Genomes & Developmental Control

Positive autoregulation of the Myocyte enhancer factor-2 myogenic control gene during somatic muscle development in *Drosophila*

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Abstract

The myocyte enhancer factor-2 (MEF2) transcription factor plays a central role in the activation and maintenance of muscle gene expression in fruit flies and vertebrates. The mechanism of action and downstream target genes of MEF2 have been defined in considerable detail, but relatively little is known about the mechanisms that regulate MEF2 expression during muscle development. Here we demonstrate that MEF2 maintains its own expression in all differentiated muscle cell types during late embryonic and larval development in *Drosophila* by binding a conserved MEF2 site in a muscle-specific regulatory enhancer. Ectopic expression of *Mef2* is sufficient to directly activate this enhancer in some, but not all, non-muscle cells. Furthermore, activation of the *Mef2* enhancer normally in muscle cells and ectopically in non-muscle cells is dependent upon the integrity of the MEF2 binding site. These findings suggest an evolutionarily conserved mechanism whereby MEF2 can stabilize the muscle phenotype by sustaining its own expression through a myogenic autoregulatory loop.

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Introduction

The process of muscle differentiation has been the subject of intense study aimed at identifying the transcriptional regulatory networks that are required for the specification, differentiation, and maintenance of the muscle lineage. Central to the formation of the musculature are members of the Myocyte enhancer factor-2 (MEF2) family of MADS (MCM1, Agamous, Deficiens, Serum response factor) domain transcription factors, which bind essential A/T-rich sites in the control regions of most muscle structural genes. There are four mammalian MEF2 factors—MEF2A, B, C, and D—and a single MEF2 factor in *Drosophila* (reviewed in Black and Olson, 1998).

The central role of MEF2 in muscle development has been demonstrated by genetic studies in mice and fruit flies. Loss-of-function mutants for murine *mef2c* show a failure of normal cardiac morphogenesis and differentiation with concomitant down-regulation of some cardiac muscle structural genes (Q Lin et al., 1997), and dominant-negative isoforms of MEF2 can inhibit myogenesis (Ornatsky et al., 1997). Similarly, terminal differentiation of all muscle lineages is abolished in *Drosophila* embryos lacking MEF2 (Bour et al., 1995; Lilly et al., 1995; Ranganayakulu et al., 1995).

In vertebrate skeletal muscle, MEF2 cooperates with the myogenic basic helix–loop–helix (bHLH) transcription factors—MyoD, myogenin, Myf5, and MRF4—to activate the muscle phenotype (Black et al., 1995; Li and Capetanaki, 1994; Molkentin et al., 1995; Naidu et al., 1995). In addition to activating subordinate downstream genes in the skeletal muscle differentiation pathway, MEF2 and myogenic bHLH factors activate and maintain the expression of each other. Expression of myogenic bHLH proteins in fibroblasts results in up-regulation of MEF2 genes and conversion to the muscle phenotype (Cserjesi and Olson, 1991; Lassar et al., 1991; Martin et al., 1993). The promoters of the *myogenin* and *myf4* genes also contain MEF2 binding sites that are critical for muscle-specific expression (Black et al., 1995; Cheng et al., 1993; Edmondson et al., 1992; Naidu et al., 1995; Yee and Rigby, 1993). While MEF2 cannot activate these promoters in non-muscle cells in the absence of...
myogenic bHLH factors, the MEF2 binding sites are associated with E-boxes through which the myogenic bHLH factors and MEF2 collaborate to regulate gene expression (Molkentin et al., 1995; Ormatsky et al., 1997; Yu et al., 1992).

Taken together, these studies have generated a robust model to explain how myogenic factors initiate and maintain the muscle phenotype by both cross-regulation and positive autoregulation. However, much remains to be learned about the mechanisms that control expression of MEF2 in different myogenic lineages. In Drosophila, we have shown that the bHLH factor Twist is a direct and essential activator of MEF2 expression in the skeletal muscle lineage (Cripps et al., 1998). However, since twist expression levels decline during myogenesis (Baylies and Bate, 1996), other factors presumably act to maintain Mef2 transcription at later stages of development. Here, we describe a muscle-specific enhancer that directs Mef2 transcription in all differentiated somatic and visceral muscles of the Drosophila embryo and in all differentiated muscles during larval development. This enhancer is a direct target of MEF2 and requires a single conserved MEF2 binding site for activity. Our results demonstrate that maintenance of MEF2 expression in mature muscle lineages results from a direct positive feedback mechanism and suggest an evolutionarily conserved mechanism for maintaining the muscle phenotype.

