Proc. Natl. Acad. Sci. USA
Vol. 93, pp. 9366–9373, September 1996
Colloquium Paper

This paper was presented at a colloquium entitled "Biology of Developmental Transcription Control," organized by Eric H. Davidson, Roy J. Britten, and Gary Felsenfeld, held October 26–28, 1995, at the National Academy of Sciences in Irvine, CA.

Combinatorial control of muscle development by basic helix–loop–helix and MADS-box transcription factors

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ABSTRACT **Members of the MyoD family of muscle-specific basic helix–loop–helix (bHLH) proteins function within a genetic pathway to control skeletal muscle development. Mutational analyses of these factors suggested that their DNA binding domains mediated interaction with a coregulator required for activation of muscle-specific transcription. Members of the myocyte enhancer binding factor 2 (MEF2) family of MADS-box proteins are expressed at high levels in muscle and neural cells and at lower levels in several other cell types. MEF2 factors are unable to activate muscle gene expression alone, but they potentiate the transcriptional activity of myogenic bHLH proteins. This potentiation appears to be mediated by direct interactions between the DNA binding domains of these different types of transcription factors. Biochemical and genetic evidence suggests that MEF2 factors are the coregulators for myogenic bHLH proteins. The presence of MEF2 and cell-specific bHLH proteins in other cell types raises the possibility that these proteins may also cooperate to regulate other programs of cell-specific gene expression. We present a model to account for such cooperative interactions.**

Cell lineage determination and differentiation during embryonic development involves the establishment of unique regulatory programs that direct cell type-restricted patterns of gene expression. Members of the basic helix–loop–helix (bHLH) family of transcription factors have been shown to control determination and differentiation of a variety of cell types, including skeletal muscle, neurons, and hematopoietic cells. In the skeletal muscle lineage, the four myogenic bHLH factors, MyoD, myogenin, Myf5, and MRF4, compose a regulatory pathway that establishes myoblast identity and controls terminal differentiation (reviewed in refs. 1–4). When introduced into nonmuscle cell types, each of these factors can activate the entire program for skeletal myogenesis. Biochemical and genetic experiments have suggested that the myogenic bHLH factors rely on a coregulator to activate muscle gene transcription. Here we review evidence indicating that members of the myocyte enhancer binding factor 2 (MEF2) family of MADS-box transcription factors act as coregulators for myogenic bHLH factors, and we consider the possibility that this type of combinatorial control may represent a more general mechanism for the regulation of cell type-specific transcription.

Regulation of Muscle Transcription by Myogenic bHLH Proteins

Members of the bHLH family of transcription factors share homology within a basic domain and an adjacent helix–loop–helix motif. Cell-specific bHLH factors like the myogenic regulators dimerize preferentially with a ubiquitous class of bHLH proteins known as E proteins, which includes E12, E47, and HEB (5, 106). The resulting heterodimers bind the consensus E-box DNA sequence CANNTG (reviewed in refs. 6–8). E-boxes have been identified in the control regions of many skeletal muscle-specific structural genes where they are required for activation by myogenic bHLH factors (9–20). However, a number of skeletal muscle genes that can be activated by the myogenic bHLH factors lack E-boxes in their control regions, suggesting that these factors can also act through indirect mechanisms to activate muscle-specific gene expression (21–24).

Mutational analysis of the myogenic bHLH factors has revealed several structural domains that cooperate to initiate muscle gene expression (Fig. 1). The bHLH region is required for DNA binding and dimerization of myogenic bHLH factors with E proteins, but this region alone does not efficiently activate myogenesis. Transcription activation domains are located in the N and C termini of the myogenic factors and are important for muscle gene activation (reviewed in ref. 3). These activation domains do not confer muscle specificity to transcription and can be replaced with the activation domain of the viral coactivator VP16 (25–27).

Evidence for a Coregulator that Recognizes the Basic Regions of Myogenic bHLH Factors

The basic regions of the myogenic factors have been the focus of intense interest. There is a 12-amino acid segment of the basic regions of these factors that is necessary and sufficient for DNA binding with the HLH region (Fig. 1). Eight of these 12 residues are conserved in E proteins. Among the nonconserved residues, an alanine and a threonine in the center of the DNA binding domain are required for muscle gene activation, but these residues are not required for DNA binding (28, 29). Mutants of the myogenic factors in which these residues are replaced with asparagines, which are found at the corresponding positions in the DNA binding domains of E proteins, retain the ability to bind DNA, but they cannot activate muscle transcription. Conversely, if the asparagines in the basic region of E12 are replaced with alanine-threonine and an aspartic acid at the junction of the basic region and of helix-1 of E12 is replaced with a lysine, which is found at that position in the myogenic factors, these residues confer upon E12 the ability to activate myogenesis (30). The fact that DNA binding activity is not affected by these substitutions suggests that these amino acids mediate an event subsequent to DNA binding that is essential for activation of muscle gene expression; it has been

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Abbreviations: MEF2, myocyte enhancer binding factor 2; bHLH, basic helix–loop–helix; SRF, serum response factor; E, embryonic day.

