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Domains Outside of the DNA-Binding Domain Impart Target Gene Specificity to Myogenin and MRF4

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Myogenin and MRF4 belong to the MyoD family of muscle-specific transcription factors, which can activate myogenesis when introduced into nonmyogenic cells. These proteins share homology within a basic-helix-loop-helix motif that mediates DNA binding and dimerization, but they are divergent in their amino and carboxyl termini. Although myogenin and MRF4 bind the same sequence within the muscle creatine kinase enhancer, only myogenin efficiently transactivates this enhancer. By creating chimeras of myogenin and MRF4, we show that the specificities of these factors for transactivation of the muscle creatine kinase enhancer can be interchanged by swapping their amino and carboxyl termini. Within these chimeras, strong cooperation between the amino and carboxyl termini was observed. These findings suggest that myogenin and MRF4 discriminate between muscle-specific enhancers and that target gene specificity is determined by domains surrounding the basic-helix-loop-helix region.

The MyoD family includes MyoD (12), myogenin (13, 35), myf5 (2), and MRF4 (1, 19, 24), each of which is expressed exclusively in skeletal muscle and can activate myogenesis when introduced into a variety of nonmyogenic cell types (reviewed in references 22 and 31). These factors share extensive homology within a segment of about 70 amino acids that encompasses a basic-helix-loop-helix (bHLH) motif that mediates DNA binding and dimerization (4, 11, 17, 20); however, these factors show little or no homology outside of this region. Members of the MyoD family bind DNA preferentially as heterooligomers with the widely expressed HLH proteins E12 and E47 (3, 5, 8, 9, 11, 20, 21). These heterooligomers bind with high affinity to the sequence CANNTG, referred to as an E-box, which is present in the control regions of numerous muscle-specific genes (3–6, 8, 9, 11, 14, 17, 23, 25, 34). Cooperative interactions among multiple E-boxes and binding sites for other muscle-specific and ubiquitous factors are required for muscle-specific transcription (14, 17, 18, 23, 25, 33, 34).

A key question that remains unanswered is whether each member of the MyoD family is functionally distinct. Indeed, the ability of all of these factors to bind the same DNA sequence raises the question of whether they are functionally redundant or exhibit unique properties separable from DNA binding. In this regard, MRF4 has been shown to bind the muscle creatine kinase (MCK) and troponin I enhancers; however, unlike myogenin, MyoD, or myf5, it fails to activate transcription from these enhancers (9, 36). The differential transcriptional activities of myogenin and MRF4 could, in principle, be mediated by their divergent amino or carboxyl termini or by amino acid differences within the conserved bHLH region or both. To distinguish among these possibilities, we created tripartite chimeras of myogenin and MRF4 and tested their abilities to transactivate the MCK enhancer. Here we show that the amino and carboxyl termini of myogenin cooperate with the bHLH region to activate the MCK enhancer and that the specificities of myogenin and MRF4 for transactivation of the MCK enhancer can be interchanged by swapping their amino and carboxyl termini. Although myogenin and MRF4 differentially regulate the MCK enhancer, they activate a reporter gene linked to a multimerized E-box to equivalent levels, suggesting that protein-protein interactions among factors that bind sites surrounding the E-box contribute to enhancer-specific transactivation.

MATERIALS AND METHODS

Cell culture and transfections. C3H10T1/2 (10T1/2) cells were transfected by calcium phosphate precipitation as described previously (29). Twenty-four hours after transfection, cultures were transferred to differentiation medium (Dulbecco's modified Eagle's medium with 2% horse serum) for 48 h, after which time cells were harvested and chloramphenicol acetyltransferase (CAT) activity in aliquots of cell extracts containing equivalent amounts of protein was determined. In some experiments, transfections were normalized by using 1 μg of Rous sarcoma virus-β-galactosidase as an internal standard. The MCK-CAT reporter gene, referred to previously as pCKCATe4, contains the 246-bp MCK promoter and the 300-bp 5′ enhancer inserted into the *Bam*HI site 3′ of CAT (29). MCK-tkCAT, also referred to as 4R-tkCAT (33), contains four copies of the MCK high-affinity, or right, E-box upstream of the thymidine kinase basal promoter and the CAT gene. Wild-type and mutant myogenin and MRF4 cDNAs were contained in the expression vector EMSV (12). Transfections were performed at least three times with the same results each time.

