MOLECULAR AND CELLULAR BIOLOGY, Oct. 1992, p. 4334–4346
0270-7306/92/104334-13$02.00/0

Vol. 12, No. 10

The MRF4 Activation Domain Is Required To Induce Muscle-Specific Gene Expression

KAM-LEUNG MAK, ROBERT Q. TO, YANFENG KONG, AND STEPHEN F. KONIECZNY*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana 47907

Received 13 May 1992/Returned for modification 24 June 1992/Accepted 2 July 1992

MRF4 is a member of the basic helix-loop-helix muscle regulatory factor family that also includes MyoD, myogenin, and Myf-5. Overexpression of MRF4 or the other muscle regulatory factors in fibroblasts converts the cells to differentiated muscle fibers and transcriptionally activates expression of endogenous and cotransfected muscle genes. Although these factors induce a similar phenotype, they also exhibit some distinct biological activities. For example, MyoD *trans* activates α-actin and troponin I reporter genes to very high levels, whereas MRF4 efficiently activates only α-actin expression. Since these proteins have a common basic helix-loop-helix domain, it is likely that portions of the proteins outside of this region impart some specificity to the activity of each muscle regulatory factor. As an initial step in determining the mechanism by which MRF4 and MyoD activate gene transcription, the transcriptional activation domain of MRF4 has been characterized. Experiments utilizing chimeric proteins containing the yeast GAL4 DNA-binding domain and portions of the MRF4 protein indicate that the MRF4 activation domain is located within amino acids 10 to 30. This amino terminus is both necessary and sufficient to elicit a transcriptional response in transfected cells. The MRF4 activation domain and the related amino-terminal MyoD activation domain are capable of substituting for one another in converting fibroblasts to a myogenic phenotype and in activating expression of an α-actin reporter gene, although the MRF4 and MyoD activation domains on these chimeric proteins also dictate the specificity of transcriptional activation. The different primary amino acid sequences of these regions leave open the possibility that different coregulator proteins interact with the muscle regulatory factors to elicit their correct transcriptional activity during skeletal muscle development.

Skeletal myogenesis provides an excellent model system in which to study the regulatory mechanisms which coordinate the transcriptional activation of sets of unrelated genes during development. Skeletal myoblasts can be maintained in vitro in a proliferative, undifferentiated state by providing the cells with growth medium containing high levels of serum or containing one of several peptide growth factors such as basic fibroblast growth factor or transforming growth factor-β (reviewed in reference 38). Switching cells to a serum-free medium, however, leads to rapid withdrawal of the cells from the cell cycle followed by the formation of multinucleate myofibers and the transcriptional activation of ~40 muscle-specific genes, many of which encode proteins that assemble to form a functional sarcomere.

Recent advances in isolating genes that control this terminal differentiation process have led to the identification of four related mammalian genes referred to as MyoD (14), myogenin, (16, 55), Myf-5 (5), and MRF4 (43) (also known as herculin [31] and Myf-6 [4]). These proteins share a central basic helix-loop-helix (bHLH) domain that is involved in DNA binding and protein-protein interactions (reviewed in references 17, 37, and 50). This shared ~60-amino-acid region likely accounts for several of the common properties that characterize this protein family. These include the abilities of MyoD, myogenin, Myf-5, and MRF4 to form heterodimers with the widely expressed bHLH factor E12 (3, 9, 18, 27, 29, 35), to bind as heterodimers to an E-box DNA consensus sequence (CANNTG) (8, 13, 26, 34), to *trans* activate expression of cotransfected contractile-protein reporter genes (7, 13, 19, 30, 57), and to efficiently convert nonmuscle cells to a myogenic lineage (5, 14, 16, 43, 55).

Although there exist many similarities between the four bHLH muscle regulatory factors, these factors also exhibit several unique properties. During embryonic development, the genes encoding the muscle regulatory factors are not expressed simultaneously. Myf-5 is detected in rostral somites as early as 8.0 days post coitum, whereas myogenin is detected at 8.5 days, MRF4 is detected at 9.0 days, and MyoD is not detected until 10.5 days (24, 39, 47). Additionally, these factors exhibit slightly altered binding affinities for different contractile-protein gene enhancers (10, 44) and different *trans*-activation potentials for contractile-protein reporter genes (10, 57). Several laboratories have shown, for instance, that MyoD and myogenin efficiently *trans* activate expression of cotransfected reporter genes containing the regulatory elements associated with the muscle creatine kinase (MCK) (10, 13), myosin light chain 1/3 (44, 54), troponin I (TnI) (30, 57), and acetylcholine receptor α-subunit and α-actin (19, 40, 41, 46, 57) genes, whereas MRF4 efficiently *trans* activates only the acetylcholine and α-actin gene constructs (41, 57). This difference in *trans* activation occurs despite the ability of MRF4-E12 heterodimers to efficiently bind to the enhancer elements associated with the MCK, myosin light chain 1/3, and TnI genes (29, 30). Since MyoD, myogenin, Myf-5, and MRF4 are unrelated in their amino- and carboxyl-terminal ends (43), it is likely that these regions play important roles in dictating the unique specificities that are associated with each muscle regulatory factor.

Although the muscle regulatory factors bind to the E-box elements associated with most contractile-protein gene enhancers, how they transcriptionally activate expression of muscle-specific genes is unclear. Several studies have suggested that MyoD, for example, binds cooperatively to

* Corresponding author.

adjacent E-box sequences prior to eliciting a transcriptional response (21, 40, 52, 54). However, many contractile-protein gene enhancer elements contain only a single E-box site and yet are efficiently *trans* activated in MyoD cotransfections (19, 30, 46). Additionally, although muscle regulatory factor-E12 heterodimers are essential in activating contractile-protein gene expression, the heterodimers by themselves are not sufficient to produce a full transcriptional response. Mutations within protein-binding sites that are adjacent to the E-box elements in the TnI, MCK, and α-actin regulatory regions lead to large decreases in the ability of MyoD or myogenin to *trans* activate expression of these reporter genes, even though the muscle regulatory factors efficiently bind to the respective E-box element (23, 30, 46). Therefore, the muscle regulatory factors must interact with additional transcription factors which are associated with the enhancer elements during terminal differentiation. The nature of these protein-protein interactions remains unknown.

To begin characterizing the mechanisms by which MRF4 activates transcription in cells maintained in low serum concentrations and exhibits different *trans*-activation abilities when compared with MyoD, myogenin, and Myf-5, we set out to identify and characterize the transcriptional activation domain of the MRF4 protein. In addition, a series of MRF4 deletions and MRF4-MyoD chimeric proteins were generated in order to determine whether the parallel protein regions from MRF4 and MyoD have functions in common. Our results demonstrate that the MRF4 activation domain is located within the amino terminus of the protein, since amino acids 10 to 30 are sufficient to generate a transcriptional response when ligated in frame to the yeast GAL4 DNA-binding domain. Deletion of this region reduces the ability of the MRF4 protein to convert fibroblasts to a myogenic lineage and to *trans* activate expression of chloramphenicol acetyltransferase (CAT) reporter genes. Replacement of the MRF4 activation domain with the corresponding MyoD activation domain restores the full myogenic capacity of the truncated MRF4 protein but alters the specificities by which MRF4 and MyoD activate different target reporter genes. These results suggest that the MRF4 and MyoD activation domains, although functionally equivalent in GAL4 transcription assays, dictate several unique specificities that characterize the respective muscle regulatory factors.

MATERIALS AND METHODS

Cell culture and DNA transfections. C3H10T1/2, NIH 3T3, and COS cells were obtained from the American Type Culture Collection. C3H10T1/2 and COS cells 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), while NIH 3T3 cells were maintained in growth medium supplemented with 10% calf serum. Transient DNA transfections of C3H10T1/2, NIH 3T3, and COS cells were performed as described by Yutzey et al. (57). For GAL4 assays, DNA precipitates consisted of 5 μg of the reporter plasmid $(GAL4)_5$-E1bTATA-CAT (28), 5 μg of GAL4(1-147) (28), or GAL4(1-147) containing portions of the MRF4 cDNA cloned in frame to the GAL4 DNA-binding domain, and 5 μg of the control β-galactosidase expression vector RSVLacZ (25). Precipitates were added to 10^6 C3H10T1/2 or NIH 3T3 cells per 100-mm dish. Four hours after the addition of DNA, the cultures were subjected to a 2-min osmotic shock in serum-free medium containing 20% glycerol and then fed growth medium. Two days after transfection, cell extracts were prepared and β-galactosidase activity was determined as described previously (36, 56). The amount of extract used for each CAT assay was normalized to the specific activity of β-galactosidase in each protein sample. CAT assays were carried out as described by Gorman et al. (22), and the percentage of acetylated chloramphenicol was determined by liquid scintillation counting. A minimum of three independent transfections was performed for each experimental gene construct.

trans-Activation assays were performed as described above with the following exceptions. For each dish of C3H10T1/2 cells, 1 μg of the control pEMSVscribeα2 expression vector (14) or 1 μg of the expression vector containing an MRF4, MyoD, or MRF4-MyoD chimeric cDNA were mixed with 5 μg of the cardiac α-actin reporter LK359CAT (33) or with 5 μg of the TnI reporter TnICAT1 (56) gene. Four hours after the addition of DNA, the cultures were subjected to an osmotic shock as described above for the GAL4 experiments and fed growth medium containing basal medium Eagle and 15% fetal bovine serum. Twenty-four hours after transfection, cultures were induced to differentiate by the addition of the serum-free medium ITS (30, 56). After 2 days in differentiation medium, protein extracts were prepared and analyzed as described above with the exception that 10 μg of protein was used in each assay.

