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Mutational Analysis of the DNA Binding, Dimerization, and Transcriptional Activation Domains of MEF2C

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There are four members of the myocyte enhancer factor 2 (MEF2) family of transcription factors in vertebrates, MEF2A, -B, -C, and -D, which have homology within a MADS box at their amino termini and an adjacent motif known as the MEF2 domain. These factors activate muscle gene expression by binding as homo- and heterodimers to an A/T-rich DNA sequence in the control regions of muscle-specific genes. To understand the mechanisms of muscle gene activation by MEF2 factors, we generated a series of deletion and site-directed mutants of MEF2C. These mutants demonstrated that the MADS and MEF2 domains mediate DNA binding and dimerization, whereas the carboxyl terminus is required for transcriptional activation. Amino acids that are essential for MEF2 site-dependent transcription but which do not affect DNA binding were also identified in the MEF2 domain. This type of positive-control mutant demonstrates that the transcription activation domain of MEF2C, although separate from the MEF2 domain, is dependent on this domain for transcriptional activation through the MEF2 site. MEF2 mutants that are defective for DNA binding act as dominant negative mutants and can inhibit activation of MEF2-dependent genes by wild-type MEF2C.

The myocyte enhancer factor 2 (MEF2) family of transcription factors comprises a group of transcriptional activators, MEF2A, -B, -C, and -D, that show homology in a MADS (MCM1, Agamous, Deficiens, serum response factor [SRF]) box and an adjacent motif known as the MEF2 domain (6, 25, 31–33, 44, 57; reviewed in reference 49). MEF2 factors form homo- and heterodimers and bind to the consensus site, C/TTA(A/T)$_4$TAG/A, which is found in the control regions of numerous muscle-specific genes and has been demonstrated to be important for skeletal and cardiac muscle gene expression (1, 3, 5, 10–13, 15, 17, 21, 22, 24–27, 36–38, 52, 56, 58). The four vertebrate *mef2* gene products, also referred to as RSRFs (related to serum response factors) (44), have greater than 85% amino acid identity within the MADS domain and an adjacent 27-amino-acid region referred to as the MEF2 domain. This homology in the MADS and MEF2 domains is also present in D-MEF2, the single MEF2 protein in *Drosophila melanogaster* (29, 39, 50), and the characterized MEF2 proteins in *Xenopus laevis* (9, 54).

During embryogenesis, MEF2 transcripts appear initially in precursors of the cardiac and skeletal muscle lineages and are subsequently expressed at high levels in these differentiated muscle cell types (9, 16, 17, 25, 31, 33, 57). Mutations of the *D-mef2* gene in *D. melanogaster* suggest that MEF2 is an essential cofactor for differentiation of skeletal, cardiac, and visceral muscle cells (4, 30). In the absence of D-MEF2, myoblasts are correctly specified and positioned, but they fail to differentiate. These results have led to the notion that MEF2 may be a cofactor for other myogenic regulators that control muscle gene expression in different myogenic lineages (41a).

The exact role of MEF2 in skeletal muscle cells has been unclear. Kaushal and coworkers (23) reported that MEF2 factors have the ability to activate the complete program for skeletal muscle differentiation with an efficiency comparable to that of the myogenic basic helix-loop-helix (bHLH) factors MyoD and myogenin. In contrast, we have found that MEF2 factors lack myogenic activity on their own, but that they potentiate the activity of myogenic bHLH factors (34). This potentiation appears to be mediated by direct protein-protein interactions between MEF2 factors and heterodimers formed between myogenic bHLH factors and E proteins (34). This type of protein-protein interaction allows either type of factor to activate transcription through the other factor's DNA binding site when only one of the factors is bound to DNA.

Despite the importance of MEF2 factors in the control of muscle gene expression, very little is known about the mechanism by which these proteins activate transcription. However, the related MADS-box-containing factor, SRF, has been extensively characterized, and the crystal structure of its DNA binding region has recently been deduced (43). A minimal 91-amino-acid region of SRF containing the MADS box is sufficient for dimerization and site-specific recognition of the serum response element, CC(A/T)$_6$GG (40, 47). The N-terminal region of the MADS box of SRF is predicted to form an α-helix that contacts DNA, while an adjacent hydrophobic region of the MADS box, predicted to form a β-strand, mediates dimerization (43, 47). The MADS box of SRF is also sufficient for transcriptional activation of some SRF-dependent genes because it mediates interactions with accessory factors that activate transcription (20).

A comparison of the MADS boxes of SRF and MEF2 proteins demonstrates a relatively high degree of similarity between amino acids 1 and 38, with more divergence between amino acids 39 and 56 (reviewed in reference 49). It is also interesting that the MADS box in SRF begins at amino acid 141, whereas in all MEF2 proteins, the MADS box is located at the extreme N terminus. Deletion of the N-terminal amino acids preceding the MADS box in SRF results in relaxed DNA binding specificity, such that the MEF2 consensus site can be

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recognized, suggesting that amino acids N terminal to the MADS box influence DNA binding specificity of SRF (48). These data also suggest that MEF2 proteins and SRF are characterized by different structural constraints that result in unique DNA recognition and dimerization functions.

In this study, we characterized the regions of MEF2C that are responsible for transcriptional activation, DNA binding, and subunit dimerization. Our results show that the MADS box is essential for DNA binding and dimerization and that the MEF2 domain plays an important role in DNA binding affinity and an indirect role in dimerization. There are also specific residues within the MEF2 domain that are required for activity of the transcriptional activation region which is located near the C terminus of the protein. Intriguingly, these specific residues in the MEF2 domain do not affect dimerization or DNA binding. The ability of mutants in the MEF2 domain to affect activity of the C-terminal transactivation domain demonstrates that these domains are interdependent. MEF2C mutants that dimerize but fail to bind DNA function as dominant negative mutants and inhibit activation of MEF2-dependent reporter genes in C2C12 myotubes. However, these mutants retain the ability to synergize with myogenic bHLH factors to activate E-box-dependent transcription (34). These results demonstrate that MEF2 factors act through multiple mechanisms to control muscle gene expression.

MATERIALS AND METHODS

Assays for DNA binding. To determine the DNA binding characteristics of either the MEF2C deletion constructs or site-specific mutant constructs, electrophoretic mobility shift assays (EMSAs) were performed. Two microliters of a coupled in vitro transcription-translation product (TNT kit; Promega, Madison, Wis.) was incubated with 40,000 cpm of a ^{32}P-labeled, double-stranded oligonucleotide corresponding to the consensus MEF2 binding site from the muscle creatine kinase (MCK) gene (17) in the presence of 1 μg of poly(dI-dC) · (dI-dC) for 10 min at room temperature. The EMSA buffer and electrophoresis conditions are described elsewhere (35).

Site-directed mutagenesis. Mutations were introduced into the pCDNAI-MEF2C expression vector by rolling-circle PCR as described earlier (18). PCR conditions were as described previously (35). The initial mutant construct was cut with *Hin*dIII and *Xba*I, and the 1.4-kb fragment corresponding to the entire MEF2C cDNA was subcloned into pCDNAI/amp (Invitrogen) and sequenced. Each mutant construct was tested for integrity by in vitro coupled transcription-translation (TNT kit; Promega) in the presence of [^{35}S]methionine and then subjected to analysis by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (Fig. 3D).