Mutagenesis. Chimeras were created by the introduction of *Spe*I and *Nsi*I sites on the amino- and carboxyl-terminal boundaries, respectively, of the bHLH regions of myogenin and MRF4. Site-directed mutagenesis was performed on single-stranded templates as described previously (4). All mutations were confirmed by sequencing. Introduction of the *Spe*I and *Nsi*I sites in myogenin resulted in substitution of lysine with threonine at codon 76 and substitution of leucine-serine with methionine-histidine at codons 136 and 137. The corresponding amino acid substitutions in MRF4 were lysine for threonine at codon 88 and leucine for

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methionine at codon 148. The oligonucleotides used for mutagenesis contained the following mutations, indicated by lowercase letters, with the restriction sites underlined: mutant myo-*Spe*I, 5′-CCGGTCCACCGACACACtagtCCTCTT ACACACCTT-3′; myo-*Nsi*I, 5′-CTCCTGGTTGAGGGAat gcatCAAGGCCTGTAGGCG-3′; MRF4-*Spe*I, 5′-ACGATC TGTGGGGGCACtagTCTCTTGCAAGTCTT-3′; and MRF4-*Nsi*I, 5′-TTGCTGATCCAGCCGaTGCAtCAGGTCCTGCA GACG-3′.

Gel mobility shift assays. cDNAs encoding myogenin, MRF4, and myogenin-MRF4 chimeras were transcribed in vitro with T3 polymerase, and the corresponding RNAs were translated in a rabbit reticulocyte lysate in the presence of [^{35}S]methionine as described previously (4). Gel mobility shift assays were performed as described previously (4) by using an end-labeled probe that encompassed the MEF-1 site from the MCK enhancer. The binding was performed in the presence of 2 μg of poly(dI-dC) as a nonspecific competitor and excess labeled probe.

RESULTS

Myogenin and MRF4 activate a reporter plasmid containing a multimerized E-box to equivalent levels but differentially activate the MCK enhancer. Full activity of the MCK enhancer requires two E-boxes (5, 6, 16, 17, 29), a binding site for the myocyte-specific transcription factor MEF-2 (10, 15), and binding sites for ubiquitous and cell-type-restricted transcription factors that are not yet fully defined (28). Because myogenin and MRF4 bind with high affinities to one of the MCK E-boxes (9), known as the MEF-1 (6) or KE-2 site (CACCTGC), we imagined that the inability of MRF4 to transactivate the MCK enhancer could reflect the factor's nonproductive interaction with this site or its inability to cooperate with transcription factors that bind other essential sites in the enhancer. In an attempt to distinguish between these possibilities, we compared the abilities of myogenin and MRF4 to transactivate MCK-tkCAT, which contains four copies of the MCK MEF-1 site linked immediately upstream of the thymidine kinase promoter (33). Weintraub and coworkers have shown that MyoD can transactivate the thymidine kinase promoter when it is linked to two or more MEF-1 sites (33). This reporter plasmid is expected to be less dependent on cooperative interactions between myogenic regulatory factors and other enhancer-binding factors for activation than the complete MCK enhancer.

Expression vectors encoding myogenin and MRF4 were cotransfected with CAT reporter plasmids into 10T1/2 fibroblasts. As shown in Fig. 1, both myogenin and MRF4 strongly transactivated MCK-tkCAT; however, MCK-CAT, which contains the complete MCK 5′ enhancer (16, 29), showed differential responsiveness to these regulatory factors. The differences in activation of the MCK enhancer by myogenin and MRF4 occur over a plasmid concentration range of at least 20-fold and are not attributable to differences in the levels of expression of these factors (9). Although myogenin and MRF4 can activate the endogenous myogenic regulatory factor genes in 10T1/2 cells (1, 19, 24, 32), the level of expression of the endogenous genes is minimal in the time frame of these transient transfection assays (9), which allows the activities of the exogenous factors to be compared directly. Differential transactivation of the MCK enhancer by myogenin and MRF4 is also observed in NIH 3T3 cells (9), which are unable to autoactivate the endogenous myogenic regulatory factor genes (19). These results indicate that the inability of MRF4 to transactivate MCK-CAT does not reflect a general lack of transcription activation potential in MRF4 and that the differential responsiveness of the MCK enhancer to myogenin and MRF4 is not mediated by the MEF-1 site.