Immunocytochemistry. Transfected cell cultures were rinsed twice in cold phosphate-buffered saline (PBS) and fixed for antibody staining. Cells to be stained with the antimyosin mouse monoclonal antibody MF-20 (2) were fixed in a 20:2:1 solution of 70% ethanol-formalin-acetic acid for 1 min at 4°C. Cells to be stained with rabbit antibodies directed against glutathione-*S*-transferase fusion proteins containing the complete MRF4 or MyoD coding sequence (42) were fixed in freshly prepared 2% formaldehyde in PBS for 10 min, permeabilized by incubation in Triton X-100 for 20 min, and incubated overnight at 4°C with a 1:500 dilution of the MRF4 or MyoD rabbit serum. After incubation with primary antibodies, cells were rinsed in PBS and treated with the appropriate biotinylated immunoglobulin secondary antibody. Immunoreactivity was visualized by using a Vectastain ABC reagent (Vector Laboratories, Inc.).

Experimental gene constructions. (i) GAL4-MRF4 chimeric genes. GAL4MRF4(1-242) was generated by cloning the MRF4 *Eco*RI insert from pEM-cRS4-1 (43) in frame to the GAL4(1-147) expression vector (28). The MRF4 cDNA inserts for GAL4MRF4(1-85), GAL4MRF4(1-30), GAL4 MRF4(10-30), GAL4MRF4(10-40), GAL4MRF4(1-49), GAL 4MRF4(10-49), GAL4MRF4(20-49), GAL4MRF4(30-49), and GAL4MRF4(50-85) were generated by polymerase chain reaction (PCR) amplification of the respective fragments using DNA primers that were complementary to the MRF4 cDNA template (43). Each 5′ primer contained the leading sequence 5′-GGAATTC-3′ (*Eco*RI site), while the 3′ primers contained the sequence 5′-GTCTAGA-3′ (*Xba*I site). The amplified fragments then were digested with *Eco*RI and *Xba*I and cloned in frame into the *Eco*RI-*Xba*I sites of GAL4(1-147). GAL4MRF4(148-242) similarly was generated by PCR amplification using the appropriate 5′ primer with an *Eco*RI recognition sequence and a 3′ primer complementary to the flanking plasmid sequence in GAL4MRF4(1-242). GAL4 MRF4(1-180) was produced by digestion of the MRF4 clone pEM-cRS4-1 with *Fsp*I, ligation of an *Xba*I linker (NEB 1062), and subsequent digestion with *Eco*RI and *Xba*I to produce an MRF4 cDNA containing amino acids 1 to 180.

GAL4MRF4(180-242) was produced as described above for GAL4MRF4(1-180) except that an *Eco*RI linker (NEB 1078) was added to the *Fsp*I end to generate MRF4 amino acids 180 to 242. Construction of GAL4MRF4(1-60) involved digesting the pEM-cRS4-1 MRF4 cDNA with *Xmn*I and then ligating *Xba*I linkers. Subsequent digestion with *Eco*RI and *Xba*I yielded an MRF4 fragment containing amino acids 1 to 60. The MRF4 amino acid 1 to 180, 180 to 242, and 1 to 60 inserts then were cloned into a GAL4(1-147) vector previously digested with *Eco*RI and *Xba*I. A similar PCR-based cloning strategy was employed to construct GAL4MyoD(1-66) by using pEMC11s (14) as the DNA template. All constructs were sequenced to verify that the correct reading frame and amplified products were produced.

(ii) MRF4-MyoD deletions and chimeric genes. The following gene constructs were produced by generating the correct MRF4-MyoD fragments and inserting them into an *Eco*RI-digested pEMscribeα2 expression vector. Briefly, MRF4Δ3′ was produced by digesting the MRF4 cDNA with *Fsp*I at amino acid position 181. After ligation of *Eco*RI linkers (NEB 1078), the truncated MRF4 insert was cloned into pEMscribeα2. MRF4Δ3′MyoD was generated by digesting the MyoD cDNA with *Nar*I and treating the 5′ and 3′ overhangs with S1 nuclease. The blunt-ended cDNA then was digested with *Eco*RI, and the fragments containing MyoD amino acids 174 to 318 were ligated to the MRF4Δ3′ fragment as described above. MRF4Δ5′ was produced by digestion of pBS-MRF4 with *Ava*II followed by treatment with Klenow polymerase and the addition of *Hin*dIII linkers (NEB 1022). Digestion with *Eco*RI released an *Eco*RI–blunt-end fragment that contained the MRF4 5′ untranslated region plus amino acids 1 to 4. This fragment was used in a three-part ligation with an *Xmn*I-digested pBS-MRF4 fragment (yielding amino acids 61 to 242). To generate MyoD-MRF4Δ5′, the *Xmn*I fragment of MRF4 (amino acids 61 to 242) was ligated to a PCR-generated MyoD fragment that contained MyoD amino acids 1 to 66. MyoDΔ3′ was produced by digesting the MyoD cDNA with *Nar*I, treating the fragment with Klenow polymerase, ligating it with *Eco*RI linkers (NEB 1078), and subsequently digesting it with *Eco*RI. MyoDΔ3′MRF4 was generated by using the MyoDΔ3′ *Eco*RI-*Nar*I insert and an MRF4 *Fsp*I-*Eco*RI fragment containing amino acids 182 to 242. To produce MyoDΔ5′, an *Eco*RI-*Alu*I fragment containing the 5′ untranslated region and amino acids 1 and 2 of MyoD was ligated to a *Bss*HII (S1-treated)-*Eco*RI fragment of MyoD containing amino acids 84 to 318. MRF4MyoDΔ5′ was generated by PCR amplification of MRF4 amino acids 1 to 50, leaving an *Apa*LI site at the 3′ end. This fragment subsequently was ligated to a MyoD fragment generated by *Apa*LI and *Eco*RI that contained amino acids 69 to 318. All cloning orientations and correct ligation junctions were confirmed by DNA sequencing.

In vitro transcription and translation. m^7GpppG-capped RNAs were synthesized in vitro from linearized DNA templates (5 μg) by using T3 or T7 RNA polymerase and a MAXIscript in vitro transcription kit (Ambion). Reaction mixtures were incubated at 37°C for 30 min, after which DNase I was added for an additional 10 min. Newly synthesized RNA was extracted with phenol-chloroform, ethanol precipitated, and suspended in 25 μl of TE (10 mM Tris [pH 7.8], 1 mM EDTA). Linear DNA templates were generated from mouse MyoD (14), rat MRF4 (43), and MRF4-MyoD chimerae cloned into the pEMSVscribeα2 vector or from the GAL4 gene constructs cloned into the pBluescript KS^+ vector (Stratagene).

For in vitro translations, 2 μl (~2 ng) of RNA transcribed in vitro was added to a 48-μl reaction mixture containing a rabbit reticulocyte lysate (Promega) and incubated at 30°C for 1 h. Aliquots of the translation products were stored at −80°C. Parallel radioactive translation products were generated by supplementing the amino acid mixtures with L-[^{35}S]methionine (>800 Ci/mmol; Amersham). Synthesis of the ^{35}S-labeled protein products was monitored by electrophoresis through a 12% discontinuous sodium dodecyl sulfate–polyacrylamide gel. The gel was fixed and stained in 50% methanol–10% acetic acid–0.2% Coomassie blue for 2 min in a microwave oven and then destained for 5 min in 30% methanol–7% acetic acid followed by 10% methanol–7% acetic acid. Fixed gels were treated with En^{3}Hance (New England Nuclear) for 1 h, vacuum dried, and exposed to Kodak XAR film.

Gel mobility shift assays. The DNA probes used in this study were generated by standard procedures (1). Briefly, the TnI enhancer (56) and GAL4 fragments containing one or five GAL4 binding sites (28) were treated with alkaline phosphatase (Boehringer Mannheim) and labeled with T4 polynucleotide kinase (New England BioLabs) and [γ-^{32}P] ATP (New England Nuclear). Oligonucleotide probes containing the TnI E-box site (30) were generated by labeling one DNA strand with T4 polynucleotide kinase and [γ-^{32}P] ATP. Unincorporated [γ-^{32}P]ATP was removed from all probes by passing the reactions through G-50 (Pharmacia) spun columns. The single-stranded labeled DNA was mixed with a 10-fold molar excess of the unlabeled complementary oligonucleotide strand, heated to 100°C for 5 min, and allowed to anneal at room temperature to generate double-stranded oligonucleotides.

Gel mobility shift assays were conducted by incubating end-labeled probes (2 to 13 fmol; ~5,000 cpm) with 0.5 μg of poly(dI-dC) (Sigma) and 3 to 8 μl of in vitro-translated products in a total volume of 20 μl. Binding reaction mixtures also contained 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; pH 7.9), 50 mM KCl, 0.5 mM dithiothreitol, 5 mM $MgCl_2$, and 10% glycerol and were incubated at room temperature for 20 min. The binding reaction mixtures then were loaded onto a 5% polyacrylamide gel (40:1 acrylamide-bisacrylamide) containing 2.5% glycerol and electrophoresed at room temperature at 8 V/cm for 3 to 3.5 h in 12.5 mM Tris (pH 8.5)–95 mM glycine–0.5 mM EDTA. After electrophoresis, the gels were fixed in 10% methanol–10% acetic acid, vacuum dried, and exposed to Kodak XAR film.

RESULTS

Identification of the MRF4 activation domain. The ability of MRF4 to bind to E-box regulatory sequences and yet differentially *trans* activate expression of cotransfected contractile-protein genes suggests that MRF4 utilizes a specific transcriptional activation domain that is distinct from the activation domains characterized for MyoD (53), myogenin (49), and Myf-5 (6). As an initial step in identifying potential activation domains in the MRF4 protein, we utilized the GAL4 DNA-binding system derived from *Saccharomyces cerevisiae* in which a CAT reporter gene containing five GAL4 binding sites [$(GAL4)_5$-E1bTATA-CAT] was introduced into C3H10T1/2 or NIH 3T3 cells with expression gene constructs containing portions of the MRF4 cDNA cloned in frame to the GAL4 DNA-binding domain.