Transfections and plasmids. The MEF2C expression vector, described previously (32), contains the 1.4-kb mouse cDNA cloned into the *Hin*dIII-*Xba*I sites of the cytomegalovirus promoter-directed expression vector pCDNAI (Invitrogen). To assess the activities of the MEF2C deletion and site-specific mutants generated with this construct, transient-transfection assays were performed with the MEF2-dependent chloramphenicol acetyltransferase (CAT) reporter construct pE102MEF2×2CAT, which contains two tandem copies of the MEF2 site from the MCK enhancer upstream from the basal promoter of the embryonic myosin heavy-chain gene, which drives expression of the CAT reporter gene (57). This plasmid is responsive to activation by MEF2 proteins. For assessment of dominant negative MEF2 activity conferred by the point mutations R3T and R24L, transfections were controlled by titrating an equal amount of empty vector, pCDNAI, such that the same amount of expression plasmid was used in all cases.

The activity of each of the mutant MEF2C proteins was analyzed by transfection assays performed with 10T1/2 cells grown in Dulbecco's modified Eagle's medium with high glucose and L-glutamine and 10% fetal bovine serum (growth medium). The cells were grown to 60% confluence in 6-cm-diameter plates, transfected by calcium phosphate precipitation for 16 h, washed, and harvested 48 h afterwards. Ten micrograms of the pE102MEF2x2CAT reporter construct (57) was used along with 5 μg of the MEF2C test construct and 1 μg of pRSVβGAL to control for transfection efficiency.

Analysis of the dominant negative MEF2C protein R3T (Arg-3 changed to Thr) in C2C12 cells was performed by transient transfection of confluent plates of fully differentiated myotubes. Cells were grown in growth medium for 2 days until confluent and then switched to differentiation medium, consisting of Dulbecco modified Eagle medium with high glucose and L-glutamine and 2% horse serum, for 6 days. On day 6, the myotubes were transfected with 5 μg of pE102MEF2×2CAT and 3 or 10 μg of a mutant expression vector encoding the R3T MEF2C protein. Forty-eight hours after transfection, cells were harvested and CAT activities were determined in aliquots of extract containing equivalent amounts of protein.

For analysis of the GAL4 fusion proteins, 10 μg of the GAL4-dependent reporter construct pG5E1bCAT (28) was transiently cotransfected into 10T1/2 fibroblasts with 5 μg of the indicated MEF2C-GAL4 fusion construct as described above. The MEF2C-GAL4 fusions were generated by blunt-end cloning of MEF2C PCR-generated DNA segments corresponding to the indicated amino acid sequences into the expression plasmid pSG424 at the *Sma*I site so that the DNA binding domain of GAL4 (amino acids 1 to 147) is fused to MEF2C. Extracts and CAT assays were performed as previously described (35).

In vitro translation and immunoprecipitation. The dimerization potential of each site-directed mutant was assessed by coimmunoprecipitation of in vitro translation products of the indicated full-length mutant construct and a truncated MEF2C construct (amino acids 1 to 105). The truncated MEF2C construct contained a FLAG (Kodak IBI, New Haven, Conn.) epitope at the C terminus so that anti-FLAG antibody could be used for specific immunoprecipitation. In vitro transcription-translation was performed in a total reaction volume of 25 μl with 0.5 μg of each construct. Five microliters of this reaction mix was immunoprecipitated as recommended by the manufacturer (Kodak IBI) in a total volume of 100 μl with 1 μl of anti-FLAG monoclonal antibody and 25 μl of protein G-agarose. The precipitated products were subjected to SDS-PAGE and autoradiography.

Western blotting (immunoblotting). The stabilities of the mutant MEF2C proteins were assessed by Western blotting of extracts made from transiently transfected 10T1/2 cells. Constructs encoding wild-type MEF2C and mutants R3T, RKK3-5TNQ, R15L, R17V, KR23,24ID, K25N, K30H, K31L, LSVL35-38QSSM, CDC39-41SDD, LI45,46RN, IF47,48DS, STDMD59-63RAVMH, VLL65-67ASR, and KYTEY68-72ECNDN were separately transfected into 6-cm-diameter dishes of 10T1/2 cells as described above. Only these mutant constructs were assayed because they demonstrated a reduced function compared with wild-type MEF2C. The transfected cells were harvested 48 h later by lysis in 50 μl of 2× loading buffer (125 mM Tris [pH 6.8], 4% SDS, 200 mM β-mercaptoethanol, 20% glycerol, 0.0025% bromophenol blue) and boiled for 5 min. Fifteen microliters of this lysate was electrophoresed on a standard 10% Laemmli gel and blotted to nitrocellulose. Hybridization and washing were performed as described previously (2). Detection of MEF2C was performed by incubation with a primary antibody against MEF2C used at a dilution of 400:1. The secondary antibody was a goat anti-rabbit alkaline phosphatase-conjugated immunoglobulin G-antibody used at a working concentration of 3,000:1 (Boehringer Mannheim, Indianapolis, Ind.). Visualization of alkaline phosphatase activity was performed with 5-bromo 4-chloro-3-indolylphosphate and nitroblue tetrazolium as described previously (2).

Analysis of the stability of the GAL4-MEF2C fusion proteins by Western blotting was performed on extracts of transiently transfected COS cells as described above. The primary antibody was a mouse monoclonal antibody against the DNA binding domain of GAL4 (Santa Cruz Biotechnology, Santa Cruz, Calif.) and was used at a working concentration of 800:1; a goat anti-mouse alkaline phosphatase-conjugated immunoglobulin G secondary antibody (Boehringer Mannheim) was used at a dilution of 2,000:1. The results of both sets of Western blot analyses demonstrated equivalent stabilities of wild-type and mutant MEF2 proteins, suggesting that the differences in transactivation are not due to differences in protein stability (data not shown).

RESULTS

The MADS and MEF2 domains lack transcriptional activity. To identify the regions of MEF2C that were involved in transcriptional activation, we generated a series of deletion mutants and assayed their abilities to support transcriptional activation of a MEF2-dependent reporter gene (pE102ME F2x2CAT) in 10T1/2 cells (Fig. 1A). The reporter gene was efficiently transactivated by MEF2C, whereas the same reporter containing mutated MEF2 sites was not transactivated (not shown). Deletion of amino acids 199 to 465 resulted in a partial loss of transcriptional activity, whereas C-terminal deletions to amino acid 143 or 117 reduced transcriptional activity to a basal level. These results suggested that a strong transcriptional activation domain was located in the C terminus of MEF2C and that the MADS and MEF2 domains, which are located in the amino terminus, lacked transcriptional activation potential. To confirm that residues 1 to 117 retained the ability to bind DNA in vivo, we fused this region of MEF2C to the activation domain of the viral coactivator VP16. This MEF2-VP16 chimera (1-117/VP16) was more potent than full-length MEF2C in activating transcription of the MEF2-dependent reporter, which confirmed that residues 1 to 117 of

