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The Basic Region of Myogenin Cooperates with Two Transcription Activation Domains To Induce Muscle-Specific Transcription

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Myogenin is a skeletal muscle-specific transcription factor that can activate myogenesis when introduced into a variety of nonmuscle cell types. Activation of the myogenic program by myogenin is dependent on its binding to a DNA sequence known as an E box, which is associated with numerous muscle-specific genes. Myogenin shares homology with MyoD and other myogenic regulatory factors within a basic region and a helix-loop-helix (HLH) motif that mediate DNA binding and dimerization, respectively. Here we show that the basic region-HLH motif of myogenin alone lacks transcriptional activity and is dependent on domains in the amino and carboxyl termini to activate transcription. Analysis of these N- and C-terminal domains through creation of chimeras with the DNA-binding domain of the *Saccharomyces cerevisiae* transcription factor GAL4 revealed that they act as strong transcriptional activators. These transcription activation domains are dependent for activity on a specific amino acid sequence within the basic region, referred to as the myogenic recognition motif (MRM), when an E box is the target for DNA binding. However, the activation domains function independent of the MRM when DNA binding is mediated through a heterologous DNA-binding domain. The activation domain of the acidic coactivator VP16 can substitute for the myogenin activation domains and restore strong myogenic activity to the basic region-HLH motif. Within a myogenin-VP16 chimera, however, the VP16 activation domain also relies on the MRM for activation of the myogenic program. These findings reveal that DNA binding and transcriptional activation are separable functions, encoded by different domains of myogenin, but that the activity of the transcriptional activation domains is influenced by the DNA-binding domain. Activation of muscle-specific transcription requires collaboration between the DNA-binding and activation domains of myogenin and is dependent on events in addition to DNA binding.

Differentiation of skeletal myoblasts to terminally differentiated myotubes is associated with the transcriptional activation of a set of genetically unlinked muscle-specific genes that encode proteins required for the specialized functions of the myofiber. Myogenin is a nuclear phosphoprotein that belongs to a family of skeletal muscle-specific regulatory factors that can activate the muscle differentiation program when expressed artificially in a variety of nonmuscle cell types (15, 50). This family of regulatory factors, often referred to as the MyoD family, also includes MyoD (13), myf5 (3), and MRF4 (also called herculin and myf6) (2, 30, 35) (reviewed in references 33 and 44). Each of these proteins shares homology within a segment of about 70 amino acids that encompasses a basic region and adjacent helix-loop-helix (HLH) motif (together referred to as the bHLH region) that mediate DNA binding and dimerization, respectively. Similar structural motifs have been identified in the *myc* family of oncogene products, in several gene products that specify cell fate during *Drosophila* embryogenesis, and in a growing number of transcription factors that are ubiquitously expressed (31).

Activation of muscle-specific transcription by members of the MyoD family is dependent on their binding to a conserved DNA sequence motif known as an E box (CANNTG), which is located in the regulatory regions of many muscle-specific genes, including MCK (6, 20, 22, 41), MLC1/3 (49), acetylcholine receptor α-subunit (34), cardiac α-actin (36), and troponin I (27). Myogenic HLH proteins bind weakly to the E-box consensus sequence as homooligomers in vitro, but their affinity for this site increases dramatically upon heterooligomerization with the ubiquitous HLH proteins E12 and E47, which arise by differential splicing of the E2A gene (4, 6, 9, 12, 27, 32). The inefficiency with which myogenin binds DNA in the absence of E2A gene products has been shown to be attributable to its inability to efficiently homooligomerize (8). E2A gene products have also been shown to oligomerize with myogenic HLH proteins in vivo and to be essential for the activation of the muscle program (23).

Deletion mutagenesis of MyoD has shown that the bHLH region is necessary and sufficient for activation of myogenesis (43). Because MyoD normally oligomerizes with E2A products in vivo (23), these results suggest either that the bHLH region of MyoD contains a transcription activation domain or that it recruits this function from its partner to induce muscle-specific transcription. Indeed, transcription activation domains have been identified within the amino termini of E12 and E47 (4, 17). MyoD and myf5 have also been shown to contain strong transcriptional activation domains, but it is not yet clear how these domains contribute to muscle-specific gene activation (4, 47).

An intriguing paradox is how different HLH proteins bind the same DNA consensus sequence but activate diverse sets of genes. A clue to the mechanism of transcriptional specificity of HLH proteins was provided by experiments with MyoD (12) and subsequently with myogenin (5), in which their basic regions were replaced with those of E12, E47, or the *Drosophila* neurogenic HLH protein achaete scute T4. These substitution mutations did not significantly diminish binding of MyoD or myogenin to the E box of the MCK enhancer in vitro, but they abolished all myogenic activity of

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these proteins. Systematic mutagenesis of the basic regions of MyoD and myogenin revealed that two adjacent amino acids, alanine and threonine, which do not substantially affect DNA binding, are required for muscle-specific gene activation (5, 12, 47). These residues are specific to and conserved in all members of the MyoD family, suggesting that they constitute part of an ancient protein motif required for activation of the myogenic program. We refer to this motif as the myogenic recognition motif (MRM). Together, these results show that binding of an HLH protein to an E box in a muscle-specific enhancer is not sufficient to activate transcription and suggest that events in addition to DNA binding are required for the transcriptional activity of these proteins to be manifested. How the MRM directs muscle-specific transcription in addition to DNA binding is unknown.

To further define the mechanism through which myogenin activates muscle-specific transcription, we have analyzed the functions of its bHLH motif and surrounding domains. We approached this problem in several ways. First, we assayed the abilities of myogenin deletion mutants, which retain the capacity to bind DNA as heterooligomers with E2A products, to *trans*-activate E-box-containing target genes. Second, we created chimeras of myogenin and the DNA-binding domain of the *Saccharomyces cerevisiae* transcription factor GAL4 and assayed for *trans*-activation of a reporter gene containing GAL4-binding sites. Use of GAL4-myogenin chimeras allowed us to assay for activating domains independent of myogenin's normal DNA response element, its oligomerization partner, and its potential interactions with heterologous enhancer-binding factors or potential coactivators (4, 17). Third, we created chimeras between myogenin and the acidic coactivator VP16 and investigated whether the VP16 transcriptional activation domain could substitute for the corresponding region of myogenin. We show that the bHLH region of myogenin is devoid of transcriptional activity and that activation of muscle-specific transcription requires cooperative interactions between the DNA-binding and transcriptional activation domains of myogenin.