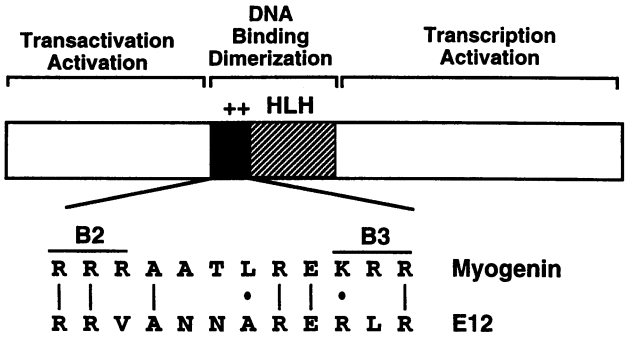

FIG. 1. Functional domains of myogenin. Myogenin contains transcription activation domains near the N and C termini, which are required for activation of muscle gene transcription. The bHLH region is necessary and sufficient for dimerization and DNA binding. The sequences of the basic regions of myogenin and E12 are shown. The alanine and threonine residues in the center of the myogenin basic region are required for muscle gene activation but not for DNA binding. B2 and B3 denote the second and third clusters of basic amino acids in the DNA binding domain (29).

proposed that theses amino acids are necessary for interaction with a coregulator required for muscle gene activation.

The crystal structure of MyoD bound to DNA predicts that the alanine and threonine residues in the basic region are buried in the major groove of the DNA binding site and are inaccessible for interaction with a putative coregulator (31). Intriguingly, however, when MyoD is bound to DNA, these residues induce a conformational change in an adjacent region of the protein that could create a recognition surface for an accessory factor. E proteins do not undergo this conformational change upon DNA binding.

The residues in the basic region required for muscle gene activation are conserved in all known myogenic bHLH factors in species ranging from *Drosophila* (107, 108) and sea urchin (35) to humans (36), and they are not found at the corresponding positions of the more than 50 other known bHLH proteins. These residues have, therefore, been proposed to constitute part of an ancient regulatory motif required for muscle-specific gene activation. Assuming these residues mediate interaction with a myogenic cofactor, this cofactor would also be predicted to be evolutionarily conserved (see below).

The MEF2 Family of Myogenic Regulatory Factors

Recent studies suggest that members of the MEF2 family of transcription factors may act as the coregulators that recognize the basic regions of myogenic bHLH factors. MEF2 was first described as a muscle-specific DNA binding activity that bound a conserved A+T-rich element in the muscle creatine kinase gene enhancer (ref. 37 and reviewed in ref. 38). MEF2 DNA binding activity is present at high levels in skeletal, cardiac, and smooth muscle cells, as well as in neurons (39), and it has also been detected at lower levels in a variety of other cell types (40, 41).

In vertebrates, MEF2 DNA binding activity is encoded by four genes, referred to as *mef2a–d* (39, 40, 42–48). There is also a single MEF2 gene in *Drosophila* (32–34) and *Caenorhabditis elegans* (M. Krause, personal communication).

The MEF2 factors contain a conserved MADS-box at their N termini (Fig. 2), named for the first four members of the family to be identified, MCM1 in yeast, Agamous and Deficiens in plants, and serum response factor (SRF) in vertebrates (reviewed in ref. 49). Immediately C-terminal to the MADS-box is a 29-amino acid domain known as the MEF2 domain, which is unique to the MEF2 factors. Every MEF2 factor described to date has been shown to bind the same DNA consensus sequence, $CTA(A/T)_4TAG$. It was reported that human MEF2B was unable to bind the MEF2 consensus sequence unless the C terminus was deleted (40, 42). However, mouse MEF2B was subsequently shown to bind DNA with the same sequence specificity as the other MEF2 factors (50). Most muscle-specific genes examined to date have been shown to contain MEF2 binding sites in their control regions (23, 24, 37, 51–59). MEF2 sites have also been implicated in directing serum-inducible gene expression (40, 60, 61).

Mutational analyses of the MEF2 proteins have demonstrated that the MADS and MEF2 domains are necessary and sufficient for dimerization and DNA binding (reviewed in ref.