Cells transfected with the CAT reporter gene and a construct expressing only the GAL4 DNA-binding domain

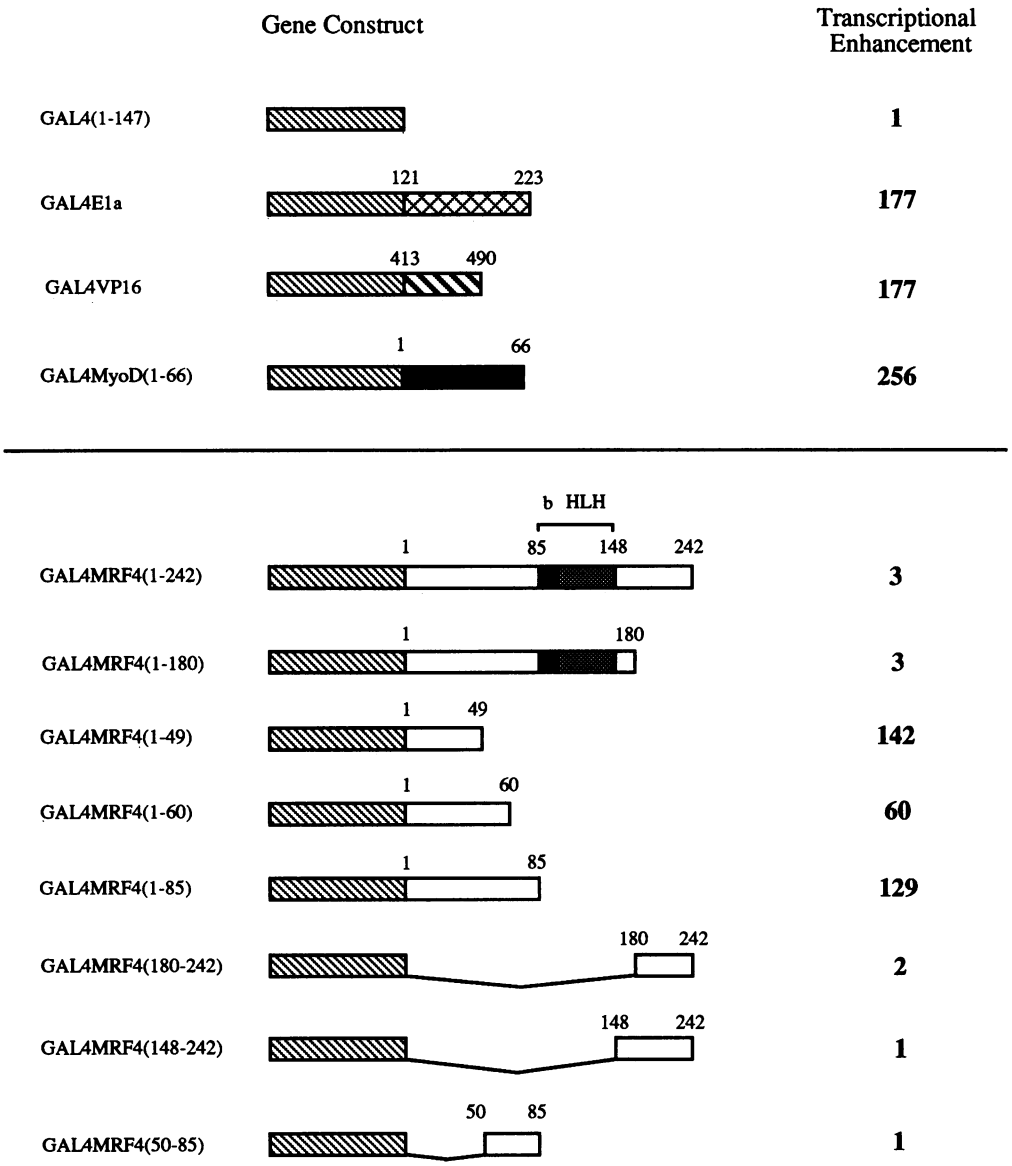

FIG. 1. The MRF4 activation domain maps to the amino terminus of MRF4. C3H10T1/2 or NIH 3T3 cells were cotransfected with the $(GAL4)_5$-E1bTATA-CAT reporter gene and each of the indicated GAL4 chimeric genes in which the GAL4(1-147) DNA-binding domain was ligated in frame with the indicated MRF4 fragments (see Materials and Methods for details). The hatched boxes represent the GAL4(1-147) portions of the protein, whereas the numbers above each construct indicate the amino acids derived from the MRF4 protein. The bHLH region of MRF4 also is indicated. Transcriptional enhancement refers to the fold increase in acetylated chloramphenicol compared with the basal level detected with GAL4(1-147), which typically was less than 0.5% conversion. All values are the average of 4 to 11 independent DNA transfections.

[GAL4(1-147)] expressed CAT at very low levels (Fig. 1). Conversely, when the activation domain of the adenovirus E1a protein (amino acids 121 to 223) or the herpes simplex virus VP16 transcription factor (amino acids 413 to 490) was ligated in frame to the GAL4 DNA-binding region, a strong transcriptional response was detected. Similar high levels of transcriptional activation were obtained when we tested a GAL4 gene construct containing the first 66 amino acids of the muscle regulatory factor MyoD, which contains the functional MyoD activation domain (53). Surprisingly, when GAL4 chimeric genes containing the complete MRF4 protein (amino acids 1 to 242) were tested in this system, only low levels of CAT expression were detected, even though the MRF4 protein functions as a tissue-specific transcriptional activator (43, 57). Low levels of expression also were observed with GAL4MRF4(1-180), which lacks the carboxyl region but retains the bHLH domain of MRF4. Further carboxyl deletions of the MRF4 protein, however, revealed a strong activation domain in GAL4MRF4(1-85), GAL4 MRF4(1-60), and GAL4MRF4(1-49) that supported a level of CAT activity similar to the levels obtained with the control GAL4E1a and GAL4VP16 chimeric proteins (Fig. 1). Again, no transcriptional activation was detected when regions flanking the bHLH domain of MRF4 were tested in this system. Two carboxyl gene constructs containing MRF4 amino acids 148 to 242 and 180 to 242 as well as GAL4

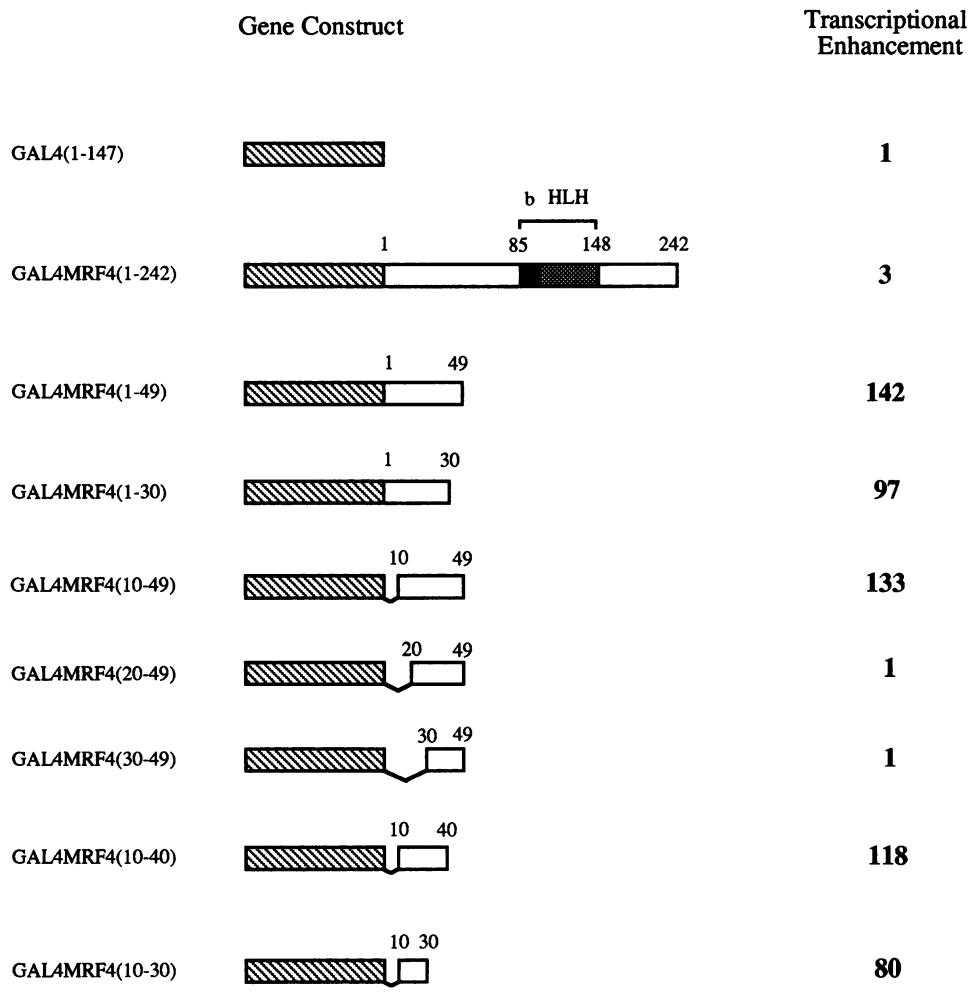

FIG. 2. The major MRF4 activation domain is located within amino acids 10 to 30. C3H10T1/2 or NIH 3T3 cells were cotransfected with the $(GAL4)_5$-E1bTATA-CAT reporter gene and the indicated gene constructs as described in the legend to Fig. 1. All values are the average of four or five independent transfections.

MRF4(50-85) containing the 36 amino acids which precede the bHLH domain produced levels of CAT activity comparable to control background levels. These results demonstrate that the amino terminus of MRF4 is sufficient to elicit a transcriptional response.

To further delineate the MRF4 activation domain, additional GAL4 gene constructs were generated in order to identify the minimum MRF4 region that functions as a transcriptional activator. As shown in Fig. 2, chimeric gene constructs in which the last 19 or first 9 amino acids of the MRF4 activation domain were deleted still retained significant transcriptional activity. GAL4MRF4(1-30) and GAL4 MRF4(10-49) exhibited the same high transcriptional activities as GAL4MRF4(1-49). Further deletions of amino acids 10 to 19 [GAL4MRF4(20-49)] and 10 to 29 [GAL4MRF4 (30-49)], however, completely abolished the transcriptional activity of the amino MRF4 peptide, whereas GAL4MRF4 (10-40) and GAL4MRF4(10-30) continued to function as strong transcriptional activators in these assays. Thus, the major transcriptional activation domain of MRF4 is contained within a 21-amino-acid region located within residues 10 to 30.