Materials and methods

DNA methods

The -12500/-9112 construct was generated by purification of a 3.5-kb EcoRI fragment from the cosmid CosD6, which contains the entire Mef2 region (Lilly et al., 1995; O’Brien et al., 1994). The 3.5-kb fragment was then cloned directly into the promoter-lacZ transformation vector CHAB (Thummel and Pirrotta, 1992). Mef2 enhancer constructs within the -9117/-8079 region were generated by PCR using genomic DNA as a template, as was a construct spanning -8159/-5906. Primers used for each construct were as follows: -9117/-8079, IIB-1 (5’-GAAATTCAGAATGGTTTTTCATT-3’); and Bin82 (5’-CGGGTCTTGAGGGGAAGGAGGC-3’); -9117/-8467, IIB-1 and Bin81 (5’-TCTCTCACACCTTCTCGCC-3’); -8543/-8079, Bin71 (5’-GTTCACTGCCGCCCCTTCTC-3’) and Bin82; -8371/-8079, 2T-4 (5’-AAGGTGGTGGGGAAGACTC-3’) and Bin82; -8233/-8079, 2T-5 (5’-CAGATGGGACGGAGGACG-3’) and Bin82; -8159/-5906, Bin72 (5’-ATGCAAGACAGCCGAGGACG-3’) and Bin100 (5’-GTGTTCTGTTCATCTGTCATG-3’). Mutagenesis of enhancer sequences was performed as described by Horton (1993) using the mutagenic primers MEFMUT+ (5’-GACTTGTCCGGGCCGATACCCGAATATC-3’) and MEFMUT- (5’-TTCCGGGCTGTTTCTGTATCTG-3’); mutated sequences are underlined. All PCR products were ligated into the pGem-T Easy vector (Promega Life Science, Madison, WI). Fragments were then excised using appropriate restriction enzymes and inserted into CHAB for generation of transgenic lines. Transgenic lines carrying other enhancer regions fused to a lacZ reporter were described previously (Cripps et al., 1999; Lilly et al., 1995).

The pUAST/Mef2 plasmid was generated by cloning a 2.6-kb EcoRI fragment comprising the entire Mef2 coding region (Lilly et al., 1995) into the pUAST vector (Brand and Perrimon, 1993).

Fly stocks and crosses

Flies were maintained on Carpenter’s medium (Carpenter, 1950) at 25°C unless indicated. Transgenic lines were generated according to Rubin and Spradling (1982) using the Delta2–3 helper plasmid (Robertson et al., 1988), and at least three independent lines of each construct were tested for enhancer activity. For ectopic expression of Mef2, lines were generated which carried an X-linked UAS-Mef2 insert as well as an autosomal copy of either the wild-type or mutant Mef2 enhancer-lacZ constructs. For the wild-type enhancer, flies had the genotype P[w+ UAS-Mef2]WT26A; P[w+ -9117/-8079 Mef2-lacZ]82-8; for the mutant enhancer, flies had the genotype P[w+ UAS-Mef2]WT26A; P[w+ -9117/-8079mutant Mef2-lacZ]82M-55. Virgin females from these lines were crossed to males homozygous for the ectodermal driver 69Bgal4 (Brand and Perrimon, 1993). Progeny embryos of the following genotypes were represented at 100%: w P[w+ UAS-Mef2]WT26A/+ Y; P[w+ -9117/-8079 Mef2-lacZ]82-8/69Bgal4; for the mutant enhancer: w P[w+ UAS-Mef2]WT26A/+ Y; P[w+ -9117/-8079mutant Mef2-lacZ]82M-55/69Bgal4. Embryos were collected at 29°C for 16 h, and then processed for immunofluorescence as indicated below.