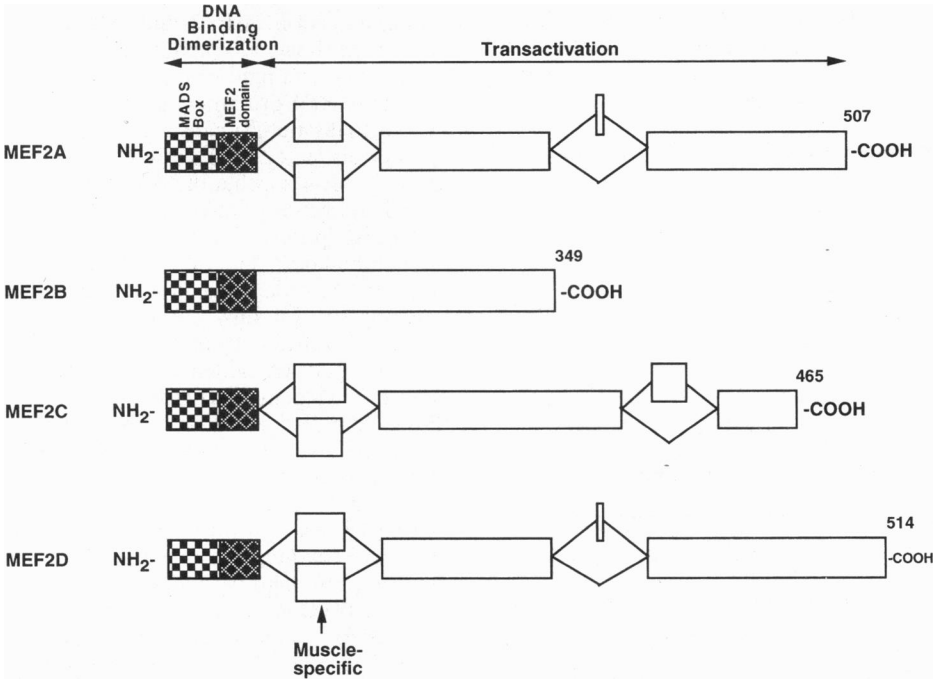

FIG. 2. Schematic representation of the four MEF2 factors. The DNA binding and dimerization region consisting of the MADS and MEF2 domains are shown at the N terminus of each factor. Alternatively spliced exons are also shown.

49; also see ref. 62). The first 28 amino acids of the MADS domain confer DNA binding site specificity, whereas amino acids 35–56 confer dimerization specificity (62–64). These conclusions are supported by the crystal structure of the DNA binding region of SRF with its DNA binding site (65). The DNA binding domain of SRF is composed of three distinct subdomains. The MADS-box contains an extended α-helical region at the N-terminal end that mediates DNA binding, whereas the C-terminal region adopts a β-strand conformation that is oriented away from the DNA and is involved in subunit dimerization (65). Immediately C-terminal to the MADS-box of SRF is a region of β-strand that is also oriented away from the DNA. This region, which is located in the same position as the MEF2 domain in the MEF2 factors, is not conserved in other MADS-box proteins and has been shown to mediate interaction of SRF with accessory factors (reviewed in ref. 66).

The C-terminal regions of the MEF2 proteins direct transactivation (reviewed in ref. 38). These regions are subject to complex patterns of alternative splicing. In transfection assays, there have been no significant differences detected among the different MEF2 isoforms with respect to transcriptional activity. Whether they might possess different specificities *in vivo* or might interact with different accessory factors remains to be determined.

MEF2 and Myogenic bHLH Factors Show Overlapping Expression Patterns in the Skeletal Muscle Lineage

During mouse embryogenesis, the myogenic bHLH factors are expressed in precursors of the skeletal muscle lineage and in developing muscle fibers (reviewed in ref. 1) (Fig. 3). Within the myotomal region of the developing somite, Myf5 is the first of the myogenic bHLH factors to be expressed, beginning at embryonic day 8 (E8). Myogenin is expressed in the myotome at E8.5, followed by MRF4 and MyoD at E9.0 and E10.5, respectively.

The MEF2 factors are also expressed in overlapping patterns in the skeletal muscle lineage, but in contrast to the myogenic bHLH factors, they are also expressed in other lineages. MEF2C is the first member of the MEF2 family to be expressed in the developing myotome at about E8.5 and is followed by expression of MEF2B at about E9.0 and MEF2A and MEF2D at E9.5 (50, 67) (Fig. 3). The MEF2 factors are also expressed in early cardiogenic precursors and in the developing heart, as well as in smooth muscle cells (61, 67, 68).

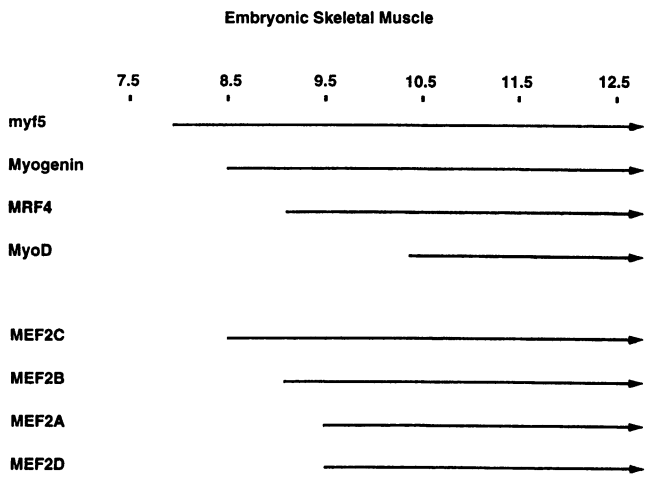

FIG. 3. Embryonic expression patterns of the myogenic bHLH and MEF2 genes in the developing somite of the mouse. The line drawings represent mRNA expression as detected by *in situ* hybridization in the developing somite myotome of the mouse. The numbers represent days post coital.

During brain development, MEF2 factors show highly localized patterns of expression that correlate with differentiation of multiple neuronal cell types (69). Late in embryogenesis, MEF2A, MEF2B, and MEF2D transcripts become expressed in a wide range of cell types, whereas MEF2C expression remains restricted primarily to skeletal muscle, brain, and spleen (43, 44).

MEF2 and Myogenic bHLH Factors Regulate the Expression of Each Other

Forced expression of the myogenic bHLH factors can induce MEF2 DNA binding activity in cells that undergo myogenic conversion, such as 10T1/2 cells, as well as in cells that are refractory to myogenic conversion, such as CV-1 kidney cells (70, 71). These results suggest that MEF2 factors lie in a regulatory pathway downstream of the myogenic bHLH factors (Fig. 4). However, MEF2 sites in the promoters of the mouse myogenin (14, 72–74) and MRF4 genes (19, 20) and the Xenopus MyoDa gene (75) are required for expression in muscle cells, indicating that MEF2 also plays a role in the regulation of the myogenic bHLH genes. The region of the Quail MyoD promoter that is required for tissue-specific expression also contains MEF2 binding sites (76), but their role in MyoD regulation has not yet been determined. Since MEF2 factors are expressed in the skeletal muscle lineage after the myogenic bHLH factors, it is most likely that they are involved in amplification or maintenance of myogenic bHLH gene expression rather than in the initial activation of these genes.