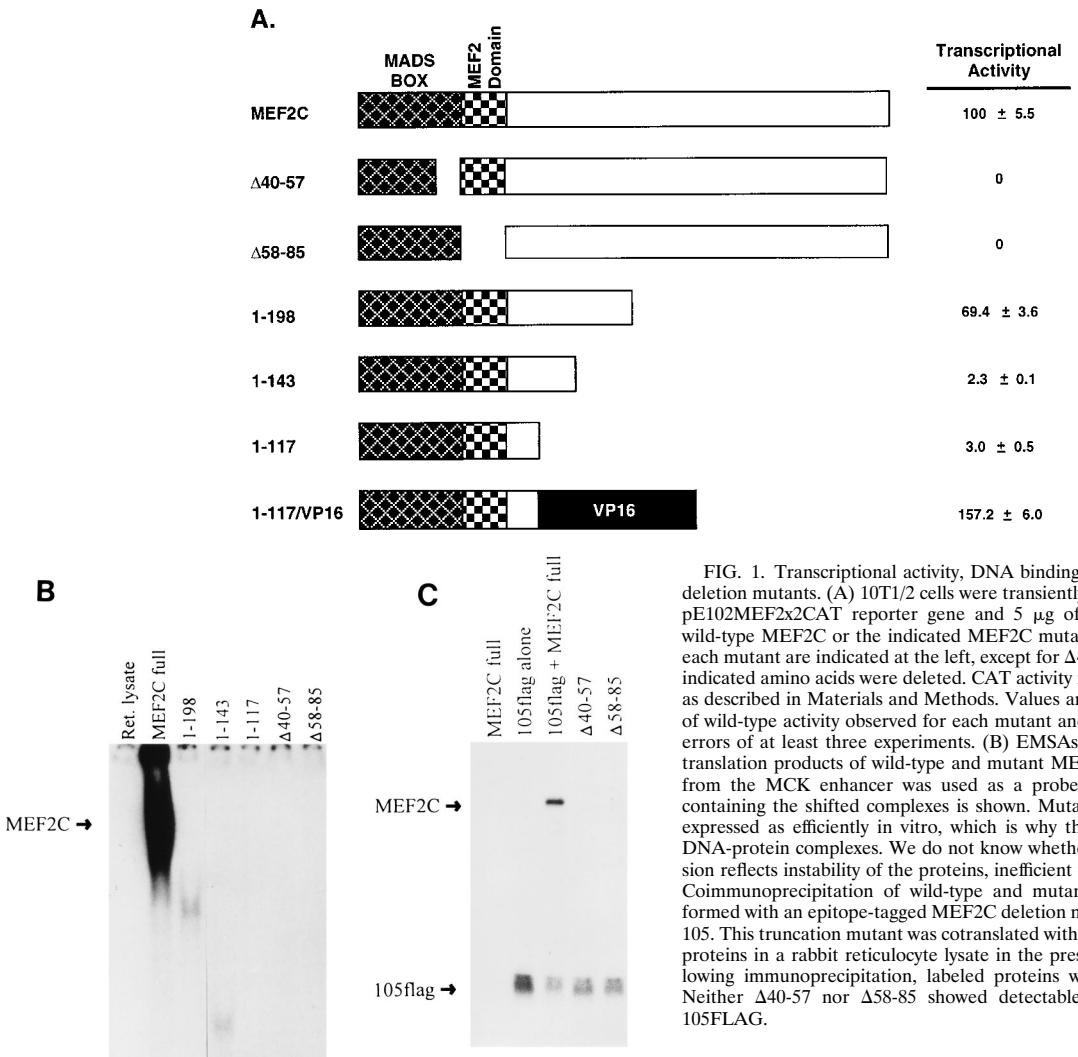


FIG. 1. Transcriptional activity, DNA binding, and dimerization of MEF2C deletion mutants. (A) 10T1/2 cells were transiently transfected with 10 μg of the pE102MEF2x2CAT reporter gene and 5 μg of expression vectors encoding wild-type MEF2C or the indicated MEF2C mutants. Amino acids contained in each mutant are indicated at the left, except for Δ40-57 and Δ58-85, in which the indicated amino acids were deleted. CAT activity in cell extracts was determined as described in Materials and Methods. Values are expressed as the percentage of wild-type activity observed for each mutant and are the averages ± standard errors of at least three experiments. (B) EMSAs were performed with in vitro translation products of wild-type and mutant MEF2C proteins. The MEF2 site from the MCK enhancer was used as a probe. Only the region of the gel containing the shifted complexes is shown. Mutants 1-198 and 1-143 were not expressed as efficiently in vitro, which is why they resulted in relatively faint DNA-protein complexes. We do not know whether their relatively poor expression reflects instability of the proteins, inefficient translation, or insolubility. (C) Coimmunoprecipitation of wild-type and mutant MEF2C proteins was performed with an epitope-tagged MEF2C deletion mutant containing residues 1 to 105. This truncation mutant was cotranslated with wild-type and mutant MEF2C proteins in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Following immunoprecipitation, labeled proteins were resolved by SDS-PAGE. Neither Δ40-57 nor Δ58-85 showed detectable interaction with MEF2C/1-105FLAG.

MEF2C were sufficient to support DNA binding and dimerization in vivo (Fig. 1A).

The MADS box encompasses residues 1 to 56, and the MEF2 domain encompasses residues 57 to 85. We introduced into MEF2C internal deletions that removed the C-terminal portion of the MADS box (Δ40-57) and the MEF2 domain (Δ58-85). Neither of these mutants was able to activate transcription (Fig. 1A).

The DNA binding activity of each mutant was assessed by EMSA with in vitro-translated protein and the MEF2 binding site from the MCK enhancer as a probe (Fig. 1B). Dimerization potential was also determined by translation of each mutant protein in vitro with an MEF2C truncation mutant containing amino acids 1 to 105 fused to a seven-amino-acid C-terminal FLAG epitope. The resultant heterodimeric complex was immunoprecipitated with FLAG antibody and subjected to SDS-PAGE (Fig. 1C). Wild-type MEF2C was able to dimerize and bind DNA, whereas mutants Δ40-57 and Δ58-85 were not. In light of the role of the MADS box in DNA binding, we anticipated that Δ40-57 would be defective in dimerization and/or DNA binding. These results indicate that the MEF2 domain, which was deleted in Δ58-85, also plays a role in dimerization. Together, these results demonstrate that the dimerization and DNA binding functions in the N terminus of MEF2C can be separated from the transactivating functions in the C-terminal region.

Mapping of the MEF2C transcriptional activation domain by using GAL4-MEF2C chimeras. To further define the boundaries of the transcription activation domain of MEF2C, we fused the DNA binding domain of yeast GAL4 (amino acids 1 to 147) to portions of MEF2C and tested the resulting chimeric proteins for their abilities to activate a GAL4-dependent reporter gene in 10T1/2 cells (Fig. 2). Full-length MEF2C fused to GAL4 (GALM2C 1-465) had the strongest transcriptional activity. Deletion of amino acids 175 to 465 (GALM2C 1-174) had only a small effect on transcriptional activity. An intermediate deletion of amino acids 198 to 465 (GALM2C 1-198) resulted in a greater decrease in transcriptional activity, suggesting the presence of an inhibitory domain between amino acids 175 and 198. Further C-terminal deletions to amino acid 143 or 93 (GALM2C 1-143 or GALM2C 1-93) resulted in a complete loss of transcriptional activity. These results are consistent with the deletion analyses of MEF2C and

