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Myogenin and MEF2 Function Synergistically To Activate the *MRF4* Promoter during Myogenesis

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The basic helix-loop-helix muscle regulatory factor (MRF) gene family encodes four distinct muscle-specific transcription factors known as MyoD, myogenin, Myf-5, and MRF4. These proteins represent key regulatory factors that control many aspects of skeletal myogenesis. Although the MRFs often exhibit overlapping functional activities, their distinct expression patterns during embryogenesis suggest that each protein plays a unique role in controlling aspects of muscle development. As a first step in determining how *MRF4* gene expression is developmentally regulated, we examined the ability of the *MRF4* gene to be expressed in a muscle-specific fashion in vitro. Our studies show that the proximal *MRF4* promoter contains sufficient information to direct muscle-specific expression. Located within the proximal promoter are a single MEF2 site and E box that are required for maximum *MRF4* expression. Mutation of the MEF2 site or E box severely impairs the ability of this promoter to produce a muscle-specific response. In addition, the MEF2 site and E box function in concert to synergistically activate the *MRF4* gene in nonmuscle cells coexpressing MEF2 and myogenin proteins. Thus, the *MRF4* promoter is regulated by the MEF2 and basic helix-loop-helix MRF protein family through a cross-regulatory circuitry. Surprisingly, the *MRF4* promoter itself is not *trans*activated by MRF4, suggesting that this *MRF* gene is not subject to an autoregulatory pathway as previously implied by other studies. Understanding the molecular mechanisms regulating expression of each *MRF* gene is central to fully understanding how these factors control developmental events.

Embryonic skeletal muscle development has become a paradigm for understanding the molecular basis of how cell lineages are established and how cells differentiate into specialized structures (reviewed in reference 38). Nearly all vertebrate muscles derive from individual somites, which produce two distinct muscle populations. The myotomal muscles consist of individual mononucleated myocytes that form axial muscle structures, while a second migratory cell population produces muscles of the developing limbs. In both instances, myogenic differentiation is accompanied by expression of muscle-specific genes encoding myosin, actin, troponins, and tropomyosins (reviewed in reference 25).

Although the embryological origin of muscle cells is fairly well established, the molecular pathways which control myogenic precursor cell populations and terminal differentiation events are just beginning to be understood. Part of the molecular regulatory network controlling muscle development involves a family of muscle regulatory factors (MRFs) that share a common DNA-binding and dimerization motif referred to as the basic helix-loop-helix (bHLH) domain (13, 37). The four members of the MRF family (MyoD, myogenin, Myf-5 and MRF4) (2, 3, 9, 11, 34, 42, 48) are capable of converting nonmuscle cells into myogenic lineages, forming heterodimers with related bHLH factors such as E12, E47, and HEB (22, 26, 35), and directly activating expression of many muscle-specific genes including desmin, M-creatine kinase, troponin I, α-actin, and the acetylcholine receptor subunit genes (reviewed in references 25, 37, and 46).

Although the MRFs share many common features, the temporal and spatial expression patterns of the individual mammalian genes are distinct (reviewed in references 14 and 44). For example, *Myf-5* expression initially is detected in somites from day 8.5 postcoitum embryos. Approximately 6 to 12 h later, myogenin transcripts appear in the myotome, followed by the transient expression of *MRF4*. Somitic expression of *MyoD* is delayed a full day after *Myf-5*, myogenin, and *MRF4* transcripts are detected. In the developing limb, transcription of the *Myf-5*, myogenin, and *MyoD* genes is closely linked, occurring at approximately 11 days postcoitum. In contrast to somite expression, *MRF4* expression in the limb occurs very late, appearing only after secondary-fiber formation and innervation events have initiated at 13 to 15 days postcoitum. In the adult musculature, *Myf-5*, *MyoD*, and myogenin gene transcription becomes reduced while *MRF4* transcription continues at a high level, making MRF4 the predominant MRF in adult muscle (21). These unique expression patterns strongly suggest that each MRF protein has a different role in controlling muscle formation. In support of this hypothesis, gene ablation studies in mice have suggested that the MyoD, Myf-5, and myogenin proteins play distinct but overlapping roles in regulating muscle development (19, 36, 43).

Although a great deal is known about the protein function of the MRFs, the *cis* and *trans* regulatory elements that control their expression in myotomal as well as differentiated muscle cells are just beginning to be understood. Studies have shown that the MRFs may be part of a feedback regulatory network in which several members of the MRF family can activate expression of other MRF genes as well as autoregulate their own expression (reviewed in reference 46). The complexity of this regulatory network is further augmented, however, by the addition of a second family of muscle-specific transcription factors, referred to as the MEF2 family, which also plays a pivotal role in controlling aspects of skeletal myogenesis (4, 12, 33, 50). MEF2 DNA-binding sites, located within the proximal promoter regions of the mouse and human myogenin genes,

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are crucial to obtain the correct myogenin expression patterns in tissue culture model systems as well as in transgenic mice (5, 7, 8, 49). Similar MEF2-binding sites are located within the chicken *MyoD* promoter. The role of the MEF2 site in this promoter remains unclear, however, since deletions of these regions have no effect on *MyoD* expression (10). At present, there are no reports describing whether MEF2 and/or the MRFs play a direct role in regulating expression of the *Myf-5* and *MRF4* genes, although the promoters of these genes are sufficient to recapitulate at least some of their normal embryonic expression patterns in transgenic mice (39, 45).

In an effort to identify the *cis* and *trans* regulatory elements controlling expression of the *MRF4* gene (20), we examined various portions of the *MRF4* promoter in different tissue culture model systems. Our studies demonstrate that the 5′-flanking sequence of the *MRF4* promoter produces a myotube-specific expression pattern in myogenic L6A1 and C2 cells as well as in primary chicken muscle cultures. Deletions of the *MRF4* promoter region show that 336 or 61 bp of the 5′-flanking sequence is sufficient to generate muscle expression in transiently transfected cells. The −336 and −61 *MRF4* promoters also can be activated in nonmuscle cells by overexpression of MyoD, myogenin, or Myf-5. Interestingly, overexpression of MRF4 does not activate the *MRF4* promoter, indicating that the *MRF4* gene is not subject to autoregulation. Our studies also show that MEF2 plays a key role in activating expression of the *MRF4* gene, since a proximal MEF2 site is required for normal *MRF4* expression. Although MEF2 and myogenin (for example) activate *MRF4* transcription independently, they also synergistically activate the *MRF4* promoter when coexpressed, suggesting that MEF2 and the MRFs form tertiary protein-DNA complexes that are required to regulate expression of the muscle regulatory factor genes. The synergistic activity of the MRFs and MEF2, along with their roles in regulating *MRF4* expression, provides direct evidence that the development of mature muscle cells involves a regulatory circuitry in which MRF and MEF2 must closely modulate expression of all members of these important gene families.

MATERIALS AND METHODS

Cell culture. L6A1 myoblasts and C3H10T1/2 (10T1/2) fibroblasts were maintained in growth medium containing basal medium Eagle (GIBCO) supplemented with 10% fetal bovine serum plus penicillin (100 U/ml) and streptomycin (100 μg/ml). C2 myoblasts were maintained in high-glucose Dulbecco's modified Eagle's medium containing 15% fetal bovine serum. Muscle differentiation was induced by incubating the cultures for 48 h in low-glucose Dulbecco's modified Eagle's medium containing 5 μg of insulin per ml, 5 μg of transferrin per ml, and 5 ng of selenium per ml (ITS medium) plus penicillin and streptomycin (51). In some instances, 10 ng of fibroblast growth factor-2 (FGF-2; R&D) per ml was added to the cultures as described previously (18). Primary chicken myoblasts were isolated from the hind limbs of 11-day chicken embryos by standard procedures (24). Primary myoblasts were plated at 1.5×10^6 cells per 100-mm collagen-coated dish in low-glucose Dulbecco's modified Eagle's medium containing 10% horse serum, 2.5% chicken embryo extract (GIBCO), penicillin, and streptomycin. For some experiments, chicken embryo fibroblasts also were isolated from 11-day embryos by standard procedures (10) and maintained as described above. All cultures were provided with fresh medium every 2 days.

Stable DNA transfections. L6A1 cells were plated at a density of 5×10^5 cells per 100-mm plate. The following day, 1 mM chloroquine was added for 2 h, after which a calcium phosphate precipitate containing 2 μg of *MRF4*-n*LacZ* plasmid DNA, 100 ng of PKO-neo DNA, and 50 μg of carrier DNA was added. The medium was changed 5 h after the addition of DNA. The following day, cells were transferred to new dishes containing complete medium supplemented with 400 μg of G418 per ml. After 14 days individual colonies were isolated and analyzed as described below.

Transient DNA transfections and CAT assays. Transient DNA transfections were carried out by calcium phosphate precipitation as described previously (18, 51). For *trans*-activation studies, 10T1/2 fibroblasts were transfected with DNA precipitates containing 5 μg of each *MRF4*-CAT reporter gene construct and 5 μg of the appropriate expression plasmid (pEMscribeα2) (9) containing rat myogenin (48), mouse *MyoD* (9), human *Myf-5* (3), or rat *MRF4* (42) cDNAs. In some experiments, the human myocyte-specific enhancer factor 2A (*hMEF2A*) cDNA, which was cloned into the expression vector pMT2 (50), was used. Each DNA precipitate was added to 10^6 10T1/2 fibroblasts per 100-mm dish. At 5 h after the addition of DNA, cultures were subjected to an osmotic shock for 2 min in serum-free medium containing 20% glycerol and then incubated in normal growth medium. Differentiation medium was added after 24 h. At 48 h later, protein extracts were prepared and chloramphenicol acetyltransferase (CAT) assays carried out as described by Gorman et al. (17). The amount of cell extract used for CAT assays was normalized to protein concentrations.