MEF2 and the Myogenic bHLH Proteins Act Cooperatively to Regulate Muscle-Specific Gene Expression

Analysis of the control regions of a number of muscle-specific genes has demonstrated that myogenic bHLH proteins cooperate with MEF2 proteins to activate transcription in skeletal muscle cells. A well-characterized example of this cooperativity is the desmin promoter, which is regulated by an MEF2 site and two E-boxes (17). Li and Capetanaki (17) showed that desmin transcription depends on cooperative interactions between these sites, and they proposed that this type of cooperativity could provide a mechanism for enhancer–promoter communication. MEF2 and myogenic bHLH proteins have also been shown to synergistically activate the muscle creatine kinase, myosin light chain-1/3, and myogenin genes (77).

The MRF4 promoter also contains an MEF2 site and an E-box that synergistically activate transcription in the presence of cotransfected MEF2 and myogenin or MyoD (19, 20). Mutagenesis of either the E-box site or the MEF2 site does not eliminate synergy between MEF2 and myogenin (19), whereas all transcriptional activation is lost when both sites are mutated in combination. This suggested that myogenin or MEF2 can cooperate to activate transcription even when there is a binding site for only one of the two factors and raised the possibility of direct protein–protein interactions between the factors. Further evidence for an interaction between MEF2 and the myogenic bHLH factors came from a study in which Funk and Wright showed that myogenin and MEF2 from myotube nuclear extracts bound DNA cooperatively (78). In this same study, an oligonucleotide containing an E-box and an adjacent MEF2 site was cloned upstream of a minimal promoter and was shown to synergistically activate transcription.

There are muscle genes that lack E-boxes in their control regions but can be induced by myogenic bHLH factors. Transactivation of the myogenin promoter by myogenin and MyoD, for example, requires an MEF2 site, but not an E-box (14, 72). The skeletal muscle-specific enhancer from the troponin C gene also contains a single MEF2 site but no E-boxes (24), yet this enhancer can be strongly activated by either MyoD or myogenin. Mutagenesis of the MEF2 site

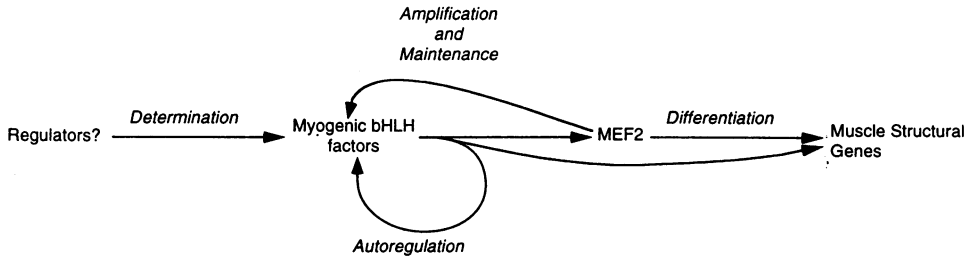

FIG. 4. MEF2 and the myogenic bHLH proteins regulate the expression of each other. The first events in myogenesis are the determination of mesodermal progenitor cells to the myogenic lineage which express myogenic bHLH proteins. The myogenic bHLH proteins then directly activate a number of muscle-specific strutural genes as well as other transcription factors. One of these factors is MEF2, which can then feedback on the promoters of the myogenic bHLH genes to potentiate their expression as well as to directly up-regulate a number of skeletal muscle-specific structural genes. Together, these two factors amplify the expression of each other to augment muscle-specific gene expression.

abolishes enhancer activity, suggesting that the myogenic bHLH proteins are capable of acting indirectly through the MEF2 site.

MEF2 Synergizes with the Myogenic bHLH Proteins During Myogenesis

To determine whether MEF2 factors were able to act like the myogenic bHLH factors to activate muscle gene expression in nonmuscle cells, we have expressed cDNAs encoding the four vertebrate MEF2 factors in transiently and stably transfected 10T1/2 and NIH 3T3 fibroblasts. However, we observed no evidence of muscle gene activation in the presence of any of the MEF2 factors (79). These results differ from another study which reported that MEF2 acted as a myogenic determination factor that could induce conversion of fibroblasts to skeletal muscle with an efficiency comparable to that of MyoD and myogenin (77). The basis for these differences has not been resolved.

Although MEF2 factors, in our hands, are unable to induce myogenesis alone, they enhanced the ability of the myogenic bHLH factors to induce muscle gene expression, suggesting that they might function as cofactors for myogenic bHLH proteins. Based on these results, we initially reasoned that mutants of MEF2 that were able to dimerize but not bind DNA would act in a dominant negative manner to block the ability of myogenic bHLH factors to activate myogenesis. However, these mutants also potentiated the myogenic activity of myogenin and MyoD to the same extent as wild-type MEF2 (79). This suggested that this potentiation between myogenic bHLH factors and MEF2 did not require binding of MEF2 to DNA and raised the possibility that these two types of factors might interact. Indeed, subsequent immunoprecipitation experiments showed that MEF2 can interact with heterodimers formed between myogenic bHLH factors and E12, but it cannot recognize either of these types of bHLH proteins alone. The interaction between myogenic bHLH factors, E12, and MEF2 can also be demonstrated *in vivo* using a GAL4-based tri-hybrid assay. The inability of MEF2 to recognize myogenic bHLH factors or E proteins alone suggests that heterodimerization of these bHLH factors creates a specific determinant for MEF2 recognition.