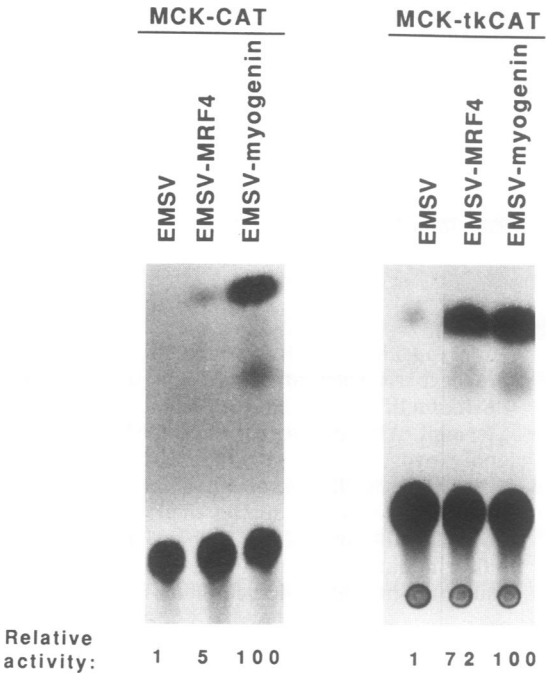


FIG. 1. Transactivation of target genes by myogenin and MRF4. 10T1/2 cells were transfected transiently with 5 μg of MCK-CAT or MCK-tkCAT (also referred to as 4R-tkCAT [33]) and 10 μg of EMSV, EMSV-myogenin or EMSV-MRF4, and CAT activity in cell extracts was determined as described in the text. Values for CAT activity are expressed relative to the activity observed in the presence of EMSV-myogenin, which was set at 100%. Transfections were performed at least three times, with the same results each time.

Generation of tripartite chimeras of myogenin and MRF4. To map the domains in myogenin and MRF4 responsible for differences in transactivation of the MCK enhancer, we introduced unique restriction sites at the amino- and carboxyl-terminal boundaries of the bHLH regions of these proteins and created tripartite chimeras by swapping their amino and carboxyl termini (Fig. 2). In naming the chimeras, we used the number 8 to refer to myogenin, because our original myogenin cDNA clone was designated myo8 (13), and the number 4 to refer to MRF4. Chimeras were assigned three numbers to indicate the origins of the three separate regions: amino terminus, bHLH region, and carboxyl terminus. Amino acid substitutions that resulted from the introduction of restriction sites did not alter the reading frames or properties of myogenin or MRF4. We confirmed that all chimeras were in frame by in vitro transcription and translation of the corresponding cDNAs and analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 3A). Like the wild-type proteins, the chimeras migrated anomalously on SDS-PAGE. By comparison of chimeras and wild-type proteins, it appears that the anomalous migration is due to the C termini, with the myogenin C terminus contributing to anomalously slow migration and the MRF4 C terminus contributing to anomalously fast migration. The influence of the C termini on apparent molecular weight can be seen by comparison of myogenin (8:8:8) and