Transfection of the C2 myogenic cell line with *MRF4*-CAT was performed essentially as described above. Myoblast (10^5 cells per 100-mm dish) or myotube (10^6 cells per 100-mm dish) cultures were transfected with 5 μg of *MRF4*-CAT reporter DNA and 5 μg of the constitutively expressed reference plasmid RSV-*LacZ*. After glycerol treatment, myoblasts were incubated in growth medium whereas myotube cultures were incubated in ITS medium to induce differentiation. Cells were harvested 72 h posttransfection and cell extracts were assayed for β-galactosidase (β-Gal) activity as previously described (51). For primary chicken muscle cultures, cells were plated at 1.5×10^6 cells per 100-mm dish and then transfected 48 h postplating with 9 μg of the appropriate *MRF4*-CAT reporter and 1 μg of RSV-*LacZ* reference plasmids as described above. Cells were harvested 48 h later and assayed for both β-Gal and CAT activities. The amount of cell extract used in each CAT assay was normalized to the specific activity of β-Gal for each transfection group, which typically ranged from 20 to 50 U of β-Gal per μg of protein. A minimum of four independent transfections were performed for each set of experiments.

β-Gal assays. Stable L6A1 −8500-n*LacZ* and −336-n*LacZ* clones were propagated in growth medium. At confluency, cultures were induced to differentiate with ITS medium as described above. At each time point, plates were fixed with 2% paraformaldehyde for 1 h and rinsed several times with phosphate-buffered saline. Cultures subsequently were stained for 1 to 5 h in 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) solution as described by Ausubel et al. (1). In some cases, transiently transfected primary chicken embryo myoblast and fibroblast cultures were stained for β-Gal activity as well. Quantitative β-Gal assays were performed as described for the control RSV-*LacZ* test gene.

Gene constructions. The −8500 to +71, −336 to +71, and −61 to +71 portions of the rat *MRF4* promoter (20) were directionally cloned into the *Hin*dIII and *Bam*HI sites of either pNL (15), containing the *lacZ* gene with a nuclear localization signal sequence, or pBLCAT3 (31). Additional 5′ unidirectional deletions of the *MRF4* promoter were generated with the Erase-a-Base (Promega) as specified by the manufacturer. A *Hin*dIII linker was added to the 5′ end of each deletion, and then the *Hin*dIII and *Bam*HI fragments were cloned into the pBLCAT3 or pNL vectors by standard techniques. For some experiments, a *TnI*-n*LacZ* gene (constructed by D. Goldhamer), containing the quail troponin I promoter and first intron enhancer (30), was used as a positive, muscle-specific control.

Mutagenesis of the *MRF4* MEF2, TATA, and E1 E-box sites. The MEF2 site, located at positions −26 to −15, the TATA site, located at positions −21 to −16, and the proximal E1 E box, located at +22 to +27, were mutated independently in the pBS −336 *MRF4* and pBS −61 *MRF4* constructs by PCR mutagenesis. The double MEF2/TATA mutant was created by replacing nucleotides −21 and −20 (TA→GC), using the *MRF4* mMEF2/mTATA primer (5′-GCTACTATAgcTA AAGCTG-3′) and a T7 primer. A second series of reactions were carried out with the T3 primer and the complementary *MRF4* mMEF2/mTATA oligonucleotide (5′-CAGCTTTAgcTATAGTAGC-3′) as the second primer. The PCR conditions used for these experiments were as follows: 94°C for 1 min, 45°C for 1 min, and 72°C for 1 min for 3 cycles, and then 94°C for 1 min, 50°C for 1 min, and 72°C for 1 min for 32 cycles. Each PCR product subsequently was gel purified and combined in an equimolar ratio, and three further cycles of PCR were performed without primers. A second PCR then was carried out for an additional 25 cycles after the addition of fresh *Taq* polymerase and T3 and T7 primers. The final amplified product was digested with *Hin*dIII and *Bam*HI, gel purified, and cloned into pBLCAT3. Individual MEF2 and TATA mutations were generated in a similar fashion, except that the mMEF2 site used the *MRF4* mMEF2 oligonucleotide (5′-CAGTAGCTACgggATATAAAGCTGGGTCG A-3′) and the mutant TATA site used the *MRF4* mTATA oligonucleotide (5′-CAGTAGCTACTAaAaATAAAGCTGGGTCGA-3′). The E1 E-box mutant promoter also was produced by replacing nucleotides +26 and +27 (TG→GA), using oligonucleotides *MRF4* mE1-1 (5′-TTAAATGCCATCgaGGTGG-3′) and *MRF4* mE1-2 (5′-CCACCtcGATGGCATTTAA-3′) as described above. To construct the double (mMEF2/mE1) and triple (mMEF2/mTATA/mE1) mutants, mutated MEF2 constructs were used as templates with *MRF4* mE1-1 and *MRF4* mE1-2 primers. To create −61 *MRF4*-CAT mutants, a 5′ primer (5′-CCAAGCT TGACAGCTAGAA-3′) spanning nucleotides −61 to −50 and a 3′ primer from the N-terminal portion of the CAT gene (5′-CCAAGCTTGAGTTTCAGTA-3′) were used with the respective wild-type and mutant −336 *MRF4* templates as described above. The complete promoter region of each gene was sequenced to verify all mutations.

In vitro transcription and translation reactions. Rat myogenin (48), human *E12* (35), and human *MEF2* (50) RNAs were prepared from pBS-Myogenin, E12R, and pGEM-MEF2-XR, respectively, by in vitro transcription reactions as previously described. *MEF2* or myogenin plus *E12* transcripts (5 μg of each