To determine whether cooperativity between myogenic bHLH and MEF2 factors requires direct protein-protein interactions, we have examined an extensive series of MEF2 mutants. Point mutations in the MADS-box that eliminate DNA binding activity of MEF2 do not significantly affect the ability to interact with the myogenin/E12 heterodimer or the ability to synergize with myogenin in myogenic conversion. Both the MADS and the MEF2 domains are required for interaction with the myogenic bHLH/E12 heterodimer. However, we have identified no single point mutation that significantly diminishes this interaction, suggesting that the binding surface is widely distributed over an extended region of the MADS and MEF2 domains. In every case examined thus far, the ability to interact with the myogenic bHLH/E12 heterodimer correlates with the ability of an MEF2 mutant to synergize with myogenin or MyoD in myogenic conversion.

We have also examined the potential of several myogenin and MyoD mutants to cooperate with MEF2 factors in myogenic conversion. Whereas the bHLH region of myogenin is unable to initiate myogenesis in transfected 10T1/2 cells (26), when expressed with MEF2, it acquires full myogenic potential (79) (Fig. 5). The finding that MEF2 can confer myogenic potential to the bHLH region of myogenin suggests that the bHLH domain of the myogenic factor bound to DNA *in vivo* recruits MEF2, resulting in synergistic activation of myogenesis because of the presence of the transactivation domain provided by MEF2. That a transcription activation domain is required in one of the two factors is demonstrated by the failure of an MEF2C mutant lacking the C-terminal transactivation domain to confer myogenic potential to the bHLH region of myogenin. We believe that the cooperativity between myogenic bHLH and MEF2 factors in muscle gene activation reflects a specific recognition event and not simply a greater degree of transcriptional activity in the presence of the two factors because the MADS and MEF2 domains of MEF2C lack transcriptional activity on their own, but they are able to augment the myogenic potential of full-length myogenin or MyoD.

The synergy between myogenic bHLH and MEF2 factors in activation of the endogenous myogenic program or artificial reporters (see below) depends on the myogenic residues (alanine and threonine) in the DNA binding domains of myogenic bHLH proteins and is not observed with a MyoD mutant containing the E12 basic region (79). Thus, the same residues in the myogenic bHLH factors that were originally predicted from mutational analyses to mediate interaction with a myogenic coregulator are required for synergy between the DNA binding domains of myogenic bHLH factors and MEF2. Whether the myogenic residues in the basic region of MyoD are required for interaction with MEF2 or whether interaction and synergy are separable events remains to be determined. It is conceivable, for example, that MyoD mutants lacking the alanine and threonine residues in the basic region can form a complex with MEF2 but that they cannot adopt a transcriptionally active conformation.

To further define the potential interactions between myogenic bHLH and MEF2 factors, we have examined the ability of these factors to activate a series of artificial reporter genes containing binding sites for one factor or the other. Deletion mutants of myogenin and E12 containing only the bHLH regions are unable to activate an artificial E-box-dependent reporter gene when cotransfected in fibroblasts because they lack functional transactivation domains. However, if these mutants are expressed with a full-length MEF2 protein, strong transactivation is seen, presumably because the myogenin/E12 heterodimer acts as a platform to recruit MEF2 and its

