MINI-REVIEW

Regulation of Muscle Differentiation by the MEF2 Family of MADS Box Transcription Factors

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INTRODUCTION

There has been dramatic progress in recent years toward understanding the molecular mechanisms that regulate skeletal muscle development. In contrast, relatively little is known of the mechanisms that give rise to cardiac and smooth muscle during embryogenesis. These different muscle cell types express many of the same muscle-specific genes. However, they are each unique in several respects, including the spectrum of muscle isoforms expressed, morphology, contractile properties, and ability to divide. In principle, the expression of muscle genes in skeletal, cardiac, and smooth muscle cells could be controlled by a shared myogenic regulatory program, which is modified within each lineage to confer the unique identities of each muscle cell type. Alternatively, there could be myogenic regulatory factors unique to each myogenic lineage, which act through different cis-acting DNA sequences to activate muscle structural genes. This review will consider recent evidence that suggests the existence of a common myogenic program, controlled by the myocyte enhancer factor-2 (MEF2) family of MADS box transcription factors.

Embryonic Origins of the Three Muscle Cell Types

During vertebrate embryogenesis, skeletal, cardiac, and smooth muscle cells arise from distinct mesodermal precursors in different regions of the embryo (Fig. 1). Skeletal muscle is derived from the somites, which form by segmentation of the paraxial mesoderm lateral to the neural tube (Wachtler and Christ, 1992). The somites appear initially as epithelial spheres, which subsequently become compartmentalized to form the dermamyotome and the sclerotome. The region of the dermamyotome adjacent to the neural tube gives rise to muscle precursors that form the myotome, the origin of the vertebral and back muscles. Cells from the ventrolateral edge of the dermamyotome migrate out to form the muscles of the limbs and body wall.

Cardiac muscle arises from cells in the anterior lateral plate mesoderm, which become committed to a cardiogenic fate soon after gastrulation. These precardial cells give rise to a heart tube, which undergoes looping, followed by formation of the atria and ventricles.

In contrast to skeletal and cardiac muscle, which arise from distinct populations of mesodermal precursors, smooth muscle arises throughout the embryo from different populations of mesenchymal cell precursors, as well as from the neural crest (Miano et al., 1994). Among the three major muscle cell types, the least is known about the mechanisms that control muscle gene expression in smooth muscle cells.

Regulation of Muscle Differentiation by Myogenic bHLH Proteins

Much of our knowledge of skeletal muscle development has come from the discovery of the MyoD family of basic helix-loop-helix (bHLH) proteins, which can activate skeletal muscle gene expression when expressed ectopically in nonmuscle cell types (reviewed in Olson, 1990; Weintraub et al., 1991; Rudnicky and Jaenisch, 1995). There are four members of this family in vertebrate species, MyoD, Myogenin, Myf5, and MRF4, which share extensive amino acid homology within their bHLH regions. During embryogenesis, the myogenic bHLH factors show overlapping, but distinct, expression patterns in the skeletal muscle lineage. Myf5 is the first member of the family to be expressed in the mouse, appearing in the dorsomedial region of the rostral uncompartmentalized somites beginning at Day 8.0 post coitum (p.c.). Myogenin is expressed in the myotome about a half-day later and MRF4 and MyoD are expressed beginning at Days 9.5 and 10.5 p.c., respectively (reviewed in...
MEF2 Family of MADS Box Transcription Factors

MEF2 was first identified as a DNA binding activity that recognized an A/T-rich element in the muscle creatine kinase (MCK) enhancer that was essential for full enhancer activity (Gossett et al., 1989). Subsequently, MEF2 sites have been found in the promoters and enhancers of the majority of skeletal and cardiac muscle structural genes (Braun et al., 1989; Ianello et al., 1991; Wentworth et al., 1991; Zhu et al., 1991; Nakatsuji et al., 1992; Nakatsuji et al., 1992; Muscat et al., 1992; Morisaki and Holmes, 1993; Hidaka et al., 1993; Molkentin and Markham, 1993; Wang et al., 1994; Li and Capetanaki, 1994; Parmacek et al., 1994). The cloning of genes encoding MEF2 factors (also called RSRFs, for related to Serum Response Factors) revealed that these proteins belong to the MADS box family of transcription factors, named for the first four factors in which this domain was identified: MCM1, which regulates mating type-specific genes in yeast, Agamous and Deficiens, which act as homeotic factors that control flower development, and Serum Response Factor, which controls serum-inducible and muscle-specific gene expression (Pollock and Treisman, 1991). Four \textit{mef2} genes, referred to as \textit{mef2A}–\textit{mef2D}, have been identified in vertebrate species (Pollock and Treisman, 1991; Yu et al., 1992; Martin et al., 1993, 1994; Brettart et al., 1993; McDermott et al., 1993; Lefter et al., 1993; Chambers et al., 1994; Wong et al., 1994). There is also a single \textit{mef2} gene in \textit{Drosophila} (Lilly et al., 1994; Nguyen et al., 1994; Taylor et al., 1995) and in \textit{Caenorhabditis elegans} (M. Krause, personal communication). The MEF2 proteins share greater than 80% amino acid homology within the 56-amino-acid MADS box at their amino termini (Fig. 2). Adjacent to the MADS box is a 29-amino-acid domain, called the MEF2 domain, that is not present in other MADS box proteins.