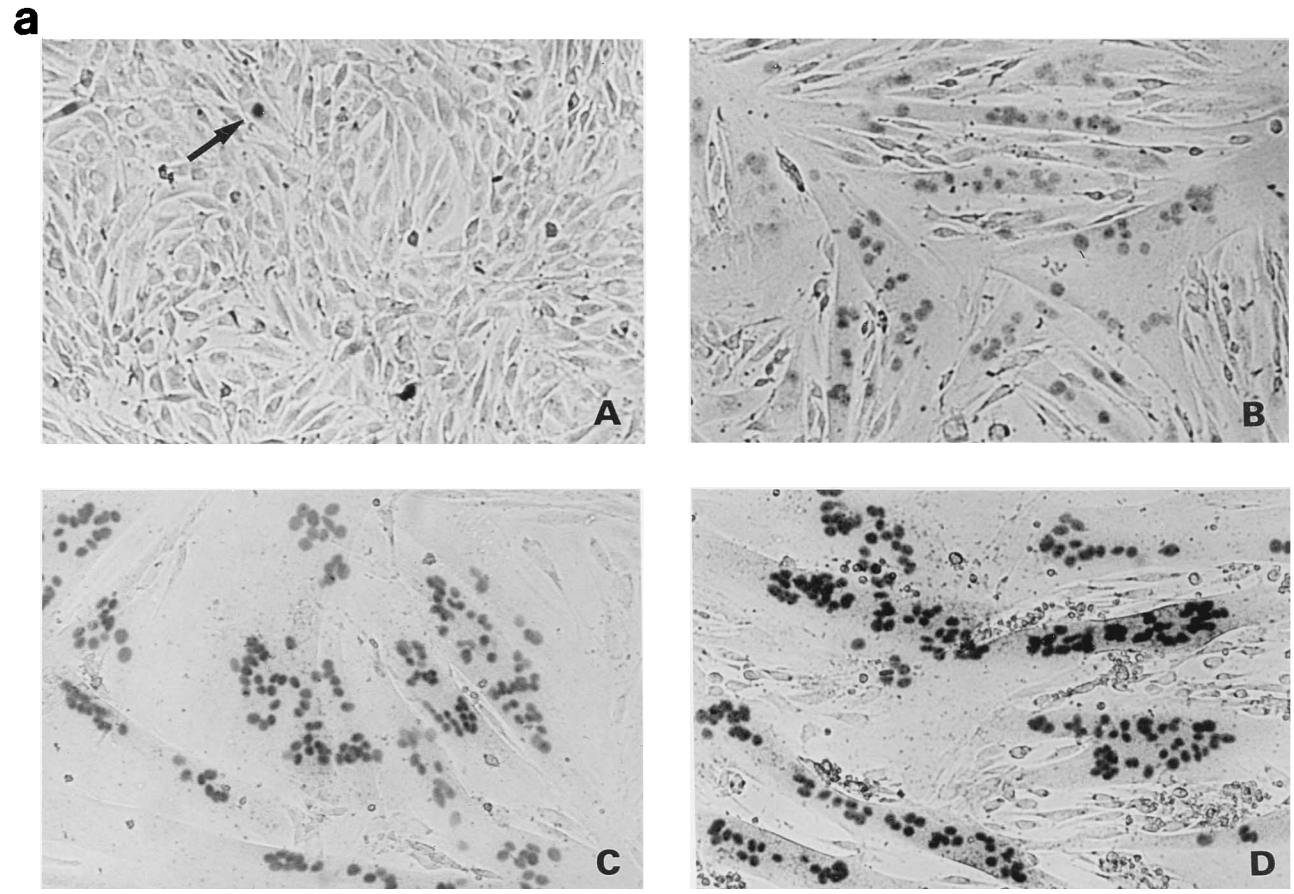

FIG. 1. Muscle-specific expression of the *MRF4* promoter in L6A1 cells. (a) A stable L6A1 cell line containing the −8500 *MRF4*-n*LacZ* gene was induced to differentiate on day 0, and then replicate plates were fixed and stained for β-Gal expression on day 0 (A), day 3 (B), day 4 (C), and day 5 (D). Nuclear β-Gal activity is restricted to differentiated myotubes and increases steadily after 3 days in differentiation medium. The arrow in panel A shows a single differentiated myocyte containing a β-Gal-positive nucleus. (b) Stable L6A1 cell lines containing the −8500 *MRF4*-n*LacZ* or −336 *MRF4*-n*LacZ* gene were induced to differentiate on day 0, and cell extracts were isolated on days 0 to 4. β-Gal assays demonstrate that the *MRF4* promoter drives expression of both the −8500 *MRF4*-n*LacZ* and −336 *MRF4*-n*LacZ* genes in a myotube-specific fashion. Error bars represent the standard errors of the means.

RNA) then were translated in vitro in a total volume of 50 μl with rabbit reticulocyte lysates (Promega) in either the presence or absence of [^{35}S]methionine as recommended by the manufacturer. The efficiencies of the in vitro translation reactions were verified by electrophoresis of the ^{35}S-labelled proteins through 12% polyacrylamide–sodium dodecyl sulfate gels.

Electrophoretic mobility shift assays. A series of double-stranded oligonucleotides containing wild-type and mutant MEF2, E1 E-box, and TATA sites were used in electrophoretic mobility shift assays as described below. The sequence of the oligonucleotides were as follows: *MRF4*-E1 box, 5′-AATTTAATTAAAT GCCATCTGGGTGGCTCC-3′; mut *MRF4*-mE1 box, 5′-AATTTAATTAAAT GCCATCgaGGTGGCTCC-3′; *MRF4*-MEF2, 5′-AATTTAGCTACTATATAT AAAGCTGGGTCG-3′; *MRF4*-mMEF2, 5′-CAGTAGCTACgggATATAAAG CTGGGTCGA-3′; *MRF4*-mTATA, 5′-CAGTAGCTACTAaAaATAAAGCTG GGTCGA-3′; *MRF4*-mMEF2/mTATA, 5′-AATTTAGCTACTATAgcTAAAG CTGGGTCG-3′; *MRF4-tata*, 5′-CCGCACTAATTAAATGCCATCTGGGTG GCT-3′; and control TATA, 5′-GCAGAGCATATAAGGTGAGGTAGGA-3′.

The troponin I enhancer E box (30) and a *MCK* enhancer MEF2 site (50) were used as positive controls for myogenin-E12 and MEF2 binding, respectively, whereas a control TATA double-stranded oligonucleotide (Promega) was used for TATA-binding protein (TBP) interaction. All oligonucleotides were labelled with T4 polynucleotide kinase and [γ-^{32}P]ATP as reported previously (18, 30). For mobility shift assays, 4 μl of in vitro translated proteins was incubated with ~15,000 cpm of each double-stranded oligonucleotide, which corresponds to ~10 fmol of [^{32}P]DNA, in a buffer containing 0.5 μg of poly(dI-dC) and 5 μl of 5× binding buffer (50 mM Tris HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 5 mM dithiothreitol, 25% glycerol). For TBP binding, 5 ng of purified human TBP (Promega) was incubated with [^{32}P]DNA as described above, except that the binding buffer consisted of 0.5 μg of poly(dG-dC) and 5 μl of 4× binding buffer (100 mM Tris HCl [pH 8.0], 200 mM KCl, 25 mM $MgCl_2$, 2 mM EDTA, 2 mM dithiothreitol, 40% glycerol). For competition assays, a 250-fold excess of the respective unlabelled double-stranded oligonucleotides was added in a final volume of 20 to 25 μl. After 30 min at room temperature, the samples were loaded onto 5% polyacrylamide gels, and electrophoresis was carried out at 100 V for 3.5 h at room temperature. The gels subsequently were dried and exposed to X-ray film (Fuji).

RESULTS

***MRF4* promoter activity in myogenic cells.** Previous studies from our laboratory demonstrated that an intact rat *MRF4* gene, when introduced into myogenic cell lines, becomes transcriptionally active in differentiated myotubes (20). To establish whether promoter sequences support this myotube-specific expression, reporter genes were generated with ~8,500 and ~336 bp of *MRF4* 5′-flanking sequences ligated to a β-Gal gene containing a nuclear localization signal (n*LacZ*) (15). Each *MRF4*-n*LacZ* gene then was introduced into the rat myogenic cell line L6A1, and stable *MRF4*-n*LacZ* clones were selected. Isolated myoblasts from each clone were induced to differentiate and subsequently analyzed for β-Gal activity. As shown in Fig. 1a, very few nuclei exhibited β-Gal activity when the −8500-n*LacZ* cells were maintained as undifferentiated myoblasts. However, upon differentiation, the number of nuclei staining positive for β-Gal increased from day 3 to day 5, during which time maximum staining and differentiation was achieved. In all cases, only myotube nuclei exhibited β-Gal activity. The few remaining undifferentiated myoblasts in the day 5 cultures remained β-Gal negative. Identical results were obtained with several independent clone isolates, as well as with L6A1 clones containing the −336-n*LacZ* construct (data not shown). As expected, the *MRF4*-n*LacZ* genes were not active in nonmuscle cells such as 10T1/2 fibroblasts (see below).

To quantitate the level of expression of the −8500-n*LacZ* and −336-n*LacZ* reporters during myogenesis, stable L6A1 cell lines containing each reporter again were induced to differentiate and *LacZ* extracts were harvested over 5 consecutive days. As expected, parental L6A1 cells expressed very low levels of β-Gal regardless of whether the cells were maintained as myoblasts or as fully differentiated myotubes (Fig. 1b). However, β-Gal activity increased steadily in the −8500-n*LacZ* and −336-n*LacZ* cell lines as cell differentiation progressed. Maximum β-Gal levels were obtained after 4 days in differentiation medium, with the −336-n*LacZ* and −8500-n*LacZ* cell lines producing ~15- and ~30-fold increases in β-Gal activity, respectively. Both reporters also were active in several additional myogenic cell lines but, as expected, were not expressed in nonmuscle cell lines such as 10T1/2 (data not shown).

The ability of the −336 *MRF4* promoter to produce a myotube-specific expression pattern in stable-transfection assays prompted us to examine the proximal *MRF4* promoter in transient-transfection assays. For these studies, C2 myoblasts were transfected with a −336 *MRF4*-CAT and −61 *MRF4*-CAT reporter gene and CAT activity in myoblast and myotube populations was measured. As shown in Fig. 2, expression in myoblasts of the −336 and −61 *MRF4*-CAT genes was minimal and was below the levels obtained from a control pCAT plasmid (data not shown). After 2 days in differentiation medium, however, CAT activity increased ~15- to 20-fold as the cells differentiated. As predicted, the −336 and −61 *MRF4*-CAT reporters remained inactive in nonmuscle cells (data not shown), demonstrating that *MRF4*-CAT expression parallels the expression pattern observed for the endogenous *MRF4* gene (20, 21). These results again suggest that the proximal *MRF4* promoter contains sufficient information to generate a muscle-specific transcription response.

The *MRF4* promoter is regulated by MEF2 and by myogenic bHLH factors. Analysis of the promoter sequence from the *MRF4* gene revealed several potential regulatory elements that have been implicated in conferring muscle-specificity. Located within 336 bp of the transcription start site are 3 E boxes (CANNTG) to which the bHLH MRFs MyoD, myogenin, Myf-5, and MRF4 potentially bind, as well as a putative MEF2-binding site located at position −26 (Fig. 3). Studies from several laboratories have suggested that the myogenic regulatory factor genes are part of a regulatory circuit in which members of this family activate each other's expression as well as autoregulate their own expression (reviewed in reference 46). To establish whether the *MRF4* gene is subject to cross-regulation by the myogenic bHLH proteins, 10T1/2 fibroblasts were cotransfected with the −336 *MRF4*-CAT reporter and cDNAs encoding each myogenic bHLH factor. Cotransfection

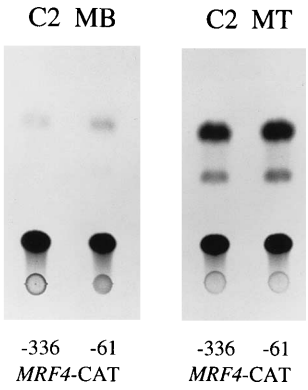

FIG. 2. C2 myoblast (MB) and myotube (MT) cultures were transiently transfected with the −336 *MRF4*-CAT and −61 *MRF4*-CAT plasmids, and CAT activity was assayed as described in Materials and Methods. High levels of −336 *MRF4*-CAT and −61 *MRF4*-CAT gene expression are restricted to differentiated myotubes.