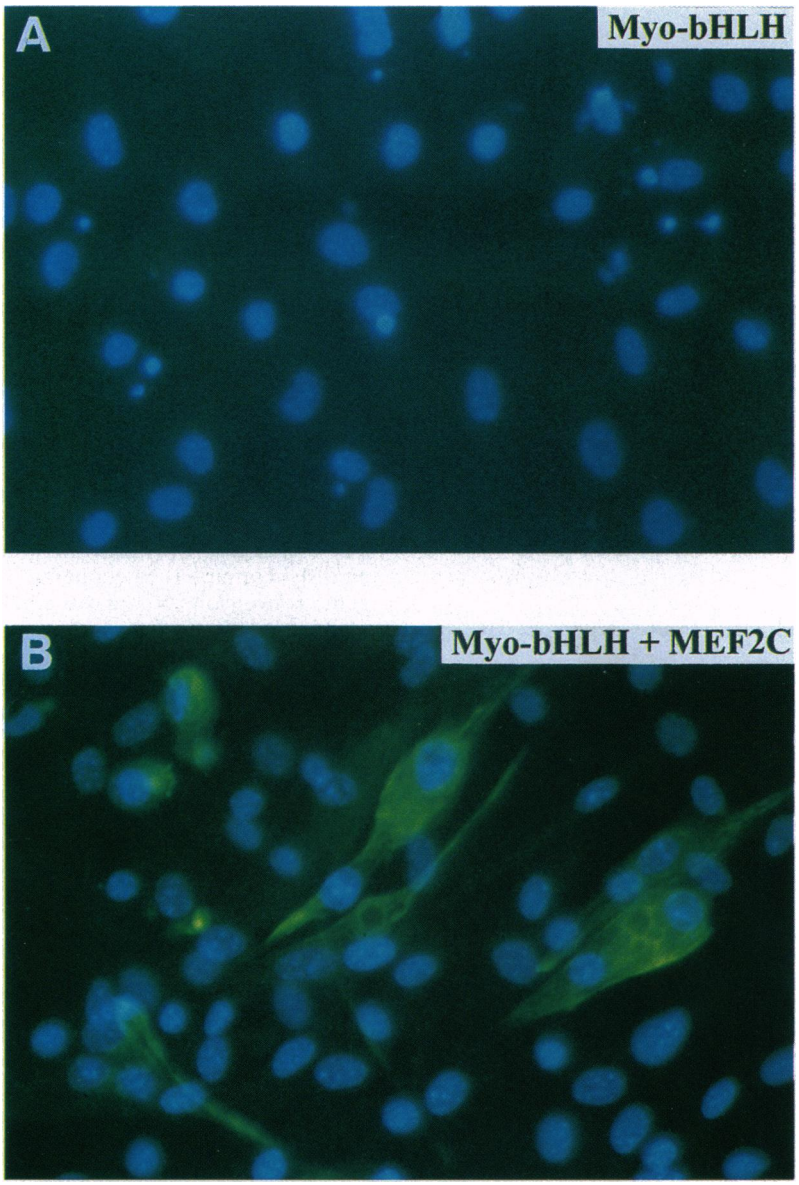

FIG. 5. Synergistic activation of myogenesis by the bHLH region of myogenin and MEF2. 10T1/2 cells were transiently transfected with expression vectors encoding the bHLH region of myogenin alone (*A*) or with MEF2C (*B*). After transfer to differentiation medium for 6 days, cells were stained with Hoechst to label nuclei (blue) and with anti-MHC antibody (green) to show myogenic conversion. Whereas neither the bHLH of myogenin nor MEF2 alone can induce myogenesis, together they lead to efficient myogenic conversion.

transactivation domain to the promoter. This type of recruitment can also occur when MEF2 is bound to DNA. A mutant MEF2 protein consisting of just the MADS and MEF2 domains is unable to transactivate an MEF2-dependent reporter. However, if full-length myogenin and E12 are also coexpressed with this type of mutant, a high level of transactivation is observed. These data demonstrate that either factor can interact with the other when one is bound to DNA.

In these types of indirect transactivation assays, it is formally possible that the factor providing the transcriptional activation domain may be interacting with the target plasmid through a cryptic binding site or that its binding specificity is altered by interaction with the factor bound to its DNA site. However, we believe this is unlikely because this transactivation occurs on several different types of basal promoters linked to multimerized E-boxes or MEF2 sites, and these different promoters do not share obvious sequence homology. Moreover, if the site for the binding factor that serves as the platform is mutated, all synergy is lost. Also, point mutants in MEF2 that eliminate its DNA binding potential do not affect its ability to interact and potentiate transactivation in these assays, providing further evidence that MEF2 does not need to bind DNA to activate transcription through an E-box when the bHLH factors are bound there.

It should be pointed out that these types of assays in which MEF2 and myogenic bHLH factors are overexpressed with reporter genes linked to multimerized E-boxes or MEF2 sites represent a highly simplified system for analyzing potential interactions between these factors and that the control of native promoters and enhancers is likely to be much more complex. The muscle creatine kinase enhancer, for example, contains two E-boxes and two MEF2 sites, and mutations in the right E-box eliminate almost all enhancer activity in muscle cells (16), which indicates that the types of protein–protein interactions we have documented with artificial promoters cannot explain the control of all muscle genes. It seems likely that the ability of native muscle genes to respond to myogenic bHLH and MEF2 factors will depend on a variety of variables, including the distance of E-boxes or MEF2 sites from the basal promoter, the presence of other positive and negative factors

that may bind DNA sequences adjacent to E-boxes and MEF2 sites, and levels of expression of the different myogenic bHLH and MEF2 factors. That the level of expression of the different factors can influence muscle gene activation is illustrated by the phenotype of the myogenin-null mouse in which MyoD, Myf5, and MEF2C are expressed normally, but there is no muscle gene activation (ref. 80; A. Rawls and E.N.O., unpublished data).

Combinatorial Control of Muscle Gene Expression

Potential combinatorial interactions between MEF2 and myogenic bHLH proteins in the control of different muscle-specific genes are schematized in Fig. 6. Some muscle-specific genes contain only E-box sequences in their control regions, yet MEF2 may still contribute to their activation by interaction with myogenic bHLH proteins bound to these sites, as depicted in model 1 (9–20). Some muscle-specific genes lack E-boxes in their control regions, but they contain MEF2 sites that may allow for indirect activation by myogenic bHLH proteins via interaction with MEF2 bound to these sites, as depicted in model 2 (22–24). Many muscle-specific genes contain both sites in their control regions and could be activated as shown in model 3. There are also muscle genes in which MEF2 binds the proximal promoter and myogenic bHLH factors bind a distal enhancer (model 4). Recent studies have shown that MEF2 and myogenic bHLH factors can bind to their target DNA sequences and to each other at the same time (unpublished data), which suggests that model 4 is a likely mechanism whereby distal enhancers might be brought in proximity of the basal promoter. In this regard, overlapping sites for MEF2 and TATA-binding protein are contained in the mouse MRF4 (19, 20) and Xenopus MyoDa promoters (75). The TATA box of the myoglobin promoter also binds MEF2 (81) and is required for muscle-specific expression; this TATA box cannot be substituted with that of the SV40 promoter (82). Myogenic bHLH factors have been shown to activate the skeletal myosin heavy chain IIB promoter through a TATA-containing promoter element that binds MEF2 (83). Together, these results are consistent with a model whereby MEF2 binds to the TATA box region of the promoter and supports an interaction with an E-box-containing distal enhancer that is bound to the myogenic bHLH proteins.

