

Muscle LIM Proteins Are Associated with Muscle Sarcomeres and Require dMEF2 for Their Expression during *Drosophila* Myogenesis

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A genetic hierarchy of interactions, involving myogenic regulatory factors of the MyoD and myocyte enhancer-binding 2 (MEF2) families, serves to elaborate and maintain the differentiated muscle phenotype through transcriptional regulation of muscle-specific target genes. Much work suggests that members of the cysteine-rich protein (CRP) family of LIM domain proteins also play a role in muscle differentiation; however, the specific functions of CRPs in this process remain undefined. Previously, we characterized two members of the *Drosophila* CRP family, the muscle LIM proteins Mlp60A and Mlp84B, which show restricted expression in differentiating muscle lineages. To extend our analysis of *Drosophila* Mlps, we characterized the expression of Mlps in mutant backgrounds that disrupt specific aspects of muscle development. We show a genetic requirement for the transcription factor dMEF2 in regulating Mlp expression and an ability of dMEF2 to bind, *in vitro*, to consensus MEF2 sites derived from those present in *Mlp* genomic sequences. These data suggest that the *Mlp* genes may be direct targets of dMEF2 within the genetic hierarchy controlling muscle differentiation. Mutations that disrupt myoblast fusion fail to affect Mlp expression. In later stages of myogenic differentiation, which are dedicated primarily to assembly of the contractile apparatus, we analyzed the subcellular distribution of Mlp84B in detail. Immunofluorescent studies revealed the localization of Mlp84B to muscle attachment sites and the periphery of Z-bands of striated muscle. Analysis of mutations that affect expression of integrins and α -actinin, key components of these structures, also failed to perturb Mlp84B distribution. In conclusion, we have used molecular epistasis analysis to position Mlp function downstream of events involving mesoderm specification and patterning and concomitant with terminal muscle differentiation. Furthermore, our results are consistent with a structural role for Mlps as components of muscle cytoarchitecture.

INTRODUCTION

Myogenesis involves a series of discrete processes beginning with specification and proliferation of the mesoderm, subdivision of functionally distinct muscle lineages, and ultimately, muscle differentiation. In vertebrates, commitment to a skeletal muscle fate requires the action of myogenic regulatory factors including members of the MyoD basic helix–loop–helix

(bHLH) family and the myocyte enhancer-binding 2 (MEF2) proteins that influence muscle development through transcriptional regulation of muscle-specific target genes (for review, see Molkenin and Olson, 1996; Yun and Wold, 1996). A third family of proteins, the LIM domain-containing cysteine-rich proteins (CRPs), have also been implicated in promoting muscle differentiation (Arber *et al.*, 1997; Louis *et al.*, 1997). The crucial involvement of CRPs in myogenic differentiation has been confirmed by genetic studies in mice. Eliminating the function of one CRP family member, CRP3 (also called the muscle LIM protein

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MLP) results in postnatal lethality caused by heart failure that is associated with dilated cardiomyopathy and severe disruptions of cardiac muscle architecture (Arber *et al.*, 1997).

Despite the dramatic consequences associated with loss of CRP3/MLP expression, the mechanistic details of CRP function in muscles remain speculative. Controversy over the precise role of CRPs stems in part from the fact that CRP isoforms have been observed in cell nuclei and in association with the actin-based cytoskeleton (Arber *et al.*, 1994; Arber and Caroni, 1996; Crawford *et al.*, 1994; Kong *et al.*, 1997; Louis *et al.*, 1997). Consistent with the hypothesis that CRP family members contribute to muscle differentiation by regulating the transcription of muscle structural genes, one study describes an interaction between CRP3/MLP and the bHLH transcription factor MyoD (Kong *et al.*, 1997). Evidence for functionally relevant interactions between bHLH factors and LIM domain proteins has been established in other systems. For example, the hematopoietic bHLH transcription factor SCL (Tal1) controls erythroid differentiation in part through interactions with the nuclear LIM protein Lmo-2 (RBTN-2) (Valge-Archer *et al.*, 1994; Wadman *et al.*, 1997).

