A Dominant-Negative Form of Transcription Factor MEF2 Inhibits Myogenesis*

(Received for publication, May 22, 1997, and in revised form, September 24, 1997)

Olga I. Ornatsky, John J. Andreucci, and John C. McDermott†‡

From the Departments of Biology and Kinesiology, Faculty of Pure and Applied Science, York University, Toronto M3J 1P3, Canada

A biological role for MEF2 (myocyte enhancer factor 2) activity during mammalian myogenesis has been inferred but not directly proven because of its role in the transcriptional activation of many muscle-specific genes. Therefore, our purpose was to determine whether MEF2 activity is absolutely required for mammalian myogenesis. Using a dominant-negative approach to address this question, we constructed a mutated MEF2A protein comprised of the amino-terminal DNA binding/dimerization domain of MEF2A without its trans-activation domain as a bacterial fusion protein (GST-131) or in a eukaryotic expression vector (pcDNA-131). GST-131 and the protein encoded by pCDNA-131 bind specifically to the MEF2 cis element and abrogate trans-activation of a MEF2-responsive luciferase reporter gene by wild type MEF2A, thus serving a role as trans-dominant inhibitors of MEF2 function. In congruence with their ability to interfere with wild type MEF2 function, microinjection of GST-131 or pCDNA-131 into L6E9 or C2C12 myoblasts inhibited myotube formation. Immunofluorescence analysis showed that the expression of myogenin, myosin heavy chain, and MEF2A were inhibited in the GST-131 or pCDNA-131-injected cells compared with GST or pCDNA-injected controls. We also document that this trans-dominant MEF2 inhibitor impairs the myogenic conversion of C3H10T1/2 fibroblasts by MyoD. Thus, these data provide evidence that the trans-activation function of the MEF2 proteins during mammalian myogenesis is required for muscle-specific gene expression and differentiation.

The MEF2 (myocyte enhancer factor 2) genes have been implicated in the complex hierarchical regulation of muscle-specific gene expression and differentiation. Vertebrate MEF2 genes (A–D) encode nuclear phosphoproteins belonging to the MADS (MCMI, agamous, deficiens, serum response factor) superfamily of DNA-binding proteins (1–10). The MEF2 proteins bind as homo- and heterodimers to a cis element with the consensus (C/T)TA(A/T)2TA(G/A) via interactions between their amino-terminal MADS/MEF2 domain and the major groove of DNA (11–13). The MEF2 proteins are also involved in complex heterotypic protein-protein interactions with members of the myogenic bHLH family (14), p300/CBP (15), and also p38 mitogen-activated protein kinase (16), and these interactions may fulfill modulatory roles and confer tissue-specific regulation.

The precise role of MEF2 proteins during myogenesis is controversial. Kaulsal et al. documented that MEF2A protein does have the ability to activate the muscle gene expression program (17), a property originally ascribed to the myogenic bHLH1 proteins as the ability to convert certain nonmuscle cell lines to muscle (18, 19). Conversely, Molkentin and co-workers found that MEF2 factors lack this ability, although they have reported that MEF2 proteins potentiate the myogenic activity of the bHLH proteins and that this potentiation is due to physical interactions between the MADS-MEF2 domain of the MEF2 proteins and the bHLH proteins (14, 20). Despite these discrepancies concerning the “myogenic potential” of the MEF2 proteins, several related lines of genetic and biochemical evidence suggest an important role for the MEF2 proteins during mammalian myogenesis: (a) the presence of the MEF2 enhancer in the regulatory regions of many muscle structural genes (reviewed in Ref. 21), (b) the synergistic trans-activation of muscle-specific target genes by the interaction of the MEF2 and myogenic bHLH proteins (13, 14, 17), (c) the role of MEF2 proteins in stabilizing myogenin expression during myogenesis (22), and (d) the observation that the functional inactivation of the single Drosophila MEF2 homologue results in a disruption of normal muscle formation (23, 24). Perhaps another approach to further characterizing the biological relevance of the MEF2 proteins for muscle formation is to ask whether the MEF2 proteins are essential for myogenesis in mammalian cells. Loss of function assays using gene targeting in mice is one elegant approach to this question, although it may be initially complicated by functional redundancy due to the overlapping function of the four MEF2 genes, as has been shown to be the case for the myogenic bHLH proteins (25–30). Alternatively, because dominant inhibitory genes are potentially useful in generating loss-of-function mutations, we approached this question by constructing a trans-dominant MEF2 inhibitor, which, when expressed in differentiating muscle cells, would abrogate the function of the endogenous MEF2 proteins, thus revealing their cumulative effect on the myogenic program.

Here we report that a GST-MEF2A fusion protein (GST-131) or an expression plasmid encoding the same polypeptide (pCDNA-131 or pMT2–131), in which the carboxyl-terminal trans-activation domain of MEF2A (amino acids 132–507) is deleted, is capable of binding specifically to the MCK MEF2 site, inhibiting trans-activation by the wild type MEF2A protein, extinguishing myogenin, myosin heavy chain and MEF2A expression, and inhibiting the myogenic differentiation program in C3H10T1/2 converted by ectopic MyoD expression.

* This work was supported by grants from the Natural Sciences and Engineering Research Council and Medical Research Council of Canada (to J. C. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† To whom correspondence should be addressed: 341 Farquharson LSB, York University, 4700 Keele St., Toronto M3J 1P3, Canada. Tel.: 416-736-2100 (Ext. 30389); Fax: 416-736-5698; E-mail: FS300557@sol.yorku.ca.

‡ The abbreviations used are: bHLH, basic helix-loop-helix; GST, glutathione S-transferase; DMEM, Dulbecco’s modified Eagle’s medium; MHC, myosin heavy chain; CAT, chloramphenicol acetyltransferase; MCK, muscle creatine kinase.
and L6E9 and C2C12 muscle cells. Thus, these experiments provide the first evidence that the trans-activation function of the endogenous MEF2 proteins during mammalian myogenesis is critical for muscle-specific gene expression and differentiation.