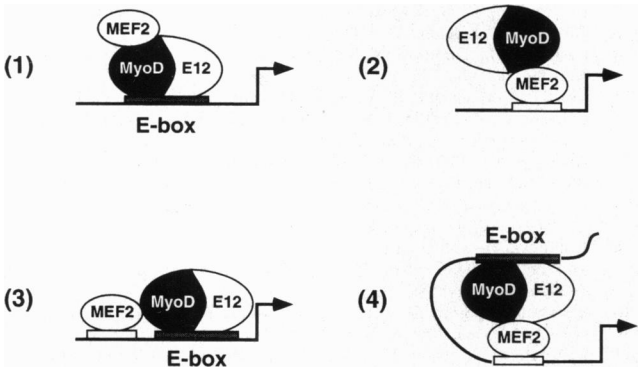

FIG. 6. Models of endogenous gene expression directed by various MEF2 and myogenic bHLH factor interactions. (Model 1) Some muscle-specific promoters only require E-box elements to direct tissue-restricted expression, yet MEF2 may still be part of this regulation via protein–protein interaction. (Model 2) A number of muscle-specific promoters that contain MEF2 binding sites have been shown to be E-box-independent. However, the MEF2 sites may recruit members of the myogenic bHLH family by protein–protein interaction with MEF2. (Model 3) Many muscle-specific promoters contain both MEF2 sites and E-box sites spaced adjacent to one another in an enhancer or in a basal promoter. A local protein–protein interaction may enhance the affinity of each factor for its site as suggested by Funk and Wright (78). (Model 4) Some muscle-specific promoters have E-box and MEF2 sites spaced far apart from one another. Examples exist in which MEF2 factors recognize the TATA box, and in this situation, it could then interact with myogenic bHLH factors bound to distal enhancer E-box sites, thereby providing a mechanism for promoter–enhancer association.

MEF2 Is Required for Myogenesis in *Drosophila*

The transfection assays described above demonstrate clearly that MEF2 factors potentiate the muscle-inducing activity of myogenic bHLH factors, but they do not indicate whether MEF2 is an essential cofactor for myogenic bHLH factors. One could imagine, for example, that myogenic bHLH factors could be incapable of initiating myogenesis in the absence of MEF2, which could account for the failure of certain cell types to be converted to muscle by MyoD. Since MEF2 factors are expressed at low levels in a variety of cell types and since there are four vertebrate MEF2 genes, it may be difficult to assay for the functions of myogenic bHLH factors in the absence of MEF2. Complete loss-of-function assays in vertebrate cells may also be difficult if MEF2 factors are essential for the regulation of serum-inducible genes (40, 60). However, analysis of the functions of MEF2 in muscle gene regulation has been facilitated in *Drosophila*, which contains a single *mef2* gene, *D-mef2* (32–34). Like the vertebrate *mef2* genes, *D-mef2* is expressed in muscle cell precursors and their descendants, as well as in regions of the central nervous system (84). The D-MEF2 protein also binds the same DNA sequence as the vertebrate factors and it can activate transcription through the MEF2 binding site in mammalian cells.

Loss-of-function mutations of *D-mef2* result in embryos completely lacking differentiated skeletal, cardiac, and visceral muscle (85–87). However, myoblasts from these lineages are correctly positioned and specified, which indicates that D-MEF2 acts at a relatively late step in the developmental pathways leading to the formation of differentiated muscle cell types. The single myogenic bHLH gene, *nautilus*, is expressed at the correct time and place in the skeletal muscle lineage of *D-mef2* mutant embryos, but muscle structural genes are not expressed. This result suggests that nautilus protein is inactive in the absence of MEF2 and is consistent with the notion that MEF2 is an essential cofactor for MyoD. The potential cofactors for MEF2 in the cardiac and visceral muscle lineages of *Drosophila* remain to be identified.

Myogenic bHLH–MEF2 Interactions as a Potential Target for Negative Control of Myogenesis

There are several situations in which myogenic bHLH factors are able to bind DNA but unable to activate muscle transcription. Exposure of myoblasts to type β transforming growth factor (88) or expression of the activated form of the transmembrane signaling protein Notch (89), for example, interfere with the transcriptional activity of MyoD and myogenin. The immediate early gene products Fos and Jun also block the transcriptional activity of myogenic bHLH factors (90). This form of repression is targeted at the bHLH region of the myogenic factors and has been proposed to occur through competition for interaction with an essential coregulator of myogenesis. Similarly, in the skeletal muscle tumor rhabdomyosarcoma, MyoD can bind DNA, but it cannot initiate the myogenic program (91). In each of these cases, inhibition of MyoD or myogenin function has been mapped to the basic region, which suggests that these inhibitory pathways may be targeted at the coregulator that recognizes the basic region. It will be of interest to determine whether MEF2 expression or activity are altered under these conditions or whether there is a block in the interaction between MEF2 and myogenic bHLH factors.