Histochemistry and immunohistochemistry

Embryos aged 0–16 h were collected and subjected to antibody staining essentially as described by Patel (1994). Primary antibodies were mouse anti-β-galactosidase (Promega, 1:500) and rabbit anti-MEF2 (Lilly et al., 1995, 1:1000). For colorimetric detection with diaminobenzidine (DAB), the secondary antibody used was biotinylated goat anti-mouse (Promega, 1:500). For fluorescent detection, the secondary antibodies were Alexa-568 goat anti-rabbit and Alexa-488 goat anti-mouse (Molecular Probes, Seattle, WA) each used at 1:2000. DAB-stained embryos were cleared in 80% (v/v) glycerol, mounted, and photographed on an Olympus BX51 microscope with DIC optics using 35-mm slide film. Slides were subsequently scanned to generate digital images. Fluorescently stained embryos were mounted in glycerol and sub-
jected to confocal microscopy using a BioRad MRC-600 confocal laser scanning microscope with 488/568 nm excitation in the dual channel mode (T1/T2A filter cubes, BioRad, Hercules, CA).

To visualize β-galactosidase activity in larval skeletal and visceral muscles, late third instar larvae were filleted and fixed as described by Molina and Cripps (2001). Larvae were then stained with an X-gal solution as described by Ashburner (1989). Stained samples were mounted and photographed using a microscope with a digital camera.

To study β-galactosidase accumulation in larval dorsal vessels, samples were processed according to Molina and Cripps (2001). Primary antibody used was mouse anti-β-galactosidase (Promega, 1:10000) and secondary antibody was Alexa-568 goat anti-mouse (1:2000). Secondary incubations also contained Alexa-488 phalloidin (Molecular Probes, diluted 1:500). Washed samples were mounted in Slofade Anti-Fade mounting medium (Molecular Probes), imaged by standard fluorescence microscopy, and captured as digital images. All figures were assembled using Adobe Photoshop.

**DNA binding assays**

Electrophoretic mobility shift assays were performed essentially as described by Sambrook et al. (1989). Probe DNA and unlabeled competitors were generated by annealing complementary oligonucleotides to generate dsDNA molecules with GG overhangs at each 5’ end. Oligonucleotides for wild-type were DM2+ (5’-GGTGTCTATATTAGCCC-3’) and DM2− (5’-GGGGCTAAATATAAGACA-3’); for mutant competitor, DM2mut+ (5’-GGTGTCGGGCCTTAAAGACC-3’) and DM2mut− (5’-GGGGCTAAACGGC GACA-3’), mutated sequences underlined; and for mck positive control, MCK+ (5’-GGGGCTCTAAAAATAAACCC-3’) and MCK− (5’-GGGGGCCGAGTTA GGC-3’). Annealed sequences were radioactively labeled with 32P-dCTP and Klenow enzyme (Roche Molecular Biochemicals, Indianapolis, IN), purified, and diluted to 50000 cpm/μl. MEF2 protein was generated from pcDNA-DMEF2 provided by Dr. Brian Black (University of California, San Francisco) using the Promega TNT coupled reticulocyte lysate kit (Promega

**Fig. 1. Identification of skeletal muscle enhancers active during late embryonic and larval development.** Enhancers active at the late embryonic stage in skeletal muscles were tested for their ability to direct muscle-specific reporter gene expression in third instar larvae. Top: map of the Mef2 upstream region showing the location of genomic fragments tested; note that only the first two exons of Mef2 are indicated. R, EcoRI site; rightward arrow indicates transcription start site; white boxes indicate untranslated sequence; black boxes indicate translated sequence; red boxes indicate enhancer regions shown by Nguyen and Xu (1988) to be active in embryonic skeletal muscles at stage 16. Bottom: (A, B) enhancer activity of the −12500/−9112 region was strong in skeletal muscles of stage 16 embryos (A), but barely detectable in L3 larvae (B); (C, D) enhancer activity of the −9117/−8079 region was strong in both embryos (C) and larvae (D). Embryos are oriented with anterior to the left and dorsal uppermost, and images depict immunohistochemical staining for β-galactosidase. Larval images are oriented with anterior to the left and dorsal midline at the top, and images show staining for β-galactosidase activity. All skeletal muscles stain in panel D, and white asterisks denote only a specific subset of the muscles to permit orientation. White asterisks denote the DA1, DA2, and DA3 muscles (top to bottom). Scale bar: 100 μm.
Corp.) and T7 RNA polymerase (Roche Molecular Biochemicals). Unprogrammed lysate was generated in the same manner except lacking the plasmid DNA.