MATERIALS AND METHODS

Cell Culture and GST Fusion Protein Production—Mammalian myoblast cell lines, C2C12 and L6E9, were maintained in DMEM supplemented with 10% fetal bovine serum in plastic dishes coated with 1% gelatin. Differentiation into myotubes was induced by changing the medium to DMEM supplemented with 5% horse serum. HeLa cells were cultured in DMEM supplemented with 10% fetal bovine serum. A cDNA encoding the amino-terminal 1–131 amino acids of MEF2A was cloned into PGEX-2T (GST-131). GST-131 and GST fusion proteins were prepared according to the manufacturer's protocol by column purification using glutathione-Sepharose 4B (Pharmacia Biotech Inc.).

DNA Binding Assays—The DNA binding assays were carried out as previously described (5, 31). Complementary oligodeoxyribonucleotides were synthesized with an Applied Biosystems synthesizer. For the DNA binding assays, the incubation reaction contained 0.2 μg of GST fusion protein (GST-131 or GST alone), 0.2 ng of radiolabeled MCK MEF2 site probe (the core nucleotide sequence of the probe being 5'-cgccttaaaata-acc-gct-3'), 100 ng of poly(dI-dC), and 0.2 ng of single stranded DNA in a total volume of 20 μl. The bound fraction was separated from the free probe by electrophoresis on a 4.5% polyacrylamide gel (acrylamide:bis, 29:1) at 4 °C. For the in vitro gel shift analysis, where appropriate, 1 μl of antisera or preimmune serum was added to the preincubation reaction.

Reporter Assays—HeLa cells were grown in 60-mm dishes in DMEM + 10% fetal bovine serum to approximately 60% confluence. For standard reporter assays the appropriate reporter/expression plasmid combination was transfected into HeLa cells by calcium phosphate coprecipitation. The cells were glycerol shocked after 24 h and harvested after 4 days. Each plate of cells was transfected with 5 μg of the appropriate luciferase or CAT reporter construct and 2 μg of pSV β-galactosidase, which served as an internal control for the transfection efficiency. For the overexpression studies 2.5 μg of an additional construct was transfected comprising either the MEF2A coding region in a pm2T expression vector or the pm2T vector alone as a control. The pCDNA-131 or pm2T–131 expression vector was added (at either 1 or 5 μg/l) to the transfection mix where appropriate (see figure legends for details in individual experiments). Cell extracts were prepared and luciferase activity was determined as described by the manufacturer (Promega). CAT activity was determined as previously reported (31). The reporters consisted of the following: the MEF2-responsive region of the c-jun promoter upstream of a basal promoter luciferase construct (nucleotides −225 to +150 of the c-jun promoter pJLuc) or a corresponding control (nucleotides −53 to +42 of the c-fos basal promoter upstream of the firefly luciferase gene pPLuc) (32).

The CAT reporter consisted of the following: the embryonic myosin heavy chain promoter (MHC emb) CAT gene downstream of two copies of the MCK MEF2 sites, inserted in a concatemerized orientation at the −102 position of the MHC emb promoter in plasmid PE102CAT (PE102CAT2×MEF2), or the same vector without the MEF2 sites present (PE102CAT). Microinjection/Reporter Assays—1000 HeLa cells for each combination of protein (0.5 ng/ml GST or GST-131) and plasmids (50 ng/ml pm2T-MEF2A and 50 ng/ml pJLuc) were injected as described below, and then growth medium was replaced with DMEM + 5% horse serum. After 48 h, cells were collected into 0.25 x Tris, pH 7.8, and freeze-thawed four times, and total protein was determined using the Bradford protein assay. Equal amounts of protein were used for estimating luciferase activity in different cell extracts, according to the luciferase assay kit (Promega).

Microinjection and Immunofluorescence Analysis—For microinjection assays, L6E9 or C2C12 muscle cells were grown on gelatin-coated coverslips for 2 days. L6E9 or C2C12 muscle cells were injected directly into the nucleus with 0.5 mg/ml solution of GST or GST-131 in 10 mM Tris, pH 7.4, 25 mM KCl, with an Eppendorf injection system attached to a Leitz Ortholux microscope. Each cell was injected for 0.2 s at constant pressure of 150 hectarose, receiving approximately 0.5 μg of protein/cell in 10 femtomoles. Dishes were rinsed after injection with serum-free DMEM and replenished with differentiation medium (DMEM + 5% horse serum). Myogenesis was evident in GST-injected cultures after 3–4 days. Confocal immunofluorescence microscopy was performed as recently described (31). Cells were washed with phosphate-buffered saline, pH 7.4, three times, and fixed for 5 min in cold methanol at −20 °C. Cells were stained with polyclonal rabbit anti-MEF2A, anti-myogenin monoclonal antibodies (F5D), or anti-myosin heavy chain monoclonal antibodies (MF20). As secondary antibodies, we used fluorescein isothiocyanate-conjugated anti-rabbit or antimouse antibodies (Sigma). Coverslips were washed in phosphate-buffered saline, and cells were counterstained for nuclei with ethidium bromide at 2 μg/ml for 2–5 min. Coverslips were examined using a Bio-Rad MRC-1000 laser-scanning confocal imaging system equipped with the Bio-Rad COMOS operating software. Photographs were made on Kodak TMAX 100 film.