The MADS box mediates DNA binding and dimerization (reviewed in Shore and Sharrocks, 1995). Consistent with the homology among MADS box proteins, different members of this family recognize similar A/T-rich DNA sequences. However, the consensus sequence for MEF2 binding, YTA(A/T)4TAR, is distinct from the binding sites of other MADS box proteins. The binding sites for MEF2 and other MADS box proteins exhibit dyad symmetry, allowing each component of the dimeric DNA binding complex to recognize half of the binding site. Mutagenesis of several MADS box proteins including MEF2 has shown that DNA binding requires the 56-amino-acid MADS box, in addition to an extension of about 30 amino acids on the carboxyl-terminal side of the MADS box, which is unique to each subclass of MADS box proteins (Pollock and Treisman, 1991; Molkentin and Olson, 1995). The three-dimensional structure of the MADS box has not yet been elucidated. However, sequence specificity of DNA binding has been shown to be mediated by basic residues that lie on the same side of a predicted \alpha-helix at the amino-terminal end of the MADS box. Replacing these 28 amino-terminal residues of the MADS box of SRF with those of...
MEF2A alters the DNA binding specificity of SRF to that of MEF2 (Sharrocks et al., 1993).

MEF2A, MEF2C, and MEF2D bind the same DNA sequence, but subtle differences in binding affinity have been observed among these MEF2 factors, suggesting that their non-conserved residues may affect their binding properties. DNA sequences surrounding the core consensus sequence also affect DNA binding (Yu et al., 1992). MEF2B binds the MEF2 consensus sequence as a homodimer in vivo or in vitro (Pollock and Treisman, 1991; Yu et al., 1992). However, binding activity is observed with a deletion mutant of MEF2B containing only the MADS and MEF2 domains, suggesting that the carboxyl-terminal region of MEF2B inhibits DNA binding. Intriguingly, MEF2B activates transcription through the MEF2 site in transfected cells, which raises the possibility that it may have a unique partner in vivo or that it potentiates the activity of other MEF2 factors.

The MEF2 binding site also binds several factors in addition to MEF2, which in principle can allow for regulation of gene expression through competition for DNA binding. In the core of the MCK enhancer, for example, is a low-affinity MEF2 site that is essential for enhancer activity in skeletal and cardiac muscle cells. This site also binds the paired-like homeodomain protein M Hox and the POU domain protein Oct-1. Mutational analysis of this site has shown that it must be bound by MEF2 for enhancer activity in muscle cells (Cserjesi et al., 1994). Since occupancy of this site by these three factors is mutually exclusive, the activity of the enhancer is dependent on which factor is bound at the site. The MEF2 site in the myosin light chain-2 (mlc-2) gene promoter also binds a tissue-restricted zinc finger protein (HF-1b), which appears to play an important role in transcriptional activation (Zhu et al., 1993), and a serum-inducible cardiac-specific factor, BBF-1 (Zhou et al., 1993). MEF2 factors can homo- and heterodimerize, but they do not interact with other known MADS box factors (Pollock and Treisman, 1991). Dimerization of MEF2 is mediated by a hydrophobic stretch of amino acids toward the C-terminal end of the MADS box, which is predicted to adopt a β-strand conformation. The MEF2 domain is also required for efficient DNA binding, but it does not affect DNA sequence recognition (Molkentin et al., 1995). Most likely, this region confers dimerization specificity and influences the orientation of the DNA binding domains of the heterodimeric partners.

The carboxyl termini of MEF2A, MEF2C, and MEF2D have been shown to function as transcription activation domains (Wong et al., 1994; Martin et al., 1994; Molkentin et al., 1995). Mutations in this region of the Drosophila MEF2 protein result in partial loss-of-function alleles (Ranganayakulu et al., 1995; discussed below). The mef2A, mef2C, and mef2D genes give rise to multiple proteins by alternative splicing within the transcription activation domain, with certain exons being muscle-specific and others being ubiquitous. Heterodimerization among different MEF2 proteins and different splice variants can in principle result in greater than 100 different heterodimeric complexes that recognize the same DNA sequence.
It remains to be determined how the different splice variants might differ functionally.