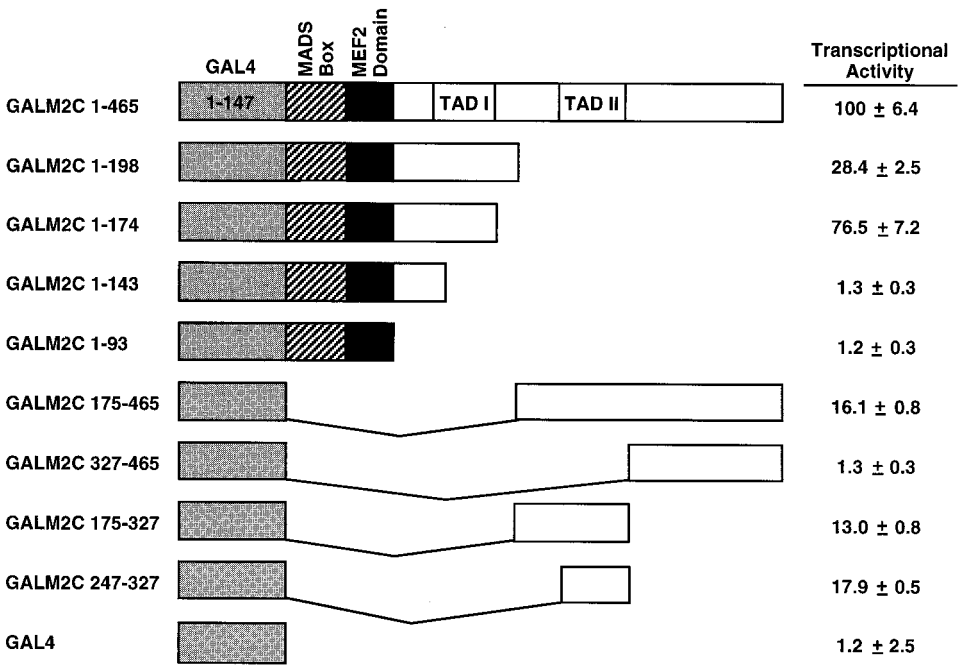


FIG. 2. Transcriptional activity of GAL4-MEF2C chimeras. 10T1/2 cells were transiently transfected with 10 μg of the pG5E1bCAT reporter gene and 5 μg of expression vector encoding the indicated GAL4-MEF2C chimeras. Amino acids of MEF2C contained in each mutant are indicated at the left. CAT activity in cell extracts was determined as described in Materials and Methods. Values are expressed as the percentage of GAL4-MEF2C activity observed for each mutant and are the averages ± standard errors of at least three experiments. TAD, transcription activation domain.

demonstrate that the MADS and MEF2 domains cannot activate transcription alone.

The more C-terminal regions of MEF2C were analyzed further by fusing a series of internal regions of MEF2C to GAL4 (Fig. 2). The region between amino acids 175 and 465 (GALM2C 175-465) could activate transcription. Dissection of this region showed that amino acids 175 to 327 (GALM2C 175-327) retained the ability to activate transcription, while a more C-terminal region (GALM2C 327-465) was inactive. Further deletions showed that transcriptional activation was directed by the region from amino acids 247 to 327 (GALM2C 247-327). Thus, the C-terminal transcription-activating region appeared to be composed of at least two subdomains that could activate transcription independently, one region between residues 143 and 174 and the other between residues 247 and 327.

To control for possible differences in transcriptional activities due to differential stability of one or more of the proteins analyzed, the expression of each protein was examined in extracts of transfected cells by Western blotting with an antibody directed against the GAL4 DNA binding domain. These experiments showed that all of the constructs were expressed at comparable levels (not shown). This finding suggests that the differences in transcriptional activity among the different proteins did not result from differences in expression or stability but reflected regions responsible for activation or repression of transcription.

Mutational analysis of the MEF2C DNA binding domain. The deletion mutations demonstrated that amino acids 1 to 117 of MEF2C were sufficient for DNA binding and dimerization, consistent with previous studies of MEF2A (RSRFC4) (44). To map more precisely the specific residues that mediate these activities, we mutated the conserved amino acids within the MADS and MEF2 domains within the full-length MEF2C protein. Mutational analysis of SRF has shown that the first 31 residues of the MADS box mediate DNA binding (41, 47). This region of SRF adopts an α-helical conformation with the basic residues making major and minor groove contacts with the DNA binding site (43). Within the corresponding region of MEF2C, there are 11 basic amino acids which may be involved in DNA-protein interactions. We therefore systematically mutated all of these residues either singly or in combinations to determine which might be required for DNA binding (Fig. 3A). Replacement of Arg-3, Lys-5, Arg-24, Lys-30, and Lys-31 with noncharged amino acids resulted in a complete loss of DNA binding activity (Fig. 3B) without a loss in dimerization potential (Fig. 3C). With the exception of mutant K30H, each of these mutations also resulted in a complete loss in transcriptional activity. The K30H mutant reproducibly transactivated at approximately 20% of the level of the wild-type protein despite an apparent lack of DNA binding capacity in vitro. The explanation for this discrepancy may be in the subtle differences in conditions between the in vitro and in vivo assays, such that K30H may retain modest DNA binding capacity in vivo.

Mutagenesis of residue Lys-4, Arg-15, Arg-17, Lys-23, or Lys-25 resulted in a partial loss of DNA binding capacity without a loss in dimerization potential. These mutations also led to a decrease in the ability to activate the MEF2-dependent reporter gene. Mutation of Arg-10 had no effect on DNA binding capacity and a minimal effect on activation potential. In vitro transcription and translation reactions were performed for each MEF2C construct in the presence of [^{35}S]methionine, and the products were subjected to SDS-PAGE (Fig. 3D). The results demonstrate that all proteins are equally stable and that the differences in DNA binding activity result specifically from the designated amino acid substitutions. Together, the results demonstrate that virtually every basic amino acid within the N-terminal MADS-box region from amino acids 1 to 31 of MEF2C is critical for protein function. That all of these basic amino acids are invariant in MEF2 proteins from human,

mouse, chicken, frog, *Drosophila* and *Caenorhabditis elegans* cells also suggests that they are functionally important.

Arg-17, Lys-23, Arg-24, Lys-30, and Lys-31 are highly conserved in all known MADS-box proteins. The amino acids corresponding to Arg-17, Lys-23, and Arg-24 in SRF are essential for DNA binding of SRF (47). Simultaneous mutation of Lys-30 and -31 also diminishes DNA binding by SRF. That mutants R17V and K23T retained residual DNA binding suggests that there are subtly different structural requirements for DNA binding by MEF2 factors and SRF.

Noncharged amino acids were substituted for each of the N-terminal MADS-box mutations so as not to disrupt the α-helical structure that is predicted to form in this region. Each of the amino acid substitutions that resulted in a significant decrease in transactivation was tested for stability in vivo by Western blotting. Western blotting was performed on extracts from 10T1/2 cells transfected with constructs encoding wild-type or mutant MEF2C proteins. The results showed no differences in protein stability for any of the constructs tested (see Materials and Methods), suggesting that the differences in transactivation shown in Fig. 3A are not the result of differences in protein stability (data not shown).

