of the E-protein E12 for the equivalent domains of MyoD abolished GST-MEF2A binding. In contrast, substitution of the myogenic basic junction domains or the basic domain alone of MyoD for the equivalent domains of E12 conferred the ability to bind GST-MEF2A. Moreover, three point mutations in the basic junction domain of E12, which convert this E-protein into a myogenic factor, impart the ability to bind GST-MEF2A. Thus, the myogenic properties of the different bHLH chimeras correlated perfectly with their ability to physically associate with MEF2A.

These results demonstrate that human MEF2A is a determination factor for the skeletal muscle cell lineage, an activity that

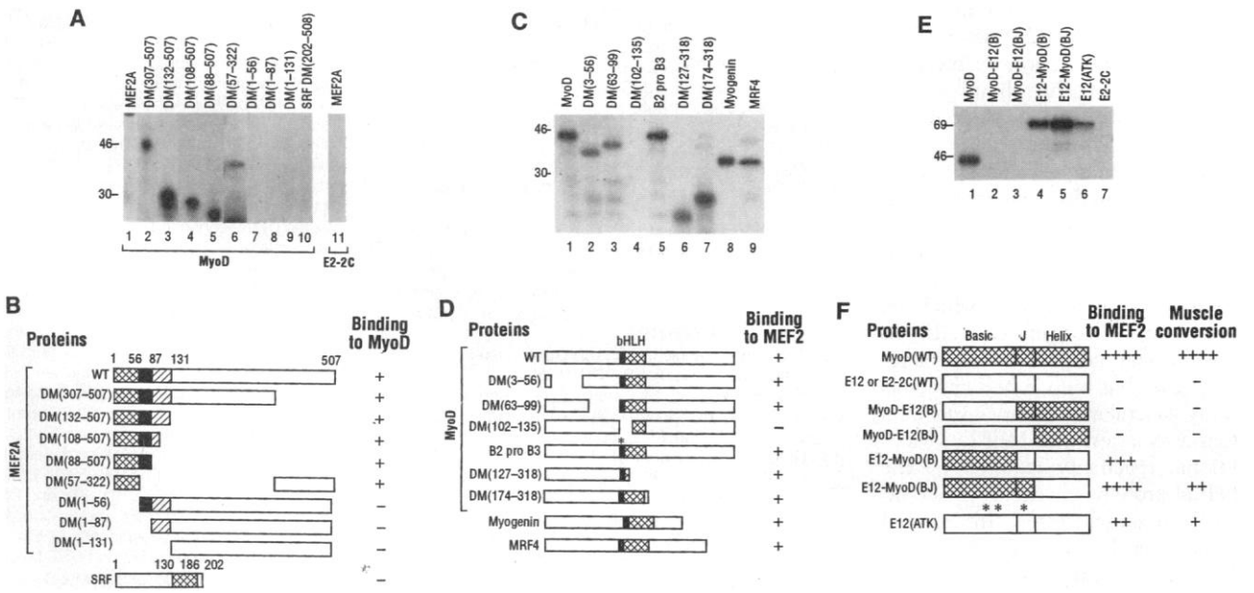


**Fig. 5.** In vitro mapping of domains required for the MEF2A and muscle bHLH protein interaction (*24*). (**A**) In vitro–translated MEF2A, various deletion mutants of MEF2A, and a truncated version of SRF retained on GST-MyoD (lanes 1 to 10) or GST-E2-2C (E-protein) resins. (**B**) Summary of results in (A). (**C** and **D**) Similar experiment as in (A) and (B) with in vitro–translated MyoD, various deletion mutants of MyoD, myogenin, and MRF4 bound to GST-MEF2A resin. (**E** and **F**) Similar experiment as in (A) and (B) with in vitro–translated E12-MyoD chimeras and E12 point mutant from (*3*) bound to GST-MEF2A resin. In (B), the domains of MEF2A are designated by crosshatch (MADS), black boxes (MEF2), and diagonal lines (alternative exon) (*8*, *9*). A "+" indicates binding; "–," no binding; muscle conversion data in (F) are from (*3*). WT, wild type; B, basic; BJ, basic junction; and ATK, point mutations. Size markers are on the left in kilodaltons.

previously distinguished the muscle bHLH family of transcriptional factors. Gain-of-function experiments in nonmuscle cells have shown that murine MEF2C and MEF2D also induce the muscle bHLH factors, thus providing evidence that myogenic activity is shared by each member of the MEF2 family (*18*). Once activated, increased amounts of muscle transcription factors are achieved by positive feedback loops that coordinately induce the expression of the genes encoding muscle bHLH and MEF2. In CV-1 cells, the ability of MEF2A to activate the endogenous myogenin but not other muscle bHLH factor or structural genes provides evidence that a portion of the regulatory feedback loop can be dissociated from the induction of myogenic differentiation. Furthermore, the results in CV-1 cells are consistent with the identification of an MEF2 DNA-binding site in the mouse myogenin gene promoter that is essential for appropriate expression of a myogenin reporter gene in cultured muscle cells and transgenic mice (*12*).