RESULTS

The Amino Terminus (amino acids 1–131) of MEF2A Protein Binds Specifically to the MEF2 Site and Inhibits Trans-activation by the Wild Type MEF2A Protein—The highly conserved MEF2 DNA-binding domain has been well characterized in terms of its location within the MEF2 proteins, amino acid sequence constraints, and its binding specificity (11, 12, 38). This information allowed us to construct a trans-dominant MEF2 inhibitor by using the DNA-binding domain of MEF2A (amino acids 1–131) with the carboxyl-terminal trans-activation region (amino acids 132–507) of the protein deleted. The rationale being that such a molecule would act in a dominant-negative manner by binding to DNA without being able to activate transcription, thus displacing the binding, and subsequently abrogate the trans-activation function of the wild type MEF2 proteins.

Initially we tested the capacity of a GST-MEF2A amino-terminal fusion protein (GST-131) to bind to a double stranded oligodeoxyribonucleotide comprising the previously characterized MCK enhancer MEF2 site (Fig. 1A, B, D, and E). The specificity of the protein-DNA complex was determined by using an unlabeled MEF2 site as a competitor and previously characterized antibody directed against a peptide corresponding to amino acids 108–122 of MEF2A (Fig. 1A). The results of these experiments show that the GST-MEF2A fusion protein can bind specifically to DNA, whereas GST alone cannot (Fig. 1A). Also a MEF2A antibody recognizes and "supershifts" the GST-131-binding complex (Fig. 1B). The GST-131 fusion pro-
MEF2 Dominant-Negative Blocks Myogenesis

The amino-terminal (1-131 amino acids) of MEF2A (GST-131) bind to the MEF2 cis element. A, binding specificity was tested by incubating the GST (lane 1) or GST-131 (lane 2) protein with the radiolabeled double stranded MCK MEF2-binding site probe in the absence or presence of a 50-fold molar excess of unlabeled binding site (lane 3). The arrow labeled with B refers to the binding complex, and the arrow labeled with F refers to the unbound binding site probe. B, to confirm that the binding complex was composed of the MEF2 fusion protein, incubations were also carried out with a preimmune serum (lane 4) or an anti-MEF2A antibody (lane 5). C, binding reactions in which the GST-131 was mixed with C2C12 myotube extracts (GST-131+ MtExtr) were performed to determine if the GST-131 could heterodimerize and thus form a complex with intermediate mobility between the low mobility (wild type MEF2 complex B’) and the higher mobility GST-131 complex (lower band, lanes 6 and 7). An intermediate complex is not observed.

Fig. 1. The amino-terminal (1-131 amino acids) of MEF2A (GST-131) bind to the MEF2 cis element. A, binding specificity was tested by incubating the GST (lane 1) or GST-131 (lane 2) protein with the radiolabeled double stranded MCK MEF2-binding site probe in the absence or presence of a 50-fold molar excess of unlabeled binding site (lane 3). The arrow labeled with B refers to the binding complex, and the arrow labeled with F refers to the unbound binding site probe. B, to confirm that the binding complex was composed of the MEF2 fusion protein, incubations were also carried out with a preimmune serum (lane 4) or an anti-MEF2A antibody (lane 5). C, binding reactions in which the GST-131 was mixed with C2C12 myotube extracts (GST-131+MtExtr) were performed to determine if the GST-131 could heterodimerize and thus form a complex with intermediate mobility between the low mobility (wild type MEF2 complex B’) and the higher mobility GST-131 complex (lower band, lanes 6 and 7). An intermediate complex is not observed.

protein does not heterodimerize with the wild type MEF2 because no intermediate DNA-binding complex was formed when the GST-131 protein and extracts from C2C12 myotubes were mixed (Fig. 1C). This is probably due to the fusion protein already existing in a stable dimeric form when it is produced. We have noticed in other assays that efficient dimerization of the MEF2 proteins requires co-translation, but it is difficult to determine if this is a peculiarity of in vitro translation and bacterial fusion protein production or whether stable dimers actually form during translation in vivo. These experiments show that GST-131 protein is capable of efficient binding to DNA but may be compromised in its ability to exchange dimerization partners.

We next attempted to assess if the GST-131 could antagonize trans-activation of a MEF2-responsive element of the c-jun promoter by the wild type MEF2A protein. To do this we microinjected a MEF2-responsive reporter construct, pJLuc (32), a MEF2A expression vector (pMT2-MEF2A) and the GST-131 protein (or GST alone) into HeLa cells. Data from these experiments show that the GST-131 protein essentially negates the trans-activation of the pJLuc reporter by wild type MEF2A, whereas GST alone does not (Fig. 2A). The data in Fig. 2B indicate that MEF2A activates pJLuc in standard reporter assays and that a basal reporter (p0FLuc) is not influenced by overexpression of MEF2A. The level of luciferase activity with pJLuc when pMT2 is co-transfected is quite high compared with the basal reporter (p0FLuc). This activity is due to the activation of the pJLuc reporter by endogenous factors and not by pMT2. In addition to the MEF2 site, the c-jun enhancer (pJLuc) also contains AP1, GC box, CAAT, and other elements. The luciferase activity of pJLuc is not different if one compares its activity with or without co-transfection with pMT2 (data not shown).

Even though the fusion protein does not contain a trans-activation domain and therefore probably does not contact the transcriptional machinery, we wanted to ascertain that the effect that we observe is not due to a general “squelching” of transcription factors associated with the basal transcription machinery. We reasoned that this type of effect would probably result in a more general inhibition of transcriptional activation. We therefore tested if overexpressed pMT2–131 affected the activation of the enhancer/promoter from the cyclin A gene that is not activated by MEF2 factors and does not contain a MEF2 site. In this assay we could not detect any inhibitory influence of pMT2–131 (the region encoding amino acids 1–131 of MEF2A cloned into the pMT2 mammalian expression vector) on the transcriptional activation of the cyclin A promoter, indicating that the effect we observe is specific to promoters containing the MEF2 site and is not due to a general squelching of basal transcription factors (Fig. 2C). Also, to independently confirm that it is indeed the MEF2 part of the GST fusion protein that is inhibiting MEF2 activity, we performed a complementary experiment in which we co-transfected pMT2–131 along with wild type MEF2A and MEF2C and observed that it inhibited the trans-activation function of the wild type MEF2A and MEF2C proteins, indicating that the effect of the MEF2A dominant-negative also inhibits MEF2C trans-activation (Fig. 2D). Together, these data show that the MEF2A-131 amino terminus can bind specifically to the MEF2 site and also inhibit the trans-activation of MEF2 site bearing target genes by the wild type MEF2A and MEF2C proteins.