Other data are consistent with a structural role for CRP isoforms in muscle. In particular, CRPs are known to distribute along the actin cytoskeleton and at integrin-rich sites of adhesion in cultured fibroblasts and muscle cells (Sadler *et al.*, 1992; Arber *et al.*, 1994; Crawford *et al.*, 1994). Moreover, two cytoskeletal proteins, zyxin and α -actinin, have been shown to interact directly with CRPs in a variety of biochemical assays (Sadler *et al.*, 1992; Pomies *et al.*, 1997). Zyxin, a protein displaying proline-rich sequences and three LIM domains, has been implicated in the control of microfilament dynamics (Golsteyn *et al.*, 1997). α -Actinin, an actin cross-linking protein, is prominent in both non-muscle and muscle cells; in the myofibril, α -actinin localizes to Z-bands that define the ends of each sarcomeric unit (McKenna *et al.*, 1986). Thus, via interactions with cytoskeletal components, CRPs may contribute to muscle differentiation as an integral part of muscle cytoarchitecture. In this context, it is noteworthy that the terminal phenotype of CRP3/MLP null mice is disorganization of cardiac muscle myofibrils (Arber *et al.*, 1997). Clearly, additional work is necessary to define more precisely the molecular mechanism by which CRPs act in myogenesis.

The availability of a genetic system for defining the pathways that require CRP function could provide significant insight into the underlying mechanisms by which CRPs participate in myogenesis. Toward that end, two *Drosophila* CRP family members, termed muscle LIM proteins Mlp60A and Mlp84B, have been identified (Arber *et al.*, 1994; Stronach *et al.*, 1996). *Drosophila* Mlps exhibit muscle-specific expression, ac-

cumulating in all of the body wall and visceral and pharyngeal muscles in the embryo. Analysis of the temporal expression profiles of Mlp60A and Mlp84B transcripts during development pointed to a potential requirement for the proteins late in the muscle differentiation process. Here, we have used molecular epistasis analysis to position the Mlps within the regulatory hierarchy that controls muscle development in *Drosophila*. Specifically, we have examined the expression and distribution of Mlps in the context of mutations that are known to disrupt specific processes in the myogenic pathway. For instance, we have evaluated the role of dMEF2, an essential myogenic transcription factor (Bour *et al.*, 1995; Lilly *et al.*, 1995), in Mlp expression. We have also determined whether myoblast fusion, a key event in somatic muscle differentiation, is required for Mlp accumulation. Finally, we analyzed at higher resolution the localization of *Drosophila* Mlp84B in various muscles at different stages of development and show that its localization is not dependent on two structural muscle proteins, α -actinin or PS2 integrin receptors.

MATERIALS AND METHODS

Drosophila Stocks and Crosses

All flies were reared at 25°C unless otherwise noted on standard cornmeal agar food plus yeast. Canton-S served as wild-type stock. For analysis of dMEF2 mutant embryos, we used the mutation *me^{f2}-2¹*, an ethyl methane sulfonate-induced null allele (Bour *et al.*, 1995). This allele was crossed into a *w¹¹¹⁸* background and balanced over a *CyO* chromosome carrying the *lacZ* gene under the control of the *actin5C* promoter (*CyO-actin1Z*), a gift from David VanVector (Harvard Medical School, Boston, MA). The *CyO-actin1Z* balancer chromosome has been used previously to distinguish embryos carrying that chromosome from those that are homozygous for recessive lethal loci (Bourgouin *et al.*, 1992; Lundgren *et al.*, 1995). In our experiments, embryos that inherit the balancer chromosome show expression of β -galactosidase during most of embryogenesis; by immunocytochemistry, the protein is detected within the nuclei of many cells that are distributed throughout the internal space of the embryo. This particular staining pattern was absent in approximately one-fourth of embryos derived from the *me^{f2}-2¹/CyO-actin1Z* stock, thus allowing for the identification of homozygous dMEF2 null mutant embryos. Aiding our identification of dMEF2 null embryos was the observation that only the portion of the population in which β -galactosidase immunoreactivity was absent also showed morphological and muscle differentiation defects consistent with loss of dMEF2 function.