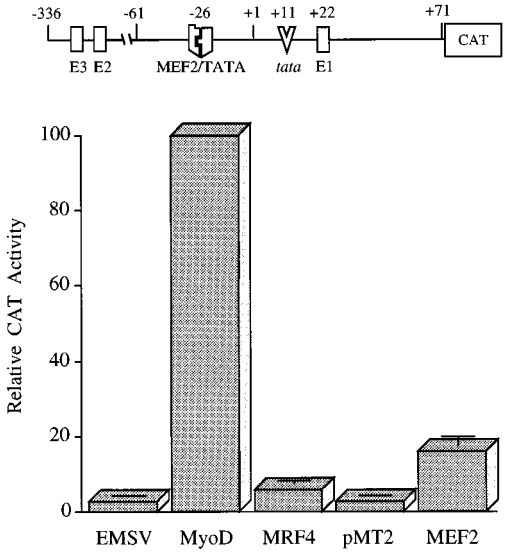

FIG. 3. *trans* activation of the proximal *MRF4* promoter by bHLH MRFs and MEF2. 10T1/2 cells were transfected with the −336 *MRF4*-CAT reporter gene and the expression plasmids pEMscribeα2 (EMSV) or pMT2 containing an MRF4, MyoD, or MEF2 cDNA. After 2 days in differentiation medium, the cells were harvested and CAT activity was quantified. The −336 *MRF4*-CAT gene is efficiently expressed in 10T1/2 cells that are cotransfected with the MyoD and MEF2 cDNA plasmids but not in 10T1/2 cells that are cotransfected with EMSV, pMT2, or EMSV-MRF4 expression plasmids. Expression levels are defined as the percentage of activity obtained compared with the MyoD value, which is set to 100%. A schematic representation of the −336 *MRF4*-CAT gene, illustrating potential regulatory elements and their positions relative to the transcription initiation start site (+1), is shown above the graph. Error bars represent the standard errors of the means.

of −336 *MRF4*-CAT and the control expression plasmid EMSV did not produce significant levels of CAT expression, whereas cotransfection of −336 *MRF4*-CAT with a MyoD expression plasmid produced very high levels of CAT activity (Fig. 3). Similar levels of CAT expression also were obtained with myogenin and Myf-5 (data not shown), demonstrating that the myogenic bHLH factors *trans* activate expression of the *MRF4* promoter in nonmuscle cells. Interestingly, cotransfection of the MRF4 cDNA did not lead to *MRF4* promoter activity (Fig. 3), even though expression of the MRF4 protein efficiently generated differentiated muscle cells. Thus, although all four MRFs converted 10T1/2 cells to a differentiated muscle phenotype, only MyoD, myogenin, and Myf-5 transcriptionally activated the *MRF4* promoter. The *MRF4* gene is not subject to direct autoregulation by the MRF4 protein, since EMSV-MRF4 does not activate expression of −336 *MRF4*-CAT. These data also agree with previous reports demonstrating that the endogenous *MRF4* gene is not significantly activated in MRF4-transfected 10T1/2 cells (references 20 and 42 and unpublished results).

Similar cotransfection experiments also were performed with a MEF2 expression plasmid to establish the importance of the putative MEF2 binding site located within the proximal *MRF4* promoter. As expected, −336 *MRF4*-CAT was not expressed in 10T1/2 cells cotransfected with the control expression plasmid pMT2 (Fig. 3). However, −336 *MRF4*-CAT was transcriptionally activated ~10-fold when the MEF2 cDNA was included in these experiments, again suggesting that MEF2, as well as the myogenic bHLH factors, plays a role in regulating MRF4 expression during development.

To establish the individual contributions of MEF2 and the myogenic bHLH proteins in activating the *MRF4* gene, 10T1/2 cells were transfected with the −336 *MRF4*-CAT or −61 *MRF4*-CAT genes and MEF2, myogenin, or MEF2 plus myogenin expression plasmids. As described above, MEF2 activated the −336 *MRF4*-CAT ~10-fold in these assays (Fig. 4). A similar result also was obtained when the −61 *MRF4*-CAT reporter was tested in the presence or absence of MEF2. Again, a 10-fold increase over basal levels was obtained. The −61 *MRF4*-CAT gene also was activated by cotransfecting cells with the myogenin plasmid, indicating that the upstream E boxes (E2 and E3) are not critical for myogenin-induced activation, since E2 and E3 are not present within this reporter. For both the −336 and −61 promoter fragments, however, maximum transcriptional activity occurred when MEF2 and myogenin were cotransfected into 10T1/2 cells (Fig. 4), suggesting that MEF2 and myogenin function synergistically to activate the *MRF4* promoter. Similar results also were obtained when MyoD or Myf-5 were coexpressed with MEF2 (data not shown). These studies indicate that the *MRF4* regulatory elements contained within the proximal 61-bp promoter confer muscle specificity, since the −61 *MRF4*-CAT gene is fully active in C2 myotubes (Fig. 2) and MEF2 and myogenin activate the −61 *MRF4*-CAT reporter to levels similar to those obtained with the −336 *MRF4*-CAT gene.

Binding of MEF2 and myogenin to the *MRF4* proximal promoter. Although MEF2 and myogenin activate the *MRF4* gene in transfection experiments, it is unclear whether these factors interact directly with the *MRF4* promoter. To examine this in greater detail, electrophoretic mobility shift assays were performed with in vitro translated proteins and labelled oligonucleotides corresponding to known MEF2- and myogenin-binding sites as well as to specific target sites derived from the *MRF4* promoter. As shown in Fig. 5a, incubation of MEF2 with an M-creatine kinase (*MCK*) enhancer MEF2 oligonucleotide produced a shifted complex that was efficiently inhibited

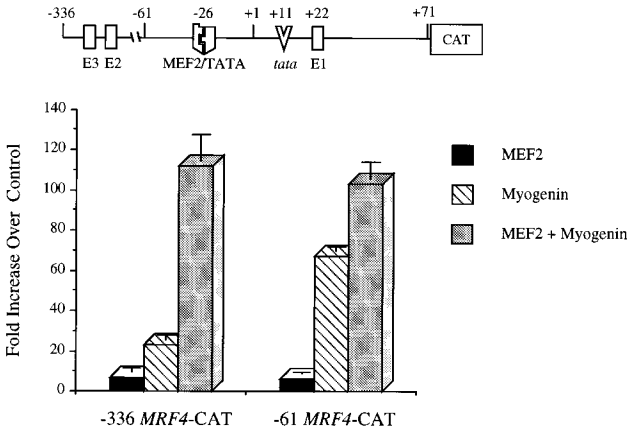

FIG. 4. Myogenin and MEF2 synergistically activate −336 *MRF4*-CAT and −61 *MRF4*-CAT gene expression. Myogenin, MEF2, or myogenin plus MEF2 cDNAs were cotransfected into 10T1/2 cells along with the −336 *MRF4*-CAT and −61 *MRF4*-CAT genes. After 2 days in differentiation medium, CAT activity in each group was measured. Coexpression of myogenin and MEF2 leads to a large increase in expression of the −336 *MRF4*-CAT and −61 *MRF4*-CAT reporter genes. The E2 and E3 E boxes appear unnecessary, since myogenin efficiently *trans* activates the −61 *MRF4*-CAT gene. CAT expression levels are presented as fold increases over expression of the *MRF4*-CAT reporter genes when cotransfected with the control EMSV expression plasmid. A schematic representation of the −336 *MRF4*-CAT and −61 *MRF4*-CAT genes is shown above the graph. Error bars represent the standard errors of the means.

by excess unlabelled MEF2 oligonucleotides. Similarly, when MEF2 protein was incubated with an oligonucleotide containing the *MRF4* proximal MEF2 site, an identical shifted complex was detected. This complex was efficiently inhibited by both the *MRF4* MEF2 site and the *MCK* MEF2 site, whereas a mutated *MRF4* MEF2 site failed to inhibit formation of this specific protein:DNA complex (Fig. 5a). As expected, the mutant *MRF4* MEF2 site also failed to bind MEF2 protein directly when tested as a probe, confirming that MEF2 binds specifically to the *MRF4* MEF2 site located within the proximal promoter. Identical MEF2 binding patterns also were obtained when muscle nuclear extracts were tested with each oligonucleotide probe (data not shown).