Microinjection of an Amino-terminal MEF2 Fragment, as a GST Fusion Protein or as an Expression Plasmid, into L6E9 and C2C12 Muscle Cells Inhibits Both the Expression of Muscle-specific Genes and Myotube Formation.—We reasoned that if MEF2 activity is absolutely required for mammalian myogenesis, the introduction of a fusion protein that interferes with MEF2 function into muscle cells might inhibit myogenesis. Having characterized GST-131 as a good candidate to fulfill a role as a trans-dominant MEF2 inhibitor, we microinjected this protein into the nucleus of L6E9 cultured muscle cells. To assess the effect of the mutated protein we studied the morphology of the GST-131-injected cells compared with GST-injected cells within the same culture dish under identical culture conditions. In these experiments, the injection per se did not interfere with the differentiation process because the GST-injected controls always formed myotubes in the normal time frame for these cells. In terms of gross morphology we found that the GST-131-injected cells were inhibited in forming myotubes compared with the GST-injected cells (Fig. 3A, Phase contrast). This experiment was repeated 14 times (with 1600–2000 cells being injected per experiment), and the same result was observed in each experiment.

As with the transcriptional inhibition assays we then sought to perform an analogous experiment with the pcDNA-131 expression vector. Again using microinjection technology we show that nuclear injection of pcDNA-131 into myogenic cells results in an inhibition of differentiation (Fig. 4, Phase contrast). In these experiments we observed a more complete block of differentiation with the expression plasmid than with the injection of the fusion protein. In these experiments a small number of GST-131- or pcDNA-131-injected cells do express muscle markers, although myotubes are not formed by these cells (Figs. 3 and 4). It is conceivable that for an unknown reason these cells are refractory to the effect of the GST-131- or pcDNA-131-encoded protein or simply that these cells were inadvertently not injected even though they were in the injection field (typically 800–1000 cells are injected for each condition). Also, the inhibition of myogenesis by the GST-131 protein is a reversible phenomena because we can eventually see myotube formation in the GST-131-injected cells. Using an antibody against the GST portion of the fusion protein, we have determined that the differentiation of the GST-131-injected...
cultures occurs 1–2 days after the loss of immunodetectable GST-131 fusion protein in the cells, thus indicating that the “escape” is due to the eventual degradation of the fusion protein (data not shown). When we inject the expression plasmid (pcDNA-131), the block of myogenesis is more complete and was apparent for at least 6 days. These data therefore suggest that the inhibition of myogenesis by GST-131 or pcDNA-131 is reversible but prevails as long as the protein is present.

To further characterize the effects of GST-131 and pcDNA-131 beyond the morphological level, we used confocal immunofluorescence microscopy to analyze the expression of markers of muscle differentiation (MEF2A, myogenin, and myosin heavy chain) in the injected cells. We found that the GST-131- or pcDNA-131-injected cells did not express detectable levels of myogenin at the same time as the GST- or pcDNA-injected cells (Figs. 3 and 4, respectively). It has previously been reported that the MEF2 site in the myogenin promoter is required for stabilizing myogenin expression during muscle formation (22). Our results indicate that the contribution of MEF2 for transcriptional regulation of myogenin expression is physiologically important. We also observed that GST-131- or pcDNA-131-injected cells did not express myosin heavy chain in parallel with the GST- or pcDNA-injected cells, consistent with the morphological and immunological data showing an inhibition of expression of important structural and regulatory proteins for myotube formation in the GST-131- or pcDNA-131-injected cells (Figs. 3 and 4, respectively).

In a further attempt to assess the anti-myogenic effect of pcDNA-131 we utilized the well documented C3H10T1/2 myogenic conversion assay, in which one of the myogenic bHLH cDNAs is transfected into C3H10T1/2 fibroblasts, which subsequently undergo myogenesis (18). Using this assay it has also been previously shown that co-transfection of MEF2A or MEF2C with MyoD results in a substantial potentiation in the

FIG. 2. The amino terminus (1–131 amino acids) of MEF2A (GST-131 or pMT2–131) inhibits trans-activation by the wild type MEF2A protein. A, to determine if MEF2 site-dependent activation of transcription could be inhibited by the GST-131 protein, we microinjected the wild type MEF2A and a MEF2-responsive luciferase reporter construct (pJLuc) along with either GST-131 or GST alone into HeLa cells (two experiments are shown, indicated by the stippled and solid bars). B, we performed standard transfections with the basal luciferase reporter gene vector (pOFluc) or the MEF2-responsive luciferase reporter gene (pJLuc) co-transfected with pMT2-MEF2A or pMT2 to show that no detectable activation of the Luc reporter in a non-MEF2 site-dependent manner by MEF2A occurred, and also that the empty expression vector (pMT2) was incapable of trans-activating the MEF2-responsive reporter gene (pJLuc). These experiments were performed at least twice with two different DNA preparations. Each data point is a mean of triplicate samples. The standard error of the mean was not more than 7.4% of the mean value for any of the data points. C, we performed transfections with the basal luciferase reporter gene vector (pOFluc) or the MEF2 “unresponsive” cyclin A-luciferase reporter gene (cyclin A-Luc) co-transfected with pMT2 or pMT2–131 (2.5 μg of each) to determine if pMT2–131 had any effect on a promoter that does not contain a MEF2 site. These experiments were performed at least twice with different DNA preparations. Each data point is a mean of triplicate samples. The standard error of the mean was not more than 9% of the mean value for any of the data points. D, graph shows CAT activity in Hela cells transfected with the embryonic myosin heavy chain promoter containing two MCK MEF2 sites (PE102CAT2x) along with either pMT2 MEF2A (1 μg) or pMT2 MEF2C (1 μg) with and without pMT2–131 (1 μg) expression vectors as indicated. Each data point is a mean of triplicate samples. The standard error of the mean was not more than 11% of the mean value for any of the data points.
We therefore transfected MyoD into C3H10T1/2 along with MEF2A and also co-transfected pMT2–131 to test if it inhibited this potentiation. The results from these experiments are presented in Table I. These assays show: (a) that MEF2 by itself is not myogenic in C3H10T1/2 consistent with a previous report (14), (b) that wild type MEF2A when co-transfected with MyoD did not potentiate the number of myogenic colonies, and (c) that co-transfection of pMT2–131 with MyoD significantly reduced the number of myogenic colonies resulting from MyoD transfection. These data indicate that the endogenous MEF2 that is induced in C3H10T1/2 cells by MyoD (19) is critical for efficient myogenic conversion.