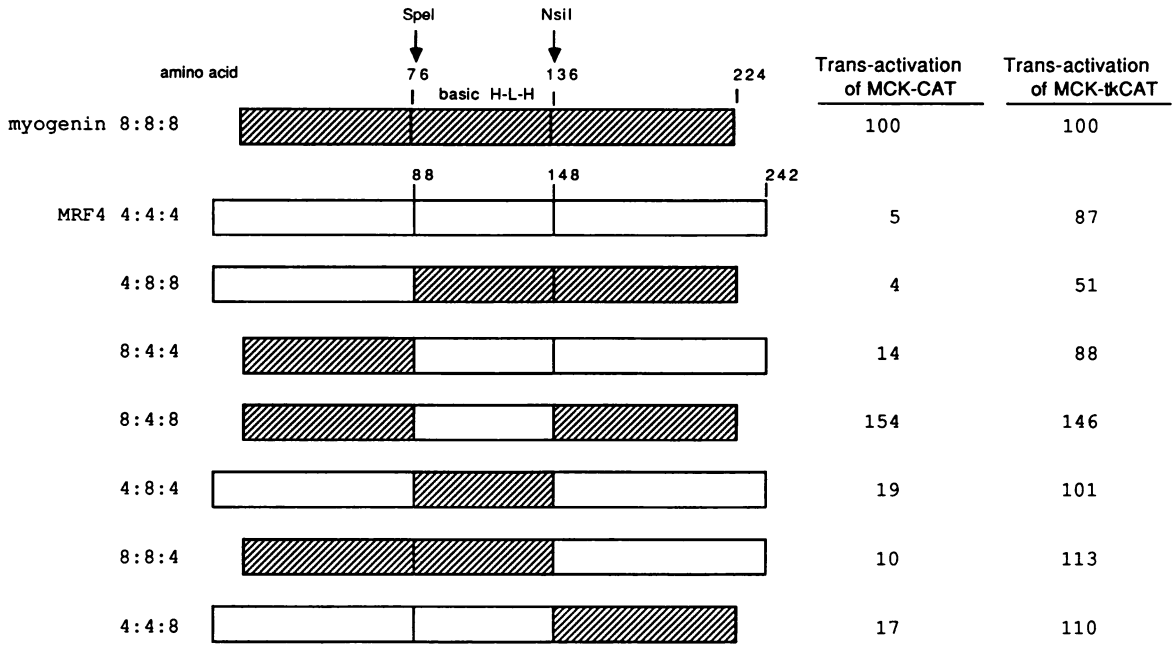


FIG. 2. Myogenin-MRF4 chimeras. Chimeras of myogenin and MRF4 were created by swapping their amino and carboxyl termini. The number 8 refers to myogenin (shaded bars), and the number 4 refers to MRF4 (open bars). The amino acids at the junctions for the swaps are indicated above myogenin and MRF4. The relative activities of each protein following transient transfection into 10T1/2 cells with MCK-CAT and MCK-tkCAT are indicated. Values are normalized to the level of CAT activity observed with myogenin, which was set at 100%. Background CAT activity when the reporter genes were cotransfected with EMSV alone was less than 5% of that with myogenin and has been subtracted from each value. Values represent the averages of at least three independent experiments and did not vary by more than 20%.

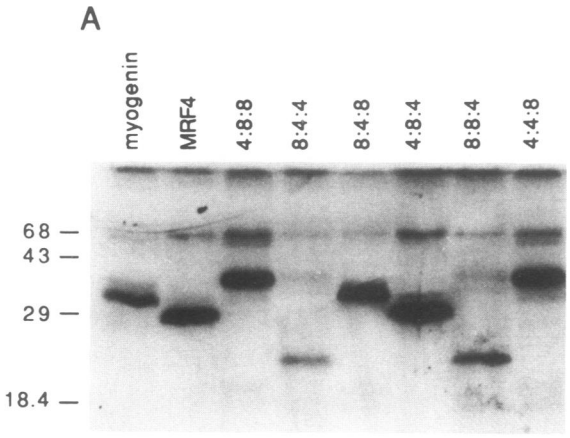
chimera 8:8:4, which differ only in their C termini. The presence of the MRF4 C terminus in chimera 8:8:4 leads to faster migration. Comparison of MRF4 (4:4:4) and 4:4:8 shows that, conversely, the myogenin C terminus retards migration.

To compare the DNA-binding properties of the chimeric proteins, the corresponding in vitro-synthesized RNAs were cotranslated with E12 RNA and DNA-binding activity was measured by gel mobility shift assays with a ^{32}P-labeled probe encompassing the MEF-1 site from the MCK enhancer. Equivalent quantities of each chimera and E12 were used in each binding reaction, as determined by parallel translations in the presence of [^{35}S]methionine. The results demonstrated that all chimeric proteins retained the ability to bind DNA in vitro (Fig. 3B). Although there were subtle differences in binding among the chimeras, these differences cannot account for their differential activities toward the MCK enhancer (see below).