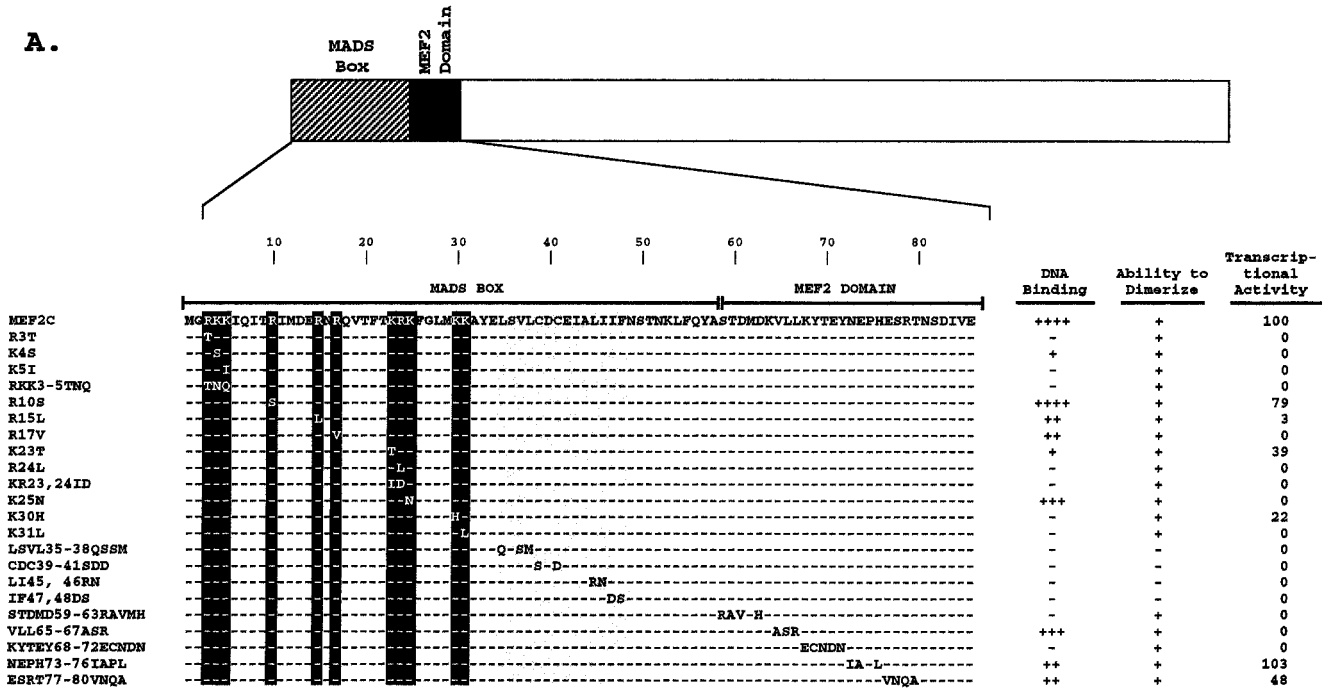
Identification of the MEF2C dimerization domain. The amino-terminal α-helical region of the MADS box is followed by a region in SRF and MEF2 factors (amino acids 28 to 56) that is predicted to adopt a β-sheet conformation. The crystal structure of SRF reveals that this region extends away from the DNA and makes relatively few DNA contacts (43). Within this region, there is a stretch of hydrophobic amino acids from residues 35 to 48. All known MADS-box proteins contain relatively similar regions of conserved hydrophobicity. To investigate the role of this region, we mutated the majority of these hydrophobic residues and tested the resulting mutants for their abilities to dimerize, bind DNA, and activate transcription. For many of the mutations, charged substitutions were introduced to disrupt the local hydrophobic pocket that is predicted to form in this region. Mutagenesis of groups LSVL35-38, LI45,46, and IF47,48 eliminated DNA binding and dimerization potential (Fig. 3B and C). The loss in DNA binding capacity is presumably due to a failure of these mutants to dimerize and is not due to a loss in protein stability (Fig. 3D and results of Western analyses [not shown]). These results suggest that the identified hydrophobic amino acids are required for dimerization and subsequent DNA binding. Surprisingly, mutation of the two cysteines at positions 39 and 41 (mutant CDC39-41SDD) also resulted in a loss in dimerization potential. This could be the result of a loss in the overall secondary structure of this region, or it could reflect a specific requirement for these residues in dimerization.

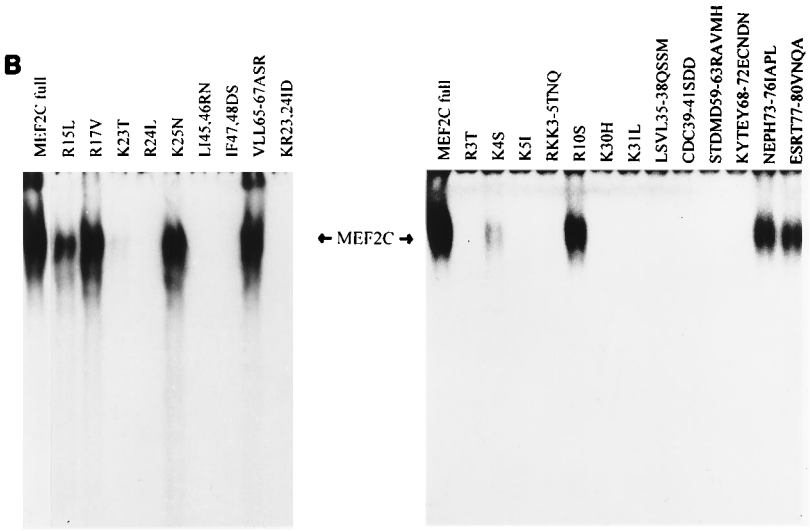
Role of the MEF2 domain. The MEF2 domain, which is adjacent to the MADS box, is conserved in and unique to members of the MEF2 family. Deletion mutation Δ58-85, which removed the MEF2 domain, resulted in a complete loss in dimerization and DNA binding capacity (Fig. 1). These results could be interpreted to indicate either that the MEF2 domain is directly required for dimerization or that it simply acted as a permissive region for dimerization directed by the hydrophobic patch in the MADS box. If the MEF2 domain contained specific amino acids that were required for directing dimerization, then mutagenesis of these amino acids should demonstrate this fact. However, if the MEF2 domain was indirectly required as a structural element, then a loss of this domain and subsequent truncation might result in a conformational change that could interfere with dimerization. To distinguish between these possibilities, we introduced a series of block mutations within this region of MEF2C (Fig. 3). None of these mutations had any effect on dimerization, suggesting that the MEF2 domain does not directly specify interactions between MEF2 monomers, but that it is required as a structural element that permits dimerization. Mutations in the MEF2 domain were also performed in blocks of four and five amino acids to scan for a region that might be involved in cofactor interaction and hence have an effect on transcriptional activation. This homologous region in SRF has been shown to interact with cofactors that are required for serum-regulatable expression (see below) (20; reviewed in reference 49).

Several mutations in the MEF2 domain impaired DNA binding and transcriptional activity. Mutation of amino acids STDMD at positions 59 to 63 and amino acids KYTEY at positions 68 to 72 eliminated DNA binding activity without affecting dimerization potential (Fig. 3B and C). Both of these mutants also lacked the ability to activate the MEF2-dependent reporter (Fig. 3A). Mutation of amino acids NEPH and ESRT at positions 73 to 76 and 77 to 80, respectively, also diminished DNA binding without affecting dimerization. Taken together, the results of these specific mutations suggest that the MEF2 domain is involved in both DNA binding and dimerization.