MATERIALS AND METHODS

Cell culture, transfection, and immunostaining. Cells were maintained in growth medium (Dulbecco's modified Eagle's medium [DMEM] containing 20% fetal bovine serum) and were transfected by calcium phosphate precipitation as described previously (41). The day after transfection, cells were refed with growth medium. To induce differentiation, cultures were transferred to differentiation medium (DMEM with 2% horse serum) the following day and were harvested 48 h later. Chloramphenicol acetyltransferase (CAT) and β-galactosidase activities were assayed in cell extracts as described previously (41). All assays were linear with respect to protein concentration.

The reporter plasmids for myogenin *trans*-activation, MCK-CAT and 4R-tkCAT, have been described previously (41, 47). Briefly, MCK-CAT, referred to previously as pCK-CATe4, contains the 246-bp MCK promoter fused immediately 5′ of CAT and the 300-bp 5′ enhancer inserted into the *Bam*HI site 3′ of CAT (41). 4R-tkCAT was a gift from A. Lassar and contains four copies of the MCK high-affinity, or right, E box upstream of the thymidine kinase basal promoter and the CAT gene (47). The GAL4 reporter plasmid pG5E1bCAT, which contains five copies of the GAL4 responsive element, has been previously described (26). Transfections also included pRSV-lacZ, which encodes β-galactosidase, as an internal control for transfection efficiency, and the level of CAT expression was normalized to β-galactosidase activity. All transfections were performed at least three times, with comparable results.

Immunostaining for myosin heavy chain (MHC) was performed with anti-MHC antibody MF-20 and the Vectastain ABC kit as described previously (15).

Mutagenesis and creation of GAL4-myogenin chimeras. Myogenin mutants containing internal deletions were made by the creation of *Xho*I sites in the myogenin open reading frame by site-directed mutagenesis. Internal regions of the open reading frame were then deleted by digestion with *Xho*I and religation. Mutations were created such that they maintained the correct reading frame and did not alter the amino acids surrounding the sites for ligation. Site-directed mutagenesis was performed on single-stranded templates as described previously (5). All mutants were confirmed by sequencing. Mutants containing premature termination codons were created by the introduction of stop codons, by site-directed mutagenesis, or by the insertion of an *Nhe*I linker (from New England Biolabs) containing stop codons in all reading frames. All myogenin deletion mutants were subcloned into the expression vector EMSV, which contains the Moloney sarcoma virus long terminal repeat (13). In all transfection experiments, the EMSV plasmid lacking a cDNA insert was transfected as a negative control to establish background levels of muscle gene expression.

VP16 chimeras were created by fusing the activating region of VP16 contained on a *Sal*I-*Bam*HI fragment into the *Nco*I site (codon 156) of myogenin or myo-E12basic. The myogenin mutant myo-E12basic, which was used to create some GAL4 and VP16 chimeras, has been described previously (5). Briefly, this mutant contains the E12 basic region substituted for that of myogenin. The amino acids that have been replaced in this mutant extend from residues 82 to 93 of myogenin and include the following sequences: for myogenin, RRRAATLREKRK; for E12, RRVANNARERLR.

The GAL4 chimera constructs GALmyo7-224, GALmyo 80-139, GALmyo142-224, and GALmyo203-224 were made by digesting the myogenin mutants containing newly created *Xho*I sites with *Xho*I and *Eco*RI, which cut into the open reading frame and 3′ untranslated region, respectively; filling in the single-stranded ends with Klenow polymerase; and ligating to *Sma*I-digested pSG424. The plasmid pSG424 contains GAL4(1-147) expressed from the simian virus 40 promoter-enhancer (26). The chimeras GALmyo33-224 and GALmyo156-224 were made by the same method, except that wild-type myogenin was used and the plasmids were digested with *Sma*I and *Nco*I, respectively, instead of *Xho*I. The chimera GALmyo156-198 was also made by ligation to the *Nco*I site of myogenin, except that the mutant DM199-224, which introduces a stop codon at codon 199, was used.

DNA binding. The ability of myogenin deletion mutants to bind DNA in the presence of E12 was determined by in vitro transcription and cotranslation of the deletion mutants and E12. The E12 cDNA used in in vitro transcription and translation, referred to as E12R (31), is truncated at its amino terminus. Gel mobility shift assays were performed with an end-labeled oligonucleotide corresponding to the high-affinity E box from the MCK enhancer, as described previously (6).