Regulation of MEF2 Expression

During mouse embryogenesis, the mef2 genes are expressed in precursors of the three myogenic lineages and their descendants. mef2C is the first member of the family to be expressed, appearing in the precardiac mesoderm at Day 7.5 p.c. (Edmondson et al., 1994). Soon thereafter, the other mef2 genes are expressed in the developing heart and the expression of cardiac muscle structural genes ensues. All of the embryonic expression studies have been performed with probes that do not distinguish between the different exons of the mef2 genes. Expression patterns of muscle-specific and ubiquitous exons of the various mef2 genes remain to be determined. The expression of mef2C in the cardiac muscle lineage co-occurs with the expression of the cardiac lineage gene Nkx-2.5 (Lints et al., 1993; Komuro and Izumo, 1993). A homologue of this gene called tinman has been identified in Drosophila, where it is required for formation of the dorsal vessel, which is analogous to the heart (Bodmer, 1995). The coexpression of MEF2 and this homeodomain protein in the early heart is especially intriguing in light of the cooperation between MADS and homeodomain proteins in other systems (see below) and suggests the possibility of direct interactions between these proteins.

In the skeletal muscle lineage, MEF2C is expressed in the somite myotome a few hours after Myogenin, making it unlikely that MEF2 is required for the genesis of myoblasts or for the initial activation of myogenin gene expression (Edmondson et al., 1994). As in the cardiac muscle lineage, the other mef2 genes are expressed in the skeletal muscle lineage after mef2C. mef2 gene expression is also observed in smooth muscle cells throughout the mouse embryo, where it precedes the expression of muscle structural genes.

By about Day 14 p.c. of mouse embryogenesis, mef2 transcripts begin to appear in a variety of nonmuscle cell types and by birth, mef2A, mef2B, and mef2D are expressed ubiquitously, except in the brain, where they show highly localized expression patterns (Lyons et al., 1995). mef2C expression remains restricted to skeletal muscle, brain, and spleen in adults. Within the developing brain, expression of the mef2 genes follows gradients of neuronal differentiation.

One of the perplexing aspects of MEF2 regulation is the disparity between the expression of MEF2 mRNAs and proteins. MEF2A, MEF2B, and MEF2D transcripts are expressed in a wide range of adult tissues and established cell lines, but MEF2 protein and DNA binding activity are largely restricted to differentiated muscle cells and neurons. The most likely explanation for this disparity is the existence of a mechanism for translational repression of MEF2 mRNAs in cell types in which the protein is undetected. Indeed, translation control of MEF2A expression has recently been demonstrated in vascular smooth muscle cells (Suzuki et al., 1995). There is evidence to suspect that translational control might be mediated by the untranslated regions of MEF2 mRNAs because exogenous MEF2 transcripts lacking sequences from the 5' and 3' untranslated regions are translated in fibroblasts, in which endogenous MEF2 transcripts are not efficiently translated. The high degree of sequence conservation of the untranslated regions of MEF2 mRNAs also suggests that they may play a regulatory role.

A Mutually Reinforcing Network of Myogenic Factors

The cell type distribution of MEF2 DNA binding activity has been the subject of much debate. While numerous studies have documented that MEF2 is highly enriched in differentiated muscle cells (Cserjesi and Olson, 1991; Muscat et al., 1992; Hidaka et al., 1993; McDermott et al., 1993; Molken and Markham, 1993), others have reported that it is ubiquitous (Horlick et al., 1990; Plochocka and Treisman, 1991; Han et al., 1992). The basis for these discrepancies is unclear. Consistent with the enrichment of MEF2 activity in muscle cells, reporter genes linked to multimerized MEF2 sites are preferentially expressed in differentiated myocytes (Gossett et al., 1989; Yu et al., 1992). The mechanisms that regulate MEF2 expression during myogenesis have been controversial. Gossett et al. (1989) reported that protein synthesis was required for the upregulation of MEF2 DNA binding activity that accompanies myoblast differentiation, whereas Goebel et al. (1994) reported that MEF2 activity was induced in the presence of cycloheximide.

During differentiation of skeletal myoblasts in vitro, the different MEF2 proteins accumulate sequentially. MEF2D has been reported to be expressed first in myoblasts, but it apparently does not activate muscle target genes until myoblasts exit the cell cycle (Breitbart et al., 1993). The mechanisms that repress MEF2D activity in myoblasts remain to be determined. Following initiation of the differentiation program by withdrawal of serum, MEF2A protein accumulates. MEF2C protein does not appear until late in terminally differentiated myotubes (McDermott et al., 1993; Martin et al., 1993). These sequential expression patterns of the MEF2 factors during myogenesis are reminiscent of the expression patterns of the myogenic bHLH proteins.