Mutation of the hydrophobic amino acids VLL at positions 65 to 67 resulted in only a minor decrease in DNA binding in vitro and had no effect on dimerization potential of the protein. However, this mutant completely lacked the ability to activate transcription. To confirm that this mutant protein was stable and was able to bind DNA in vivo, we performed EMSAs with nuclear extracts from COS and 10T1/2 cells that had each been transiently transfected with the VLL65-67ASR expression vector. DNA binding activity comparable to that of the wild-type protein was observed from extracts of each cell type (data not shown). The inability of this mutant to activate transcription demonstrates that the MEF2 domain is critical for transcriptional activity of the C-terminal transactivation domain in the context of the full-length protein and that DNA binding is necessary but not sufficient for transcriptional activation by MEF2. This result also suggests that the MEF2 domain mediates an additional event required for activation of gene expression.

Mutation of the MEF2C DNA binding domain generates dominant negative proteins. Mutations in the MADS box of MEF2C that eliminated DNA binding without affecting dimerization might be predicted to generate dominant negative proteins that can interfere with the activity of wild-type MEF2 proteins by dimerizing with wild-type MEF2 and preventing subsequent DNA binding and transcriptional activation. To test this possibility, we measured the transcriptional activity of wild-type MEF2C in the presence of increasing amounts of the DNA binding mutants R24L and R3T, which fail to bind DNA but retain the ability to dimerize (Fig. 4A). When 10T1/2 cells were transfected with expression vectors encoding these mutants at a 1:1 ratio with either wild-type MEF2C or MEF2A, we observed an ~25% decrease in activation of the MEF2-dependent reporter gene pE102MEF2x2CAT. Higher relative amounts of the mutants resulted in near-complete inhibition of reporter gene expression (Fig. 4A). These results demonstrate that the MEF2C mutants R24L and R3T are capable of acting in a dominant negative manner, presumably because they dimerize in vivo with wild-type MEF2 proteins and form inactive heterodimers. To further characterize the inhibitory activities of these dominant negative mutants, transient-transfection assays were performed with C2C12 myotubes, which contain high levels of endogenous MEF2 DNA binding activity (17). As reported previously, the MEF2-dependent reporter gene was expressed efficiently in C2C12 myotubes (Fig. 4B).


A.
MADS Box
MEF2 Domain
10
20
30
40
50
60
70
80
MADS BOX
MEF2 DOMAIN
DNA Binding
Ability to Dimerize
Transcriptional Activity
MEF2C ++++ + 100
R3T - + 0
K4S + + 0
K5I - + 0
RKK3-5TNQ - + 0
R10S ++++ + 79
R15L ++ + 3
R17V ++ + 0
K23T + + 39
R24L - + 0
KR23,24ID - + 0
K25N +++ + 0
K30H - + 22
K31L - + 0
LSVL35-38QSSM - - 0
CDC39-41SDD - - 0
LI45, 46RN - - 0
IF47,48DS - - 0
STDMD59-63RAVMH - + 0
VLL65-67ASR +++ + 0
KYTEY68-72ECNDN - + 0
NEPH73-76IAPL ++ + 103
ESRT77-80VNQA ++ + 48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MEF2C full
R15L
R17V
K23T
R24L
K25N
LI45,46RN
IF47,48DS
VLL65-67ASR
KR23,24ID
← MEF2C →
MEF2C full
R3T
K4S
K5I
RKK3-5TNQ
R10S
K30H
K31L
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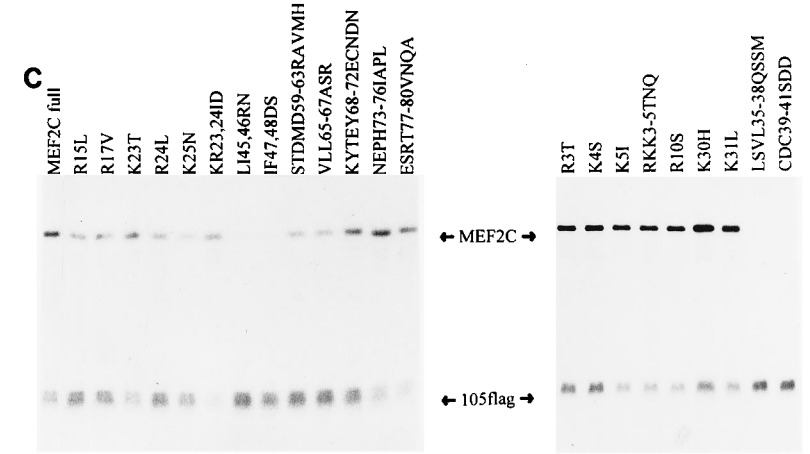
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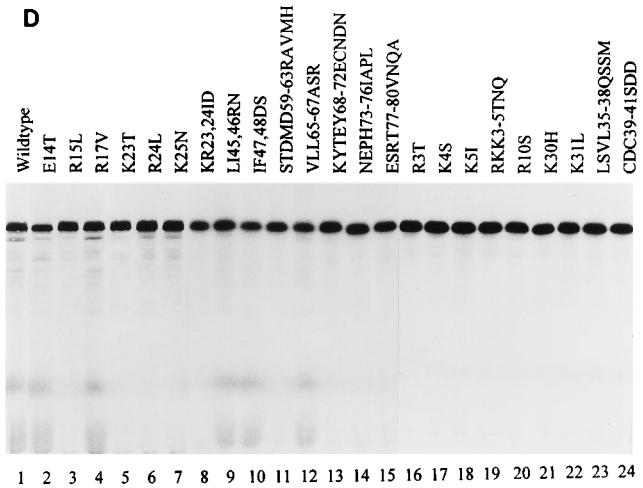

FIG. 3. Transcriptional activity, DNA binding, and dimerization of wild-type and mutant MEF2C proteins. (A) The amino acid sequences of the MADS and MEF2 domains of mouse MEF2C are shown, and the names of mutants are at the left. A dash indicates no change at that position. Basic amino acids within the DNA binding domain are indicated in black, and the hydrophobic dimerization domain is shaded. Relative transcriptional activities determined by using pE102MEF2x2CAT as a reporter in transfected 10T1/2 cells are indicated. Relative DNA binding activities were determined by PhosphorImager analysis and are indicated as ++++ (wild-type DNA binding), +++ (~75% of wild-type binding), ++ (~50% of wild-type binding), + (~25% of wild-type binding), or − (no binding). Dimerization (+) or its absence (−) is also shown. (B) EMSAs were performed with in vitro translation products of wild-type and mutant MEF2C proteins. The MEF2 site from the MCK enhancer labeled with ^{32}P was used as a probe. Only the region of the gel containing the shifted complexes is shown. (C) The MEF2C deletion mutant containing residues 1 to 105 fused to the FLAG epitope was cotranslated with wild-type and mutant MEF2C proteins in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine. Following immunoprecipitation, labeled proteins were resolved by SDS-PAGE. (D) The various MEF2C mutant constructs were in vitro translated in the presence of [^{35}S]methionine and subjected to SDS-PAGE. The results demonstrate similar stabilities for the various products.

However, in the presence of mutants R24L or R3T, expression was reduced by ~90%. No reduction in basal activity was seen for an identical construct containing mutated MEF2 sites (data not shown). This result suggests that the dominant negative proteins R24L and R3T can dimerize with endogenous MEF2 proteins in C2C12 myotubes and inhibit their activities.