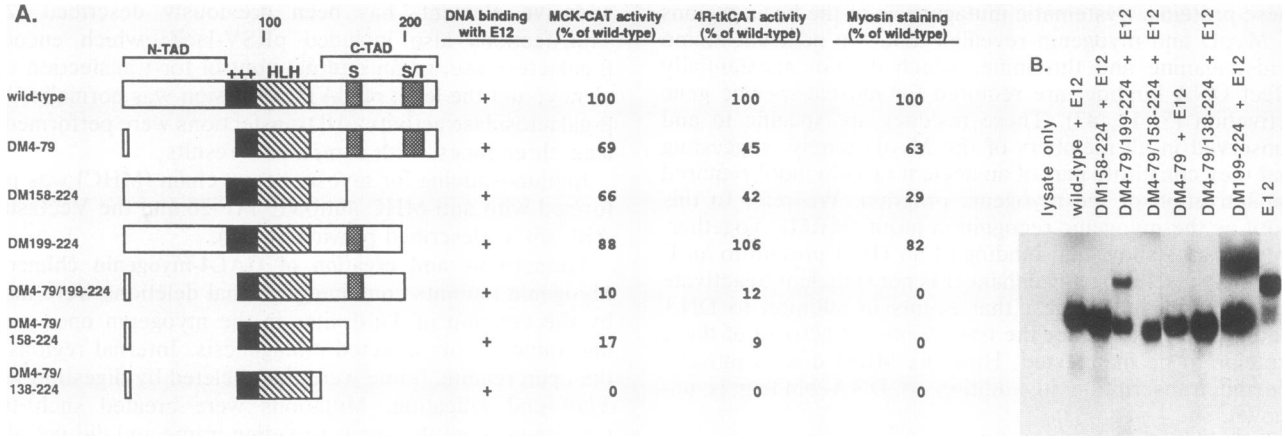


FIG. 1. Deletion mutants of myogenin reveal domains in the N and C termini that cooperate with the bHLH region to activate muscle-specific transcription. (A) A series of deletions was created within the myogenin open reading frame. Amino acids that were deleted are indicated to the left of each deletion mutant. The in vitro translation product of each mutant was tested for its ability to bind to the right E box from the MCK enhancer in the presence of E12 by using gel mobility shift assays; a plus sign indicates binding. Each mutant was also tested for its ability to *trans*-activate MCK-CAT and 4R-tkCAT and to activate the endogenous myogenic program, assayed by MHC staining, following transient transfection into 10T1/2 cells. For transfections to be assayed for MHC staining, cells were transiently transfected with 5 μg of EMSV expression vector encoding the indicated mutant. For *trans*-activation assays, 5 μg each of myogenin expression vectors and CAT reporters were used. One microgram of Rous sarcoma virus-*lacZ* was also included as an internal control for transfection efficiency. Following transfection, cultures were maintained in growth medium for 24 h and were then transferred to differentiation medium for 48 h prior to harvesting. Values for these assays are expressed relative to that of wild-type myogenin and represent the average of at least three experiments. The extent of conversion of [^{14}C]chloramphenicol to acetylated products was generally about 20% in extracts from cells transfected with wild-type myogenin, compared with 0.2% with the expression vector EMSV lacking an insert. The frequency of MHC-positive cells following transfection with wild-type myogenin was generally about 1%. TAD, transcription activation domain; S, serine-rich motif; S/T, serine-threonine-rich motif; +++, the basic region. (B) Gel mobility shift assays with unlabeled E12 and wild-type and mutant myogenin proteins obtained by in vitro translation. Amounts of myogenin and E12 in each binding reaction were normalized on the basis of parallel translations containing [^{35}S]methionine, taking into account the number of methionines in each deletion mutant. The probe corresponds to the right E box from the MCK enhancer, which was end labeled. Only the region of the gel containing the shifted probe is shown. Less than 5% of the probe was shifted with myogenin plus E12. The band of lower mobility observed with mutant DM4-79/199-224 represents binding of E12 homooligomer, which is detected because there is a slightly lower amount of this deletion mutant in the binding reaction. Other experiments indicate that this mutant binds DNA with an affinity similar to those of the other mutants.

RESULTS

The amino and carboxyl termini of myogenin cooperate with the bHLH region to activate myogenesis. To begin to define the domains of myogenin required for muscle-specific gene activation, we tested myogenin deletion mutants for their abilities to activate the endogenous myogenic program in transiently transfected 10T1/2 cells, assayed by immunostaining for MHC. Myogenin deletion mutants were also tested for their abilities to *trans*-activate a reporter gene linked to the MCK promoter and enhancer (MCK-CAT) (41) or to a multimerized myogenin-binding site upstream of the thymidine kinase basal promoter (4R-tkCAT) (47). Whereas the complete MCK enhancer requires cooperative interactions among multiple enhancer-binding factors for activation, the multimerized myogenin-binding site is expected to be less dependent on combinatorial interactions among heterologous factors for activation. Comparison of these target genes therefore allowed us to determine whether deletions that potentially inhibited activation of the complete MCK enhancer were due to altered transcriptional activity of myogenin or reflected altered interactions between myogenin and other important transcription factors that bind sites surrounding the myogenin-binding site in the enhancer.

An amino-terminal deletion that extended through the conserved cysteine-rich region and first cluster of basic amino acids in the basic domain (see reference 5 for a description of myogenin's structural features) had only a small effect on the ability of myogenin to activate myogenesis (DM4-79; Fig. 1A). Similarly, deletion of the carboxyl terminus (DM158-224) by the introduction of a termination codon 3′ of the HLH motif resulted in a partial rather than complete loss in myogenic activity. Deletion of amino acids 199 to 224 of myogenin (DM199-224) did not significantly diminish its myogenic activity. However, when this deletion was combined with the amino-terminal deletion (DM4-79/199-224), nearly all myogenic activity was lost, suggesting some redundancy in the function of the N and C termini. Immunostaining confirmed that the deletion mutants described above were expressed in transiently transfected cells (data not shown).

We also tested the ability of the bHLH domain alone (DM4-79/138-224) to activate myogenesis and found it to be completely inactive in transient assays. Because the corresponding region of MyoD has been reported to contain significant myogenic activity in stably transfected 10T1/2 cells (43), we also carried out stable transfections with DM4-79/138-224, but we observed no myogenic conversion in more than 200 independent clonal colonies (data not shown). The bHLH region plus the leucine repeat that is contiguous with helix 2 of the HLH motif (DM4-79/158-224) showed weak but measurable activity with the exogenous reporter genes, but it did not detectably activate the endogenous program measured by MHC staining in transiently transfected cells.

The DNA-binding activity of each deletion mutant was also examined by gel mobility shift assays by using the

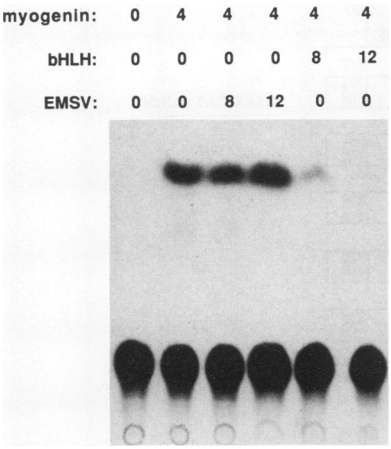


FIG. 2. The bHLH region of myogenin can inhibit *trans*-activation by wild-type myogenin. 10T1/2 cells were transiently transfected with 5 μg of 4R-tkCAT as a reporter and the indicated quantities (in micrograms) of wild-type myogenin and mutant DM4-79/138-224 (the bHLH region) or EMSV. EMSV is the expression vector in which both wild-type and mutant myogenins are cloned, and it was added as a control to demonstrate that inhibition was not the result of competition between promoters. CAT activity in cell extracts was determined as described in Materials and Methods and the legend to Fig. 1. Comparable results were obtained in three separate experiments.

corresponding in vitro-synthesized proteins in the presence of E12. Figure 1B shows that all of the deletion mutants were able to bind with similar efficiencies to a labeled DNA probe encompassing the high-affinity E box, or MEF-1 site, from the MCK enhancer (7). Although these assays do not provide a quantitative assessment of the relative affinities of the different mutants, they are sufficient to indicate that they do not exhibit dramatic differences in their DNA-binding activities. These results support the notion that the bHLH region mediates DNA binding and cooperates with domains in the amino and carboxyl termini to activate muscle-specific transcription.