It has been stated that the MADS-MEF2 domain of MEF2 proteins is necessary and sufficient for interaction with the bHLH proteins (14). We were therefore interested in determining if GST-131 was capable of interacting with the bHLH proteins when we introduce it into the cellular environment containing wild type MEF2 and bHLH myogenic factors. To test this idea we mixed GST-131 protein with extracts from muscle cells at different stoichiometries and immunoprecipitated myogenin from this incubation mix to determine if GST-131 co-immunoprecipitated with myogenin. In these assays we detected the GST-131 fusion protein with a commercially available anti-GST antibody (Fig. 5C). We then immunoprecipitated myogenin using a monoclonal antibody, which successfully detects myogenin in C2C12 extracts and also efficiently immunoprecipitates it (Fig. 5A), and under these co-incubation conditions we then determined if we could detect GST-131 in the myogenin immunoprecipitate using the GST-specific antisera. Under these experimental conditions we could not detect GST-131 in the myogenin immunoprecipitate (Fig. 5C). As a positive control for the bHLH-MEF2 interaction under these conditions, we can observe an interaction between wild type MEF2D and myogenin by probing the myogenin immunopre-
indicating rabbit immunoglobulins from the immunoprecipitation recognized by the secondary antibody. Lanes 3–5 show C2C12 cell lysates co-incubated with GST-131 (lanes 3 and 4) or GST (lane 5) immunoprecipitated (IP) with the anti-myogenin antibody and probed with anti-myogenin antibody (see “Materials and Methods” for experimental details). The arrow indicates myogenin protein. The arrowhead indicates rabbit immunoglobulins from the immunoprecipitation recognized by the secondary antibody. A, anti-myogenin (αMg) immunoblot. Lane 1 indicates molecular mass markers (kDa). Lane 2 shows detection of myogenin in C2C12 muscle cell extracts. Lanes 3–5 show C2C12 cell lysates co-incubated with GST-131 (lanes 3 and 4) or GST (lane 5) immunoprecipitated (IP) with the anti-myogenin antibody and probed with anti-myogenin antibody (see “Materials and Methods” for experimental details). The arrow indicates myogenin protein. The arrowhead indicates rabbit immunoglobulins from the immunoprecipitation recognized by the secondary antibody. B, anti-MEF2D (αMEF2D) immunoblot. Lane 1 indicates molecular mass markers (as in A). Lane 2 shows detection of MEF2D in muscle cell extracts. Lanes 3–5 show C2C12 cell lysates co-incubated with GST-131 (lanes 3 and 4) or GST (lane 5) immunoprecipitated with the anti-myogenin antibody and probed with anti-MEF2D antibody (see “Materials and Methods” for experimental details). The arrow indicates MEF2D protein. The arrowhead indicates rabbit immunoglobulins from the immunoprecipitation recognized by the secondary antibody. C, anti-GST (αGST) immunoblot. Under the same co-incubation conditions as in A and B above, the myogenin immunoprecipitates were probed with the anti-GST antibody to determine if GST-131 or GST is present in the myogenin immunoprecipitate (lanes 1 and 2). Lane 3 indicates molecular mass markers (as in A). Lanes 4 and 5 indicate a positive control showing detection of GST-131 and GST, respectively. The lower arrow denotes GST, and the upper arrow denotes GST-131. There was no detectable GST-131 band present in the myogenin immunoprecipitate (lane 1). Bands below the immunoglobulins in lanes 1 and 2 are nonspecific. The arrowhead indicates rabbit immunoglobulins from the immunoprecipitation recognized by the secondary antibody.

**FIG. 5. No detectable interaction between dominant-negative MEF2 fusion protein (GST-131) and myogenin from cell extracts.** A, anti-myogenin (αMg) immunoblot. Lane 1 indicates molecular mass markers (kDa). Lane 2 shows detection of myogenin in C2C12 muscle cell extracts. Lanes 3–5 show C2C12 cell lysates co-incubated with GST-131 (lanes 3 and 4) or GST (lane 5) immunoprecipitated (IP) with the anti-myogenin antibody and probed with anti-myogenin antibody (see “Materials and Methods” for experimental details). The arrow indicates myogenin protein. The arrowhead indicates rabbit immunoglobulins from the immunoprecipitation recognized by the secondary antibody. B, anti-MEF2D (αMEF2D) immunoblot. Lane 1 indicates molecular mass markers (as in A). Lane 2 shows detection of MEF2D in muscle cell extracts. Lanes 3–5 show C2C12 cell lysates co-incubated with GST-131 (lanes 3 and 4) or GST (lane 5) immunoprecipitated with the anti-myogenin antibody and probed with anti-MEF2D antibody (see “Materials and Methods” for experimental details). The arrow indicates MEF2D protein. The arrowhead indicates rabbit immunoglobulins from the immunoprecipitation recognized by the secondary antibody. C, anti-GST (αGST) immunoblot. Under the same co-incubation conditions as in A and B above, the myogenin immunoprecipitates were probed with the anti-GST antibody to determine if GST-131 or GST is present in the myogenin immunoprecipitate (lanes 1 and 2). Lane 3 indicates molecular mass markers (as in A). Lanes 4 and 5 indicate a positive control showing detection of GST-131 and GST, respectively. The lower arrow denotes GST, and the upper arrow denotes GST-131. There was no detectable GST-131 band present in the myogenin immunoprecipitate (lane 1). Bands below the immunoglobulins in lanes 1 and 2 are nonspecific. The arrowhead indicates rabbit immunoglobulins from the immunoprecipitation recognized by the secondary antibody.