Chimeras of myogenin and MRF4 show that enhancer-specific transactivation is determined by the amino and carboxyl termini. The regions required for transactivation of the MCK enhancer by myogenin were next examined by testing the myogenin-MRF4 chimeras in transactivation assays with MCK-CAT as the target. As shown in Fig. 2, replacement of either the amino or the carboxyl terminus of myogenin with the corresponding region of MRF4 resulted in a loss in transactivation potential (chimeras 4:8:8 and 8:8:4), suggesting that both regions of myogenin are important and that neither is sufficient for full activation of the MCK enhancer. That both the amino and the carboxyl termini of myogenin are important for efficient transactivation was further suggested by the lack of activity of chimeras containing only the amino or the carboxyl terminus of myogenin and the remainder of MRF4 (chimeras 8:4:4 and 4:4:8). However, when both the amino and the carboxyl termini of myogenin were fused to the bHLH region of MRF4 (chimera 8:4:8), high-level transactivation occurred. The observation that chimera 8:4:8 can efficiently transactivate the MCK enhancer shows that the amino acid differences between the bHLH regions of myogenin and MRF4 do not account for the failure of MRF4 to transactivate the MCK enhancer and that cooperation between the amino and carboxyl termini of myogenin is required for efficient transactivation of the MCK enhancer. In contrast to their dramatically different activities toward the MCK enhancer, each of the chimeras was able to transactivate MCK-tkCAT, in agreement with the abilities of myogenin and MRF4 to transactivate this target gene. These results imply that all of the chimeras are expressed and can bind DNA in vivo.

DISCUSSION

Our results show that domains surrounding the bHLH regions of myogenin and MRF4 can discriminate between target genes and that the transcriptional specificities of these factors can be interchanged by swapping their N and C termini. These results show that target gene specificity for transactivation by myogenic HLH proteins can be influenced by domains outside of the DNA-binding region and that events in addition to DNA binding are important for transcriptional activation. On the basis of these findings, we suggest that activation of the MCK enhancer, and presumably other muscle-specific control regions, depends on cooperative interactions between myogenic HLH proteins and other transcription factors that bind surrounding sites. While the bHLH region mediates interactions with the E-box target



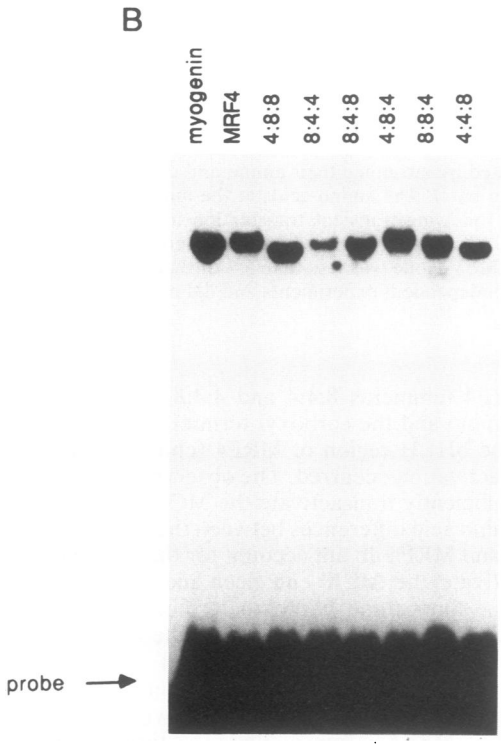


FIG. 3. In vitro translation products and DNA-binding activities of myogenin-MRF4 chimeras. (A) cDNAs encoding myogenin-MRF4 chimeras were transcribed and translated in vitro, and [^{35}S]methionine-labeled translation products were analyzed by SDS-PAGE. (B) cDNAs encoding myogenin-MRF4 chimeras and E12 were transcribed and translated in vitro in the absence of [^{35}S]methionine. Amounts of myogenin-MRF4 chimeras were normalized by comparison with [^{35}S]methionine-labeled products in panel A, taking into account the number of methionines in each protein, and were used in gel mobility shift assays with a labeled probe corresponding to the MCK MEF-1 site. Only the region of the gel containing the mobility-shifted probe is shown. Less than 5% of the probe was shifted with myogenin plus E12. Molecular weights (in thousands) are indicated at the left.

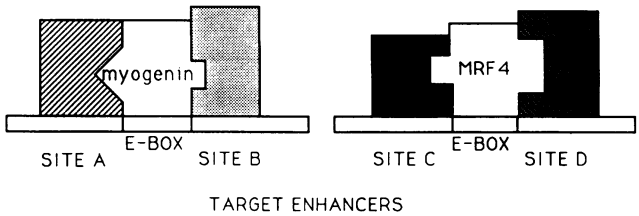


FIG. 4. Hypothetical model of the roles of the amino and carboxyl termini in differential transactivation. Myogenin and MRF4 bind the same E-box consensus sequence, which may be surrounded by binding sites for different transcription factors in different muscle-specific regulatory regions. The abilities of myogenin and MRF4 to activate a target enhancer may be determined by their abilities to cooperate with heterologous transcription factors that bind adjacent sites. These cooperative interactions may depend on the divergent amino and carboxyl termini.