The bHLH region can inhibit muscle-specific gene activation by wild-type myogenin. The lack of myogenic activity of the bHLH region (DM4-79/138-224) suggested that, if this mutant was indeed expressed at a normal level in vivo, it might function as a *trans*-dominant inhibitor of wild-type myogenin by competing for interaction with a limiting amount of E2A product or for DNA binding, or both. To further examine the importance of the N and C termini of myogenin for myogenic conversion and to confirm that the bHLH deletion mutant was indeed expressed in transfected cells in a form that could oligomerize with E2A products, we tested whether this mutant could inhibit muscle-specific gene activation by performing cotransfection assays with wild-type myogenin in the presence of DM4-79/138-224. As shown in Fig. 2, the bHLH deletion mutant was able to inhibit *trans*-activation of 4R-tkCAT by wild-type myogenin in a concentration-dependent manner, demonstrating that this mutant is expressed in vivo and further indicating that the N and C termini are essential for the activation of the myogenic program by myogenin.

Chimeras of myogenin and the DNA-binding domain of GAL4 reveal N- and C-terminal transcription activation domains. The apparent dependence of the bHLH region on the N and C termini of myogenin for the activation of myogenesis led us to test whether the latter regions might contain transcription activation domains that could function independently of the DNA-binding site. To test for such domains, we created a series of chimeras by fusing portions of myogenin to amino acids 1 to 147 of the *S. cerevisiae* transcription factor GAL4, which encodes its DNA-binding domain. The transcriptional activities of GAL4-myogenin chimeras were then assayed in transiently transfected 10T1/2 cells by using a reporter gene containing CAT linked to the TATA box of the E1b gene and five GAL4-binding sites (26). As shown in Fig. 3, the GAL4 DNA-binding domain alone was inactive in this assay, whereas the fusion of amino acids 7 to 224 of myogenin to the GAL4 DNA-binding domain was sufficient to activate the transcription of the reporter plasmid to high levels [compare GAL4(1-147) and GALmyo7-224]. Further deletions revealed that a 71-amino-acid segment of the amino terminus (amino acids 7 to 77) and a 69-amino-acid segment of the carboxyl terminus (amino acids 156 to 224) were able to function as strong transcriptional activators (GALmyo7-77 and GALmyo156-224). Consistent with the failure of the bHLH region to activate myogenesis (Fig. 1A), this region failed to activate transcription when fused to GAL4 (Fig. 3, GALmyo80-139).

In order to more finely map the C-terminal activating domain, we created additional deletions within the C terminus. These deletions indicated that the transcription activation domain was located between amino acids 156 and 224 and was apparently dependent on cooperative interactions between the regions encompassing amino acids 156 to 198 and 203 to 224 (Fig. 3; compare GALmyo156-224 with GALmyo156-198 and GALmyo203-224). Neither of these subdomains showed transcriptional activity alone, whereas together they showed strong activity. These results are consistent with those obtained from the myogenin deletion mutants tested for activation of myogenesis (Fig. 1) and suggest that the C-terminal activation domain is bipartite and requires cooperative interactions among both subdomains for activity. The requirement of the region between residues 203 and 224 for transcriptional activity of the C-terminal activation domain can explain why deletion of this region from a myogenin mutant lacking the N terminus severely impairs myogenic activity (Fig. 1A, DM4-79/199-224). We cannot rule out the possibility that the C-terminal activation domain is contiguous, extending from amino acids 156 through 224, and is inactivated when it is bisected at amino acid 198. This seems unlikely, however, because myogenin deletion mutants tested for myogenic activity (Fig. 1) indicate that the region between residues 139 and 198 can cooperate with the amino terminus or the region from amino acids 203 to 224 to activate muscle-specific transcription. Within the C-terminal activation domain are two regions rich in serine and threonine, the more C-terminal of which is conserved in the four vertebrate myogenic factors: myogenin, MyoD, myf5, and MRF4. We are currently investigating whether these regions are critical for activity of the C-terminal domain as well as the role of phosphorylation in this activity.

The activation domain of VP16 can substitute for the N- and C-terminal activation domains of myogenin. We next asked whether the activation domains of myogenin were important for the muscle specificity of transcription or whether specificity was derived solely from the bHLH region. To answer this question, we investigated whether the powerful activation domain of the acidic coactivator VP16 could substitute for the activation domains of myogenin (11). Similar studies with MyoD recently revealed that the VP16 activation domain could functionally replace the N-terminal transcription

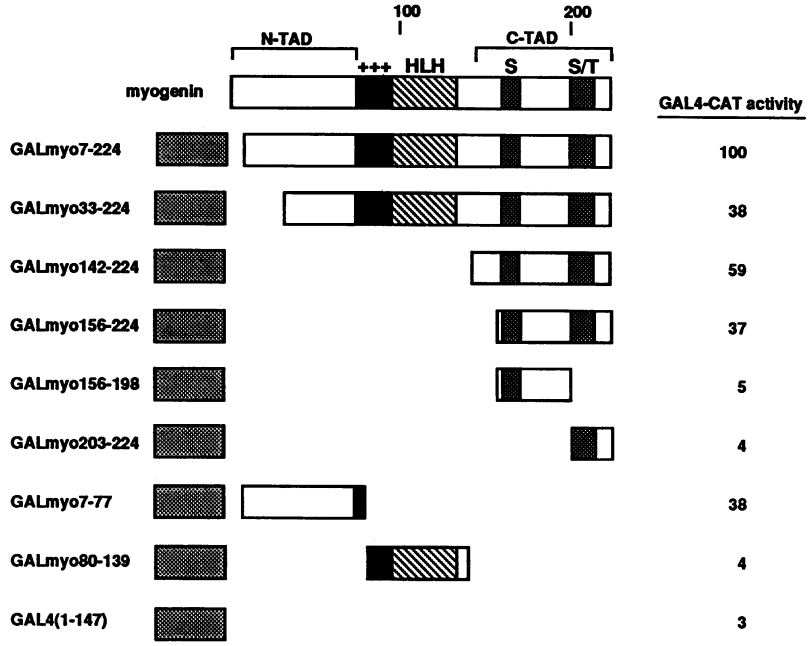
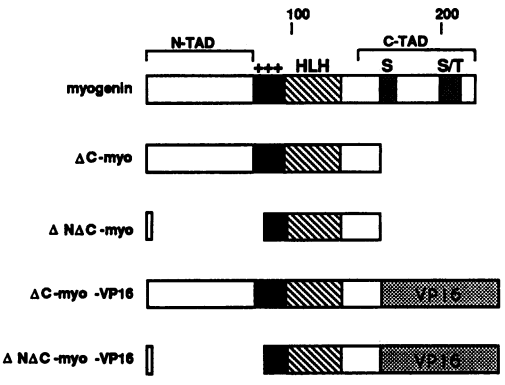