To further characterize the effect that was mediated by these dominant negative proteins, EMSAs were performed with extracts of transfected 10T1/2 cells (Fig. 4C). Untransfected 10T1/2 cells showed almost no MEF2-like activity (lane 1); however, transfection of 2 μg of the wild-type MEF2C expression vector resulted in a robust shift (lane 2). If a 10-fold excess of the mutant R3T MEF2C expression vector was cotransfected, an 80% decrease in the shifted band was observed (lane 3). These results demonstrate that the decrease in transcriptional activation directed by these dominant negative MEF2C proteins is due to heterodimerization and subsequent sequestration of wild-type MEF2 proteins in vivo.

DISCUSSION

Members of the MADS-box family of transcription factors have been identified in plants, the yeast *Saccharomyces cerevisiae*, invertebrates, and vertebrates. The primary amino acid sequences of the MADS boxes from the different members of the family show extensive homology, suggesting a common secondary structure as well as related DNA binding sites (Fig. 5). Indeed, most MADS-box proteins have been reported to recognize the DNA consensus sequence $CC(A/T)_6GG$, which is similar to the consensus sequence for the MEF2 factors $CTA(A/T)_4TAG$ (reviewed in reference 49). There is greater than 85% sequence identity between the MADS and MEF2 domains of MEF2 proteins from human (6, 33, 44, 57), mouse (31, 32), chicken (30a), *Xenopus* (9, 54), *Drosophila* (29, 39, 50), and *C. elegans* (23a) cells. The MEF2 domain is also highly conserved among MEF2 factors, but it is not present in other MADS-box proteins.

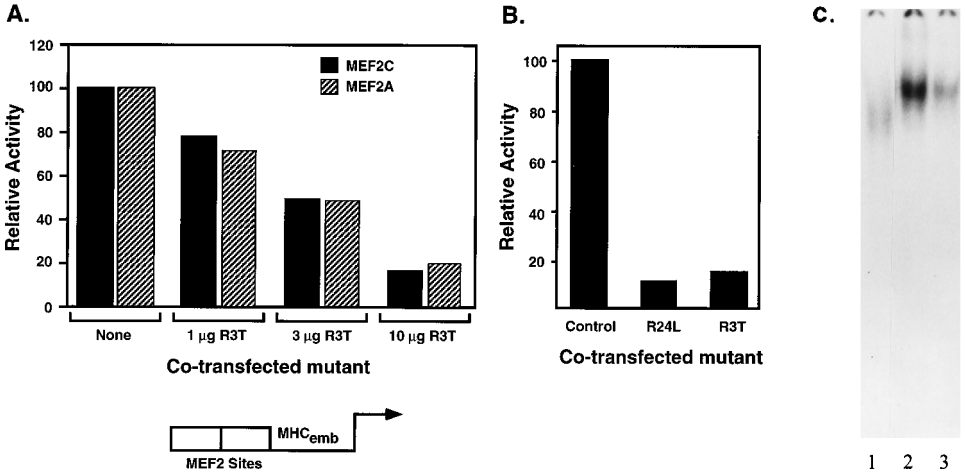

FIG. 4. Inhibition of MEF2-dependent promoters by dominant negative MEF2C proteins. (A) Dose-dependent inhibition of transactivation in the presence of increasing amounts of R3T expression plasmid. 10T1/2 cells were transiently transfected with pE102MEF2x2CAT, 1 μg of MEF2A or MEF2C expression vector, and the indicated amounts of mutant R3T expression vector and empty vector so that the total amount of pCDNAI expression vector remained constant. (B) Inhibition of endogenous MEF2 activity in C2 myotubes in the presence of 3 μg of the indicated mutant expression vectors. Forty-eight hours after transfection, cells in the experiments shown in panels A and B were harvested, and CAT activities were determined in aliquots of extract containing equivalent amounts of protein. In panel B, the degree of reduction in activity was the same when either 3 or 10 μg of dominant negative construct was used, indicating that transient transfection with 3 μg was sufficient to fully saturate endogenous MEF2 activity. No reduction in activity was seen upon transfection with 10 μg of empty expression vector. MHC_{emb}, embryonic myosin heavy-chain promoter. (C) EMSA of extracts from transfected 10T1/2 cells with an oligonucleotide corresponding to the MCK MEF2 site. Lane 1, untransfected; lane 2, transfected with 2 μg of wild-type MEF2C expression vector and 20 μg of empty pCDNAI vector; lane 3, transfected with 2 μg of wild-type MEF2C expression vector and 20 μg of MEF2C R3T dominant negative expression vector.

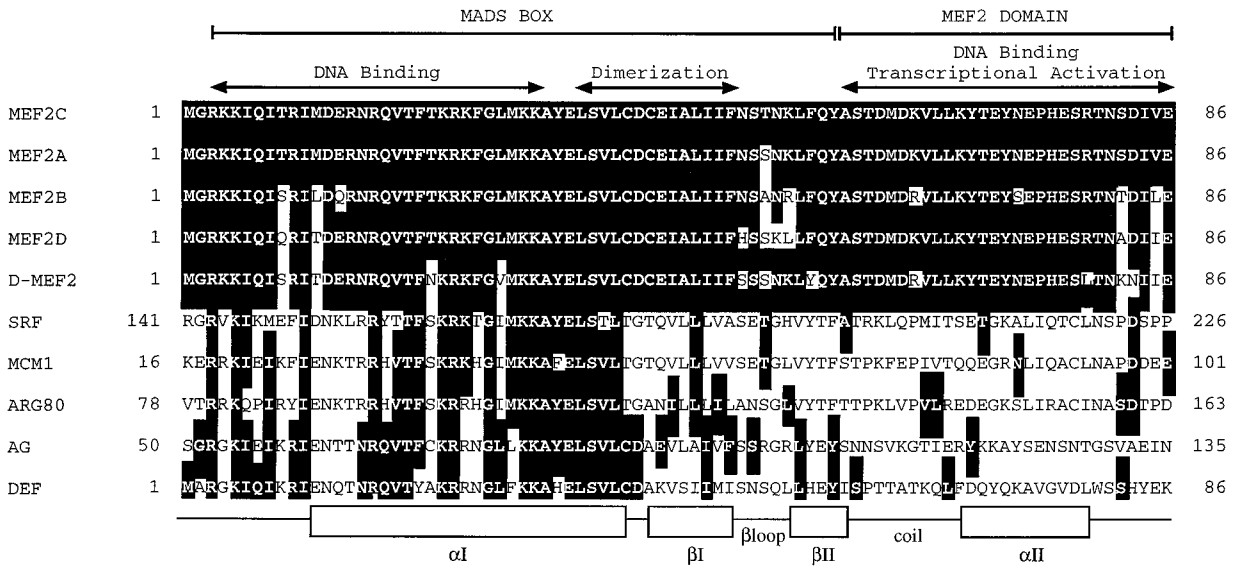

FIG. 5. Identities among MADS-box proteins. Sequences of different MADS-box proteins are shown. Sequences (references) are as follows: MEF2C (32); MEF2A (44); MEF2B (44); MEF2D (31); D-MEF2 (29); SRF (40); MCM1 (42); ARG80 (14); AG (Agamous) (55); DEF (46). Regions of secondary structure in SRF are indicated at the bottom (43). Within the MADS domain, MEF2 factors are similar in secondary structure to SRF, whereas the MEF2 domain does not show predicted structural similarity to the corresponding region of SRF.