For dMEF2 overexpression studies, we used *w;GawB 69B*, a homozygous viable enhancer trap line that expresses GAL4 in the epidermis at germ band extended stages (Brand and Perrimon, 1993). Males from this stock were crossed to virgins from the homozygous viable stock *yw; UAS-MEF 1*, which carries the dMEF2 cDNA under the control of GAL4 target upstream activating sites (UASs) (Lin *et al.*, 1997). All embryos derived from this cross expressed dMEF2 in the epidermis. For analysis of myoblast fusion, a severe *rollingstone* allele was used, *rost²³*, balanced over the *CyO* chromosome (Paululat *et al.*, 1995). Mutant embryos were recognized by fusion defects. α -Actinin mutant alleles used in this study include ethyl methane sulfonate-induced *l(1)EA43* and *l(1)EA82* and x-ray-induced *l(1)HC288*, *l(1)HC207*, and *l(1)C212* (Perrimon *et al.*, 1985; Flybase, 1994). All of these alleles are larval lethal. Larvae from

each stock were picked for midgut dissection. The integrin mutant stocks used were *mys¹*/FM4, carrying a null allele of the β_{PS} subunit (Flybase, 1994), and *y v jf^{B4}*/FM6, carrying a null allele of the α_{PS2} subunit (Brown, 1994). These mutant embryos were recognized by morphological criteria including their specific muscle defects.

Larval Midgut Preparation

Wild-type first or third instar larvae for dissection were picked out of Canton-S stock bottles. Hemizygous α -actinin mutant larvae were identified by their progressively paralyzed, flaccid phenotype among a population of developing larvae. Larvae were dissected, and midguts were processed as described (Saide *et al.*, 1989). Primary antibodies were B50 (rabbit anti-Mlp84B) preabsorbed against early stage fixed embryos and diluted to 1:200 (Stronach *et al.*, 1996) and 3A1 (mouse anti- α -actinin) culture supernatant diluted 1:2 (Saide *et al.*, 1989). Secondary antibodies were affinity-purified Texas Red-conjugated goat anti-mouse IgG and fluorescein-conjugated goat anti-rabbit IgG used at 1:250 (Cappel Laboratories, Durham, NC). Samples were viewed using confocal microscopy.

Fluorescent Embryo Staining and Confocal Analysis

For immunofluorescence, embryos were collected and fixed according to standard procedures (Patel, 1994) except the Triton X-100 concentration was raised to 0.3% in all wash and blocking buffers. In all cases, egg collections were performed for no more than 15 h to minimize the presence of older embryos with impermeable cuticles. The following antibodies and dilutions were used in this study: rabbit anti-Mlp60A (B49) at 1:250, rabbit anti-Mlp84B (B50) at 1:250–500, rabbit anti- β -galactosidase at 1:5000 (Cappel Laboratories), mouse anti- β_{PS} (CF6G11) at 1:1000 (Brower *et al.*, 1984), and rabbit anti-muscle myosin at 1:500 (Kiehart and Feghali, 1986). In the analysis of Mlp expression in *dMEF2* mutant embryos, both primary antibodies (anti-Mlp and anti- β -galactosidase) were derived from rabbit and thus were visualized using a single secondary antibody. Because the distributions of Mlps and β -galactosidase are nonoverlapping (our unpublished results), genotypes can be unambiguously assigned based on the presence or absence of β -galactosidase staining. All fluorochrome-conjugated secondary antibodies were obtained from either Cappel Laboratories or Jackson ImmunoResearch Laboratories (West Grove, PA) and used at 1:250–500. Images of embryos were captured as described previously (Stronach *et al.*, 1996). Images of visceral muscle were obtained using a 60 \times objective and represent 1- μ m-thick optical sections. For assessment of protein colocalization, we were cognizant of the alignment of tissue and degree of distortion in the Z dimension with respect to the axis of the laser during optical sectioning.

Genomic Sequence Analysis

For studying the genomic region comprising the *Mlp84B* gene, we first isolated clones from the *Drosophila melanogaster* genomic library "D.m. isox234" constructed in λ EMBL3, a kind gift from John Tamkun (University of California, Santa Cruz, CA). Approximately 400,000 plaque-forming units were screened with the entire *Mlp84B* cDNA (Stronach *et al.*, 1996) using standard techniques (Sambrook *et al.*, 1989). Genomic sequence was obtained primarily from phage clone 4B2, because it fully spans the noncoding and coding portions of the *Mlp84B* transcript. Sequencing was performed by the DNA Sequencing Core Facility at the University of Utah using *Mlp84B* gene-specific primers. Either ABI dRhodamine dye terminators or ABI Prism BigDye terminators were used during cycle sequencing with *Taq* FS DNA polymerase. DNA sequence was collected and analyzed on an ABI Prism 377 automated DNA sequencer (Applied Biosystems, Foster City, CA). Approximately 5000 bp of sequence have been submitted to GenBank under accession number AF090832. Sequences were analyzed using DNASTAR software (DNASTAR, Madison, WI). The positions of each of the six regions