The inability of GAL4MRF4(1-242) and GAL4MRF4(1-180) to activate expression of the $(GAL4)_5$-E1bTATA-CAT reporter gene was unexpected, since each contains the MRF4 activation domain (amino acids 10 to 30). These constructs, however, also contain the MRF4 bHLH domain, which is involved in DNA binding as well as in the formation of protein heterodimers (29). Since the muscle regulatory factors exhibit relatively short half-lives (15, 51), it is conceivable that the low levels of transcriptional activation obtained from these chimeric proteins are due to protein instability or to an inability of specific chimeric proteins to efficiently enter the nucleus. To examine these possibilities, COS cells were transfected with various GAL4MRF4 chimeric gene constructs, and the relative abundance and cellular location of each chimeric protein were determined by using polyclonal antibodies directed against MRF4 (29, 42). As shown in Fig. 3, cells transfected with the GAL4(1-147) control plasmid did not react with anti-MRF4 antibodies. However, cells transfected with expression constructs containing the entire MRF4 cDNA [GAL4MRF4(1-242)] or portions of the MRF4 cDNA such as GAL4MRF4(1-180) and GAL4MRF4(1-85) exhibited intense nuclear staining. Identical results also were obtained when C3H10T1/2 or NIH 3T3 cells were used as recipients for these chimeric gene con-

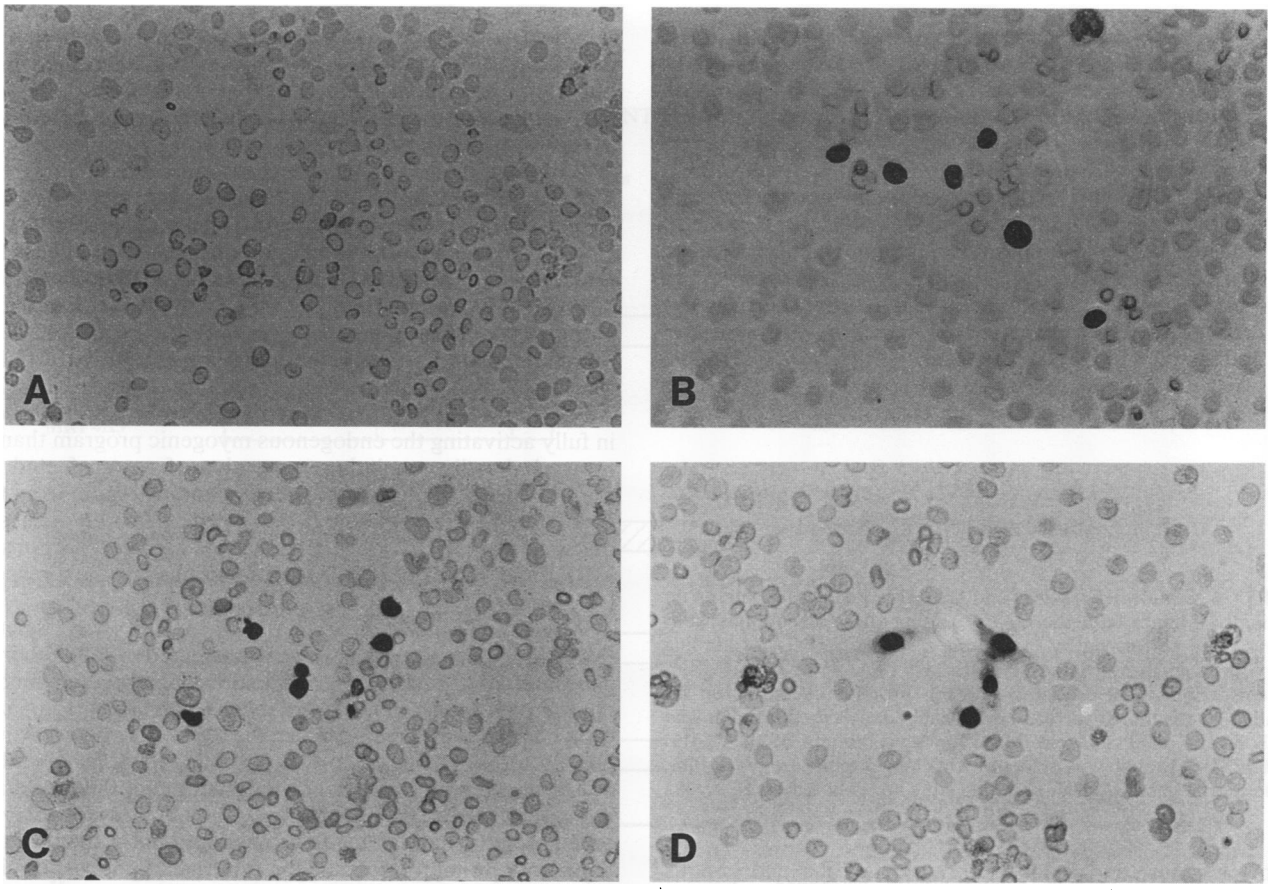

FIG. 3. GAL4MRF4 proteins efficiently translocate to the nucleus. COS cells were transiently transfected with GAL4(1-147) (A), GAL4MRF4(1-242) (B), GAL4MRF4(1-180) (C), and GAL4MRF4(1-85) (D) expression vectors. After transfection, the cells were stained with anti-MRF4 rabbit sera as described in Materials and Methods. Nuclear staining is detected with all GAL4MRF4 proteins, regardless of whether the proteins are transcriptionally active (see text for details).

structs (data not shown). Thus, the inability of GAL4MRF4 (1-242) and GAL4MRF4(1-180) to activate expression of the CAT reporter gene is not a result of in vivo protein instability or of an inappropriate cellular location.

Since GAL4MRF4(1-242) and GAL4MRF4(1-180) contain the MRF4 DNA-binding domain in addition to the GAL4 DNA-binding domain, we next examined by electrophoretic mobility shift assays whether each chimeric protein efficiently interacts with the GAL4 DNA-binding site. For these studies, equivalent amounts of in vitro-synthesized GAL4 MRF4 chimeric proteins were incubated with a ^{32}P-labeled DNA probe containing one or five GAL4 binding sites (see Materials and Methods for details) and then subjected to electrophoretic mobility shift assay. As expected, GAL4 (1-147) and also GAL4MRF4(1-49), GAL4MRF4(10-49), and GAL4MRF4(148-242) proteins efficiently bound to the ^{32}P-labeled DNA probe regardless of whether these chimeric proteins elicited transcriptional activation responses when expressed in vivo (Fig. 4). Conversely, the GAL4MRF4(1-180) and GAL4MRF4(1-242) chimeric proteins, which exhibit no significant transcriptional activation in the GAL4 CAT assays (Fig. 1), interacted very weakly with the GAL4 site compared with all other proteins in this DNA-binding assay (Fig. 4). Although the reason for this weak binding is unknown, it is likely that the bHLH domains contained within GAL4MRF4(1-180) and GAL4MRF4(1-242) interfere with the GAL4 DNA-binding domain associated with these chimeric proteins. The bHLH domain found within the GAL4MRF4(1-242) and GAL4MRF4(1-180) proteins, however, is nonfunctional, since overexpression of these chimeric proteins in C3H10T1/2 fibroblasts did not generate detectable myosin-expressing cells (data not shown). Therefore, the inability of GAL4MRF4(1-180) and GAL4MRF4 (1-242) to efficiently bind to the GAL4 target gene likely accounts for the lack of CAT expression obtained from these chimeric proteins, even though they both contain the major MRF4 transcriptional activation domain located within amino acids 10 to 30.

MRF4 activation domain is required to efficiently convert fibroblasts to a myogenic lineage. Overexpression of MRF4 or MyoD in C3H10T1/2 fibroblasts leads to their conversion to myogenic cells which, when maintained in a serum-free medium, rapidly differentiate by expressing the contractile-protein gene set. In order to examine the role of the MRF4 and MyoD activation domains in this conversion process, we constructed several MRF4 and MyoD deletion mutants as well as MRF4-MyoD chimeric proteins to determine whether the MRF4 and MyoD activation domains are essential to producing a fully differentiated muscle phenotype. All constructs were tested for their abilities to bind to muscle-specific E-box sequences, translocate to the nucleus, *trans* activate expression of cotransfected α-actin and TnI CAT

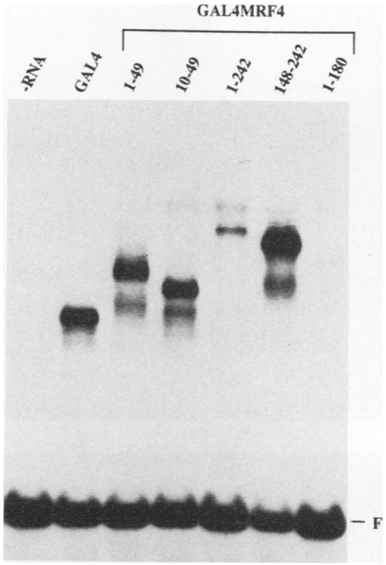

FIG. 4. GAL4MRF4(1-242) and GAL4MRF4(1-180) proteins inefficiently interact with the GAL4 binding site. Gel mobility shift assays were performed by incubating a ^{32}P-labeled GAL4 binding site with in vitro-translated GAL4(1-147) (GAL4) or GAL4MRF4 proteins. Equivalent amounts of each protein, as measured by parallel ^{35}S reactions, were used for each assay (see Materials and Methods for details). Weak but detectable binding of the GAL4 MRF4(1-180) protein was evident with a longer exposure of the film. Unbound ^{32}P-labeled GAL4 binding sites are denoted by F.

reporter genes, and induce expression of the endogenous contractile-protein genes. Initially, N-truncated and C-truncated MRF4 proteins were generated to examine the roles of the carboxyl and amino portions of the MRF4 protein in regulating skeletal myogenesis. Additionally, two gene constructs in which the amino- and carboxyl-terminal ends of MRF4 were replaced with the corresponding portions of the MyoD protein were generated (Fig. 5A). These constructs, however, retained the MRF4 bHLH domain. Similarly, N-truncated and C-truncated MyoD proteins were produced along with gene constructs in which the amino- and carboxyl-terminal ends of MyoD were replaced with the corresponding regions of MRF4. In this instance, each chimeric protein retained the MyoD bHLH domain (Fig. 5B).