Examination of the *MRF4* promoter revealed that the TATA box at positions −21 to −16 is positioned within the *MRF4* MEF2 site located at positions −26 to −15, suggesting that this region of the *MRF4* promoter contains overlapping contact sites for two essential transcription factors, MEF2 and TBP. A similar shared MEF2/TATA site is present within the *Xenopus MyoDa* promoter and has been shown to be essential for full activity of the *XMyoDa* gene (27). To establish whether the MEF2/TATA region of the *MRF4* promoter is capable of binding both MEF2 and TBP, electrophoretic mobility shift assays again were performed. As shown in Fig. 5b, both TBP and MEF2 bound to an oligonucleotide containing the −36 to −7 region of the *MRF4* promoter. Individual point mutations within the MEF2 site (mMEF2/TATA) (see Materials and Methods for details) abolished MEF2 binding but retained TBP binding. Likewise, mutations that simultaneously alter the TATA and MEF2 sites (mMEF2/mTATA) abolished both MEF2 and TBP binding, demonstrating that the binding sites of MEF2 and TBP, although overlapping, are distinguishable with appropriate mutations. The converse mutation, in which the TATA site is destroyed but the MEF2 site is retained (MEF2/mTATA), also has been generated and tested. In this instance MEF2, but not TBP, bound to the mutated promoter. Interestingly, the MEF2/mTATA mutation generated a MEF2

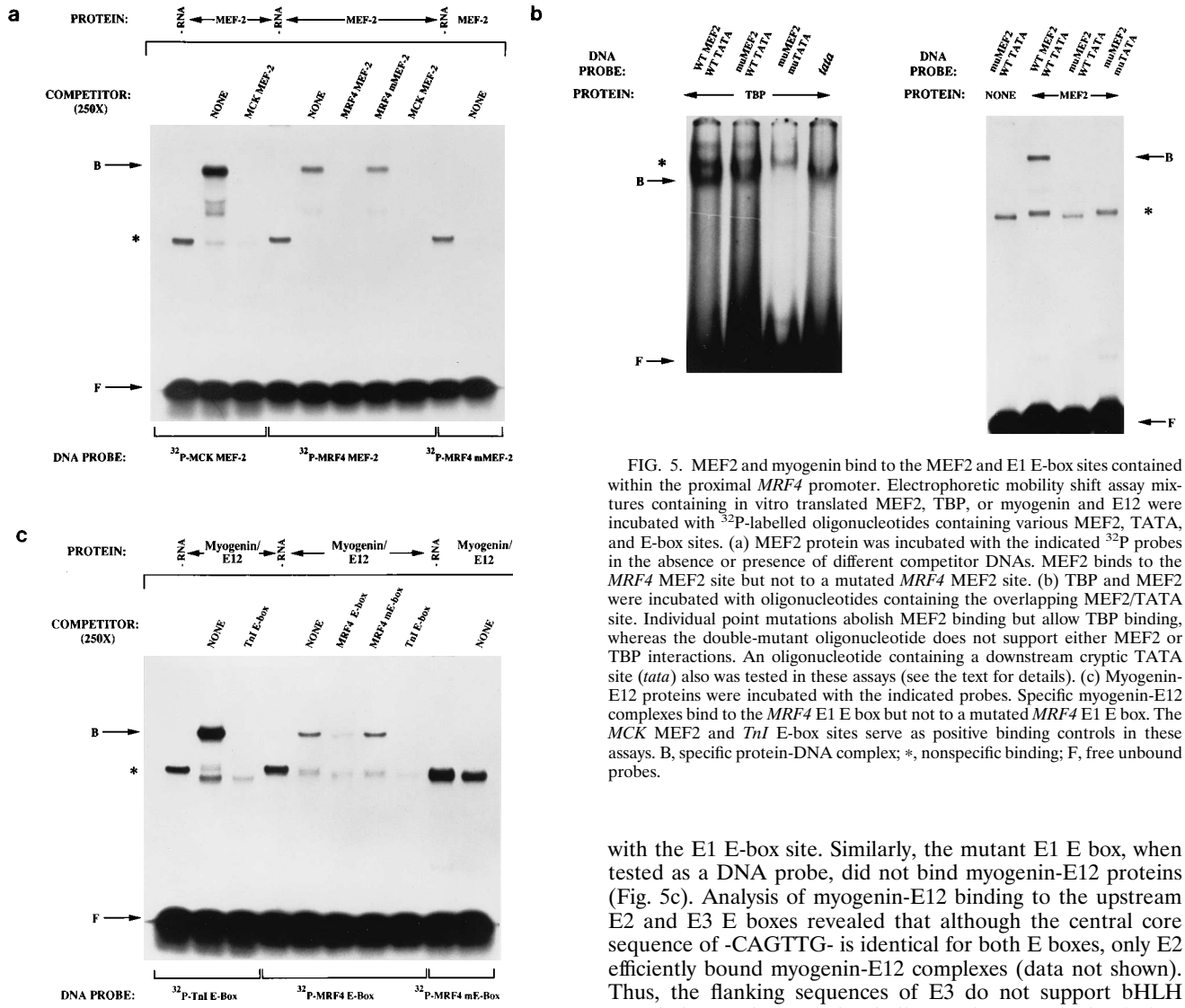

FIG. 5. MEF2 and myogenin bind to the MEF2 and E1 E-box sites contained within the proximal *MRF4* promoter. Electrophoretic mobility shift assay mixtures containing in vitro translated MEF2, TBP, or myogenin and E12 were incubated with ^{32}P-labelled oligonucleotides containing various MEF2, TATA, and E-box sites. (a) MEF2 protein was incubated with the indicated ^{32}P probes in the absence or presence of different competitor DNAs. MEF2 binds to the *MRF4* MEF2 site but not to a mutated *MRF4* MEF2 site. (b) TBP and MEF2 were incubated with oligonucleotides containing the overlapping MEF2/TATA site. Individual point mutations abolish MEF2 binding but allow TBP binding, whereas the double-mutant oligonucleotide does not support either MEF2 or TBP interactions. An oligonucleotide containing a downstream cryptic TATA site (*tata*) also was tested in these assays (see the text for details). (c) Myogenin-E12 proteins were incubated with the indicated probes. Specific myogenin-E12 complexes bind to the *MRF4* E1 E box but not to a mutated *MRF4* E1 E box. The *MCK* MEF2 and *TnI* E-box sites serve as positive binding controls in these assays. B, specific protein-DNA complex; *, nonspecific binding; F, free unbound probes.

site that bound MEF2 more efficiently than did the wild-type MEF2 site (data not shown). Finally, we examined the ability of a second potential TATA site (referred to as *tata*) at position +11 on the *MRF4* gene to bind TBP. Although the *tata* site represents a very poor TATA consensus sequence and is not readily utilized by the endogenous *MRF4* gene (20), the *tata* element bound TBP in these assays (Fig. 5b). Thus, it is conceivable that under some experimental conditions, the downstream *tata* element may serve as a binding site for TBP and TFIID in generating a preinitiation transcription complex (see below). Unlike the upstream TATA, however, the *tata* site did not bind MEF2 (data not shown).

A similar analysis of myogenin binding to the E1 E box located within the *MRF4* 5′ untranslated region revealed that myogenin-E12 heterodimers interact with the E1 site. As shown in Fig. 5c, myogenin-E12 proteins produced identical shifted complexes when either the E1 E box or the control troponin I (*TnI*) enhancer E box were used as probes. In both cases, excess *TnI* E-box or *MRF4* E1 E-box oligonucleotides efficiently competed for myogenin-E12 binding, whereas a mutated E1 E box did not inhibit myogenin-E12 from interacting with the E1 E-box site. Similarly, the mutant E1 E box, when tested as a DNA probe, did not bind myogenin-E12 proteins (Fig. 5c). Analysis of myogenin-E12 binding to the upstream E2 and E3 E boxes revealed that although the central core sequence of -CAGTTG- is identical for both E boxes, only E2 efficiently bound myogenin-E12 complexes (data not shown). Thus, the flanking sequences of E3 do not support bHLH protein interactions. Again, similar results were obtained when nuclear extracts from differentiated muscle cells were tested in these assays (data not shown), confirming that myogenin binds to the E1 and E2 E boxes located in close proximity to the *MRF4* transcription start site. Interestingly, MRF4-E12 heterodimers also bound to the E1 and E2 E boxes (data not shown), even though MRF4 does not readily activate the *MRF4* promoter (Fig. 3).

MEF2 and E-box mutations alter *MRF4* promoter activity. We have demonstrated that 336 or 61 bp of the *MRF4* promoter supports muscle-specific transcription when tested with various reporter genes. In addition, myogenin (or MyoD or Myf-5) plus MEF2 activates expression of the *MRF4* promoter in a synergistic fashion, presumably through direct interactions with the proximal *MRF4* promoter, since both proteins bind to their respective sites in vitro. To ascertain the importance of the E1 E box, E2 E box, and MEF2/TATA-binding sites for full *MRF4* activity, we generated individual point mutations as well as the corresponding double and triple mutations in the −336 *MRF4*-CAT and −61 *MRF4*-CAT reporter genes. Each mutant reporter, as well as the wild-type genes, was cotransfected into 10T1/2 cells with expression plasmids containing MEF2, myogenin, or both cDNAs as described above. In this series of experiments, expression of MEF2 or myogenin alone *trans*