MEF2 binding activity can be induced in nonmuscle cells by forced expression of myogenic bHLH factors (Lassar et al., 1991; Cserjesi et al., 1994). This upregulation occurs in 10T1/2 fibroblasts in which the complete myogenic program is induced, as well as in CV-1 cells, which are refractory to myogenic conversion. These findings led to the initial conclusion that MEF2 was present in a regulatory pathway "downstream" of myogenic bHLH proteins. However, it was recently reported that forced expression of MEF2A in fibroblasts can activate expression of Myogenin and MyoD, resulting in formation of multinucleated myotubes and muscle differentiation (Kaushal et al., 1994). The efficiency of
FIG. 3. Myogenic bHLH proteins and MEF2 factors in the skeletal muscle lineage. Early mesodermal regulators induce the expression of myogenic bHLH factors during myoblast determination. Myogenic bHLH factors autoregulate their expression and induce the expression of MEF2, which binds the promoters of several myogenic bHLH genes, amplifying and maintaining their transcription. MEF2 and myogenic bHLH factors collaborate to induce muscle structural genes during differentiation. While MEF2 can be induced by myogenic bHLH factors in tissue culture, this has not yet been demonstrated in vivo. Other regulators might also initially induce MEF2 expression in skeletal muscle cells independent of myogenic bHLH factors. This schematic model is highly simplified and does not take into account differences in expression of individual myogenic bHLH and MEF2 factors during embryogenesis.

myogenic conversion by MEF2A was similar to MyoD. These results suggest that skeletal muscle determination and differentiation are not controlled by a linear genetic pathway, but rather by two classes of regulators that regulate each others’ expression in a mutually reinforcing regulatory network (Fig. 3). It should be pointed out that other investigators have been unable to demonstrate stable conversion of fibroblasts to differentiated muscle cells by forced expression of MEF2 (J. Martin, T. Firulli, and E. Olson, unpublished). The basis for these differences is unclear.

The ability of MEF2 factors to regulate muscle gene expression has also been examined in the Xenopus animal cap system. Animal pole cells from blastula stage embryos normally differentiate as ectoderm and neural tissues. Specification of the developmental fate of these cells can be changed by exposure to growth factors that induce the differentiation of mesodermal derivatives, such as muscle. Although forced expression of MEF2D in animal caps was unable to induce expression of endogenous myogenic bHLH genes, activation of the endogenous cardiac-specific gene mlc-2 was detected in isolated animal pole explants dissected from early blastula stage embryos that were injected as fertilized eggs with synthetic MEF2D transcripts (Chambers et al., 1994). This result is consistent with the expression of MEF2D in cardiac muscle cell precursors and supports a role for MEF2 in the differentiation of cardiac muscle cell lineages. Expression of the mlc-2 gene was activated by ectopic expression of MEF2D but not by expression of MEF2A, suggesting that functional differences exist between members of the MEF2 protein family.

MEF2 Factors Regulate Some Myogenic bHLH Genes

Several recent studies have revealed a role for MEF2 in the regulation of the myogenic bHLH genes. The myogenin promoter, for example, contains a MEF2 site that is required for high level transcription in cultured muscle cells (Edmondson et al., 1992; Buchberger et al., 1994). This site is also required for expression of a myogenin–lacZ transgene in the limb buds and somites of transgenic mice (Cheng et al., 1993; Yee and Rigby, 1993). In the absence of this site, transgene expression in the limb buds and the dorsal regions of the somites is lost. However, myogenin transcription in the ventral regions of the somites does not require the MEF2 site. These results suggest that Myogenin is expressed in two distinct populations of muscle cells; one of which requires MEF2 and the other of which is independent of MEF2, for expression of myogenin. The existence of two populations of somitic muscle cell precursors has also been demonstrated by somite transplantation experiments (Ordahl and Le Douarin, 1992). It is possible that the myogenin–lacZ transgene containing a mutated MEF2 site is able to distinguish between these two populations. Because MEF2 gene expression is initiated after myogenin expression in the somites and limb buds, it is likely that MEF2 participates in an indirect autoregulatory loop to amplify and maintain myogenin gene expression, rather than initially activating myogenin expression (Fig. 3).

MEF2 has also been shown to regulate expression of the Xenopus MyoDα gene. In this case, a consensus MEF2 binding site overlaps precisely with the XMyoDα TATA box, the binding site for the multisubunit transcription factor TFIID (Leibham et al., 1994). Binding of both factors to the specialized TATA motif is required since transactivation is abolished by promoter mutations that selectively prevent binding of either factor. Activation of XMyoDα promoter by MEF2 requires only the MADS/MEF2 domains; this activation is independent of a region toward the C-terminus of MEF2 which is required to activate transcription of promoters with separate binding sites for MEF2 and
TFIID (Wong et al., 1994). Presumably, different types of interactions occur between MEF2 and the transcriptional machinery depending on whether MEF2 is bound to the TATA box or elsewhere.