Binding reactions contained polydI.dC (1 μg), MEF2, or unprogrammed lysate (3 μl), 1 × buffer (Gossett et al., 1989), competitor DNA (100 × molar ratio), and water to

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Fig. 2. Deletion analysis of the -9117/-8079 enhancer region. (A) Summary of enhancer-lacZ constructs tested and their activities in stage 16 embryos. The location of a consensus MEF2 binding site is indicated. (B–D) Representative images of transgenic embryos carrying the indicated Mef2-lacZ constructs, immunohistochemically stained with anti-β-galactosidase. Note that the full-length construct consistently showed the highest level of reporter gene expression, but that sequences in the 3′ half of the enhancer were sufficient for some muscle-specific activity (D). sm, skeletal muscles; scale bar, 100 μm.

Fig. 3. Expression profile of the -9117/-8079 enhancer during embryonic and larval development. (A–D) Transgenic embryos carrying the -9117/-8079 Mef2-lacZ construct showed dynamic reporter gene expression during embryonic development. Expression was first detected at late stage 12 in precursors of the longitudinal visceral muscles (A, vm), and was detected by stage 14 in skeletal myoblasts (B, sm). By stage 16, activity was strong in visceral muscles (C), but no reporter gene expression was detected in the dorsal vessel (D, dv). In third instar larvae, the enhancer was active in both longitudinal and transverse visceral muscle fibers (E, lvm and tvm, respectively), and in the dorsal vessel (F). Panels A–D are immunohistochemical stains for β-galactosidase protein. Panel E is a histochemical stain for β-galactosidase activity. Panel G is an immunofluorescent detection of β-galactosidase protein, and panel H is a fluorescent phalloidin stain of the sample in (F) to show the location of the dorsal vessel. All panels are oriented with anterior to the left. (A, B) Sagittal views; (C) ventral view; (D) dorsal view; (E) whole mount of larval gut; (F, G) ventral view of the dorsal midline of a filleted larva. Scale bar, 100 μm.
bring the volume to 10 μl. Reactions were incubated at room temperature for 20 min and then electrophoresed on a 3% (w/v) non-denaturing polyacrylamide gel. The gel was dried and exposed to autoradiography film overnight.

Results

Identification of a Mef2 enhancer active during larval muscle development

Previous studies demonstrated that transcription of Mef2 in Drosophila embryos is regulated by a complex set of independent enhancer elements dispersed throughout approximately 15 kb of upstream DNA (Cripps et al., 1998, 1999; Gajewski et al., 1997, 1998; Lilly et al., 1995; Nguyen and Xu, 1998; Schulz et al., 1996). To understand how Mef2 expression is maintained in fully differentiated muscle lineages, we analyzed this region for enhancers able to direct expression of a lacZ transgene in larval muscles. While there is a strong late embryonic skeletal muscle enhancer contained within a 3.5-kb EcoRI fragment upstream of Mef2 called III-F (Nguyen and Xu, 1998; Schulz et al., 1996; Fig. 1A, construct /C0 12500/C0 9112), the 3.5-kb fragment containing III-F displayed only weak enhancer activity in the skeletal muscles of third instar larvae (Fig. 1B).

By contrast, an adjacent 1-kb region (/C0 9117/C0 8079) contains a late skeletal muscle enhancer called II-E (Nguyen and Xu, 1998; Fig. 1C). The 1-kb region maintained high levels of enhancer activity in all of the skeletal muscles of third instar larvae (Fig. 1D; the three dorsal acute muscles are indicated with asterisks for orientation purposes). We also tested additional enhancer constructs spanning the region from /C0 8079 to the EcoRI site at +521 for activity in larval skeletal muscles; however, no activity was observed for any additional enhancer fragment (data not shown). These data indicate that the region between /C0 9117 and /C0 8079 contains a powerful muscle-specific enhancer that functions as the predominant regulator of Mef2 expression in somatic muscle during late embryonic and larval life.