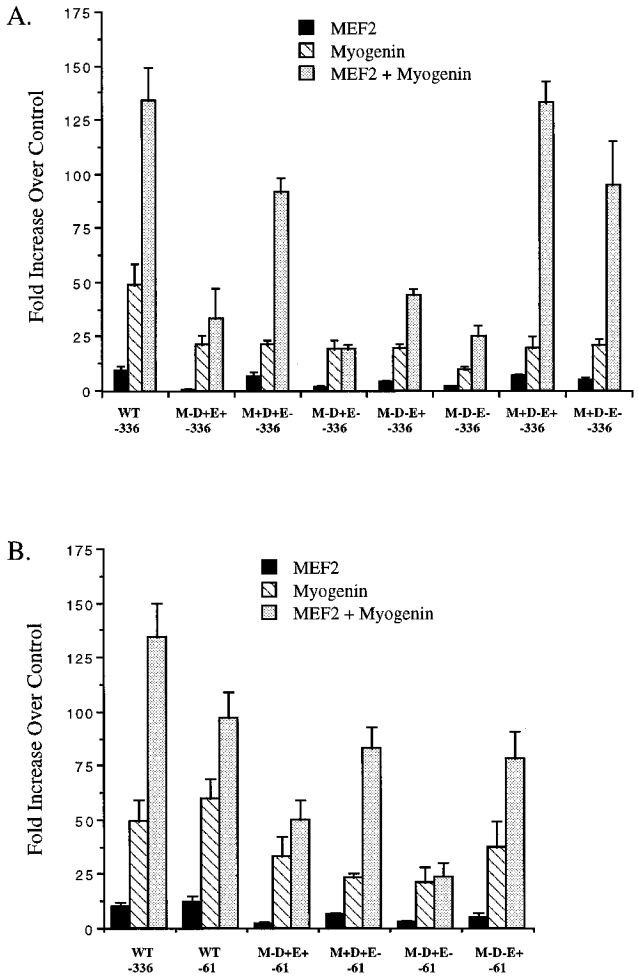

FIG. 6. MEF2 and myogenin synergistically activate the *MRF4* promoter, even in the absence of a functional E box. Wild-type (WT) and mutant −336 (A) and −61 (B) *MRF4*-CAT genes were cotransfected into 10T1/2 cells along with MEF2, myogenin, or MEF2 plus myogenin expression plasmids. After 2 days in differentiation medium, CAT activity was quantified. Maximum synergistic activity is detected only when an intact MEF2 site is present (see the text for details). Wild-type (+) and mutant (−) regulatory elements are represented as follows: M, MEF2 site; D, TATA site; E, E1 E-box (E). Values represent fold increases over control as in Fig. 4. Error bars represent the standard errors of the means.

activated the wild-type −336 *MRF4* promoter 10- and 49-fold, respectively, whereas coexpression of MEF2 and myogenin led to the expected synergistic effect, activating the wild-type −336 *MRF4* promoter approximately 130-fold over basal levels (Fig. 6A). Similar results were obtained with the −61 *MRF4* promoter (Fig. 6B), although the synergistic effect of expressing both MEF2 and myogenin was not as pronounced. Mutation of the MEF2 site (M−), TATA box (D−), and E1 E box (E−) confirmed the importance of each of these regulatory elements in generating the full activity of the *MRF4* promoter. For example, the mutant MEF2 site (M−D+E+) promoter was not efficiently activated in 10T1/2 cells expressing the MEF2 protein (Fig. 6). This mutation also reduced the ability of myogenin to *trans* activate the *MRF4* promoter, even though the −336 promoter contains functional E1 and E2 E boxes and the −61 promoter contains a functional E1 E box. This result supports earlier studies demonstrating that myogenin protein activates the endogenous *MEF2* genes in 10T1/2 cells (33). Since the *MRF4* MEF2 site is destroyed in this construct, the overall effect of myogenin on *MRF4*-CAT expression is reduced. Coexpression of both MEF2 and myogenin with the M−D+E+ mutant also did not lead to a synergistic level of CAT expression compared with the wild-type promoters, suggesting that intact MEF2 and E1 sites are required to obtain high levels of *MRF4* expression. As predicted, the M+D+E− mutation exhibited wild-type MEF2-dependent activation but a dramatically reduced myogenin-dependent activation. Interestingly, however, this construct exhibited a synergistic response when MEF2 and myogenin proteins were coexpressed. This response occurred despite the fact that the E1 E box was mutated, which for the −61 promoter abolishes all myogenin-binding sites. In all constructs in which the MEF2 site was mutated (M−D+E+, M−D+E−, M−D−E+, and M−D−E−) the normally high (~100-fold) synergistic activity obtained when both MEF2 and myogenin proteins are present was not observed. Thus, the synergistic effect of overexpressing both MEF2 and myogenin requires an intact MEF2 site but not necessarily an intact E box, suggesting that the myogenin protein is capable of interacting with a MEF2-DNA complex to transcriptionally activate the *MRF4* gene without actually binding to an E-box site (see Discussion).

The requirement for both the MEF2 and E1 E-box sites also was evident when the *MRF4* mutant M−D+E− was examined. In this instance, the *MRF4* promoter exhibited only low levels of expression when *MEF2* or myogenin expression plasmids were cotransfected into 10T1/2 cells (Fig. 6). As expected, no difference in expression existed when myogenin or myogenin plus MEF2 was coexpressed, confirming that MEF2 and myogenin probably interact in vivo with the *MRF4* promoter to activate this muscle regulatory gene.

As a final set of experiments, we examined the role of the combined MEF2/TATA site in regulating *MRF4* expression. Mutation of the TATA site alone (M+D−E+) had little effect on the ability of the *MRF4* promoter to respond to MEF2 and/or to myogenin proteins in these assays (Fig. 6). This expression was detected despite the fact that TBP does not interact with the TATA element in this mutant (Fig. 5b). We interpret these results to indicate that either MEF2 binding can substitute for TBP binding or the downstream *tata* element, which binds TBP but normally is not the preferred TATA site in the promoter (20), is now used efficiently. In all cases, these mutants exhibited the synergistic activity that is obtained when MEF2 and myogenin are coexpressed in the cells as long as the MEF2 site remains intact. Taken together, our results indicate that the MEF2 and E1 E-box sites have a direct and major role in controlling the transcriptional activity associated with this developmentally regulated gene.

To examine how the wild-type −336 and −61 promoters function in normal primary muscle cells, as well as to compare the effects of various point mutations on these promoters, chicken primary fibroblasts and myoblasts were transfected with various *MRF4*-n*LacZ* and *MRF4*-CAT gene constructs. As shown in Fig. 7, the control pNL-n*LacZ* plasmid was not expressed in either fibroblast or muscle cell cultures, whereas a *TnI*-n*LacZ* gene exhibited the predicted muscle-specific expression pattern. Likewise, wild-type −8500 and −336 *MRF4*-n*LacZ* genes were not expressed in nonmuscle cells but were efficiently expressed in differentiated myotubes. In contrast, fibroblasts, undifferentiated myoblasts, and myotubes all expressed the control RSV-*LacZ* gene, confirming that the *MRF4* promoter is expressed exclusively in a muscle-specific fashion in these primary cultures. Identical muscle-specific expression also was obtained when the −336 and −61 *MRF4*-CAT genes were tested in this system, with myotube cultures