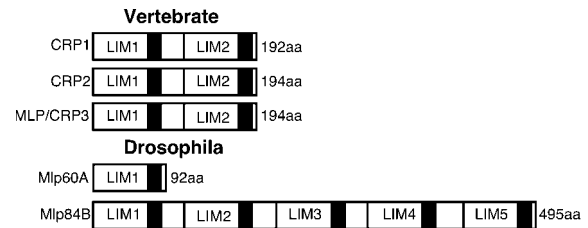


Figure 1. Schematic representation of the CRP family of LIM domain proteins. In vertebrates, there are three conserved isoforms, CRP1, CRP2, and CRP3/MLP, encoded by unique genes. All share the same molecular architecture with two LIM domains, each followed by short glycine-rich regions (black box). In *Drosophila*, there are two proteins related to the vertebrate CRPs. Mlp60A exhibits a single LIM-glycine motif. Mlp84B comprises five tandem LIM-glycine modules.

that exactly match the MEF2 consensus binding site (Olson *et al.*, 1995) are noted in the accession, as are four other sites that have a 9/10 match to the consensus.

The genomic region comprising the *Mlp60A* gene was sequenced by the Berkeley *Drosophila* Genome Project (Celniker *et al.*, unpublished results) and obtained via GenBank accession number AC004642. A region of ~5000 bp constituting the gene was analyzed for potential MEF2 binding sites using the same criteria.

Gel Mobility Shift Assays

Gel mobility shift assays were performed with dMEF2 protein synthesized using the Promega (Madison, WI) TNT rabbit reticulocyte lysate in vitro transcription and translation system (Lilly *et al.*, 1994). For each 20- μ l reaction, 3 μ l of lysate containing PCite-dMEF2 or the control PCite vector alone were used. The lysates were incubated for 10 min at room temperature with 1.5 μ g of poly(dI-dC), 1 \times binding buffer (40 mM KCL, 15 mM HEPES, pH 7.9, 1 mM EDTA, 0.5 mM DTT, and 5% glycerol), and the indicated competitor oligonucleotides. ³²P-labeled probe (20,000 cpm) was then added and incubated for an additional 10 min before loading onto a 6% polyacrylamide gel in 0.5 \times Tris borate-EDTA buffer. The competitor oligonucleotides were added at 100-fold molar excess to the labeled probe. The sequences of the sense strand of the oligonucleotides used for probes and competitors were as follows (linker nucleotides added for end labeling are shown as lowercase): muscle creatine kinase (MCK) MEF2, gatcGCTCTAAAAATAACCCTGTCC; Mutant 6, gatcGCTCTAAACATAACCCTGTCC; Mlp60A-B, gatcGCCCTC-TATTTATAGATATG; and Mlp84B-D, gatcCACTATTATTAATA-GATTCCG.

RESULTS

The CRP family of LIM domain proteins currently consists of three vertebrate isoforms, CRP1, CRP2, and CRP3/MLP, and two *Drosophila* members, Mlp60A and Mlp84B (Figure 1) (Weiskirchen and Bister, 1993; Weiskirchen *et al.*, 1995; Arber *et al.*, 1994; Crawford *et al.*, 1994; Stronach *et al.*, 1996; Louis *et al.*, 1997). The vertebrate proteins share a common molecular architecture with two copies of the LIM domain, each followed by a small glycine-rich segment. Although the *Drosophila* family members diverge in the number of LIM-glycine repeats, they show significantly high sequence identity (46–60%) when compared with their