Similarities between the DNA binding and dimerization domains of MEF2 and SRF. The crystal structure of SRF indicates that the DNA binding region is composed of three structural domains (Fig. 5). The MADS box encompasses a coiled-coil that interacts with DNA and a central β-sheet involved in protein dimerization that also contacts DNA. The amino acid sequence identity between the MADS boxes of SRF and the MEF2 factors suggests that the DNA binding region of the MEF2 factors adopts a secondary structure similar to that of SRF. Immediately C terminal to the MADS box of SRF is a region that is oriented away from the DNA (43) and has been implicated in interactions between SRF and accessory factors (20; reviewed in reference 49). The MADS boxes of SRF and MEF2 proteins have the greatest identity within amino acids 1 to 38, with more divergence between amino acids 39 and 56. The N-terminal region of the MADS box is predicted to adopt an α-helical conformation. The basic residues in the α-helical region of SRF make extensive contacts with the major and minor grooves of the DNA binding site (43). Mutagenesis of this region of MEF2C demonstrated that the majority of the basic amino acids in this region are important for DNA binding.

The C-terminal region of the MADS box (residues 39 to 56) contains a hydrophobic cluster of amino acids predicted to form a β-strand. Our results demonstrate that these residues are involved in dimerization of MEF2 proteins. A similar hydrophobic region has been shown to direct dimerization of SRF (47). However, the spacing and identity of the hydrophobic amino acids are different in SRF and the MEF2 proteins, which may explain the observation that MEF2 proteins do not dimerize with SRF (44).

Mutagenesis of the MEF2 domain demonstrated that it directly influences DNA binding but not dimerization. However, deletion of the MEF2 domain resulted in a mutant MEF2 protein that was deficient in its ability to dimerize, suggesting that this region is structurally required to allow dimerization which is directed by the characterized amino acids in the C-terminal half of the MADS box. Mutations in the MEF2 domain that eliminate DNA binding activity are likely the result of an influence on the MADS box and the manner in which it interacts with DNA. The corresponding region of SRF is oriented distally when bound to DNA and contains a short amphipathic α-helix (αII in Fig. 5), which serves as an interface for dimerization of SRF monomers (43). Yet this region is not in intimate contact with DNA, suggesting that it does not specify DNA interaction. There is no amino acid sequence or secondary structural similarity between these regions of SRF and MEF2, which suggests that the function of the MEF2 domain may not be shared with the corresponding region of SRF. This is consistent with the observation that these regions of SRF and MEF2 factors mediate interactions with different accessory factors (20, 34).

Interdependence of the MEF2 domain and the transcription activation domains. Our results demonstrate that the C terminus of MEF2C acts as a transcription activation domain. The first 105 amino acids of MEF2C, which encompass the MADS and MEF2 domains, can dimerize and bind DNA, but this region is unable to activate transcription through the MEF2 site in the absence of a C-terminal transcription activation domain. The C termini of mouse MEF2D and MEF2B and *Xenopus* MEF2A (XMEF2A) are also required for transcriptional activation by these factors (31, 33a, 54). In MEF2C, residues 143 to 174 and 247 to 327 in the C terminus can activate transcription independently. Both of these regions are rich in serine, threonine, and proline residues, which are often found in the activation domains of transcription factors, and correspond to regions of similarity among different MEF2 factors (32). The region from residues 143 to 174 in MEF2C also corresponds to the position of a transactivation domain in XMEF2A, whereas the transcription-activating function contained within residues 247 to 327 of MEF2C appears to be absent from XMEF2A (54).

Mutagenesis of the MEF2 domain yielded an intriguing mutant, VLL65-67ASR, that dimerized and bound DNA but failed to activate transcription. This type of positive-control mutant is similar to mutants that have been generated in the myogenic bHLH factors (7, 13, 45, 53) and indicates that DNA

binding is not by itself sufficient for MEF2 to activate transcription. The ability of mutant VLL65-67ASR to bind DNA without activating transcription suggests that there is an interdependence between the MEF2 domain and the C-terminal transcription activation region. How residues in the MEF2 domain might influence activity of the transcription activation domain, which is located in a separate region of the protein, is unclear. One possibility is that binding of MEF2 to DNA results in an allosteric change in the protein that unmasks the activation domain; such a conformational change might not occur in mutant VLL65-67ASR. Alternatively, the residues VLL at positions 65 to 67 might mediate interactions with other transcription factors, which are disrupted by the mutation. In this regard, other MADS-box proteins such as SRF and yeast MCM1 require interactions with cofactors for activation of their target genes (reviewed in references 19 and 49). The regions of these factors that mediate interactions with accessory proteins are located immediately adjacent to the MADS box, similar to the location of the MEF2 domain.

The MEF2 domain is required for interaction of MEF2 factors with heterodimers formed between myogenic bHLH factors and E proteins (34). We have tested mutant VLL65-67ASR for its ability to interact with myogenin-E12 heterodimers in an in vivo one-hybrid assay and have found that it interacts as efficiently as wild-type MEF2C (data not shown). Similarly, mutant VLL65-67 can synergize with the bHLH region of myogenin to induce transcription through the E-box sequence. Thus, these residues do not appear to affect formation of a MEF2C-myogenin-E12 ternary complex. Whether they might mediate interaction with cofactors involved in general transcriptional initiation remains to be determined.

While residues VLL at positions 65 to 67 of MEF2C are required for transcriptional activation through the MEF2 site, these residues are not required for activity of the C-terminal transcription activation domains when they are fused to the DNA binding domain of GAL4. This observation may indicate that the creation of GAL4-MEF2C fusions constitutively activates the activation domains by altering the conformation of the protein or that this type of fusion protein interacts with different cofactors to activate transcription through the GAL4 binding site. Similar observations have been made with myogenin and MyoD, which require specific amino acids in the basic region to activate transcription through an E-box binding site but not through a GAL4 site when these factors are fused to the GAL4 DNA binding domain (45, 51).

Because MEF2 factors function as homo- and heterodimers and interact with myogenic bHLH factors to regulate muscle gene expression (34), we tested MEF2 mutants for possible dominant negative effects on the activities of wild-type MEF2 factors. Indeed, MEF2 mutants defective for DNA binding interfered with the ability of wild-type MEF2C and MEF2A to activate transcription through the MEF2 site. Dominant negative mutants were capable of dimerizing with wild-type MEF2C to sequester it from binding DNA, as determined by EMSAs of extracts cotransfected with constructs encoding both proteins.

Whereas MEF2 DNA binding mutants cannot activate MEF2 site-dependent transcription alone and can block transcriptional activation by wild-type MEF2 factors, these mutants retain the ability to synergize with the bHLH region of myogenin or MyoD to activate E-box-dependent transcription (34). These results suggest that MEF2 can regulate at least two types of target genes: those which are activated directly by MEF2 and lack E boxes and those that lack MEF2 sites and are activated indirectly by interaction of MEF2 with myogenic bHLH proteins that are bound to E boxes. Although residues 1 to 117 of MEF2C are incapable of activating transcription through the MEF2 site, in the presence of myogenic bHLH factors, this region of MEF2C can efficiently activate transcription (34). These results demonstrate that the transcription activation domain of MEF2C is not essential for MEF2 site-dependent transcription when myogenic bHLH proteins are present. Since MEF2 factors are expressed more widely than myogenic bHLH factors, it will be interesting to determine if there are factors in other cell lineages that can collaborate with the MADS and MEF2 domains of MEF2 to regulate gene expression.

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