FIG. 3. GAL4-myogenin fusions reveal transcription activation domains in the N and C termini of myogenin. Segments of myogenin were fused in frame to amino acids 1 to 147 of GAL4, which contains its DNA-binding domain. Names of chimeras and amino acids from myogenin contained in each chimera are shown to the left. GAL4-myogenin chimeras were assayed for their abilities to *trans*-activate pG5E1bCAT, which contains five copies of the GAL4-binding site and the E1b promoter upstream of CAT. Activities of each GAL4-myogenin chimera are expressed relative to the activity of GALmyo7-224. Transfections in 10T1/2 cells were performed with 2 μg of the indicated chimera and 1 μg of Rous sarcoma virus-*lacZ*. CAT activity was assayed in cell extracts prepared from cells in growth medium 48 h following transfection. Values represent the averages of at least three independent experiments.

activation domain of MyoD (47). As shown in Fig. 4, fusion of the VP16 activation domain to the C terminus of mutant DM158-224 (ΔC-myo) or DM4-79/158-224 (ΔNΔC-myo) dramatically augmented the ability of these deletion mutants to activate the endogenous myogenic program, as assayed by MHC staining. Indeed, both myogenin-VP16 chimeras (designated ΔC-myo-VP16 and ΔNΔC-myo-VP16) were significantly more active than wild-type myogenin in this assay.

When assayed for their abilities to *trans*-activate exogenous reporter plasmids, both myogenin-VP16 chimeras were able to *trans*-activate 4R-tkCAT. However, only ΔC-myo-VP16 was able to efficiently *trans*-activate MCK-CAT, suggesting that activation of a complex muscle-specific enhancer such as that of MCK requires either the N or C terminus of myogenin. Because ΔC-myo-VP16 could activate MCK-CAT, at least some of these determinants of



	10T1/2 cells			HeLa cells	
	MCK-CAT activity (% of wild-type)	4R-tkCAT activity (% of wild-type)	Myosin staining (% of wild-type)	MCK-CAT activity (% of wild-type)	4R-tkCAT activity (% of wild-type)
myogenin	100	100	100	3	100
ΔC-myo	66	42	29	NT	NT
Δ NΔC-myo	17	9	0	NT	NT
ΔC-myo -VP16	127	454	293	3	230
Δ NΔC-myo -VP16	14	333	421	5	107

FIG. 4. The transcriptional activation domain of VP16 can substitute for the N- and C-terminal activation domains of myogenin. 10T1/2 or HeLa cells were transiently transfected with 5 μg of MCK-CAT or 4R-tkCAT as reporters and 5 μg of wild-type myogenin, myogenin deletion mutants, or myogenin-VP16 chimeras in EMSV. Deletion mutants were ΔC-myo (DM158-224) and ΔNΔC-myo (DM4-79/158-224). Myogenic conversion was also assayed by MHC immunostaining following transient transfection with the indicated expression vectors. CAT activity in cell extracts was determined as described in Materials and Methods and the legend to Fig. 1. Values are expressed as the percentage of wild-type activity observed with each plasmid. The data for ΔC-myo (DM158-224) and ΔNΔC-myo (DM4-79/158-224) are also shown in Fig. 1A but are repeated here for ease of comparison with the corresponding VP16 chimeras. Because MCK-CAT was not significantly induced in HeLa cells, values for its expression are normalized to the level of expression of 4R-tkCAT in the presence of myogenin in HeLa cells.

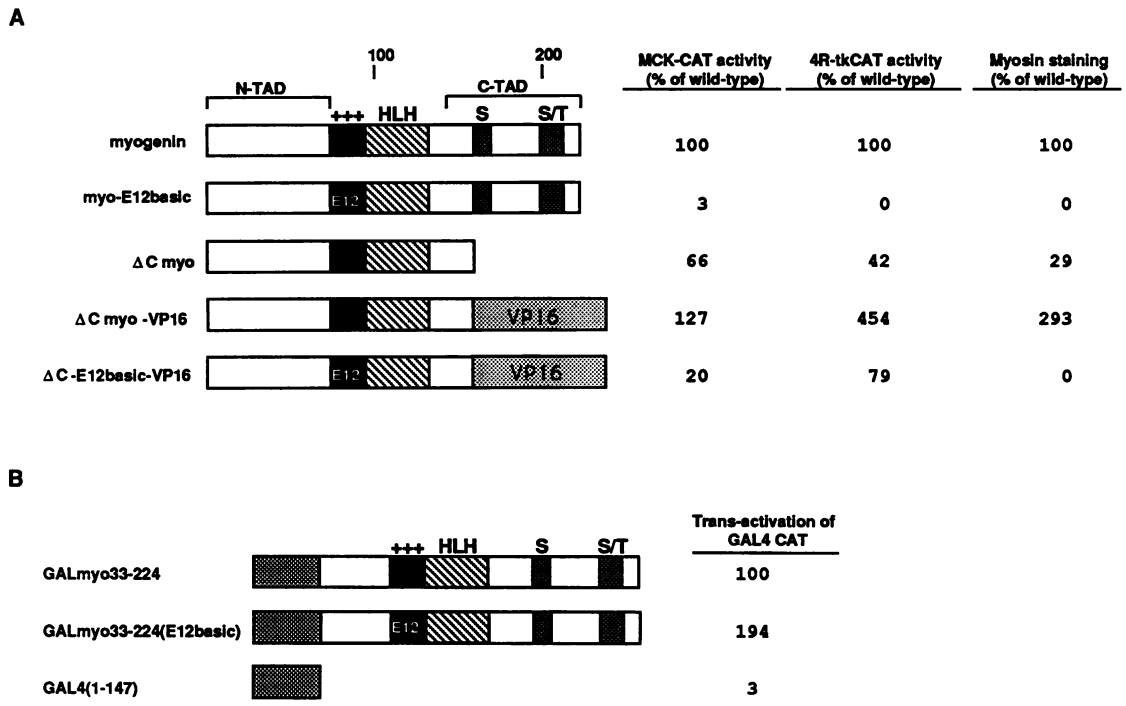


FIG. 5. The myogenin basic region is important for activity of the transcriptional activation domains only when an E box is the target for transcriptional activation. (A) 10T1/2 cells were transiently transfected with 5 μg of MCK-CAT or 4R-tkCAT as a reporter and 5 μg of wild-type myogenin or the indicated myogenin mutants in EMSV. Myogenic conversion was also assayed by MHC immunostaining following transient transfection with the indicated expression vectors, and CAT activity in cell extracts was determined as described in Materials and Methods and the legend to Fig. 1. Values for each mutant are expressed as the percentage of wild-type activity observed with each plasmid. The data for ΔC-myo and ΔC-myo-VP16 are also shown in Fig. 4; they are repeated here for comparison with the E12basic constructs. (B) 10T1/2 cells were transiently transfected with G5E1bCAT and the GAL4 expression vectors shown. Values for CAT activity are expressed relative to the level observed with GALmyo33-224.

target gene specificity must be located in the N-terminal region. In this sense, the activation domains function not only to induce transcription but also to discriminate between potential target genes. It is also apparent from deletion mutant ΔNΔC-myo-VP16 that *trans*-activation of the complete MCK enhancer and induction of myosin expression have different requirements, since this mutant is nearly inactive against MCK-CAT but is more active than wild-type myogenin in inducing myosin.