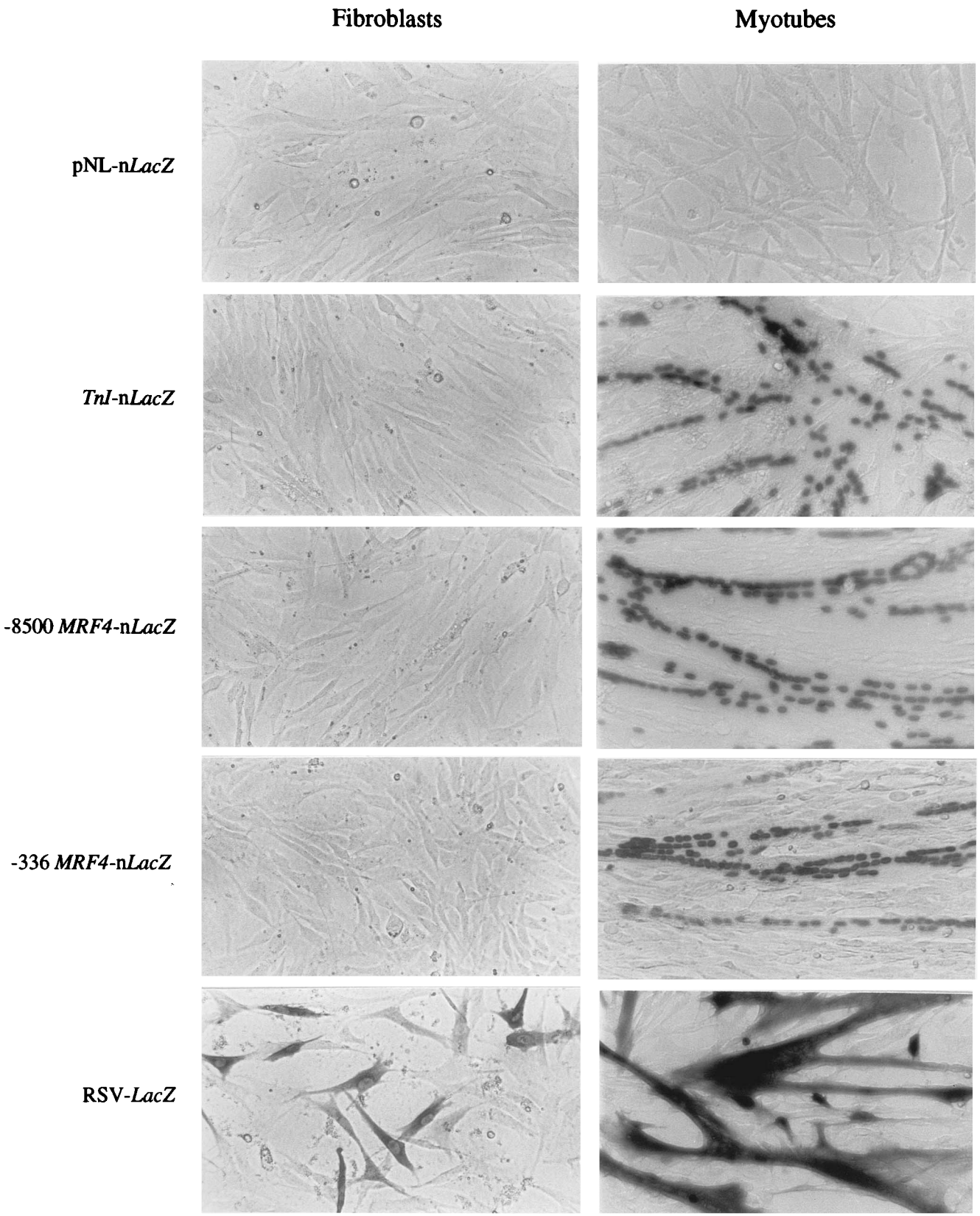

FIG. 7. Expression of *MRF4*-n*LacZ* promoter constructs in chicken primary cells. Chicken embryo fibroblasts and myoblasts were transfected with the indicated expression plasmids and stained 48 h later for β-Gal activity. The *TnI*-n*LacZ* and *MRF4*-n*LacZ* genes are expressed exclusively in differentiated myotubes, whereas the RSV-*LacZ* gene is expressed in both muscle and nonmuscle cells. As expected, the control pNL-n*LacZ* plasmid lacking a promoter is not expressed in either cell type.

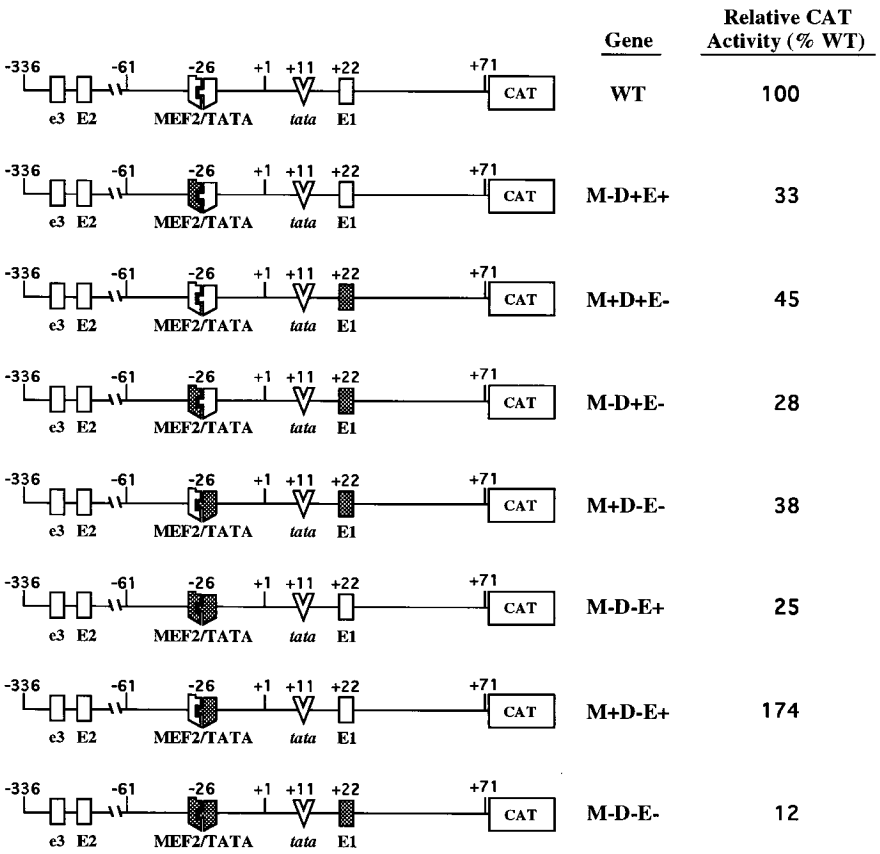

FIG. 8. Expression of −336 *MRF4*-CAT promoter constructs in chicken primary myotube cultures is dependent on the MEF2 and E1 E-box sites. Chicken embryo myoblasts were transfected with the indicated constructs and harvested 48 h later. Values are relative to the wild-type (WT) promoter expression level, which was set to 100%. All MEF2 and E1 E-box mutations reduce MRF4-CAT expression in these assays. The indicated shaded elements represent point mutations in protein-binding sites as described in the text. M, D, and E indicate the MEF2 site, TATA site and E1 E box, respectively, as in Fig. 6.

expressing at least 30-fold-higher levels than control fibroblast cultures (data not shown).

Since expression of the *MRF4*-CAT genes is muscle specific, we wished to examine how each of the *MRF4* mutant promoters would respond to normal skeletal muscle differentiation events. Therefore, the −336 *MRF4*-CAT and various point mutants were introduced into primary muscle cultures and CAT activity was measured. As predicted from the *lacZ* experiments described above, the −336 wild-type *MRF4* promoter exhibited high levels of CAT expression in differentiated myotube cultures (Fig. 8). Mutation of the MEF2 site (M−D+E+) resulted in an approximate 70% loss of activity. Similarly, mutation of E1 (M+D+E−) also resulted in a decrease in promoter activity but in this case not to the extent observed with the MEF2 mutation. Again, the double mutant M−D+E− was significantly impaired in its ability to generate full transcriptional activity in muscle cells.

Mutations of the TATA site, in conjunction with mutations in the E1 (M+D−E−) or MEF2 (M−D−E+) site, exhibited approximately the same reduced levels of expression as those observed with the single E1 (M+D+E−) or MEF2 (M−D+E+) mutant, again suggesting that the TATA site is not absolutely required for *MRF4* expression (Fig. 8). This is particularly evident with the M+D−E+ mutant, in which approximately twice as much activity as that in the wild-type *MRF4* promoter was observed. In this instance, mutation of the TATA site creates a new MEF2 site that binds MEF2 more efficiently than does the wild-type MEF2 site (unpublished observations). Thus, with M+D−E+, it is possible that TBP now interacts with the downstream *tata* site and that MEF2 binds more efficiently to the promoter, resulting in a higher expression level than that observed with M+D+E+. As expected, however, when the MEF2, TATA, and E1 sites were mutated simultaneously (M−D−E−), only basal expression levels were detected (Fig. 8). These results confirm the importance of the MEF2 and E-box regulatory sites in obtaining a correct *MRF4* gene expression pattern.

FGF inhibits myogenin from activating the *MRF4* promoter. The developmental timing of *MRF4* gene expression in muscle cells in culture follows the myogenin gene and *MEF2* and occurs only after myogenic cells terminally differentiate (4, 21, 48). Terminal differentiation, however, is inhibited when the cells are maintained in various serum growth factors, including FGF-2 (18, 29, 51). To examine the role of FGF-2 in inhibiting *MRF4* gene expression directly, 10T1/2 cells were cotransfected with the −336 *MRF4*-CAT gene and MEF2, myogenin, or MEF2 plus myogenin expression plasmids in the absence or presence of FGF-2. Addition of FGF-2 to the culture medium produced only a marginal effect on the ability of MEF2 to activate expression of the −336 *MRF4*-CAT gene (Fig. 9), suggesting that MEF2 activity is not inhibited in cells exposed to FGF-2. Very different results were obtained, however, when myogenin protein was tested. In this instance, FGF-2 inhibited the ability of myogenin to activate the *MRF4* promoter by approximately 90% (Fig. 9). A similar level of FGF-2 inhibition also was obtained when myogenin and MEF2 expression

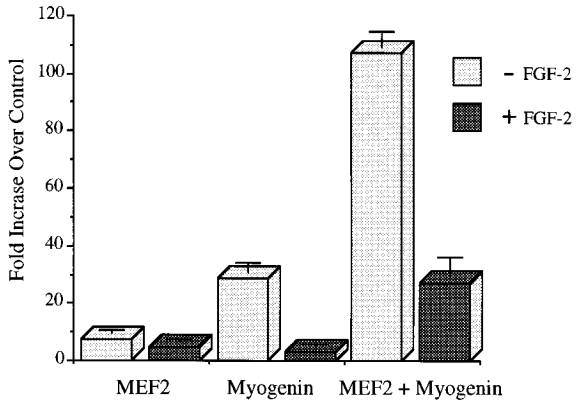

FIG. 9. FGF-2 represses the ability of myogenin to activate *MRF4* expression. The −336 *MRF4*-CAT gene was cotransfected into 10T1/2 cells with MEF2, myogenin, or MEF2 plus myogenin expression plasmids in the presence or absence of 10 ng of FGF-2 per ml. After 2 days in differentiation medium, cells were harvested and expression from the −336 *MRF4*-CAT gene was measured. FGF-2 severely inhibits myogenin-induced *MRF4* expression but has little effect on MEF2-induced activation of this reporter gene. Error bars represent the standard errors of the means.

plasmids were cotransfected into the cells, indicating that the myogenin and MEF2 synergistic activities respond to FGF-2 signal transduction pathways. These results suggest that FGF-2 inhibition of *MRF4* gene expression involves primarily the myogenic bHLH factors and not MEF2.