In order to localize sequences important for activity of the /C0 9117/C0 8079 enhancer, deletion constructs were tested for enhancer activity in transgenic embryos (Fig. 2). Deletion of approximately 400 bp of 3′ sequence completely abolished enhancer activity in the embryo (Figs. 2A, C; construct /C0 9117/C0 8467), whereas deletion of 574 bp from the 5′ end of the region resulted in a reduction in activity but not a complete loss of enhancer function (Figs. 2A, D; construct /C0 8543/C0 8079). The latter construct was also active in larval muscles (data not shown). Larger deletions from the 5′ end abolished all enhancer activity (Figs. 2A, E). These findings indicated that muscle-specific activity of the enhancer could be conferred by the 3′ sequences, although the 5′ region of the complete enhancer is important for high levels of expression. Therefore, subsequent analyses concentrated on the activity of the entire enhancer region (−9117/−8079) rather than on deletion constructs with reduced activity.

Expression profile of the −9117/−8079 enhancer

To determine the complete expression pattern of the −9117/−8079 enhancer, transgenic flies harboring the lacZ probe:

Fig. 4. MEF2 protein binds to a conserved consensus sequence within the −9117/−8079 enhancer. (A) Location of the conserved MEF2 binding site in the −9117/−8079 enhancer, and comparison of this sequence to similar regions in the genomes of D. melanogaster (Dm), D. pseudoobscura (Dp), and D. virilis (Dv). MEF2 consensus binding site is highlighted. (B) Electrophoretic mobility shift assay to determine if MEF2 could bind to the consensus site. Free probe had a high mobility in the gel in the presence of lysate lacking MEF2 protein (Unp). A bound complex (MEF2) was formed in the presence of MEF2 lysate, which was competed by a 100-fold excess of nonradioactive wild-type sequence (WT) but not by a 100-fold excess of nonradioactive mutant sequence (Mut). As a positive control, MEF2 was shown to bind to the muscle creatine kinase (MCK) canonical MEF2 site.
reporter controlled by this region were stained for β-galactosidase at embryonic and larval stages. lacZ expression was first detected in the visceral muscle cells of late stage 12 embryos (Fig. 3A), followed shortly by expression in skeletal myoblasts at stage 13 (Fig. 3B). By stage 16, the enhancer showed strong activity in all skeletal (Nguyen and Xu, 1998; Fig. 1C) and visceral muscles (Fig. 3C). However, during embryogenesis, the enhancer showed no detectable activity in the dorsal vessel (Fig. 3D). To determine if this enhancer was also active in tissues other than skeletal muscle later in development, the alimentary canal was dissected out of third instar larvae, fixed, and stained with X-gal. Strong staining was observed in both the longitudinal and transverse muscle fibers of the gut mesoderm (Fig. 3E). β-Galactosidase was also detected in the dorsal vessel of filleted third instar larvae (Fig. 3F).

These findings indicate that the −9117/−8079 enhancer displays a strikingly broad spectrum of activity in all of the differentiated muscle lineages of the larva. It is also interesting that the onset of activity of this enhancer matches the onset of terminal muscle differentiation in each lineage: visceral muscle cells activate markers of terminal differentiation earliest during embryogenesis, followed by skeletal muscle cells, and finally by cells of the dorsal vessel (Herranz et al., 2004; Kelly et al., 2002; Zhang and Bernstein, 2001).

Identification of a conserved MEF2 binding site in the −9117/−8079 enhancer

To begin to understand the molecular basis for the activity of the −9117/−8079 enhancer, we analyzed the sequence for binding sites of known muscle regulatory factors. In the 3′
region of the enhancer, shown to be essential for transcriptional activity, we identified a 10-bp sequence located at −8209/−8200 that perfectly matched the consensus binding site for MEF2 proteins (Andres et al., 1995; Fig. 4A). Examination of the upstream regions of the Mef2 genes of two related Drosophila species, D. pseudoobscura (MegaBLAST at URL: http://www.ncbi.nih.gov/blast/mntrace.html) and D. virilis (Cripps et al., 1999), revealed that the MEF2 consensus binding sequence was also conserved for these genes, suggesting an important function.