In order to examine the DNA-binding properties of the truncated and chimeric muscle regulatory factors, gel mobility shift assays were performed. Incubation of the related bHLH protein E12 with the full-length MRF4 and MyoD proteins as well as with the amino- and carboxyl-truncated MRF4 and MyoD proteins revealed that all formed heterodimers and bound to the E-box regulatory sequence found within the TnI muscle-specific enhancer (Fig. 6). Identical binding also was observed with the MRF4Δ3′ MyoD and MyoDMRF4Δ5′ chimeric proteins and with the MRF4MyoDΔ5′ chimeric protein, confirming that the bHLH domains present in each truncated or chimeric protein mediate these specific protein-DNA interactions.

Transfection of C3H10T1/2 fibroblasts with expression vectors containing the wild-type MRF4 or MyoD clones and their corresponding mutants coupled with immunocytochemistry using anti-MRF4 and anti-MyoD antibodies (42) revealed that all deleted and chimeric proteins were expressed stably and were transported efficiently to the nucleus (Fig. 5). In addition, all constructs activated expression of the endogenous myosin genes and *trans* activated cotransfected α-actin reporter genes. The efficiency by which each protein activated the myogenic program, however, differed significantly. For example, removal of the carboxyl portion of MRF4 (MRF4Δ3′) led to a modest decrease in the ability of this protein to activate expression of a cotransfected α-actin gene. At the same time, however, 84% fewer cells expressed the endogenous myogenic program compared with the levels obtained with the full-length MRF4 cDNA (Fig. 5A). Addition of MyoD amino acids 174 to 318 to this C-truncated MRF4 protein (MRF4Δ3′MyoD) produced a slight increase in α-actin and myosin expression, although the degree of myogenic conversion by this chimeric protein did not approach the levels obtained with MRF4. These results indicate that the carboxyl-terminal 182 to 242 amino acids of MRF4 are more crucial to the MRF4 protein in fully activating the endogenous myogenic program than in transcriptionally regulating expression of cotransfected reporter genes. Addition of the corresponding region from the MyoD protein did not significantly rescue this myogenesis-deficient phenotype, suggesting that the carboxyl portions of MRF4 and MyoD serve distinct functions for their respective proteins.

When the activation domain of MRF4 was deleted (MRF4Δ5′), the activity of the cotransfected α-actin reporter gene and the number of cells expressing the endogenous contractile-protein genes decreased 62 to 73% (Fig. 5A). This decrease occurred despite the ability of the truncated protein to bind to targeted E-box sequences (Fig. 6) and to be transported to the nucleus, suggesting that the activation domain of MRF4, as defined by GAL4 CAT assays, plays a crucial role in maintaining the transcriptional activity of this regulatory protein. Interestingly, full transcriptional activity, plus the ability to activate the endogenous myogenic program, was restored to the MRF4 N-truncated protein when the activation domain of MRF4 was replaced with the activation domain of MyoD (MyoDMRF4Δ5′). Thus, the MRF4 activation domain is required to elicit a full myogenic conversion response. In addition, the MRF4 and MyoD activation domains are interchangeable with respect to *trans* activating cotransfected α-actin reporter genes or to activating the endogenous contractile-protein genes and myogenic program.

In an effort to establish the importance of the MyoD activation domain in myogenic conversion, we also tested the corresponding truncated MyoD and chimeric proteins for their abilities to activate cotransfected α-actin reporter genes and skeletal myogenesis. As shown in Fig. 5B, truncation of the carboxyl-terminal region of MyoD (MyoDΔ3′) did not affect the *trans* activation of cotransfected α-actin reporter genes or the activation of the endogenous myogenic program. Interestingly, substitution of the MyoD carboxyl domain with the corresponding region from MRF4 (amino acids 182 to 242) generated a chimeric muscle regulatory factor (MyoDΔ3′MRF4) which produced a twofold-higher level of α-actin expression as well as a twofold increase in the number of converted myogenic cells compared with the levels obtained with the wild-type MyoD protein. These results further suggest that the carboxyl domain of MRF4 plays an important role in establishing the myogenic program.

As expected, deletion of the MyoD activation domain (MyoDΔ5′) led to an approximately 70% reduction in α-actin expression, although the number of differentiated muscle cells that were produced decreased only 25% compared with the number produced with the full-length MyoD protein (Fig.

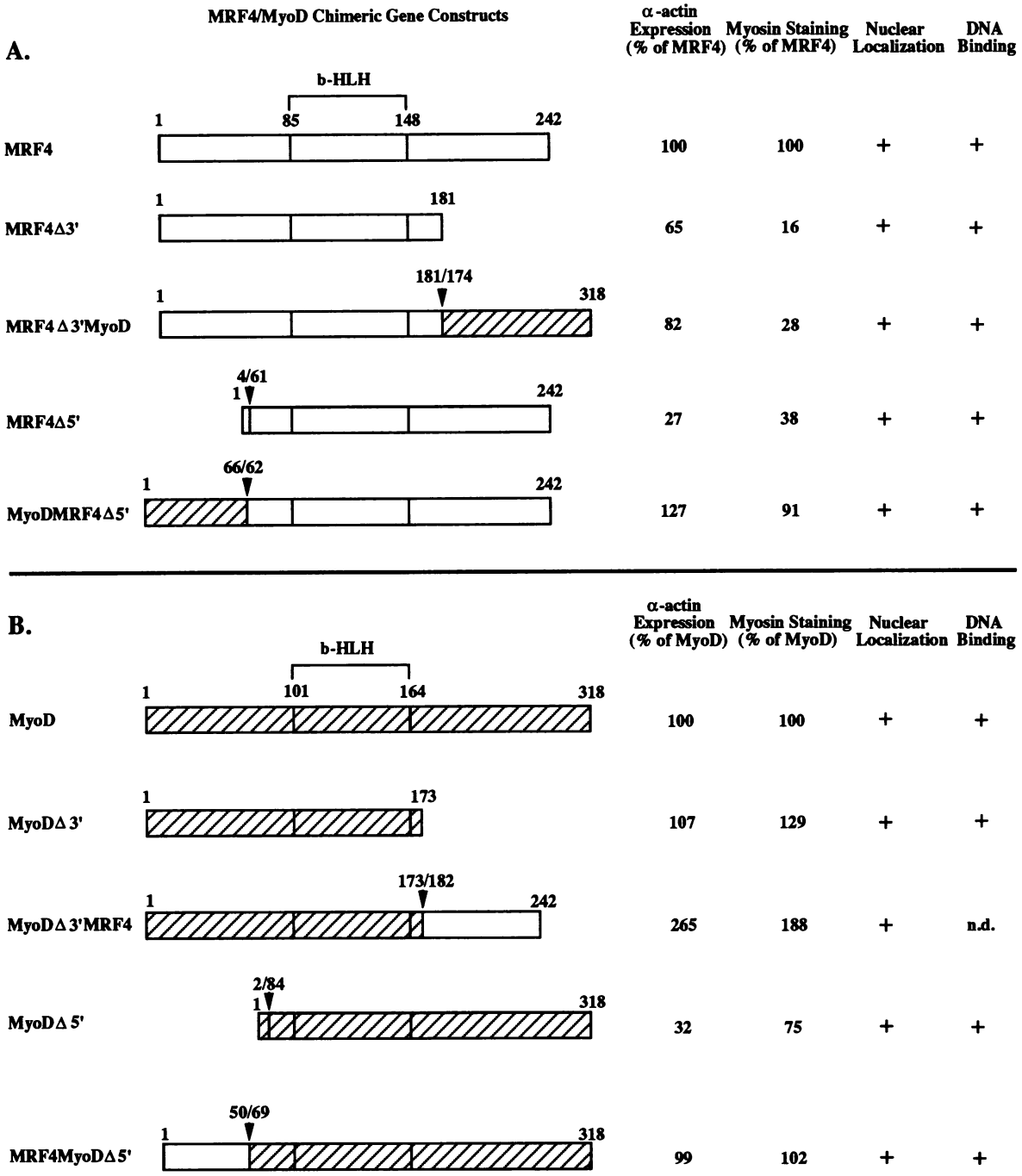

FIG. 5. The amino-terminal regions of MRF4 and MyoD can substitute for each other to confer a complete myogenic response. MRF4 and MyoD deletions plus MRF4-MyoD chimeric protein expression vectors were introduced into C3H10T1/2 fibroblasts with an α-actin CAT reporter gene. After differentiation, parallel dishes were processed for CAT activity, endogenous myosin expression, and muscle regulatory factor nuclear localization as described in Materials and Methods. All values were normalized to those obtained in parallel experiments with the wild-type MRF4 protein (A) or the wild-type MyoD protein (B) as controls. Each value is the average of three to seven independent transfections with the exception of the myosin staining values obtained with the MyoD chimeric proteins (B), which were derived from a single experiment. n.d., not determined.

5B). Addition of the MRF4 activation domain to an N-truncated MyoD protein that lacked an activation domain, however, restored full activity with respect to both α-actin and myosin expression, again suggesting that the activation domains of MyoD and MRF4 are necessary for the proteins to fully activate myogenesis. Thus, in the context of these assays, the MRF4 and MyoD activation domains can be substituted for each other to yield functional muscle regulatory factors.