Because of the remarkable potency of the myogenin-VP16 chimeras described above, we also tested these chimeras for their abilities to *trans*-activate MCK-CAT and 4R-tkCAT in HeLa cells, which are refractory to myogenic conversion by MyoD. As reported previously by Weintraub and coworkers for MyoD (48), myogenin was unable to *trans*-activate MCK-CAT in HeLa cells (Fig. 4). However, myogenin efficiently activated 4R-tkCAT in HeLa cells, as did chimeras ΔC-myo-VP16 and ΔNΔC-myo-VP16.

Amino acids in the myogenin basic region regulate activity of the N-terminal activation domain but not of a heterologous transcription activation domain. Activation of muscle-specific transcription by myogenin and MyoD has been shown to depend on a conserved motif within their basic regions, the MRM (5, 8, 12, 47). Replacement of the basic region of myogenin or MyoD with the corresponding region of E12 results in a complete loss in transcriptional activity without substantially affecting DNA binding.

The observation that the MRM within the DNA-binding domain of myogenin could regulate the transcriptional activation domains when activation was mediated through the E-box motif led us to test whether a heterologous activation domain fused to myogenin would also be dependent on this specific amino acid sequence in the basic region for transcriptional activity. We therefore replaced the C-terminal activation domain of the myogenin mutant myo-E12basic with the VP16 activation domain. myo-E12basic contains the E12 basic region in place of the myogenin basic region and lacks myogenic activity (Fig. 5A). Replacement of the C-terminal activation domain of myo-E12basic with the activation domain of VP16 was sufficient to restore the ability of the mutant to *trans*-activate 4R-tkCAT (Fig. 5A; compare myo-E12basic and ΔCmyo-E12basic-VP16). However, this mutant was only marginally active on MCK-CAT and failed to activate the endogenous myogenic program. In all assays, ΔC-E12basic-VP16 was significantly weaker than ΔC-myo-VP16.

The observation that the amino and carboxyl termini of myogenin could activate transcription in the absence of the myogenin basic region when they were fused to the GAL4 DNA-binding domain suggested that, within this context, these domains were released from their dependence on the basic region for "activation." To investigate whether the myogenin basic region was able to modulate transcriptional activity of the activation domains within the complete myogenin protein fused to GAL4, we next replaced the basic region of myogenin in GALmyo33-224 with the basic region of E12. As shown in Fig. 5B, this GAL4-myogenin chimera, referred to as GALmyo33-224(E12basic), was able to activate the GAL4 target gene with an efficiency greater than that of the wild-type protein GALmyo33-224. These results

indicate that the activation domains of myogenin can function independently of the MRM when activation is mediated by a heterologous DNA-binding site.

DISCUSSION

Activation of muscle-specific transcription by myogenin and other members of the MyoD family requires heterooligomerization with E2A products E12 and E47 and binding to the E-box motif in muscle-specific control regions (6, 23). Our results show that myogenin contains two transcription activation domains that act in conjunction with the DNA-binding domain to activate muscle-specific transcription. The activities of these transcriptional activation domains, which lie amino and carboxyl terminal of the bHLH region, are dependent on a specific amino acid sequence within the basic region, the MRM, when the E-box motif is the target for DNA binding, but they are constitutively active when fused to the DNA-binding domain of GAL4 and assayed for transcriptional activation through the GAL4 DNA-binding site. Deletion of one or the other of these activation domains results in a partial loss in transcriptional activity, whereas deletion of both abolishes the ability of myogenin to induce myogenesis. Deletion mutants of myogenin lacking the transcriptional activation domains retain the ability to bind DNA with E2A products but function as *trans*-dominant inhibitors of myogenesis.

The N- and C-terminal activation domains of myogenin appear to be functionally similar with respect to their relative potencies and their dependence on the myogenin basic region for activity when transcriptional activation is mediated by the E-box motif, but they do not show obvious structural similarities. The N-terminal activation domain, located between amino acids 7 and 77, contains many acidic residues and might therefore function as an acidic activator (28, 29). MyoD and myf5 have also been shown to contain activation domains in their amino termini (4, 47). Although there is no obvious homology between the amino termini of myogenin and these other factors, they are also relatively acidic in this region, suggesting that these activation domains may function similarly.

Analysis of the C-terminal activation domain of myogenin through GAL4 fusions and deletion mutagenesis of the wild-type protein suggests that this domain is bipartite and contains subdomains between amino acids 156 and 198 and amino acids 203 and 224 that are essential for activity. In contrast to the N terminus, the C-terminal activation domain does not contain a preponderance of acidic residues, but it contains two regions rich in serine and threonine that map to the two essential subdomains. Between residues 159 and 171 is a stretch of alternating serines bounded by prolines. The region from residues 200 to 214, which is conserved in all vertebrate myogenic HLH proteins described thus far, is also rich in serines and threonines. Recent studies indicate that this region contains multiple sites for phosphorylation by casein kinase II (25). The possibility that phosphorylation plays a role in transcriptional activation by this domain is currently under investigation. Phosphorylation has, for example, been shown to be important for activity of transcriptional activation domains in c-Jun (1), in the homeodomain protein Pit-1 (19), and in the cyclic AMP-response element binding protein CREB (16).