To determine if MEF2 protein was capable of interacting with the putative MEF2 site, an electrophoretic mobility shift assay was performed using a radioactively labeled double-stranded oligonucleotide corresponding to the putative MEF2 site plus five flanking nucleotides. MEF2 protein generated in vitro bound strongly to the radioactive probe DNA sequence, and binding was competed by an identical unlabeled oligonucleotide but not by an oligonucleotide in which the MEF2 site had been mutated (Fig. 4B). The canonical MEF2 site from the mck gene (Cserjesi et al., 1994) was included as a positive control. These findings suggested that the −9117/−8079 Mef2 enhancer might be activated, at least in part, by the product of the Mef2 gene itself.

The MEF2 binding site is required for enhancer activity in all muscle lineages

The data presented in Fig. 2 suggested that the conserved MEF2 site was important for muscle-specific expression, since its deletion eliminated enhancer activity. However, the MEF2 site and adjacent sequences are not sufficient for activity since neither the −8371/−8079 nor the −8233/−8079 regions, which contain this site, showed activity (see Fig. 2).

Therefore, to determine the functional significance of the MEF2 binding site, the sequence was mutated in the context of the −9117/−8079 enhancer to the same sequence used as

![Fig. 6](image-url)

Fig. 6. The −9117/−8079 enhancer can be activated by ectopic MEF2 in vivo. Embryos carrying wild-type or mutant −9117/−8079 enhancers were subjected to ectopic Mef2 expression and tested for the activation of Mef2 and reporter gene expression via immunofluorescence and confocal microscopy. (A, D) Accumulation of MEF2 protein (red) in the ectoderm and amnioserosa of transgenic animals; (B, E) accumulation in the same embryos of β-galactosidase protein (green) occurred in embryos carrying the wild-type enhancer, but not in those carrying the mutant enhancer. (C, F) Merge of previous images shows cells of the amnioserosa that activated the reporter gene in response to MEF2 protein. White dashed line delimits the amnioserosa; white arrows in A–C indicate the nucleus of an amnioserosa cell. All panels show dorsal views with anterior to the left. Scale bar, 100 μm.
mutant competitor in the DNA binding assays. Whereas the wild-type enhancer was active strongly in all differentiated muscle lineages (Figs. 5A–D), the mutant enhancer showed no activity at any stage of embryogenesis or larval development (Figs. 5F–I).

**Ectopically expressed Mef2 can ectopically activate the −9117/−8079 enhancer**

To determine if the −9117/−8079 enhancer could be activated in non-muscle cells by MEF2, we used the Gal4-UAS system to ectopically express Mef2 by using the ectodermal driver 69B-gal4 and an X-linked UAS-Mef2 construct (see Materials and methods for details). Flies were stained for the co-expression of ectopic MEF2 protein and β-galactosidase from the enhancer-lacZ reporter.

MEF2 was expressed at a high level in the ectoderm and amnioserosa of w P[w+ UAS-Mef2]WT26A/+ or Y; P[w+ −9117/−8079 Mef2-lacZ]82-8/69Bgal4 embryos, as visualized by confocal microscopy (Fig. 6A). The same animals showed strong expression of lacZ in ectopic locations (Figs. 6B, C), although only in a subset of the cells of the amnioserosa and no cells in the ectoderm. By contrast, the enhancer with the MEF2 site mutation was not activated by ectopic MEF2 expression in w P[w+ UAS-Mef2]WT26A/+ or Y; P[w+ −9117/−8079 mutant Mef2-lacZ]82M-55/69Bgal4 embryos (Figs. 6D–F). These findings demonstrated that MEF2 could activate the autoregulatory enhancer in non-muscle cells to varying degrees and suggested that the amnioserosa is a more permissive environment for ectopic activation of the enhancer than the ectoderm.