MRF4 and MyoD activation domains partially dictate differential *trans*-activation responses. Previous studies have shown that MRF4 and MyoD differentially activate expres-

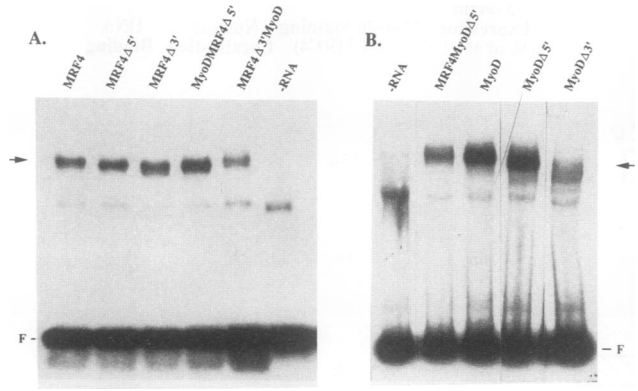

FIG. 6. MRF4 and MyoD deletions and MRF4-MyoD chimeric proteins bind to the TnI E-box sequence in the presence of E12. A ^{32}P-labeled TnI E-box oligonucleotide (A) or the entire TnI enhancer (B) was incubated with in vitro-synthesized E12 proteins plus the indicated in vitro-synthesized MRF4 and MyoD proteins. The relatively weak binding of the MyoDΔ3′ protein is due to the low concentration of the protein used in this reaction and does not reflect a difference in binding affinity. Unbound ^{32}P-labeled GAL4 binding sites are denoted by F.

sion of cotransfected reporter genes despite a relatively equal myogenic conversion frequency (10, 57). MyoD activates expression of the α-actin, TnI, and MCK genes, whereas MRF4 efficiently activates only the α-actin gene, even though the MRF4 protein binds to the enhancer elements driving expression of the TnI and MCK genes (29). The reason for the different activation potentials of these two muscle regulatory factors remains unknown. One possibility that we explored was that their respective activation domains, which are unrelated at the primary amino acid sequence (43), may dictate how each protein transcriptionally activates expression of muscle-specific genes. In order to examine this possibility further, we compared the ability of each truncated and chimeric MRF4 and MyoD protein to activate expression of cotransfected TnI and α-actin genes.

Transfection of C3H10T1/2 fibroblasts with 1 μg of the MyoD expression vector and 5 μg of the TnI CAT reporter gene produced a level of CAT activity approximately 20 fold higher than that obtained with the MRF4 expression vector (Fig. 7). Conversely, cotransfection of MyoD or MRF4 with the α-actin reporter gene produced roughly equivalent levels of CAT expression. Deletion of the 3′ terminus of MRF4 (MRF4Δ3′) led to a slight decrease in α-actin expression and a slight increase in TnI expression compared with expression by the full-length MRF4 protein. Addition of the carboxyl region of MyoD (amino acids 174 to 318) did not significantly alter the ability of the MRF4Δ3′ protein to *trans* activate expression of the α-actin and TnI reporter genes. As reported above, deletion of the 5′ terminus of MRF4 (MRF4Δ5′) reduced the amount of α-actin expression by approximately 70% but had no effect on the low level of TnI expression obtained with the full-length MRF4 protein, again indicating that the amino terminus of MRF4 is required to elicit a full transcriptional response when tested with the α-actin reporter. Substitution of the MyoD activation domain onto this MRF4 deletion mutant, however, not only restored α-actin expression but also produced a chimeric protein (MyoDMRF4Δ5′) that exhibited an appropriate fivefold-higher level of TnI expression than was obtained with the wild-type MRF4 protein (Fig. 7). Thus, the MyoD activation domain is capable of conferring partial transcriptional specificity on the MRF4 protein when tested with the TnI reporter gene.

Similar tests of the MyoD deletions and chimeric proteins produced results which were consistent with the hypothesis that the amino-terminal domains of MRF4 and MyoD are involved in controlling the specificity of their respective proteins. Again, the full-length and carboxyl-truncated MyoD proteins exhibited high and relatively equal levels of *trans* activation for both the α-actin and TnI reporter genes (Fig. 7). Substitution of the carboxyl domain of MRF4 onto the MyoDΔ3′ protein produced a twofold-higher level of α-actin expression but did not result in a similar increase in TnI activation compared with the C-truncated or wild-type MyoD proteins, suggesting that the carboxyl domain of MRF4 also imparts some specificity to the transcriptional activity of the MRF4 protein.

As expected, deletion of MyoD amino acids 3 to 83 resulted in an approximately 70% reduction in α-actin and TnI expression in these assays (Fig. 7). Interestingly, substitution of the MRF4 activation domain onto an N-truncated MyoD protein restored full *trans*-activation functions when the α-actin reporter gene was tested, whereas only a twofold increase in TnI expression was detected compared with expression with MyoDΔ5′. Thus, although the MRF4 and MyoD activation domains are interchangeable with respect to their abilities to activate the α-actin reporter gene, these activation domains exhibit different specificities in their abilities to activate the TnI reporter gene. The different primary amino acid sequences associated with these two domains suggest the possibility that the domains interact with different protein partners to assemble an active transcription complex on muscle-specific enhancer elements.

DISCUSSION

MRF4, MyoD, myogenin, and Myf-5 are muscle-specific transcription factors that contain a common structural motif referred to as the bHLH domain. Although these proteins exhibit over 85% similarity within this region, their respective amino and carboxyl termini are divergent (reviewed in reference 37). In an effort to examine how the amino and carboxyl portions of the MRF4 protein regulate myogenic activity, we examined these regions for the presence of a functional transcriptional activation domain. Utilizing a heterologous GAL4 assay system, we established that MRF4 amino acids 10 to 30 are sufficient to confer a strong activation response when ligated in frame to the GAL4 DNA-binding domain. Similarly, deletion of the MRF4 amino terminus produced a truncated protein that is impaired in its abilities to activate expression of cotransfected α-actin genes and to activate the endogenous myogenic program. Thus, the amino terminus of MRF4 contains a functional activation domain that is required and sufficient to elicit a transcriptional response.

The 21-amino-acid MRF4 activation domain has an overall net negative charge due to the presence of 4 acidic residues, which is a common feature in activation domains (reviewed in reference 32). Several studies have shown that the presence of acidic amino acids often is a critical component of activation domain structures (20). An additional feature of the MRF4 activation domain is that the acidic residues are positioned on the hydrophilic face of the peptide when tested in a helical-wheel model (48). Interestingly, the MyoD, myogenin, and Myf-5 activation domains also share this characteristic (30a). Thus, the MRF4 activation domain

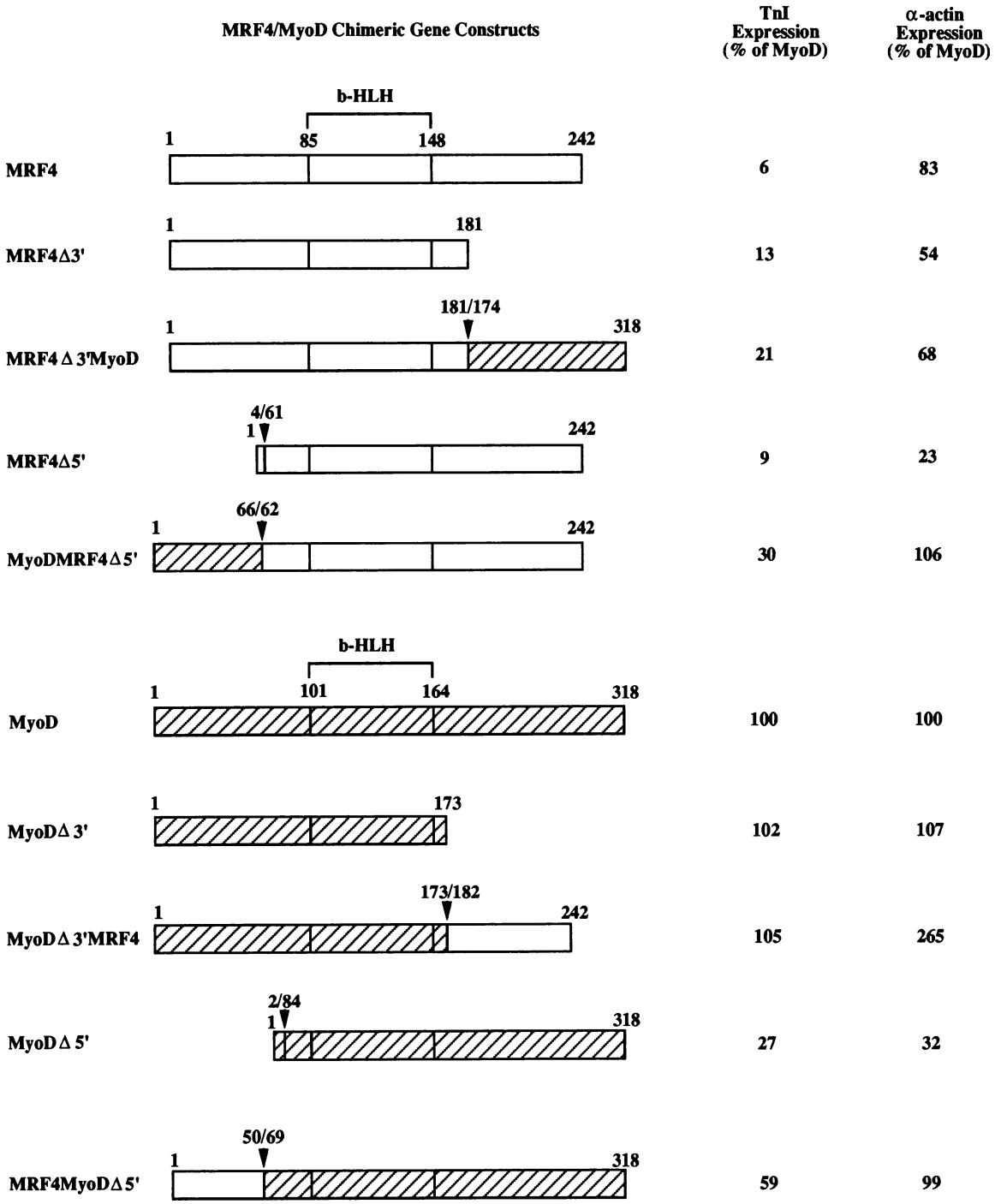

FIG. 7. MRF4, MyoD, and MRF4-MyoD chimeric proteins differentially *trans* activate cotransfected TnI and α-actin CAT reporter genes. Each indicated muscle regulatory factor expression vector was cotransfected with TnI or α-actin CAT reporter genes into C3H10T1/2 fibroblasts and assayed for CAT activity as described in the legend to Fig. 5. All values are normalized to the CAT expression levels obtained with the wild-type MyoD protein, which was set to 100%. Each value is the average of three to seven independent transfections.

contains both a net negative charge and a specific arrangement of hydrophobic residues which characterize several known activation domains, including those found within the VP16, SP1, GAL4, and GCN4 transcription factors (12, 45). Understanding the significance of these ionic and structural motifs to the function of the MRF4 activation domain will require a detailed in vitro mutagenesis approach to establish the importance of each residue within this 21-amino-acid region.