**TABLE I**

| Plasmid          | pMT2-MyoD | pMT2-MEF2A | pMT2-MyoD + pMT2-MEF2A dominant-negative | pMT2-MEF2A dominant-negative | pMT2-MyoD + pMT2-MEF2A dominant-negative | pMT2-MEF2A dominant-negative |
|------------------|-----------|------------|-----------------------------------------|-----------------------------|-----------------------------------------|-----------------------------|
| Field            |           |            |                                         |                             |                                         |                             |
| 1                | 70        | 0          | 68                                      | 22                          | 0                                       |                             |
| 2                | 80        | 0          | 77                                      | 21                          | 0                                       |                             |
| 3                | 79        | 0          | 72                                      | 18                          | 0                                       |                             |
| 4                | 71        | 0          | 93                                      | 17                          | 0                                       |                             |
| 5                | 89        | 0          | 75                                      | 25                          | 0                                       |                             |
| ± mean           | 77.8 ± 7.7| 0          | 77 ± 9.6                                | 22 ± 5.0′                   | 0                                       |                             |
| Total number of myotubes | 389 | 0          | 385                                     | 103                         | 0                                       |                             |

*a* *p* < 0.05 compared with MyoD alone.

**DISCUSSION**

Orchestration of myogenesis in vertebrates involves at least two families of transcriptional regulatory proteins that coordinately activate the large number of tissue-specific genes required for the differentiated phenotype. The proteins of the basic helix-loop-helix family of myogenic regulators (MyoD, MRF4, Myf5, and myogenin; reviewed in Ref. 33) were initially identified as key regulators of myogenesis with the ability to activate the muscle differentiation program. Using a gene targeting strategy it has been shown that the activity of the bHLH proteins is indispensable for myogenesis, although this analysis was complicated by some functional redundancy between the different family members (25–30). MEF2 Proteins May Function as Co-regulators for the Myogenic Basic Helix-Loop-Helix Factors during Myogenesis—Based on a number of biochemical and genetic studies, it has been suggested that the bHLH proteins require a co-regulator that is critically important during myogenesis (14). Subsequent work has revealed that very likely candidates for this role are the proteins encoded by the myocyte enhancer factor 2 (MEF2A-D) family (14, 17, 20).

In these studies we employed a dominant-negative approach to block MEF2 function to study the role of MEF2 activity during myogenesis in vitro. These data provide further evidence that MEF2 is an important co-regulator for the myogenic factors. Moreover, we report that MEF2 activity is absolutely required for mammalian myogenesis in vitro, because abrogation of MEF2 activity using a dominant-negative mutation inhibits myogenesis. These studies provide the first evidence indicating that the MEF2 factors are required for vertebrate myogenesis.

**Properties of the MADS-MEF2 Domain and the Mechanism of Action of MEF2A-131 as a Dominant-Negative Mutation—** The MADS box transcription factor family comprises of a large family of genes (>40) from a spectrum of eukaryotic organisms ranging from yeast to humans (34). The high degree of sequence similarity exhibited by MADS box transcription factors suggests that family members exhibit similar structural and DNA binding properties. However, there are clearly subfamilies within the MADS “superfamily” that have divergent prop-
MEF2 Dominant-Negative Blocks Myogenesis

Table II
Summary of functional properties of the MADS/MEF2 domains of MEF2 proteins

| MEF2 factor studied | DNA binding activity | Transcriptional activity | Protein-protein interaction | Synergistic activation | Potentiates MyoD-10T1/2 conversion | Reporter used/cell type |
|---------------------|----------------------|--------------------------|-----------------------------|------------------------|-----------------------------------|------------------------|
| MEF2C aa 1–117 (14) | Active               | Inactive                 | (+) MyoD                    | Positive (with MyoD)   | Positive                           | PE102CAT 2x MEF2/10T1/2 |
| MEF2A aa 1–87 (37)  | Active               | Inactive                 | (+) THR                     | Negative (with THR)    | NR                                | αMHC gene/CV1           |
| MEF2A aa 1–131 (Present study) | Active               | Inactive                 | (−) Myogenin                | Negative (with MyoD)   | Negative (inhibits)                | PE102CAT 2x MEF2 c-jun enhancer/HeLa |

THR, thyroid hormone receptor; aa, amino acids; NR, not reported.

DNA binding activity refers to the ability to interact with a consensus MEF2 site. Transcriptional activity refers to the ability to activate transcription of a reporter construct containing one or multimerized MEF2 sites. Protein-protein interaction indicates proteins with which this fragment of MEF2 has been shown to directly interact. Synergistic activation refers to the ability of this region of MEF2 to activate transcription synergistically with the factor indicated (THR, thyroid hormone receptor). Potentiates MyoD-10T1/2 conversion refers to the ability of this fragment of MEF2 to enhance the number of C3H10T1/2 fibroblast cells converted to muscle by MyoD. The last column refers to the reporter constructs that were tested and the cells that were used in these studies for the reporter assays.