To extend these observations, we looked at enhancer activity in additional cells that express Mef2 during the larval stage. The wing imaginal discs express high levels of MEF2 at the end of the third larval instar (Ranganayakulu et al., 1995; Cripps et al., 1998; Fig. 7A); however, the

![Fig. 7. Expression of the −9117/−8079 enhancer is restricted to differentiated skeletal muscles of third instar larvae.](image-url)

(A, B) Immunofluorescence revealed that wing imaginal discs from −9117/−8079 Mef2-lacZ transgenic lines accumulated MEF2 in the adip epithelial cells (A, ac) but did not accumulate β-galactosidase protein in those cells (B). (C, D) Similarly, cells of the mushroom body of the larva accumulated MEF2 (C, mb) but did not accumulate β-galactosidase. Scale bar, 100 μm.
autoregulatory enhancer was inactive in the wing discs at this stage of development (Fig. 7B). These results are consistent with our earlier observation that an enhancer closer to the Mef2 promoter is solely responsible for Mef2 activation in the wing discs (Cripps et al., 1998). The autoregulatory enhancer was also inactive in the mushroom body cells of the brain, which express Mef2 in late third instar larvae (Schulz et al., 1996; Figs. 7C, D). Thus, the enhancer is not a simple reporter of Mef2 expression but is specifically active in a subset of MEF2-positive cells.

Discussion

Cellular phenotypes can be stabilized by transcriptional feedback mechanisms as well as by chemical and structural modifications of the DNA (reviewed in Dillon and Grosveld, 1994), which maintain expression of cell-specific regulatory and structural genes. Examples of positive autoregulation of transcription are found in muscle development in vertebrates (reviewed in Yun and Wold, 1996; Ludolph and Konieczny, 1995) and in proneural gene expression in Drosophila (Helms et al., 2000; Sun et al., 1998). Furthermore, recent genome-scale analyses of gene regulatory networks reveal that positive autoregulation is a common maintenance mechanism for the expression of regulatory genes (Davidson et al., 2002).

Positive autoregulation by MEF2

The results of the study presented here demonstrate that MEF2 directly and positively autoregulates transcription during the late stages of muscle development in Drosophila. We show that the autoregulatory enhancer is capable of providing readout for the presence of MEF2: the enhancer is not expressed in non-muscle cells in the embryo, it can be activated by ectopic MEF2, and the activity of the enhancer is critically dependent upon a MEF2 binding site. Together these results provide compelling evidence of a positive autoregulatory role for MEF2 in differentiated muscles.

Since Mef2 is essential for muscle differentiation in Drosophila via direct activation of muscle structural genes (Lin et al., 1996; Ranganayakulu et al., 1995; Kelly et al., 2002; Arredondo et al., 2001), it is likely that sustained Mef2 expression is required for muscle maintenance and growth during the larval stage. Furthermore, since the Mef2 autoregulatory enhancer is active relatively late in embryonic development and remains active throughout the larval stage, the enhancer is likely to account, at least in part, for maintenance of the muscle phenotype via sustained activation of Mef2.

The cells in which the −9117/−8079 enhancer is active share the common feature of being late in the differentiation pathway. The temporal pattern of activation of the enhancer (visceral, then skeletal, then cardiac muscles) matches the generally accepted order in which these muscle lineages begin to express markers of terminal muscle differentiation (Herranz et al., 2004; Kelly et al., 2002; Zhang and Bernstein, 2001). By contrast, the early mesoderm, despite expressing Mef2, does not show enhancer activity; and the adipesimal cells of the wing disc are also myoblasts that have yet to initiate the differentiation program (Fernandes et al., 1991). Therefore, one possibility is that a MEF2 cofactor critical for activity of the autoregulatory enhancer is induced specifically in differentiating cells.