As discussed above, the activation domains of MyoD (53), myogenin (49), and Myf-5 (6) recently were identified. The activation domain of MyoD is located within the amino terminus (amino acids 3 to 53), while the activation domain of Myf-5 is found within the carboxyl region (amino acids

135 to 255). In contrast, myogenin contains two equally active activation domains that are present in both the amino (amino acids 7 to 77) and carboxyl (amino acids 156 to 224) regions. In all cases, the conserved bHLH domain present in each muscle regulatory factor is required for myogenesis but does not serve as a transcriptional activator. Thus, the nonconserved N- and C-terminal regions of these related proteins exhibit abilities to transcriptionally activate expression of specific reporter genes. Since the activation domains are unrelated at the primary amino acid sequence, it is conceivable that these distinct activation domains serve different roles in controlling gene expression during skeletal muscle development.

Although the MRF4 and MyoD activation domains are unrelated, our studies have shown that they can substitute for one another. Deletion of the MRF4 activation domain, for example, renders the MRF4 protein ineffective in activating the endogenous myogenic program and in activating expression of cotransfected α-actin genes. Addition of the MyoD activation domain (amino acids 1 to 66), however, restores full activity. Interestingly, the MRF4 and MyoD activation domains impart some specificity, since MyoDMRF4Δ5′ activates expression of a cotransfected TnI reporter gene to higher levels than are obtained with MRF4 or MRF4Δ5′ cotransfections. Parallel experiments replacing the MyoD activation domain with the MRF4 activation domain also support the hypothesis that the two activation domains have different specificities. Although MRF4MyoD Δ5′ efficiently activates expression of α-actin, this chimeric protein does not activate expression of the TnI gene to the levels attained with the wild-type MyoD protein. Nonetheless, it is clear that the activation domains of MRF4 and MyoD are not solely responsible for this differential *trans*-activation level. Substitution of the MyoD activation domain onto the N-truncated MRF4 protein restores only partial TnI *trans* activation compared with the levels obtained with the complete MyoD protein.

Construction of MRF4-MyoD chimeric proteins sometimes produced muscle regulatory factors that were more active than the wild-type MRF4 or MyoD proteins. The most potent chimeric protein in these studies was MyoDΔ3′ MRF4, which activated α-actin CAT and the endogenous myogenic program approximately twofold more than did MRF4 or MyoD. These data correlate with the decrease in myogenic activity observed by deleting the carboxyl portion of MRF4, suggesting that MRF4 amino acids 182 to 242, although not transcriptionally active when tested in GAL4 assays, nonetheless serve an important role in controlling myogenesis. A similar increase in myogenic activity also has been observed with myogenin-VP16 chimeric proteins in which the carboxyl domain of myogenin was replaced with the VP16 activation domain (49). Studies to establish the mechanisms by which the MyoDΔ3′MRF4 protein potentiates myogenesis more efficiently than the normal muscle regulatory factors currently are under way.

The results from our studies indicate that the domains of MyoD and MRF4 that lie outside of the bHLH region play an important role in regulating the activity of these proteins. In agreement with this finding, Chakraborty and Olson (11) demonstrated that the MRF4 and myogenin regions flanking the bHLH domain also have a role in regulating the myogenic activity of these proteins. Substitution of the myogenin amino domain onto a truncated MRF4 protein led to an approximately threefold increase in CAT activity for the MCK enhancer, which normally is efficiently activated by myogenin but not by MRF4 (11, 57). Similarly, substitution of the MRF4 amino terminus onto an N-truncated myogenin protein produced a chimeric transcription factor that no longer activated MCK expression even though it contained the bHLH and carboxyl regions of the myogenin protein. Thus, the activation domains of MRF4, MyoD, and myogenin impart some transcriptional specificity, but the mechanisms of action or the targets that these regions must interact with remain unknown.

Although it is clear that the activation domains of the muscle regulatory factors are essential to eliciting a myogenic response, these regions must function in concert with their associated bHLH domains. The muscle regulatory factors contain two highly conserved residues in their basic region (alanine 98 and threonine 99 for MRF4) that are essential for myogenesis (7). Mutation of these residues in MRF4, myogenin, and MyoD completely blocks the ability of the proteins to activate muscle-specific genes or to convert C3H10T1/2 fibroblasts to a myogenic lineage (7, 53; unpublished results). Substitution of the myogenin or MyoD basic domains with the related basic domain from E12 also inhibits the protein from activating the myogenic program (7, 53). These and other studies support the notion that the basic domain of the muscle regulatory factors interacts with additional nuclear proteins to produce the correct transcriptional response when the cells are presented with growth factor-depleted medium. Whether the putative coregulator(s) interacts with a muscle regulatory factor basic domain, activation domain, or both is open to speculation. However, since the activation domains of MRF4, MyoD, myogenin, and Myf-5 are unrelated at the amino acid level and often are situated in different regions of the protein, it is conceivable that a common coregulator could interact with the basic domain while other, distinct factors interact with a subset of the muscle regulatory factors.

Trying to understand how these disparate activation domains function in development will require further mutagenesis studies. Although truncated and MRF4-MyoD or MRF4-myogenin chimeric proteins have shed some light on the role that the amino and carboxyl regions play in controlling the specific transcriptional activities of these proteins, these experiments must be interpreted with caution. For example, MRF4Δ5′ and MyoDΔ5′ lack their respective activation domains and yet retain substantial *trans*-activation capabilities. Since these truncated proteins dimerize with E12 and bind to E-box regulatory sequences, the residual transcriptional activity may be due to the E12 activation domain when E12 is positioned within the context of these truncated muscle regulatory factors. Additionally, it is unclear whether the modest differential *trans*-activation level obtained with MyoDMRF4Δ5′ is due to the MyoD activation domain not fully supporting *trans* activation of the TnI reporter gene or to an incompatibility of the MyoD activation domain with the rest of the MRF4 protein. In either case, it remains possible that the activation domains associated with each muscle regulatory factor interact with different transcriptional coregulators to elicit a correct transcriptional response. Identifying these putative coregulator targets will be essential to fully understanding the molecular mechanisms by which the muscle regulatory factors control skeletal myogenesis.

ACKNOWLEDGMENTS

We thank M. Green and S. Triezenberg for generously providing control GAL4 gene constructs, T. Hinterberger for generating GAL4MyoD(1-66), and S. Rhodes for the preparation and characterization of the anti-MRF4 and anti-MyoD sera. We are very

grateful to Lea Longcor for excellent technical assistance and to Connie Philbrook for the preparation of the manuscript.

This work was funded by grants to S.F.K. from the National Institutes of Health. S.F.K. is an Established Investigator of the American Heart Association.