Properties and fulfill very different functions in the physiology of the cells in which they are expressed (34). Most MADS domain proteins contain a minimal conserved core DNA binding domain (56 amino acids) followed by a carboxyl-terminal extension (approximately 30 amino acids), which in many cases is essential for DNA binding and dimerization. The carboxyl-terminal extension does diverge considerably between the different subgroups of MADS domain proteins but is usually highly conserved within subgroups such as the MEF2 family (34–36, 38, 40). In the present studies we have utilized this detailed knowledge of MADS domain structure and function to construct a truncated MEF2A protein that contains the MADS-MEF2 domain and functions as a trans-dominant inhibitor of MEF2 by virtue of its ability to bind to the MEF2 site without activating transcription. All of our data so far are consistent with the idea that the amino-terminal fragment of MEF2A blocks the activity of MEF2 factors by binding to the MEF2 site without activating transcription. However, given the complex role of this region of the MEF2 proteins and based on the properties of the MADS domain as a protein-protein interaction domain, it is also possible that this truncated protein physically associates with and sequesters a MEF2 co-activator from the endogenous transcription complex. There are several potential MEF2 interacting proteins, but the most obvious candidate is the interaction with the bHLH proteins. We think this mode of action unlikely given that we cannot detect an interaction between the truncated protein and myogenin, under conditions when we can see an interaction between wild type MEF2 and myogenin. Also, the dominant-negative has no effect on the ability of MyoD to activate a MyoD-responsive reporter gene. These experiments are thus suggestive that the MEF2 dominant-negative does not exert its effect by inhibiting the bHLH proteins. There are, however, other possibilities. We are in the process of cloning factors that interact with the amino terminus of MEF2, and it is possible that an unidentified co-activator could be titrated away by the dominant-negative protein.

Functional Properties of the Amino Termini of MEF2A and MEF2C—Our studies show that the amino terminus of MEF2A (amino acids 1–131) is able to bind to DNA, lacks transcriptional activity, does not synergize with MyoD, and also inhibits MyoD-induced myogenic conversion of C3H10T1/2. In studies reported by Molkentin and co-workers (14), there is partial agreement with our data in that the amino-terminal 1–118 amino acids of MEF2C is shown to be sufficient to bind to DNA but is transcriptionally silent. However, in contrast to our work with MEF2A, they show that this amino-terminal fragment of MEF2C can recruit the trans-activation function of the myogenic bHLH proteins to a target gene by protein-protein interaction and also potentiate MyoD-induced myogenic conversion of C3H10T1/2 cells (see Table II for a comparison of MADS domain properties of MEF2A and MEF2C). Examination of the amino termini of these two proteins reveals that both contain the highly conserved MADS-MEF2 domain; although they exhibit different properties, presumably these differences must be attributed to the nonconserved regions of these molecules.

In our work we have not observed a synergistic activation of transcription by the amino terminus of MEF2A with the myogenic bHLH proteins. There are several lines of evidence suggesting that the amino-terminal MADS-MEF2 domain is insufficient to activate transcription by recruiting intact bHLH proteins to the MEF2 site. First, in the experiments reported here, if the myogenic bHLH proteins could contribute an activation domain to the amino terminus of MEF2A, MEF2-responsive genes could be activated because the bHLH proteins are present in these cells, and there would presumably be no effect of GST-131 or pcDNA-131 on muscle differentiation, which is contrary to our observations. In another context, we have been studying the regulation of the c-jun promoter in myogenic cells, and MyoD can synergize with MEF2 on this promoter without the presence of an E box. However, this synergy requires that both proteins are intact and transcriptionally active. We observe no synergy between MyoD and the truncated MEF2A used in this study.2

In studies performed by Lee et al. (37), it was shown that MEF2 proteins and the thyroid hormone receptor can physically interact but the trans-activation domains of both MEF2 and the thyroid hormone receptor are absolutely required for synergistic activation of the c heart cardiac MHC gene. Again an amino-terminal truncated MEF2A protein was incapable of supporting any synergy with the thyroid hormone receptor. Also, a recent report from Lassar's group has documented that ectopic expression of mouse Twist (Mtwist) inhibits the trans-activation function of the MEF2 proteins (38), indicating that physiological targeting of this function is an evolved strategy for inhibition of myogenesis in the “non-myotomal” epithelial somite. The lines of evidence cited above are entirely consistent with our data and indicate that in several contexts the amino terminus of MEF2 is incapable of mediating synergistic activation of target genes by recruiting the bHLH proteins to the MEF2 site.

Endogenous MEF2 Is Required for Efficient Myogenic Conversion of C3H10T1/2 Fibroblast Cells—It is known that MEF2 activity is induced by the myogenic bHLH proteins in fibroblast cells (e.g., C3H10T1/2) that are permissive for myogenesis (18, 19). We therefore reasoned that if MEF2 is down-

2 J. Andreucci and J. McDermott, unpublished observation.
stream of the bHLH proteins and is required for myogenesis, our MEF2 dominant-negative should interfere with MyoD-induced myogenic conversion of C3H10T1/2 cells. In these experiments we observed that the number of MyoD-induced myogenic colonies was significantly reduced in the presence of our pMT2–131 dominant-negative. However, when MEF2 is over-expressed along with MyoD in C3H10T1/2 cells, we did not observe a potentiation of myogenic conversion. Thus, our interpretation of these data is that the endogenous MEF2 induced by MyoD is sufficient to fully co-operate in the activation of myogenesis and that additional ectopic expression of MEF2 above the level induced by MyoD is without effect. These data, therefore, fully support the role of MEF2 as a key biochemical co-regulator for the bHLH proteins during myogenesis.

In summary, we have constructed a trans-dominant MEF2 inhibitor that, when microinjected into muscle cells, inhibits the myogenic differentiation program. These data indicate that the trans-activation function of MEF2 proteins is required during mammalian myogenesis.