Protein kinase C is also a potent inhibitor of muscle gene activation. One mechanism by which protein kinase C inhibits myogenesis is through phosphorylation of the threonine in the basic region of myogenin, which prevents DNA binding (92). If this threonine is replaced with aspartic acid to mimic phosphorylation, the ability of myogenin to synergize with MEF2 in transcriptional activation of MEF2-dependent reporter genes is lost (79). This suggests that in addition to blocking DNA binding, phosphorylation of myogenin by protein kinase C may interfere with the ability of myogenin to interact with MEF2.

Possible Mechanisms for Synergy

The transfection assays and coimmunoprecipitation experiments demonstrate that the DNA binding domains of myogenic bHLH and MEF2 factors play a dual role in DNA–protein and protein–protein interactions; but how do the interactions between these factors actually result in cooperative activation of muscle transcription? Because both types of factors can bind DNA independently and contain transcription activation domains, it is perhaps surprising that they rely on each other to coregulate the myogenic program. One possible explanation for this interdependence is that the ternary complex formed between the myogenic bHLH factor, E12, and MEF2 creates a unique surface that interacts with the transcriptional machinery more efficiently than any of the individual factors alone. Alternatively, MEF2 might induce a conformational change in the myogenic bHLH factor, enabling it to activate transcription. In this regard, it has been reported that the activation domain of MyoD is normally cryptic and that the alanine and threonine residues in the basic region are required for this domain to be unmasked upon DNA binding (30). It is also conceivable that the three proteins, MyoD, E12, and MEF2, act through a concerted mechanism to activate transcription more efficiently together than alone. Resolution of these questions will require identification of the targets for the ternary complex in the transcriptional machinery.

MEF2 as a Potential Cofactor for Other Tissue-Restricted bHLH Proteins

The *D-mef2* loss-of-function phenotype suggests that in addition to its role in skeletal muscle, MEF2 is required for differentiation of cardiac and visceral muscle. The notion that MEF2 acts as a cofactor for myogenic bHLH proteins, but does not itself activate skeletal muscle transcription, is consistent with the fact that MEF2 factors are expressed at high levels in cardiac and smooth muscle and in neurons. Based on the inability of cardiac and visceral muscle cells to differentiate in *Drosophila* embryos lacking MEF2, we propose that MEF2 may serve as a cofactor for other cell-specific transcription factors, bHLH or otherwise. Although our studies indicate that the myogenic amino acids in the basic regions of myogenic bHLH factors are required for synergy with MEF2, the possibility that MEF2 might also recognize the basic regions of other bHLH factors cannot be ruled out.

MEF2 DNA binding sites are present in the control regions of numerous cardiac-specific promoters and enhancers, such as the α-MHC, MLC-2v, and the ANF genes. Detailed studies on the MLC-2v (23, 93–96) and α-MHC promoters (97) has shown that binding sites for the bHLH protein USF are closely associated with the MEF2 site. Whether MEF2 and USF might interact to control cardiac muscle transcription remains to be determined. Two cardiac-restricted bHLH factors, dHAND and eHAND (98–100), have recently been identified and shown to be important for cardiac looping during chicken embryogenesis. It will be of interest to determine whether these factors might also interact with MEF2 to control a specific program of gene expression during cardiac morphogenesis.

In the neural lineage, members of the Achaete-scute family of bHLH factors (101) and Neuro-D (102) have been shown to regulate cell determination and differentiation. Since many of the MEF2 factors demonstrate unique patterns of expression in restricted regions of the brain (69), it will be of interest to determine if MEF2 acts as a regulator for these neurogenic bHLH factors. Indeed, recent studies suggest that MEF2 interacts strongly with the basic region of MASH1 (B. Black and E.N.O., unpublished data). This suggests that MEF2 recognizes a secondary structural conformation of the basic region and possibly the HLH of different bHLH factors and that the myogenic residues are not an obligatory component of this recognition.

The finding that MEF2 acts as an accessory factor for myogenic bHLH factors is consistent with the functions of MADS-box proteins in other systems. SRF, for example, interacts with the homeodomain protein Phox, resulting in an increase in SRF's DNA binding activity (103). SRF also interacts with a number of ETS-domain proteins that form ternary complexes with adjacent binding sites (reviewed in ref. 66). In *Saccharomyces cerevisiae*, the MADS-box factor MCM1 interacts with the accessory factors α1 and α2 (104) to control cell type-specific genes and with the factor STE12 to control pheromone responsiveness (105). Given that other MADS-box proteins cooperate with regulatory factors that bind DNA sequences adjacent to the MADS-box binding site, it will also be of interest to analyze the sequences that flank MEF2 sites to determine if they play a role in determining MEF2 specificity in cell lineages other than skeletal muscle.

We thank A. Tizenor for assistance with graphics and T. Davis for editorial assistance. J.D.M. was supported by a National Institutes of Health postdoctoral fellowship. E.N.O. was supported by grants from the National Institutes of Health, The Muscular Dystrophy Association, The Robert A. Welch Foundation, and The Human Science Frontiers Program and in part by the National Cancer Institute.

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