Since the DNA binding component of TFIID (TBP) interacts with the minor groove and MEF2 with the major groove of DNA, both factors could, in principle, occupy the same site simultaneously. However, it has not yet been possible to demonstrate simultaneous binding of recombinant TBP and MEF2 to the XMyoDa TATA box in vitro. This might reflect a requirement for additional components (TAFs?) necessary for a stable complex or it could indicate that binding of these factors to the XMyoDa TATA motif is mutually exclusive. In the latter case, prior binding of MEF2 might prevent inactivation of the promoter by chromatin assembly or other inhibitory events. Because activation of XMyoD expression precedes that of MEF2 in early Xenopus embryos, MEF2 appears to function downstream of XMyoD in the myogenic pathway. The binding of MEF2 to the XMyoDa TATA box might constitute a simple and direct mechanism for stabilizing and amplifying XMyoDa expression in differentiating muscle cells. MyoD genes from other species appear to be regulated by different mechanisms than XMyoDa, since the mouse and chicken myoD genes do not contain MEF2 sites in their proximal promoters (Tapscott et al., 1992; Dechesne et al., 1994).

Binding of MEF2 to the TATA boxes of muscle-specific genes may explain the observation that certain muscle genes require a specific TATA box for expression. Overlapping binding sites for MEF2 and TBP are present in the promoters of the mouse and rat MRF4 genes (Naidu et al., 1995; Black et al., 1995). Since MEF2 can also transactivate the MRF4 promoter upon binding to the TATA box, this architecture appears to be biologically meaningful rather than fortuitous. The presence of overlapping binding sites for MEF2 and TBP is not restricted to the promoters of myogenic regulatory genes. The myoglobin gene is efficiently expressed in muscle cells only with its native TATA box, but not when that sequence is replaced with the TATA box of the SV40 promoter (Wefald et al., 1990). The myoglobin TATA box has been recently shown to bind MEF2 (Grayson et al., 1995). Analogous situations exist with the chick ß-globin gene promoter, which is activated by binding of the erythroid-specific protein cGATA-1 to the TATA motif (Fong and Emerson, 1992) and the pituitary specific regulatory gene PIT-1/GHF-1 TATA box, which binds a pituitary-specific factor (McCormick et al., 1990). These and other examples are illustrative of a general control mechanism involving the direct interaction of regulatory factors with specialized TATA boxes.

Genetic Analysis of MEF2 Function in Drosophila

Given the overlapping expression patterns and possible redundancy of the vertebrate MEF2 genes, it may be difficult to address their functions by gene targeting without simultaneously inactivating multiple loci. However, the genetic analysis of MEF2 has been facilitated by the mutagenesis and phenotypic analysis of the single mef2 gene of Drosophila, called D-mef2. The MADS and MEF2 domains of the D-MEF2 protein share greater than 85% amino acid identity with the corresponding regions of the mammalian MEF2 factors. Outside of these conserved motifs, D-MEF2 diverges significantly from the vertebrate proteins, but like those factors it is rich in glutamine, serine, threonine, and proline in its carboxyl-terminal region. D-MEF2 binds the same DNA sequence as its vertebrate homologues and can activate transcription in Drosophila and mammalian cells through the MEF2 binding site (Lilly et al., 1994; Nguyen et al., 1994). Within the conserved region of the gene, the positions of D-mef2 introns map to the same codons as in the mammalian mef2 genes. Thus, this structural and functional conservation suggests that the Drosophila and mammalian mef2 genes evolved from a common ancestral gene more than 600 million years ago.

D-mef2 is expressed during embryogenesis in a profile that is strikingly similar to the MEF2 expression patterns observed in early mouse development. Gene transcripts are first detected in cells of the ventral furrow in the late blastoderm/early gastrula and are restricted to the mesodermal cell layer during germ band extension (Lilly et al., 1994; Nguyen et al., 1994; Taylor et al., 1995). During the reorganization of the mesoderm during stage 10 and thereafter (Bate, 1993), there is a dynamic pattern of D-mef2 expression in the external and internal mesoderm cell layers and in the precursors of the heart. During germ band retraction and dorsal closure of the embryo, the muscles of the body wall, gut, and heart are formed. D-mef2 transcripts are present in all of these muscle types during their differentiation into the final muscle structures. Immunolocalization experiments using a D-MEF2 antibody shows the expression of a nuclear protein that faithfully follows the accumulation of D-mef2 RNA in the mesoderm, muscle cell lineages, and differentiated muscles (Fig. 4) (Lilly et al., 1995; Bour et al., 1995).