Acknowledgments—We thank Ron Prywes for kindly providing the pJLuc and pOFLuc reporter constructs and antisera to MEF2A and MEF2D and Rik Derynck for the cyclin A-Luc reporter construct. We also thank Robert Parsons of Zeiss, Canada for technical help with the microinjection system.

REFERENCES
1. Pollock, R., and Treisman, R. (1991) Genes Dev. 5, 2327–2341
2. Yu, Y.-T., Breitbart, R. E., Smooth, L. B., Lee, Y., Mahdavi, V., and Nadal-Ginard, B. (1992) Genes Dev. 6, 1783–1789
3. Martin, J. F., Schwarz, J. J., and Olson, E. N. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 5282–5286
4. Breitbart, R. E., Liang, C.-S., Smooth, L. B., Leifer, D., Krainc, D., and Lipton, S. A., and Nadal-Ginard, B. (1993) Development 118, 1095–1108
5. McDermott, J. C., Cardoso, M. C., Yu, Y.-T., Andre, V., Leifer, D., Krainc, D., and Lipton, S. A., and Nadal-Ginard, B. (1993) Mol. Cell. Biol. 13, 2564–2577
6. Leifer, D., Krainc, D., Yu, Y.-T., McDermott, J. C., Breitbart, R. E., Heng, J., Neve, R. L., Kosofsky, B., Nadal-Ginard, B., and Lipton, S. A. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 1546–1550
7. Chambers, A. E., Loggan, M., Kotecha, S., Towers, N., Sparrow, D., and Mohun, T. J. (1994) Genes Dev. 8, 1324–1334
8. Wong, M.-W., Plsegna, M., Lu, M.-F., Leibham, D., and Perry, M. (1994) Dev. Biol. 166, 683–695
9. Lilly, B., Galewsky, S., Firulli, A. B., Schulz, R. A., and Olson, E. N. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 5662–5666
10. Nguyen, H. T., Bodmer, R., Abmayr, S. M., McDermott, J. C., and Spoerel, N. A. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7520–7524
11. Sharracks, A. D., von Hesler, F., and Shaw, P. E. (1993) Nucleic Acids Res. 21, 215–221
12. Nurrish, S. J., and Treisman, R. (1995) Mol. Cell. Biol. 15, 4076–4085
13. Funk, W. D., and Wright, W. E. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 9484–9488
14. Molkentin, J. D., Black, B. L., Martin, J. F., and Olson, E. N. (1995) Cell 83, 1125–1136
15. Sartorelli, V., Huang, J., Hamamori, Y., and Kedes, L. (1997) Mol. Cell. Biol. 17, 1010–1026
16. Han, J., Jiang, Y., Li, Z., Kravchenko, V. V., and Ulevitch, R. J. (1997) Nature 386, 296–299
17. Kaushal, S., Schneider, J. W., Nadal-Ginard, B., and Mahdavi, V. (1994) Science 266, 1236–1240
18. Lassar, A. B., Davis, R. L., Wright, W. E., Kedes, L., Morre, C., Voronova, A., Baltimore, D., and Weintraub, H. (1991) Cell 66, 305–315
19. Cserjesi, P., and Olson, E. N. (1991) Mol. Cell. Biol. 11, 4854–4862
20. Molkentin, J. D., and Olson, E. N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 9366–9373
21. Olson, E. N., Perry, M., and Schulz, R. A. (1995) Dev. Biol. 172, 2–14
22. Edmondson, D. G., Cheng, T., Cserjesi, P., Chakraborty, T., and Olson, E. N. (1992) Mol. Cell. Biol. 12, 3665–3677
23. Bour, B. A., O'Brien, M. A., Lockwood, W. L., Goldstein, E. S., Bodmer, R., Taghert, P. H., Abmayr, S. M., and Nguyen, H. T. (1995) Genes Dev. 9, 730–741
24. Lilly, B., Zhao, B., Ranganayakulu, G., Paterson, B., Schulz, R. A., and Olson, E. N. (1995) Science 267, 688–693
25. Braun, T., Rudnicki, M. A., Arnold, H. H., and Jaenisch, R. (1992) Cell 71, 369–382
26. Hasty, P., Bradley, A., Morris, J. H., Edmondson, D. G., Venuti, J., Olson, E. N., and Klein, W. H. (1993) Nature 364, 501–506
27. Nabeshima, Y., Hanaoka, K., Hayasaka, M., Esumi, E., Li, S., Nonaka, I., and Nabeshima, Y. (1993) Nature 364, 532–535
28. Olson, E. N., and Klein, W. H. (1994) Genes Dev. 8, 1–8
29. Rudnicki, M. A., Braun, T., Hinuma, S., and Jaenisch, R. (1992) Cell 71, 383–390
30. Rudnicki, M. A., Schnegelsberg, P. N. J., Stead, R. H., Braun, T., Arnold, H. H., and Jaenisch, R. (1993) Cell 75, 1351–1359
31. Ornatsky, O. I., and McDermott, J. C. (1996) J. Biol. Chem. 271, 24927–24933
32. Han, T., and Prywes, R. (1995) Mol. Cell. Biol. 15, 2907–2915
33. Olson, E. N. (1990) Genes Dev. 4, 1454–1461
34. Shore, P., and Shanbhag, N. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 12217–12221
35. West, A. G., Shore, P., and Shanbhag, N. A. (1997) Mol. Cell. Biol. 17, 2304–2308
36. Molkentin, J. D., Li, L., and Olson, E. N. (1996) J. Biol. Chem. 271, 17199–17204
37. Lee, Y., Nadal-Ginard, B., Mahdavi, V., and Izumo, S. (1997) Mol. Cell. Biol. 17, 2745–2755
38. Spicer, D. B., Rhee, J., Cheung, W. L., and Lassar, A. B. (1996) Science 272, 1476–1480