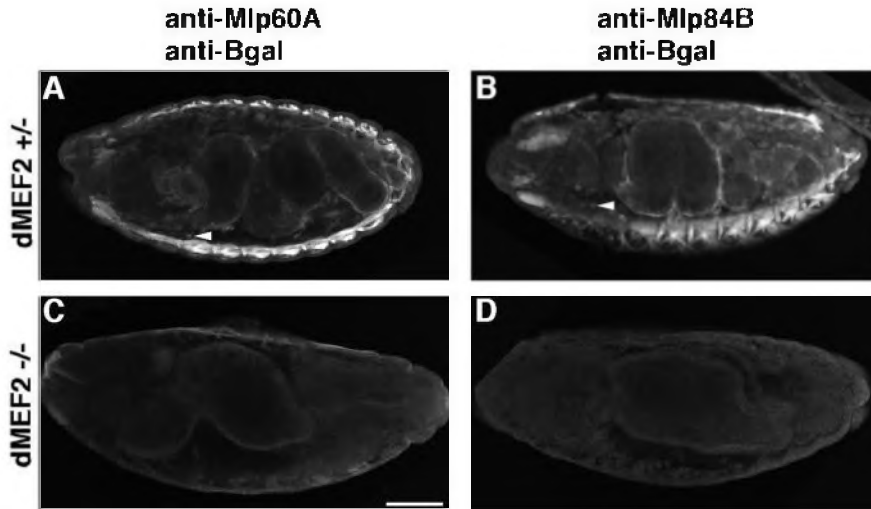


Figure 2. The transcription factor dMEF2 is essential for Mlp expression. *dMEF2* heterozygous embryos express Mlp60A (A) and Mlp84B (B) in somatic, visceral, and pharyngeal muscles. These embryos also carry the balancer chromosome from which the *lacZ* gene is expressed. Thus, by indirect immunofluorescence, we detect not only Mlp expression in these embryos but expression of β -galactosidase in single cells dispersed throughout the embryo (A and B, arrowheads). Mlp60A (C) and Mlp84B (D) fail to be expressed in the *dMEF2* null mutant embryos. Null embryos display defective midgut morphology and no β -galactosidase-positive cells. All panels show lateral views of stage 16 (13- to 16-h) embryos oriented with dorsal up and anterior to the left. Bar, 50 μ m.

vertebrate relatives. Much work suggests that CRP family members play an essential role in myogenic development by promoting muscle differentiation. Here, we address the potential requirements for *Drosophila* Mlps by positioning their function within a genetic regulatory hierarchy that controls myogenesis.

Regulation of Muscle LIM Protein Expression by dMEF2

In *Drosophila*, the dMEF2 transcriptional regulator is essential for completion of myogenesis (Bour *et al.*, 1995; Lilly *et al.*, 1995). *dMEF2* null mutant embryos exhibit a failure of muscle differentiation, although muscle specification and patterning occur fairly normally. Targets of dMEF2 activity include muscle structural genes that encode components of the myofibril, such as myosin and tropomyosin (Bour *et al.*, 1995; Lin *et al.*, 1996). To assess whether Mlps are downstream of dMEF2 activity, we used indirect immunofluorescence to evaluate the expression of Mlp60A and Mlp84B in *dMEF2* mutant embryos. In heterozygous embryos, which express β -galactosidase encoded by a *lacZ* gene carried on the balancer chromosome, Mlps are expressed appropriately in the muscles (Figure 2, A and B; cf. Stronach *et al.*, 1996). The Mlp expression pattern is quite distinct and exhibits no overlap with the expression of β -galactosidase. Mlps are found exclusively in muscle derivatives, whereas β -galactosidase is detected in many internal nonmuscle cells (Figure 2, A and B, arrowheads). Homozygous *dMEF2* null embryos were identified by morphological criteria and by lack of staining for β -galactosidase; they constituted ~25% of the total collected population. In the mutant embryos, neither Mlp60A nor Mlp84B expression was detected in any tissue (Figure 2, C and

D). From the results presented here, we conclude that dMEF2 is an essential positive regulator of Mlp60A and Mlp84B expression. This lack of expression is notable given that it has been previously shown that mesoderm is formed and specified normally in *dMEF2* mutants (Bour *et al.*, 1995).