An alternative possibility is that the MEF2 protein in the early mesoderm, in the adipesimal cells and in the mushroom body, is rendered transcriptionally inactive. It is known that in vertebrates, MEF2 function can be post-translationally regulated by class II histone deacetylases (Lemercier et al., 2000; Lu et al., 2000; Miska et al., 1999). Since MEF2 is present in the early mesoderm of Drosophila embryos from stage 6 (Bour et al., 1995; Lilly et al., 1995), yet the earliest known targets of MEF2 are not activated until stages 10–11 (Kelly et al., 2002; Taylor, 2000), one possibility is that Drosophila MEF2 does not acquire transcriptional activity until the onset of differentiation. Thus, the inability of MEF2 to activate the −9117/−8079 enhancer in the early mesoderm, the adipesimal cells, or the mushroom body, and the relative ineffectiveness of ectopic MEF2 in activating the enhancer might reflect cellular environments repressive to MEF2-mediated differentiation. The Drosophila class II histone deacetylase encoded by CG1770 is expressed broadly at low levels in the embryo (Zeremski et al., 2003) and thus could carry out this function early. Whether this gene is also expressed during the third larval instar in imaginal discs or brain remains to be determined. However, it should also be noted that the MEF2 site alone is not sufficient for enhancer activity (see Fig. 2); therefore, the full activation of this enhancer might arise from the effects of both additional trans-acting factors as well as modulation of MEF2 activity.

Requirement for factors in addition to MEF2 on the autoregulatory enhancer

The ectopic expression assays confirm that MEF2 is capable of activating the autoregulatory enhancer outside muscle cell lineages, although it is unclear why the enhancer is not activated in all cells expressing ectopic MEF2. This finding may result from subtle cell-to-cell variations in the level of MEF2 accumulation from the UAS transgene, such that the enhancer is only activated above a certain threshold of MEF2 concentration. The level of this threshold may depend upon the ability of MEF2 to overcome either effects of repressive factors, or alternatively the absence of positive co-regulators that are restricted to muscle lineages.

In vertebrates, MEF2 factors collaborate with tissue-restricted cofactors to activate target gene expression (Molkentin et al., 1995), and there is significant evidence that this is also the case in Drosophila (SC Lin et al., 1997). It is therefore likely that MEF2 collaborates with additional
factors on the autoregulatory enhancer, and it is possible that such factors could also be distinct in each muscle type. Our deletion analyses indicating that sequences in the 5′ half of the −9117/−8079 enhancer are required for full enhancer activity are consistent with this hypothesis. However, the identity of these additional factors remains unknown since no other known binding sites for muscle-specific regulators reside in this location. Furthermore, an E-box located close to the MEF2 site, while strongly conserved, is not required for enhancer activity.

Additional support for the conclusion that MEF2 alone is insufficient for enhancer activation comes from the fact that there are cells that express Mef2 in which the −9117/−8079 enhancer is not active. These include the early mesoderm of the embryo (Bour et al., 1995; Lilly et al., 1995), the adepithelial cells of the wing imaginal disc (Cripps et al., 1998), and a subset of cells of the mushroom body of the brain (Schulz et al., 1996). Thus, the −9117/−8079 enhancer is not simply an indicator of MEF2 presence in a cell but is specific to a particular subset of the mesoderm.

Possible evolutionary conservation of muscle regulatory pathways

There is significant evolutionary conservation in the transcriptional pathways for normal development, and this conservation extends to the regulatory networks used to control myogenesis. Gain-of-function assays have demonstrated that myogenic bHLH factors can activate vertebrate mef2 gene transcription (Cserjesi and Olson, 1991; Lassar et al., 1991; Martin et al., 1993); this is also the case in Drosophila, where the bHLH factor Twist can activate Mef2 expression at high levels (Taylor et al., 1995). More recent studies of vertebrate mef2c have determined that this activation occurs via a skeletal muscle enhancer that is strongly responsive to myogenic bHLH gene activation (Dodou et al., 2003; Wang et al., 2001); and this is paralleled in Drosophila by the observation that the bHLH factor Twist is a direct and positive regulator of Mef2 expression (Cripps et al., 1998). In addition, the mef2c enhancer contains an AT-rich sequence essential for activation in vivo (Dodou et al., 2003; Wang et al., 2001) and which has been shown to bind to MEF2 protein (Wang et al., 2001). This result is paralleled by the data presented here demonstrating that Drosophila Mef2 undergoes positive autoregulation and reinforces the notion that cellular specification and differentiation pathways have been strongly conserved over large periods of evolutionary time. Thus, the autoregulatory loop uncovered in this study could be a component of an evolutionarily conserved mechanism by which muscle cells maintain the muscle phenotype. However, since positive autoregulation is a common occurrence among regulatory genes, it is also possible that Mef2 autoregulation evolved independently in diverse organisms.

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