There is a lack of D-mef2 expression in twist mutant embryos and expression is severely reduced in snail mutant embryos, implicating these two transcription factors as probable regulators of early D-mef2 expression (Lilly et al., 1994; Nguyen et al., 1994). D-mef2 appears to be a direct target of the Twist protein as ectopic expression of a twist cDNA in the epidermis under the control of a heat shock promoter results in the expression of D-mef2 RNA in the same cells (Taylor et al., 1995). Intriguingly, D-mef2 is coexpressed with tinman in the ventral furrow, undifferentiated mesoderm, and cardiac muscle lineage in the Drosophila embryo; just as the mammalian homologues of these genes are coexpressed in the precardiac mesoderm in the mouse embryo. However, the early mesodermal expression of D-mef2 is independent of tinman (Lilly et al., 1994; Nguyen et al., 1994). In the future, it will be especially interesting to determine the mechanisms that regulate D-mef2 expression in the mesoderm and threomyogenic lineages. Preliminary analyses of D-mef2 regulatory sequences in Drosophila
FIG. 4. D-MEF2 expression in a stage 14 Drosophila embryo. A stage 14 Drosophila embryo was stained for D-MEF2 protein expression, filed along the ventral side, and flattened beneath a cover slip. Expression of D-MEF2 can be seen in pharyngeal (pm) and somatic muscle (sm) cells, as well as in the forming dorsal vessel (dv).

germline transformant lines have identified separable enhancer elements that properly drive the expression of a lacZ reporter gene in cells of the ventral furrow, mesoderm, and somatic, visceral, and cardiac muscle lineages (Lilly et al., 1995; C. Chromey, G. Ranganayakulu, B. Zhao, E. Olson, and R. Schulz, unpublished).

To address the possible functions of D-mef2 in mesoderm differentiation and muscle development, P-element insertionional and ethyl methane sulfonate (EMS) chemical mutagenesis screens have been carried out to recover lethal mutations in the gene. Embryos homozygous for a P-element-induced 25-kb chromosome deletion that removes essential D-mef2 regulatory sequences express the D-MEF2 protein at very low levels and fail to form normal differentiated muscles (Lilly et al., 1995). The use of molecular markers for the precursors of the somatic, visceral, and cardiac muscles showed that muscle precursor cells were specified and positioned normally. For example, the mesodermal regulators tinman and bagpipe, which are required for the formation of the heart and visceral muscle (Azpiazu and Frasch, 1993; Bodmer, 1993), are expressed correctly in mutant embryos. The formation of the somatic musculature in Drosophila is believed to occur by the fusion of founder cells with fusion-competent myoblasts (Rushton et al., 1995). Markers for the founder cells, such as the MyoD homolog nautilus (Michelson et al., 1990; Paterson et al., 1991) and the homeobox genes S59 (Dohrmann et al., 1990) and apterus (Bourgoin et al., 1992), are expressed in their normal pattern in D-mef2 mutant embryos. Thus, the phenotype of the D-mef2 deficiency embryos suggests that the gene acts at a relatively late stage within the different myogenic lineages to control cell differentiation (Fig. 5).

A more detailed understanding of the role of D-mef2 in muscle cell differentiation was obtained through the analysis of mutants containing point mutations in the gene. The phenotypic analysis of embryos homozygous for EMS-in-
FIG. 5. Myogenic lineages in Drosophila. Schematic representation of the three myogenic lineages from Drosophila and the genes that are expressed in those lineages. D-mef2 and tinman are coexpressed in the uncommitted mesoderm; both genes are dependent on twist and snail for expression. The prospective mesoderm gives rise to the somatic, visceral, and cardiac muscle lineages, all of which express D-mef2. tinman is expressed initially in the cardiac and visceral muscle lineages, but ultimately becomes restricted to the dorsal vessel. In D-mef2 mutant embryos, tinman is expressed normally. Formation of somatic muscles is believed to occur by fusion of founder cells with fusion-competent myoblasts. Both of these cell types express D-MEF2. In D-mef2 mutant embryos, nautilus and the homeobox genes S59 and apterous are expressed normally in founder cells, but there is no fusion. Activation of muscle structural genes in all three lineages is dependent on D-MEF2.

duced null (Bour et al., 1995) or severe loss-of-function (Ranganayakulu et al., 1995) alleles of D-mef2 revealed the partial differentiation of somatic muscle precursors. However, an absence of myoblast cell fusion and muscle fiber formation was observed, and a dramatic decrease in the myoblast population followed due to programmed cell death. Such a phenotype is consistent with the ability of the Drosophila embryo to eliminate cells that have failed to complete their normal differentiation program (Abrams et al., 1993). Intriguingly, apoptosis is restricted to the somatic muscle population and is not observed in cardiac or visceral muscle precursors.