The data presented here further demonstrate an intimate functional relation between the families of MEF2 and muscle bHLH proteins that is an integral aspect of the myogenic regulatory cascade. The MEF2A and muscle bHLH factors enhance each other's ability to bind adjacent MyoD and MEF2 DNA-binding sites. Moreover, once bound, these factors cooperatively activate the transcription of muscle genes. The MADS domain of MEF2A specifically recognizes those amino acid residues of the basic junction domain that encode the myogenic activity of the muscle bHLH factors. The significance of this interaction for myogenesis is emphasized by the ability of the MADS domain of MEF2 to discriminate between myogenic and nonmyogenic basic junction domains. Muscle bHLH proteins, on the other hand, can discriminate between the MADS domain of MEF2A and that of the ubiquitously expressed MADS protein SRF. Because E-box binding sites are present in a wide variety of genes (most of which are not expressed in skeletal muscle cells), at least one additional recognition event is required to specify that muscle bHLH factors activate only skeletal muscle genes. Our data suggest that association with MEF2 provides the additional specificity required by the muscle bHLH proteins for exclusive activation of skeletal muscle genes. In cultured cells, activation of the MEF2 DNA-binding site in the gene encoding myogenin is regulated by a posttranslational modification of the MEF2 proteins (*19*). Our data suggest that the direct physical association with muscle bHLH proteins might be required for this posttranslational event. This hypothesis provides for a highly complex network of combinatorial interactions among four distinct muscle bHLH proteins and numerous alternatively spliced products of the four MEF2 genes. We further propose a general model wherein the MEF2 family participates in the differentiation pathways of cardiac muscle, smooth muscle, neural, and perhaps other cells by inducing the expression of, and directly associating with, cell type–specific transcriptional regulators in these lineages.

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20. Northern (RNA) blots were done as described (*6*) with the addition of an actin complementary DNA (cDNA) probe [L. G. Garfinkel, M. Periswamy, B. Nadal-Ginard, *J. Biol. Chem.* **257**, 11078 (1982)].
21. A 52-kD segment of human MEF2A (residues 272 to 507) was produced in *Escherichia coli* as a GST fusion protein. The gel-purified fusion protein was used to raise a polyclonal antiserum that was purified on a GST-MEF2A affinity column.
22. Transcription factor binding sites were cloned into the Bam HI–Hind III site of TK-CAT with the use of the following synthetic oligonucleotides: MCK E, 5′-AGCTTAGGCAGCAGGTGTTTCTAGAA-3′; MCK E-E, 5′-AGCTTAGGCAGCAGGTGTTAGGCAGCAGGTGTTTCTAGAA-3′; MCK M, 5′-AGCTTTCGCTCTAAAAATAAACCTGTCTAGAA-3′; MCK E-M, 5′-AGCTTAGGCAGCAGGTGTTGGATCCTCGCTCTAAAAATAACCCTGTCTAGAA-3′; MCK E(mt)-M, 5′-AGCTTAGGCAGACGGGTTTGGATCCTCGCTCTAAAAATAACCCTGTCTAGAA-3′; MCK E-M(mt), 5′-AGCTTAGGCAGCAGGTGTTGGATCCTCGCTCTAAGGCTAACCCTGTCTAGAA-3′); MCK E(mt)-M(mt), 5′-AGCTTAGGCAGACGGGTTTGGATCCTCGCTCTAAGGCTAACCCTGTCTAGAA-3′; MLC1/3 E-M, 5′-AGCTTATGACAGGTGCCTAATTCCTCAGTCTTTAAAAATAACTTTTGAGAG-3′; MLC1/3 E(mt)-M, 5′-AGCTTATGAACCGTGCCTAATTCCTCAGTCTTTAAAAATAACTTTTGAGAG-3′; MLC1/3 E-M(mt), 5′-AGCTTATGACAGGTGCCTAATTCCTCAGTCTTTAAGGCTAACTTTTGAGAG-3′; MLC1/3 E-M (+5 bp), 5′-AGCTTATGACAGGTGCCTCCTGCAATTCCTCAGTCTTTAAAAATAACTTTTGAGAG-3′; MLC1/3 E-M (+10 bp), 5′-AGCTTATGACAGGTGCCTCCTGCCCTGCAATTCCTCAGTCTTTAAAAATAACTTTTGAGAG-3′; MCK M-M, as described in (*8*); p3300MCKCAT [S. L. Amacher, J. N. Buskin, S. D. Hauschka, *Mol. Cell. Biol.* **13**, 2753 (1993)]; and pMYO184CAT (*12*). The human MEF2A cDNA (containing the muscle-specific axon SEEEELEL) cloned into MEF2A was inserted into a pcDNA3 vector (Invitrogen). C2C12 myotubes and neonatal rat primary cardiocytes were prepared as described (*8*). Transfections and CAT assays were done as described (*8*).
23. The co-immunoprecipitation was done as follows. C2C12 myotubes were extracted in low-stringency buffer [50 mM tris (pH 8), 120 mM NaCl, 0.5% NP-40, 100 mM NaF, and 200 mM sodium orthovanadate] and immunoprecipitated with preimmune or MEF2A antiserum, or myogenin (F5D) or unrelated control (PAb101) mAbs (American Type Culture Collection). The immunoprecipitates were washed three times in NETN buffer [20 mM tris (pH 8), 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40], separated by 10% SDS–polyacrylamide gel electrophoresis, immunoblotted with myogenin or MEF2A Abs, and detected by the enhanced chemiluminescence system (Amersham).
24. The in vitro binding experiment was done as described [W. G. Kaelin Jr., D. C. Pallas, J. A. DeCaprio, F. J. Kaye, D. M. Livingston, *Cell* **64**, 521 (1991)]. Full-length GST-MEF2A was generated by polymerase chain reaction (PCR) amplification of pMT2-MEF2A plasmid (*8*) with the use of 5′ and 3′ amplifiers containing an Eco RI site and ligated into pGEX-2T (Pharmacia). GST–E2-2C protein contained the bHLH domain of human E2-2C. In vitro–translated MEF2A protein deletion mutants DM(307–507), DM(132–507), DM(108–507), DM(88–507), DM(57–322), DM(1–56), DM(1–87), and DM(1–131) were all generated by PCR amplification. Additional proteins included myogenin (*6*), MRF4 (*1*), E12-MyoD chimeric proteins (*3*), and GST-MyoD (*5*).


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