REFERENCES

1. **Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.).** 1987. Current protocols in molecular biology, vol. 2. John Wiley & Sons, Inc., New York.
2. **Bader, D., T. Masaki, and D. A. Fischman.** 1982. Immunochemical analysis of myosin heavy chain during avian myogenesis *in vivo* and *in vitro*. J. Cell Biol. **95**:763–770.
3. **Braun, T., and H. H. Arnold.** 1991. The four human muscle regulatory helix-loop-helix proteins Myf 3-Myf6 exhibit similar hetero-dimerization and DNA binding properties. Nucleic Acids Res. **19**:5645–5651.
4. **Braun, T., E. Bober, B. Winter, N. Rosenthal, and H. H. Arnold.** 1990. Myf-6, a new member of the human gene family of myogenic determination factors: evidence for a gene cluster on chromosome 12. EMBO J. **9**:821–831.
5. **Braun, T., G. Buschhausen-Denker, E. Bober, E. Tannich, and H. H. Arnold.** 1989. A novel human muscle factor related to but distinct from MyoD1 induces myogenic conversion in 10T1/2 fibroblasts. EMBO J. **8**:701–709.
6. **Braun, T., B. Winter, E. Bober, and H. H. Arnold.** 1990. Transcriptional activation domain of the muscle-specific gene regulatory protein myf5. Nature (London) **346**:663–665.
7. **Brennan, T. J., T. Chakraborty, and E. N. Olson.** 1991. Mutagenesis of the myogenin basic region identifies an ancient protein motif critical for activation of myogenesis. Proc. Natl. Acad. Sci. USA **88**:5675–5679.
8. **Brennan, T. J., and E. N. Olson.** 1990. Myogenin resides in the nucleus and acquires high affinity for a conserved enhancer element on heterodimerization. Genes Dev. **4**:582–595.
9. **Chakraborty, T., T. J. Brennan, L. Li, D. Edmondson, and E. N. Olson.** 1991. Inefficient homooligomerization contributes to the dependence of myogenin on E2A products for efficient DNA binding. Mol. Cell. Biol. **11**:3633–3641.
10. **Chakraborty, T., T. J. Brennan, and E. N. Olson.** 1991. Differential trans-activation of a muscle-specific enhancer by myogenic helix-loop-helix proteins is separable from DNA binding. J. Biol. Chem. **266**:2878–2882.
11. **Chakraborty, T., and E. N. Olson.** 1991. Domains outside of the DNA-binding domain impart target gene specificity to myogenin and MRF4. Mol. Cell. Biol. **11**:6103–6108.
12. **Cress, W. D., and S. J. Triezenberg.** 1991. Critical structural elements of the VP16 transcriptional activation domain. Science **251**:87–90.
13. **Davis, R. L., P.-F. Cheng, A. B. Lassar, and H. Weintraub.** 1990. The MyoD DNA binding domain contains a recognition code for muscle-specific gene activation. Cell **60**:733–746.
14. **Davis, R. L., H. Weintraub, and A. B. Lassar.** 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. Cell **51**:987–1000.
15. **Edmondson, D. G., T. J. Brennan, and E. N. Olson.** 1991. Mitogenic repression of myogenin autoregulation. J. Biol. Chem. **266**:21343–21346.
16. **Edmondson, D. G., and E. N. Olson.** 1989. A gene with homology to the *myc* similarity region of MyoD1 is expressed during myogenesis and is sufficient to activate the muscle differentiation program. Genes Dev. **3**:628–640.
17. **Emerson, C. P.** 1990. Myogenesis and developmental control genes. Cell Differ. **2**:1065–1075.
18. **Farmer, K., F. Catala, and W. E. Wright.** 1992. Alternative multimeric structures affect myogenin DNA binding activity. J. Biol. Chem. **267**:5631–5636.
19. **French, B. A., K.-L. Chow, E. N. Olson, and R. J. Schwartz.** 1991. Heterodimers of myogenic helix-loop-helix regulatory factors and E12 bind a complex element governing myogenic induction of the avian cardiac α-actin promoter. Mol. Cell. Biol. **11**:2439–2450.
20. **Gill, G., I. Sadowski, and M. Ptashne.** 1990. Mutations that increase the activity of a transcriptional activator in yeast and mammalian cells. Proc. Natl. Acad. Sci. USA **87**:2127–2131.
21. **Gilmour, B. P., G. R. Fanger, C. Newton, S. M. Evans, and P. D. Gardner.** 1991. Multiple binding sites for myogenic regulatory factors are required for expression of the acetylcholine receptor γ-subunit gene. J. Biol. Chem. **266**:19871–19874.
22. **Gorman, C. M., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
23. **Gossett, L. A., D. J. Kelvin, E. A. Sternberg, and E. N. Olson.** 1989. A new myocyte-specific enhancer-binding factor that recognizes a conserved element associated with multiple muscle-specific genes. Mol. Cell. Biol. **9**:5022–5033.
24. **Hinterberger, T. J., D. A. Sassoon, S. J. Rhodes, and S. F. Konieczny.** 1991. Expression of the muscle regulatory factor MRF4 during somite and skeletal myofiber development. Dev. Biol. **147**:144–156.
25. **Jaynes, J. B., J. S. Chamberlain, J. N. Buskin, J. E. Johnson, and S. D. Hauschka.** 1986. Transcriptional regulation of the muscle creatine kinase gene and regulated expression in transfected mouse myoblasts. Mol. Cell. Biol. **6**:2855–2864.
26. **Lassar, A. B., J. N. Buskin, D. Lockshon, R. L. Davis, S. Apone, S. D. Hauschka, and H. Weintraub.** 1989. MyoD is a sequence-specific DNA binding protein requiring a region of *myc* homology to bind to the muscle creatine kinase enhancer. Cell **58**:823–831.
27. **Lassar, A. B., R. L. Davis, W. E. Wright, T. Kadesch, C. Murre, A. Voronova, D. Baltimore, and H. Weintraub.** 1991. Functional activity of myogenic HLH proteins requires hetero-oligomerization with E12/E47-like proteins *in vivo*. Cell **66**:305–315.
28. **Lillie, J. W., and M. R. Green.** 1989. Transcription activation by the adenovirus E1a protein. Nature (London) **338**:39–44.
29. **Lin, H., and S. F. Konieczny.** 1992. Identification of MRF4, myogenin, and E12 oligomer complexes by chemical cross-linking and two-dimensional gel electrophoresis. J. Biol. Chem. **267**:4773–4780.
30. **Lin, H., K. E. Yutzey, and S. F. Konieczny.** 1991. Muscle-specific expression of the troponin I gene requires interactions between helix-loop-helix muscle regulatory factors and ubiquitous transcription factors. Mol. Cell. Biol. **11**:267–280.

30a. **Mak, K.-L.** Unpublished data.

31. **Miner, J. H., and B. Wold.** 1990. Herculin, a fourth member of the MyoD family of myogenic regulatory genes. Proc. Natl. Acad. Sci. USA **87**:1089–1093.
32. **Mitchell, P. J., and R. Tjian.** 1989. Transcription regulation in mammalian cells by sequence-specific DNA binding proteins. Science **245**:371–378.
33. **Miwa, T., and L. Kedes.** 1987. Duplicated CArG box domains have positive and mutually dependent regulatory roles in expression of the human α-cardiac actin gene. Mol. Cell. Biol. **7**:2803–2813.
34. **Murre, C., P. Schonleber McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, *daughterless*, *MyoD*, and *myc* proteins. Cell **56**:777–783.
35. **Murre, C., P. Schonleber McCaw, H. Vaessin, M. Caudy, L. Y. Jan, Y. N. Jan, C. V. Cabrera, J. N. Buskin, S. D. Hauschka, A. B. Lassar, H. Weintraub, and D. Baltimore.** 1989. Interactions between heterologous helix-loop-helix proteins generate complexes that bind specifically to a common DNA sequence. Cell **58**:537–544.
36. **Nielsen, D. A., J. Chou, A. J. MacKrell, M. J. Casadaban, and D. F. Steiner.** 1983. Expression of a preproinsulin-β-galactosidase gene fusion in mammalian cells. Proc. Natl. Acad. Sci. USA **80**:5198–5202.
37. **Olson, E. N.** 1990. MyoD family: a paradigm for development? Genes Dev. **4**:1454–1461.
38. **Olson, E. N., T. J. Brennan, T. Chakraborty, T.-C. Cheng, P. Cserjesi, D. Edmondson, G. James, and L. Li.** 1991. Molecular control of myogenesis: antagonism between growth and differentiation. Mol. Cell. Biochem. **104**:7–13.

39. **Ott, M.-O., E. Boder, G. Lyons, H. Arnold, and M. Buckingham.** 1991. Early expression of the myogenic regulatory gene, myf-5, in precursor cells of skeletal muscle in the mouse embryo. Development **111**:1097–1107.
40. **Piette, J., J.-L. Bessereau, M. Huchet, and J.-P. Changeaux.** 1990. Two adjacent MyoD1 binding sites regulate expression of the acetylcholine receptor α-subunit. Nature (London) **345**:353–355.
41. **Prody, C. A., and J. P. Merlie.** 1991. A developmental and tissue-specific enhancer in the mouse skeletal muscle acetylcholine receptor α-subunit gene regulated by myogenic factors. J. Biol. Chem. **266**:22588–22596.
42. **Rhodes, S. J.** 1991. Identification, cloning and characterization of MRF4: a new muscle regulatory factor. Ph.D. thesis, Purdue University, West Lafayette, Ind.
43. **Rhodes, S. J., and S. F. Konieczny.** 1989. Identification of MRF4: a new member of the muscle regulatory factor gene family. Genes Dev. **3**:2050–2061.
44. **Rosenthal, N., E. B. Berglund, B. M. Wentworth, M. Donoghue, B. Winter, E. Bober, T. Braun, and H.-H. Arnold.** 1990. A highly conserved enhancer downstream of the human MLC1/3 locus is a target for multiple myogenic determination factors. Nucleic Acids Res. **18**:6239–6246.
45. **Ruden, D. M.** 1992. Activating regions of yeast transcription factors must have both acidic and hydrophobic amino acids. Chromosoma **101**:342–348.
46. **Sartorelli, V., K. A. Webster, and L. Kedes.** 1990. Muscle-specific expression of the cardiac α-actin gene requires MyoD1, CArG-box binding factor, and Sp1. Genes Dev. **4**:1811–1822.
47. **Sassoon, D., G. Lyons, W. E. Wright, V. Lin, A. Lassar, H. Weintraub, and M. Buckingham.** 1989. Expression of two myogenic regulatory factors, myogenin and MyoD1 during mouse embryogenesis. Nature (London) **341**:303–307.
48. **Schiffer, M., and A. B. Edmundson.** 1967. Use of helical wheels to represent the structures of proteins and to identify segments with helical potential. Biophys. J. **7**:121–135.
49. **Schwartz, J. J., T. Chakraborty, J. Martin, J. Zhou, and E. N. Olson.** 1992. The basic region of myogenin cooperates with two transcription activation domains to induce muscle-specific transcription. Mol. Cell. Biol. **12**:266–275.
50. **Tapscott, S. J., and H. Weintraub.** 1991. MyoD and the regulation of myogenesis by helix-loop-helix proteins. J. Clin. Invest. **87**:1133–1138.
51. **Thayer, M. J., S. J. Tapscott, R. L. Davis, W. E. Wright, A. B. Lassar, and H. Weintraub.** 1989. Positive autoregulation of the myogenic determination gene MyoD1. Cell **58**:241–248.
52. **Weintraub, H., R. Davis, D. Lockshon, and A. Lassar.** 1990. MyoD binds cooperatively to two sites in a target enhancer sequence: occupancy of two sites is required for activation. Proc. Natl. Acad. Sci. USA **87**:5623–5627.
53. **Weintraub, H., V. J. Dwarki, I. Verma, R. Davis, S. Hollenberg, L. Snider, A. Lassar, and S. J. Tapscott.** 1991. Muscle-specific transcriptional activation by MyoD. Genes Dev. **5**:1377–1386.
54. **Wentworth, B. M., M. Donoghue, J. C. Engert, E. B. Berglund, and N. Rosenthal.** 1991. Paired MyoD binding sites regulate myosin light chain gene expression. Proc. Natl. Acad. Sci. USA **88**:1242–1246.
55. **Wright, W. E., D. A. Sassoon, and V. K. Lin.** 1989. Myogenin, a factor regulating myogenesis, has a domain homologous to MyoD. Cell **56**:607–617.
56. **Yutzey, K. E., R. L. Kline, and S. F. Konieczny.** 1989. An internal regulatory element controls troponin I gene expression. Mol. Cell. Biol. **9**:1397–1405.
57. **Yutzey, K. E., S. J. Rhodes, and S. F. Konieczny.** 1990. Differential *trans*-activation associated with the muscle regulatory factors MyoD1, myogenin, and MRF4. Mol. Cell. Biol. **10**:3934–3944.