Transcription activation domains, in addition to myogenin's bHLH region, are required to activate myogenesis. The importance of the activation domains for the induction of myogenesis is underscored by the observation that the bHLH motif, which is sufficient to bind DNA with an affinity comparable to that of the complete protein, functions as a *trans*-dominant inhibitor of wild-type myogenin in cotransfection assays. The inhibitory activity of this mutant is likely to reflect competition with wild-type myogenin for a limiting cellular factor (probably E12 or E47) required for muscle-specific gene activation or for binding to the E-box motif. These results differ from previous findings with MyoD, which showed that the bHLH region could activate myogenesis with an efficiency similar to that of the wild-type protein when stably expressed in 10T1/2 cells (43). How can the difference in behavior of the bHLH regions of MyoD and myogenin be explained? One possibility is that slight differences in the deletions of myogenin and MyoD account for their apparent differences in myogenic activity; the bHLH region of MyoD tested previously contained five additional residues at the N-terminal end and three additional residues at the C-terminal end of the bHLH region compared with myogenin deletion mutant DM4-79/138-224 tested in this study. However, mutagenesis studies have thus far failed to indicate a role for these residues in myogenic activity. An alternate explanation for the activity of the bHLH region of MyoD is that activation of myogenesis by this region of MyoD reflects activation of the endogenous MyoD gene via the autoregulatory loop (45). An indication that activation of myogenesis by the bHLH region of MyoD may reflect autoactivation of the endogenous MyoD locus is suggested by the fact that the bHLH region of MyoD can activate muscle-specific genes only in stably transfected cells, whereas it is inactive in transient assays in which there is little or no activation of the endogenous myogenic regulatory factor genes (43). In this regard, it is clear that myogenin and MyoD differ in their autoregulatory activities; MyoD can readily activate and maintain its own transcription in the presence of high levels of serum, whereas myogenin can neither autoregulate nor cross-activate the expression of MyoD under these conditions (14, 45). Inspection of the bHLH regions of MyoD and myogenin does not reveal obvious amino acid differences that could provide the basis for these differing functions.

Considering that E12 and E47 have been shown to contain transcription activation domains (4, 17), the failure of the bHLH region of myogenin to induce myogenesis suggests that the activation domains of these HLH proteins are insufficient by themselves to activate muscle-specific transcription when bound to an E-box target sequence as heterooligomers with only the bHLH region of myogenin. These results also suggest that transcriptional activation by a heterooligomer of myogenin and E2A products may require cooperativity between the activation domains within each partner. Further evidence that an activation domain within myogenin is essential for transcriptional activity of a myogenin-E2A heterooligomer was provided by the finding that fusion of the activation domain of VP16 to the bHLH region of myogenin was sufficient to dramatically augment transcriptional activity.

The transcription activation domains, in conjunction with the bHLH region, determine target gene specificity. In addition to activating transcription, the activation domains of myogenin appear to contribute to target gene specificity; this is suggested by the observation that ΔNΔC-myo-VP16 can efficiently *trans*-activate 4R-tkCAT but cannot activate MCK-CAT, whereas the VP16 chimera containing the N terminus in addition to the bHLH region, ΔC-myo-VP16, activates both reporters. Thus, simply having an activation domain attached to the bHLH region is not necessarily

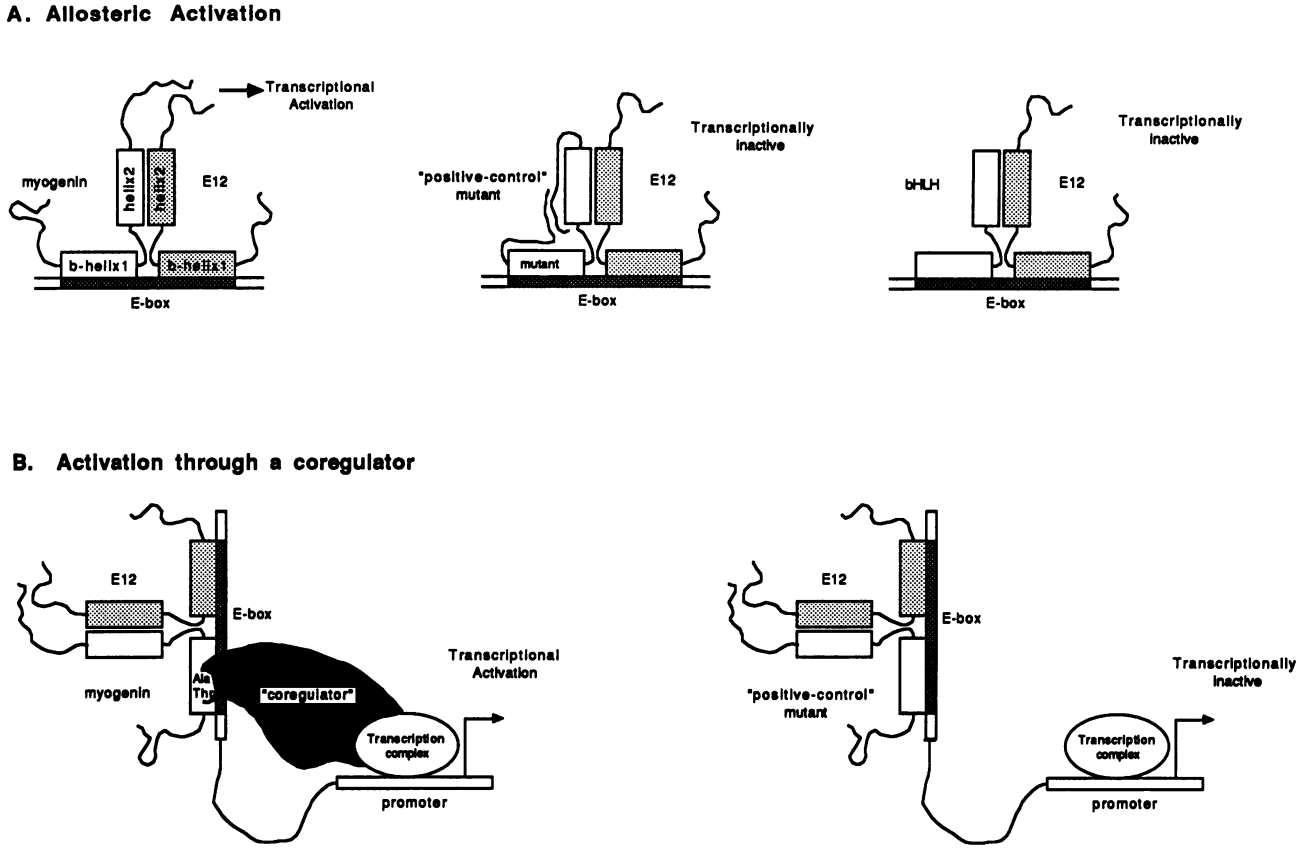


FIG. 6. Hypothetical models for the role of the activation domains and the basic region of myogenin in the activation of muscle-specific transcription. (A) Binding of a myogenin-E12 heterooligomer to an E box in a target muscle-specific enhancer allosterically unmasks the transcriptional activation domains in the N and C termini, which can then cooperate with the activation domains in E12 and in other enhancer binding factors (data not shown) to induce muscle-specific transcription. "Positive-control" mutants can bind to the E box but may be unable to allosterically unmask the transcription activation domains in response to DNA binding. The mutant myogenin-E12 heterooligomer is thus transcriptionally inactive. This model predicts that the basic region of myogenin communicates with E12 to regulate its activation domains. Heterodimers of the bHLH deletion mutant and E12 also bind to the E box but fail to activate transcription because of the absence of transcriptional activation domains in myogenin. The behavior of the bHLH mutant implies that the activation domains in E12 are by themselves insufficient to activate a muscle-specific enhancer. (B) Ala-Thr in the center of the myogenin basic region comprises the MRM, which may serve as part of an interface for interaction with a coregulator that communicates with the transcriptional machinery to activate muscle-specific transcription. Positive-control mutants can bind the E box but cannot interact with the coregulator because of mutagenesis of the MRM. The models in panels A and B are not mutually exclusive and could both be involved in the activation of muscle-specific transcription by myogenin.