sequence, the amino and carboxyl termini of myogenic HLH proteins may mediate additional protein-protein interactions required for formation of an active transcription complex. Since each member of the MyoD family is distinct in its N and C termini, each could potentially interact with distinct, albeit overlapping, sets of transcription factors. A hypothetical model of these types of interactions is shown in Fig. 4. Accordingly, myogenin might be able to activate transcription in collaboration with transcription factors A and B, which bind adjacent to the E-box in a muscle-specific enhancer, whereas MRF4 might be able to bind the same sequence but might form a productive transcription complex only when transcription factors C and D are bound nearby. This type of mechanism, in which unique domains outside of the bHLH region mediate specific protein-protein interactions within a target promoter or enhancer, could also provide a mechanism to explain the observation that other HLH proteins that bind the same E-box show different target gene specificity for transcriptional activation.

Considerable evidence indicates that activation of muscle-specific transcription requires cooperative interactions between E-boxes and binding sites for other transcription factors. The MCK (15–17, 29), troponin I (18), and myosin light-chain 1/3 enhancers (34), for example, are composed of multiple elements in addition to E-boxes that synergistically activate transcription. Similarly, induction of cardiac α-actin transcription has been shown to require cooperative interactions between MyoD, Sp1, and SRF (25; see also reference 14). The potential of domains outside of the bHLH region of myogenic HLH proteins to mediate cooperative interactions with other enhancer-binding factors is supported by the results of Weintraub et al., who showed that the amino terminus of MyoD mediates cooperative binding of MyoD to high- and low-affinity E-boxes in the MCK enhancer (33).

The differential activities of myogenin and MRF4 are reminiscent of the homeodomain proteins Oct-1 and Oct-2, which bind the same DNA sequence but differ in their activation potentials (30). Whereas Oct-2 activates beta-globin transcription upon interaction with its target sequence, Oct-1 does not. Transcription activation by Oct-2 requires two interdependent domains that lie amino and carboxy terminal to the POU domain, which mediates DNA binding. The C-terminal activation domain is absent from Oct-1. Thus, the regulation of target gene specificity by domains outside of the DNA-binding region may represent a common mechanism whereby transcription factors that bind the same site can evoke different transcriptional responses.

Members of the MyoD family show distinct patterns of expression during embryogenesis and during the differentiation of most established muscle cell lines in culture (1, 2, 12,

13, 19, 24, 26, 35), suggesting that they may play distinct roles within the myogenic lineage and might therefore be expected to show distinct, but overlapping, target gene specificities. The ability of MRF4 to activate MCK-tkCAT, which contains a multimerized MEF-1 site upstream of the thymidine kinase basal promoter, indicates that MRF4 has the potential to activate transcription in transient assays and suggests that other muscle-specific genes, unlike MCK, may be regulated by MRF4. Indeed, MRF4 can activate human cardiac α-actin-CAT (36) and mouse desmin-CAT (7) reporter genes to levels comparable to those elicited by myogenin, indicating that it is active with some muscle-specific control regions. Similarly, MRF4 efficiently activates endogenous myosin heavy-chain expression in transiently transfected 10T1/2 cells (24). Each of the myogenin-MRF4 chimeras also retained the ability to activate the endogenous myogenic program in 10T1/2 cells, assayed by myosin expression (data not shown).

The finding that each of the chimeras had the ability to transactivate MCK-tkCAT also shows that their differential activities toward the MCK enhancer do not reflect altered DNA-binding properties of these proteins in vivo. We have attempted to demonstrate differential interactions of the chimeras with other cellular factors contained within nuclear extracts but have thus far been unable to detect differences among them (data not shown). Although we favor the hypothesis that the lack of transcriptional activities of certain myogenin-MRF4 chimeras reflects an inability of their N and C termini to interact productively with heterologous enhancer-binding factors, it is also possible that some chimeras possess secondary structures that simply preclude their participation in productive transcription complexes within the MCK enhancer. As these chimeras activate MCK-tkCAT, however, their loss of function with the MCK enhancer would be enhancer specific.

In addition to their apparent potential to influence target gene specificity, the amino and carboxyl termini of myogenin contain strong transcription activation domains (27). Similar domains in myf5 have been identified (3). Studies to determine whether transcription activation and target gene specificity are related functions mediated by common amino acid sequences are in progress.

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