DISCUSSION

The *MRF* genes exhibit distinct and relatively complex expression patterns during embryogenesis, as well as later in the adult organism (44). The differential expression of the *MRF* genes suggests that the individual MyoD, myogenin, Myf-5, and MRF4 proteins play distinct roles in establishing as well as maintaining the myogenic lineage. Recent gene ablation studies of the *MyoD*, myogenin, and *Myf-5* genes support the notion that the proteins expressed by this gene family are involved in different aspects of myogenesis (19, 36, 43). Determining the molecular events that regulate expression of the *MRF* genes is crucial to fully understanding how this unique gene family operates during development.

In this study, we concentrated our efforts on examining the regulatory mechanisms that underlie *MRF4* gene regulation. We have shown that 336 or 61 bp of the rat *MRF4* proximal promoter is sufficient to confer a muscle-specific transcription response when introduced into primary muscle cultures and myogenic cell lines or when tested in 10T1/2 cells that have been converted to a myogenic lineage by overexpression of myogenin, MyoD, or Myf-5. Expression of the *MRF4* reporter gene parallels expression of the endogenous *MRF4* gene, with maximum transcription occurring several days after the cells commit and initiate differentiation events. Within the proximal *MRF4* promoter lie two regulatory elements that are essential for normal *MRF4* expression, i.e., an E box at position +22 and a combined MEF2/TATA-binding site at positions −26 and −21. Individual mutations within the proximal E-box or MEF2 site severely impair *MRF4* promoter activity in myogenic cells as well as in nonmuscle cells overexpressing MEF2 or MRF proteins. These results suggest that the bHLH myogenic factors, as well as MEF2 proteins, function in concert to transcriptionally activate the *MRF4* promoter during development.

Several studies have suggested that the myogenic bHLH factors function in an autoregulatory network in which each member is capable of activating expression of the entire muscle bHLH protein gene family as well as autoregulating their own expression (reviewed in reference 46). Although overexpression of MRF4 in 10T1/2 cells activates the endogenous *MyoD*, myogenin, and *Myf-5* genes (42), we have been unable to detect expression of the endogenous *MRF4* gene under similar conditions (unpublished observations). In agreement with these findings, MyoD, myogenin, and Myf-5 proteins activate *MRF4* reporter genes to very high levels, whereas expression of the MRF4 protein in 10T1/2 cells does not lead to *MRF4*-CAT activity. The inability of the MRF4 protein to activate *MRF4*-CAT occurs despite a very high percentage of differentiated muscle cells being produced in these cultures (18, 42) and the ability of MRF4-E12 heterodimers to bind to the E1 and E2 E boxes. In vivo, the most likely bHLH candidate for initiating *MRF4* transcription is myogenin, since myogenin expression is induced just prior to *MRF4* transcription (11, 48). In the adult, however, MRF4 is the predominant MRF, suggesting that the MRF4 protein itself, or non-bHLH factors such as MEF2, may maintain *MRF4* gene expression at these later developmental stages. Studies examining this model of *MRF4* regulation in both embryonic and adult muscle tissues are presently under way.

In addition to the involvement of bHLH MRFs in activating the *MRF4* promoter, a second muscle-specific transcription factor family is crucial to *MRF4* gene expression. The MEF2 protein family consists of several related members that all share a conserved DNA-binding and dimerization domain, referred to as the MADS box, and bind to the DNA consensus sequence $-CTA(A/T)_4TAG/A-$ (33, 50). This site is present within the promoters and enhancers of many muscle-specific genes, including myogenin, chicken *MyoD* and *Xenopus MyoDa*. Mutations in the myogenin MEF2 site or in the *XMyoDa* MEF2 site dramatically reduce myogenin and *MyoD* expression, respectively (5, 8, 27, 49). The *MRF4* promoter similarly is positively regulated by MEF2, since cotransfection of 10T1/2 cells with MEF2 and *MRF4*-CAT leads to an increase in CAT expression. Mutation of the *MRF4* MEF2 site completely abrogates the MEF2-induced expression of *MRF4*-CAT in these cells. Similarly, *MRF4* promoters containing MEF2 mutations exhibit reduced activity in both C2 myotubes (unpublished results) and primary chicken muscle cultures (Fig. 8). The ability of the myogenic regulatory factors to activate MEF2 expression in 10T1/2 cells and the coexpression of these factors in muscle cells again suggests that MEF2 and the bHLH MRFs function in cross-regulatory pathways to control expression of the *MRF* genes.

An interesting aspect of the *MRF4* promoter is the combination MEF2/TATA site in which MEF2 as well as TBP bind. This shared TATA/MEF2 region is not unique among the MRF genes, since the *Xenopus MyoDa* gene also contains a combination MEF2/TATA site that imparts muscle-specific expression on the *XMyoDa* gene during development (27). Mutations that prevent MEF2 binding but allow TBP binding substantially reduce the activity of the *MRF4* promoter but have little effect on expression of the *XMyoDa* gene. Interestingly, mutations in both promoters that prevent TBP binding but permit MEF2 binding continue to be expressed at relatively high levels in muscle cells (27). These results suggest that binding of MEF2 alone to the MEF2/TATA site may be sufficient to direct muscle-specific expression of these promoters even in the absence of direct binding of TBP and the TFIID complex. Thus, it is conceivable that MEF2 can substitute for TBP and interact with RNA polymerase II-associated factors to generate a complete transcription response. In the case of the *MRF4* promoter, however, the presence of a downstream

TATA motif (*tata*) raises the possibility that this site binds TBP when the upstream TATA site is destroyed. Studies to address these interesting possibilities are under way.

Although MEF2 and myogenin activate expression of the −336 *MRF4* promoter ~10- and ~50-fold, respectively, coexpression of the two transcription factors in 10T1/2 cells produces a level of expression that is ~130-fold greater than basal levels. A similar MRF-MEF2 cooperative response recently has been reported for the mouse desmin gene (28) as well as for reporter genes containing different combinations of multimerized MEF2 and E-box sites (23). The ability of myogenin and MEF2 to activate high levels of *MRF4* gene expression implies that these proteins probably interact with each other, or interact in concert with the basal transcription machinery, to fully activate the *MRF4* gene. In support of this hypothesis, Funk and Wright (16) have shown that myogenin interacts with several nuclear proteins, including MEF2, when a MEF2 site and E box are present in close proximity to one another. Similarly, Kaushal et al. (23) have recently reported that myogenin, MyoD, Myf-5, and MRF4 form complexes with MEF2 in vitro through their respective basic and MADS domains. MEF2 and myogenin also coimmunoprecipitate with one another in muscle nuclear extracts (23), confirming the importance of the MEF2-MRF interactions in vivo. Although MEF2 binds in vitro to all four MRF transcription factors, some specificity in protein-protein interactions may exist in vivo since coexpression of MEF2 and MRF4 does not lead to the high synergistic expression levels of *MRF4*-CAT that are obtained when MEF2 and myogenin are coexpressed (unpublished data). These individual properties of myogenin and MRF4 also correlate with their ability to differentially activate several additional muscle-specific genes, including M-creatine kinase and troponin I (51). Given that the amino-terminal activation domains of myogenin and MRF4 exhibit distinct activities (6, 32), it is conceivable that MEF2 differentially interacts with these regions of the bHLH factors to produce maximal transcription levels.

As shown in this study, mutation of the MEF2 site, E1 E box, or both interferes with *MRF4* expression in primary myotubes. However, only the MEF2 mutation interferes with the ability of MEF2 and myogenin to synergistically activate the *MRF4* promoter in transfected 10T1/2 cells (Fig. 6). MEF2 and myogenin cooperate to activate reporter genes containing a mutant E1 E box (M+D+E−) even though myogenin-E12 complexes are severely inhibited from binding to their target sites. At this time it is unclear how these synergistic activities are maintained on mutant E-box-binding sites. One possibility that we are exploring is that binding of the MEF2 protein to the *MRF4* MEF2 site can recruit myogenin and thus "anchor" myogenin to the *MRF4* proximal promoter in the presence or absence of a wild-type E-box site. An analogous model has been suggested to account for the activities associated with TBP, in which TBP activates RNA polymerase II- and polymerase III-dependent transcription and yet binds directly only to polymerase II promoters (41, 47). A similar "bridging" model also has been proposed to account for Sp1-dependent synergistic activity (40). Future studies involving protein cross-linking and double electrophoretic mobility shift assays of MEF2 and myogenin-E12 complexes should allow a direct test of this hypothesis.

The organizations of the *MRF4*, *Xenopus MyoDa*, chicken *MyoD*, and myogenin promoters are very similar in that each contains proximal MEF2 sites as well as E boxes (7, 8, 10, 27, 49). However, these genes utilize the MEF2 and E box sequences in distinct fashions. For example, myogenin and *XMyoDa* transcription is MEF2 dependent but E box independent (5, 7, 8, 27), whereas transcription of the chicken *MyoD* gene occurs through pathways that are independent of both MEF2 and MRF binding (10). In contrast, *MRF4* activity is clearly dependent on the proximal MEF2 and E1 E-box sites for activity, although the MEF2 site appears to be the major regulatory element controlling full muscle-specific transcription. Thus, the distinct expression patterns observed in vivo with the four *MRF* genes are likely to be manifested by how they respond to the muscle-specific transcription factor MEF2 as well as MyoD, myogenin, Myf-5, and MRF4. Understanding these complex regulatory events will require a further understanding of how the *MRF* promoters are regulated during each stage of muscle development.

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