To investigate whether dMEF2 activity stimulates the expression of the *Mlp* genes, we used an ectopic tissue-specific expression system (Brand and Perrimon, 1993). Ectopic dMEF2 activity in the epidermis of germ band extended embryos is achieved using the GAL4 enhancer trap line *69B* and a construct containing *dMEF2* coding sequences downstream of the GAL4 recognition sites or UAS. dMEF2 is not normally found in this tissue, and ectopic expression leads to epidermal defects and subsequent embryonic lethality (Lin *et al.*, 1997). We examined the possibility that ectopic expression of dMEF2 would result in up-regulation of muscle-specific targets such as the Mlps and myosin (Figure 3). In wild-type embryos undergoing muscle differentiation, myosin and the Mlps are observed in muscle tissues but not in epidermal cells (Figure 3, A–C); however, myosin expression is strongly induced in the epidermis by the presence of ectopic dMEF2 (Figure 3D). Mlp84B protein is also detected in the epidermis, concomitant with this ectopic expression of dMEF2 (Figure 3E). In contrast to both myosin and Mlp84B, Mlp60A protein is not detected at any appreciable level in epidermal cells of embryos that ectopically express dMEF2 (Figure 3F). We often observe a halo of staining in these embryos that is likely attributable to variable background associated with the anti-Mlp60A antibody. This staining does not appear cellular in nature, as that seen with the epidermal expression of myosin and Mlp84B, but rather as nonspecific surface staining.

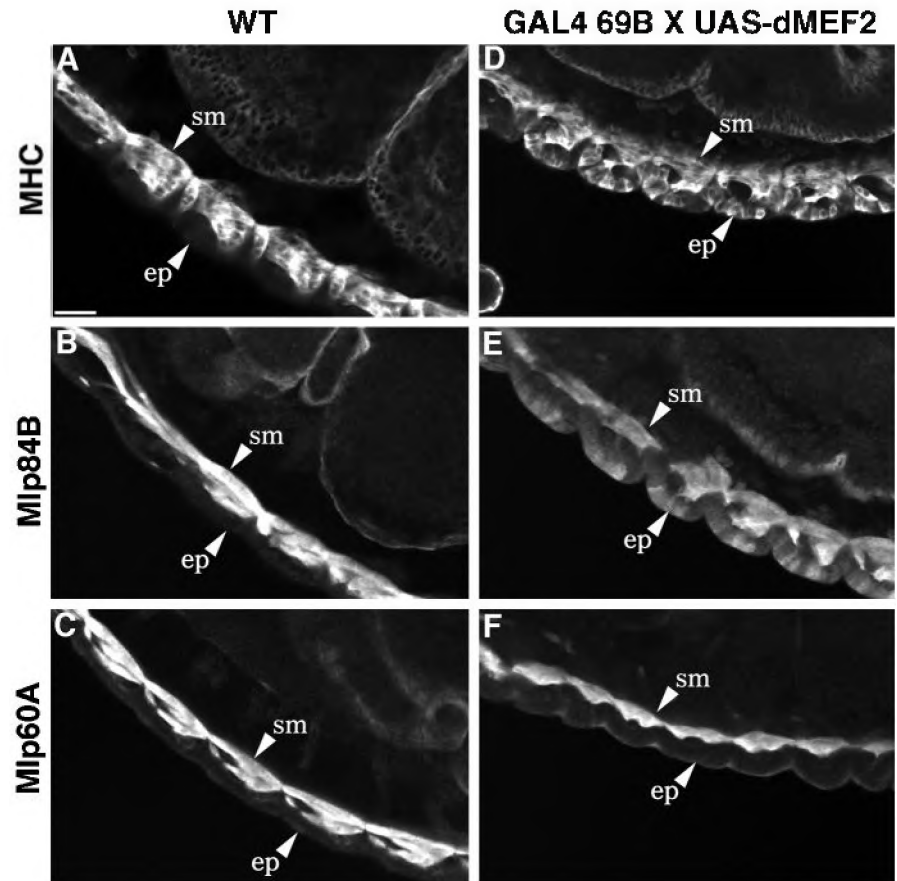


Figure 3. Ectopic dMEF2 expression stimulates expression of myosin and Mlp84B but not Mlp60A. In wild-type embryos, myosin (A), Mlp84B (B), and Mlp60A (C) are detected by indirect immunofluorescence in embryonic somatic muscles (sm) but not epidermal cells (ep). Myosin (D) and Mlp84B (E) expression can be induced in the epidermis by ectopic expression of dMEF2 under the control of the *69B* GAL4 enhancer. Unlike myosin and Mlp84B, Mlp60A is not up-regulated by ectopic expression of epidermal dMEF2 (F). All panels display lateral views of 12- to 14-h embryos oriented with dorsal up and anterior to the left. Bar, 20 μ m.