sufficient to activate a complex enhancer such as that of MCK, which requires cooperative interactions among heterologous transcription factors for activity. The most likely explanation for the failure of ΔNΔC-myo-VP16 to activate the MCK enhancer is that the activation domains of myogenin mediate protein-protein interactions essential for transcriptional activation. The results obtained with chimera ΔC-myo-VP16 suggest that the N terminus mediates at least some of these interactions because it *trans*-activates MCK-CAT, whereas the chimera without the N terminus cannot. A role for the N and C termini in mediating protein-protein interactions required for enhancer activation is consistent with the findings of Weintraub and coworkers, who showed that the N terminus of MyoD is important for cooperative binding of MyoD to the low- and high-affinity E boxes in the MCK enhancer (46).

The apparent ability of the activation domains of myogenin to influence target gene specificity is also consistent with the observation that myogenin and MRF4 bind the MCK enhancer with comparable affinities but only myogenin *trans*-activates the enhancer (9). However, MRF4, like ΔNΔC-myo-VP16, efficiently *trans*-activates 4R-tkCAT (10). Presumably, MRF4 and ΔNΔC-myo-VP16 are able to induce myogenesis by activating transcription of the other myogenic factors and probably other muscle-specific genes without being able to *trans*-activate the MCK enhancer specifically.

Cell type specificity of *trans*-activation. Previous studies have demonstrated that different cell types show various responses to the MyoD family (37, 48). HeLa cells, for example, are refractory to myogenic conversion and are nonpermissive for *trans*-activation of the MCK enhancer by MyoD (48). Our results show that myogenin also cannot *trans*-activate the MCK enhancer in HeLa cells (Fig. 4). However, myogenin and the myogenin-VP16 chimeras are highly active against 4R-tkCAT as a target gene for *trans*-activation in HeLa cells. These results reveal that myogenin retains the ability to bind DNA in cells such as HeLa cells that are refractory to myogenic conversion and indicate that

the block to muscle-specific gene activation in these cells must be independent of DNA binding.

How can the failure of myogenin to activate MCK-CAT in HeLa cells be explained? Since myogenin is active with 4R-tkCAT in this cell background, the lack of activity with MCK-CAT cannot be attributed to a lack of activity of the transcription activation domains. Indeed, all of the GAL4-myogenin chimeras that show activity in 10T1/2 cells are also active in HeLa cells (39). The lack of full myogenic activity of myogenin in HeLa cells therefore seems most likely to reflect the absence of an essential factor or the presence of a repressor for the MCK enhancer and other endogenous muscle-specific genes. Heterokaryon experiments should distinguish between these possibilities, and they are under way.

The MRM regulates muscle-specific gene activation. Collectively, our results and those of Weintraub and coworkers demonstrate that mutations in the basic regions of myogenin and MyoD can abolish the abilities of these proteins to activate muscle-specific genes without affecting their DNA-binding activities (5, 12, 47). As shown initially for MyoD (47) and confirmed here for myogenin, the VP16 activation domain can substitute for the activation domains within myogenic HLH proteins and partially restore the ability of an MRM mutant to *trans*-activate the simple promoter 4R-tkCAT. However, the VP16 activation domain is unable to restore the ability to induce the endogenous myogenic program, which further indicates the importance of the MRM for muscle-specific gene activation.

These basic region mutations which abolish transcriptional activity without affecting DNA binding are analogous to the so-called "positive-control" mutations that have been described for a variety of other transcription factors, including Oct-1 (40), yeast HAP-1 (21), the glucocorticoid receptor (38), and phage lambda repressor (18), and demonstrate that events in addition to DNA binding are required for activation of muscle-specific transcription. Two models to explain this interdependence of the basic region and activation domains for muscle-specific transcription are schematized in Fig. 6. In the first model (Fig. 6A), the transcriptional activation domains are in an inactive conformation and undergo an allosteric change that potentiates the activation domains upon binding to a muscle-specific E box. Specific amino acids within the basic region, which are not critical for DNA recognition, signal this rearrangement to the transcriptional activating domains. This rearrangement would presumably be unnecessary in the context of the GAL4 chimeras because they are active when binding DNA through the GAL4 domain. In this model, these amino acids are changed in the myogenin and MyoD positive-control mutants such that the basic domain would be unable to signal a productive rearrangement to the activating domains. There is precedent for this type of allosteric activation of a transcription factor upon DNA binding. The *S. cerevisiae* pheromone/receptor transcription factor (PRTF), for example, can bind to upstream activating sequences (UAS) of **a**-specific and α-specific genes but only acts as a transcriptional activator on the UAS of **a**-specific genes. Proteolysis experiments indicate that a conformational change occurs upon binding to the **a**-specific UAS but not the α-specific sequence, suggesting that the protein is in an inactive conformation in solution and that binding to a particular sequence causes the protein to assume an active conformation (42).

An alternate mechanism (Fig. 6B) is for activation of muscle-specific transcription to be dependent on a "coregulator" that recognizes the basic region (24). Such a coregulator might serve as a link between myogenin or MyoD and the basic transcriptional machinery. This type of coregulator would not be required for activity of the activation domains when fused to GAL4, because in this context they are independent of the basic region. An example of such a coregulator is VP16, which cooperates with the homeodomain protein Oct-1 to induce transcription. Positive-control mutations within Oct-1 have mapped the site of interaction to the homeobox domain (40).

The modular structure of myogenin, in which DNA binding and transcriptional activity are mediated by separable domains, is similar in many respects to a variety of other transcription factors. A remarkable feature of myogenin (and probably other members of the MyoD family) is that even though myogenin possesses transcriptional activating domains, the DNA-binding domain nevertheless controls transcriptional activation as well as DNA-binding specificity.

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