Further studies on embryos expressing hypomorphic D-mef2 alleles have revealed novel functions of the gene that could not be resolved with complete loss-of-function alleles (Ranganayakulu et al., 1995). In particular, certain somatic muscles are selectively lost in embryos with partial D-MEF2 activity arising from truncated D-MEF2 proteins, indicating that D-mef2 is required for both the formation and patterning of body wall muscle in the Drosophila embryo. This could be explained if the myoblasts that normally form the muscles that are absent require a higher threshold of D-MEF2 for their differentiation. Alternatively, D-MEF2 may interact with different sets of cofactors in different somatic muscle precursors and mutations in the D-MEF2 protein could selectively disrupt interactions with some cofactors and not with others, resulting in the absence of specific muscles. Whereas weak alleles of D-mef2 result in selective ablation of certain somatic muscle fibers, but not others, even relatively weak alleles that have only minor effects on the somatic musculature completely eliminate certain aspects of the differentiation of cardiac and visceral muscle cells. This suggests that these muscle cell types are more sensitive to MEF2 activity than somatic muscle cells. One combination of D-mef2 alleles allows for the occasional survival of transheterozygous mutant adults. These adults are flightless and exhibit severe defects in the patterning and organization of the indirect flight muscles present in the thorax. Therefore, D-MEF2 is required for both the larval and adult myogenic programs. D-MEF2 is expressed in adipocytic cells which are the precursors of the adult thoracic muscles. Given the simultaneous expression of Twist (Bate et al., 1991) and D-MEF2 in these cells, and the recent demonstration of the cooperativity of the myogenic bHLH
and MEF2 proteins in regulating skeletal muscle gene expression (Kaushal et al., 1994; Naidu et al., 1995), Twist and D-MEF2 may interact in the control of gene expression in adult muscle differentiation.

Information is emerging on potential targets of D-MEF2 transcriptional activity in the different muscle cell lineages. In the cardiac lineage, the dorsal vessel is formed, yet myosin protein is not detected (Lilly et al., 1995; Bour et al., 1995; Ranganayakulu et al., 1995). The individual mhc, mlc-alk, and mlc2 subunit genes are not expressed in the dorsal vessel of mutant embryos and thus serve as likely targets of D-MEF2 (Ranganayakulu et al., 1995). Both genetic and molecular studies implicate the αps2 integrin subunit gene as a target of D-MEF2 in visceral muscle. Null mutations in the inflated locus (Brabant and Brower, 1993; Brown, 1994), which encodes this integrin subunit, result in an identical midgut phenotype to that observed in D-mef2 mutant embryos. This common phenotype correlates with the lack of αps2 gene expression and muscle-specific enhancer function in the absence of D-mef2 function (Ranganayakulu et al., 1995). Given the presence of a high-affinity D-MEF2 site in the αps2 enhancer, it is probable that this essential integrin subunit gene is a direct target of D-MEF2 transcriptional activation in visceral muscle.

Future challenges in the study of the D-mef2 gene will include the analysis of its complex regulation, more detailed studies on its function in the larval and adult myogenic programs, and the identification of D-MEF2 target genes. The use of Drosophila genetics will be essential for precisely determining the position and function of this MEF2 family member in the genetic hierarchy controlling mesoderm differentiation and muscle development. Our current knowledge of the genes and events involved in this complex process is summarized in Fig. 5.

Regulation of Cell-Specific Gene Expression by MADS Box Proteins

An important unanswered question is how MEF2 can regulate muscle gene expression in multiple myogenic lineages, which express overlapping but distinct subsets of muscle-specific genes. The mlc-2A gene, for example, is controlled by MEF2 and is expressed in ventricular cardiac myocytes, but not in fast skeletal muscle (Lee et al., 1992). Similarly, the myogenin gene is expressed in skeletal, but not in cardiac or smooth muscle cells (Edmondson and Olson, 1989). Thus, the ability of MEF2 to activate its target genes must be influenced by a cell's identity or developmental history.

In thinking about how MEF2 might regulate muscle gene expression in multiple muscle cell types, it is useful to consider the mechanisms whereby other MADS box proteins regulate programs of cell-specific gene expression. There are many examples in which MADS box proteins act at the endpoints of signal transduction pathways to regulate inducible genes and to confer cell identity. In most cases, MADS box proteins exert these activities through cooperation with other regulatory factors. SRF, for example, interacts with a group of ETS-domain proteins, referred to as ternary complex factors (TCFs), which bind a site adjacent to the serum response element in the c-fos promoter (Treisman, 1994). This interaction is dependent on a region adjacent to the dimerization domain of SRF. DNA binding by SRF has also been shown to be potentiated by interaction with the homeodomain protein Phox, also called M-Hox. Enhanced DNA binding by SRF in the presence of Phox is believed to occur by acceleration of the on-rate of DNA binding (Grueneberg et al., 1992). It has not been possible to demonstrate direct physical interaction between SRF and Phox, so it is unclear precisely how this effect is achieved.