To assess whether the *Mlp60A* gene might require a higher threshold level of dMEF2 for its activation, we increased the dosage of dMEF2 in the epidermis by exploiting the temperature-sensitive nature of the GAL4 protein. At 29°C, GAL4 is reported to have higher activity resulting in greater accumulation of UAS-target gene product (Brand *et al.*, 1994). Indeed, embryos raised at the higher temperature displayed more substantial phenotypic defects, suggesting increased dMEF2 expression in the epidermis, but still did not show ectopic up-regulation of Mlp60A protein (our unpublished results).

dMEF2 Binds Muscle LIM Protein Gene Sequences *In Vitro*

To begin to address whether Mlps may require dMEF2 activity directly for their expression, we analyzed the genomic sequences of the *Mlp* genes to identify potential dMEF2 binding sites. Figure 4A displays the genomic organization of the *Mlp60A* and *Mlp84B* genes denoting intron and exon structure. The *Mlp60A* gene exhibits three exons interrupted by two small introns, one in the 5' untranslated region and another in the coding region. The *Mlp84B* gene contains one

noncoding and one coding exon separated by a single large intron. Analysis of noncoding DNA within and surrounding the *Mlp* genes revealed the presence of multiple A/T-rich sequences matching exactly the reported MEF2 target binding consensus sequence (Olson *et al.*, 1995). An alignment of the sites in the *Mlp* genes in comparison with the MEF2 consensus sequence and a bona fide mammalian MEF2 target sequence is displayed in Figure 4B. The *Mlp60A* gene contains three potential dMEF2 binding sites; two of these sites are located in the region 5' to the start of gene, whereas the third is found 3' to the coding sequence. Six putative dMEF2 binding sites are found in the *Mlp84B* gene. Four of the six are clustered in the intron, another is located 3' to the coding region of the gene, and another is contained completely within the first exon.

One potential target site from each *Mlp* gene was chosen to test directly for dMEF2 binding activity *in vitro*, in parallel with a control site derived from the mammalian MCK gene (Gossett *et al.*, 1989), which has been shown to bind dMEF2 *in vitro* (Lilly *et al.*, 1994; Nguyen *et al.*, 1994). Labeled oligonucleotides consisting of the core MEF2 binding site flanked by three to