MCM1 has been shown to regulate cell-type-specific gene expression in the budding yeast Saccharomyces cerevisiae through interaction with accessory proteins (Herskowitz, 1989). The identities of the two haploid cell types in yeast, α and a, require the activation and repression of distinct sets of genes. MCM1 controls cell-type-specific genes in yeast by acting in conjunction with two coregulators, α1 and a2, which are expressed only in α cells. In a cells, MCM1 inhibits the expression of a-specific genes by binding their promoters in collaboration with the transcriptional repressor a2. α-Specific genes are activated in the same cells by MCM1 in collaboration with the homeodomain protein α1. Neither the α2 repressor nor the a1 activator are present in a cells; α-specific genes are therefore not expressed, even though MCM1 is present. Thus, the response of a gene to MCM1 is dictated not simply by the presence of an MCM1 binding site, but also by the sites surrounding that site, as well as the affinity of MCM1 for that site.

MCM1 also mediates the actions of pheromones in yeast, which act through the cell surface receptors STE2 and STE3. Transcription activation of pheromone-responsive genes is controlled by MCM1 and the transcriptional regulator STE12, which form a complex on the promoters of these genes (Dolan and Fields, 1991). Activation of pheromone-responsive genes leads to cell cycle arrest and morphological changes prior to cell fusion. Expression of the SW15 gene, which encodes a cell-type-specific transcription factor required for mating type switching, is also controlled by MCM1 (Lydall et al., 1991). Transcriptional activation of SW15 by MCM1 is mediated by the cooperative interaction of MCM1 with another regulatory protein SFF to form a transcriptional complex on the SW15 gene control region. The functions of MADS box proteins in regulating cell-type-specific gene expression have also been well characterized in plants, in which combinations of MADS box proteins act within a genetic network that establishes floral organ identity (Weigel and Meyerowitz, 1994).

Given the propensity of MADS box proteins to act combinatorially with other classes of transactivators, it is tempting to speculate that MEF2 factors may also interact with other regulators to control muscle gene expression. Indeed, MEF2 has been shown to bind DNA cooperatively with myogenic bHLH proteins, resulting in synergistic activation of muscle-specific transcription (Funk and Wright, 1992).
functions for MEF2 factors in addition to muscle gene regulation

In addition to their expression in myogenic lineages, members of the MEF2 family exhibit highly specific expression patterns in the developing brain and appear to demarcate gradients of neuronal maturation. MEF2 gene expression is especially pronounced in the cerebellum, cerebral cortex, and hippocampus (Lyons et al., 1995). The functions of MEF2 in neural cells remain to be determined, and no target genes for MEF2 regulation have been identified in neural cells thus far. The use of Drosophila genetics may allow for the elucidation of the function of MEF2 in neuronal cell differentiation. D-MEF2 is expressed in specific neurons of the central nervous system (G. Ranganayakulu and R. Schulz, unpublished) and genetic approaches exist to generate mosaic animals that have wild-type gene function in muscle cells, yet are mutant for D-mef2 function in neuronal cells.

MEF2 has also been implicated in serum-inducible expression of the c-jun gene in HeLa cells (Han et al., 1992; Han and Prywes, 1995). The possibility that MEF2 might confer serum inducibility to certain genes is particularly interesting in light of the role of SRF in the control of muscle and serum-inducible gene expression (Treisman, 1990). Perhaps these different MADS domain proteins rely on similar mechanisms to control these seemingly disparate forms of gene expression.

Future Questions

In summary, there is convincing evidence in support of a role for MEF2 factors in the control of muscle gene expression in multiple muscle cell types. However, many important questions remain to be answered. In particular, if MEF2 controls differentiation of diverse muscle cell types as the genetic studies in Drosophila indicate, what determines whether a cell is skeletal, cardiac, or smooth muscle? We favor a model in which MEF2 provides a function that is essential for myogenesis in general and in which the additional specificity that is unique to each myogenic lineage arises from combinatorial interactions between MEF2 and other regulators that are restricted to each myogenic lineage. In skeletal muscle cells, it is likely that MEF2 cooperates with myogenic bHLH proteins to establish the skeletal muscle phenotype. Whether similar bHLH proteins are expressed in cardiac and smooth muscle remains to be determined, but it is likely that MEF2 has a conserved, common function in the differentiation of these different muscle cell lineages. Given the many parallels between muscle and neural differentiation (Jan and Jan, 1993), it will also be interesting to determine whether MEF2 collaborates with neurogenic bHLH factors such as NeuroD (Lee et al., 1995) to regulate neural differentiation.

MEF2 expression in skeletal muscle cells is also induced by myogenic bHLH proteins. It will be of interest to determine the mechanisms that lead to MEF2 expression in other muscle cell types and to determine to what extent the functions and position of MEF2 in the regulatory pathways leading to muscle formation have been conserved from Drosophila to mammals.

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