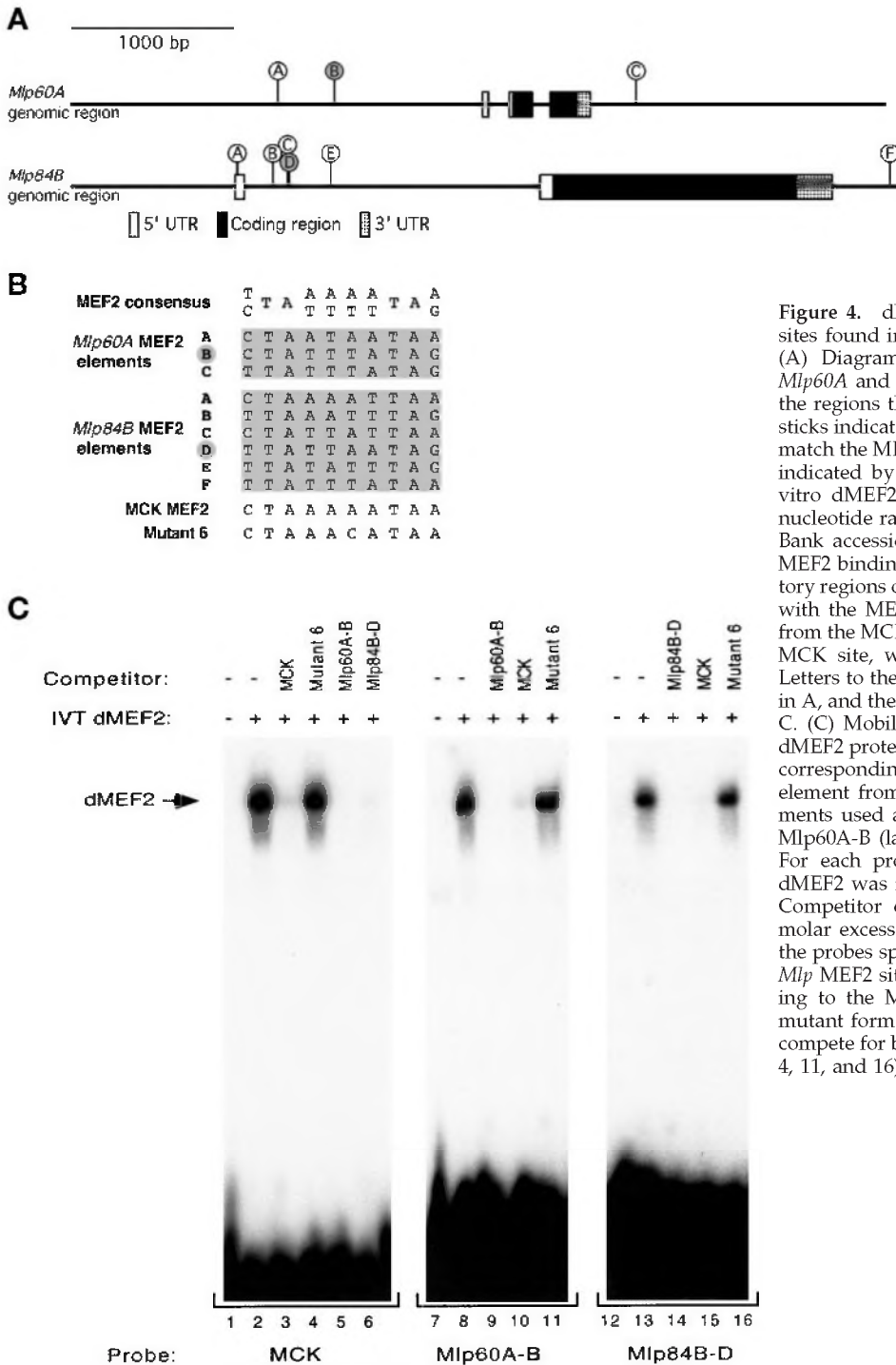


Figure 4. dMEF2 protein binds to consensus MEF2 sites found in the genomic regions of the *Mlp* genes. (A) Diagram of the genomic organization of the *Mlp60A* and *Mlp84B* genes. The boxed areas indicate the regions that correspond to cDNA sequences. The sticks indicate positions of 10-bp elements that exactly match the MEF2 consensus binding site. The two sites indicated by filled circles are those tested in the in vitro dMEF2 binding assay shown in C. The exact nucleotide range of the *Mlp84B* sites is noted in GenBank accession number AF090832. (B) The potential MEF2 binding elements found in the putative regulatory regions of *Mlp60A* and *Mlp84B* are shown aligned with the MEF2 consensus, the MEF2 regulatory site from the MCK enhancer, and the Mutant 6 form of the MCK site, which does not support MEF2 binding. Letters to the left correspond to the sites diagrammed in A, and the filled letters indicate those sites tested in C. (C) Mobility shift assays with in vitro-translated dMEF2 protein were performed with oligonucleotides corresponding to the MCK MEF2 site and one MEF2 element from each of the *Mlp* genes. The MEF2 elements used as probes were MCK MEF2 (lanes 1–6), Mlp60A-B (lanes 7–11), and Mlp84B-D (lanes 12–16). For each probe a control lysate lacking translated dMEF2 was included in parallel (lanes 1, 7, and 12). Competitor oligonucleotides were used at 100-fold molar excess of each probe. dMEF2 binds to each of the probes specifically (lanes 2, 8, and 13). Each of the *Mlp* MEF2 sites binds dMEF2 and competes for binding to the MCK element and their cognate site. A mutant form of the MCK element (Mutant 6) fails to compete for binding with any of the sites tested (lanes 4, 11, and 16).

eight additional nucleotides derived from genomic sequence were generated and used in electrophoretic gel mobility shift assays with in vitro-translated dMEF2 protein (Lilly *et al.*, 1994). Figure 4C shows a shifted complex with each of the three probes (MCK, Mlp60A-B, and Mlp84B-D) dependent on the addition

of dMEF2 translation product. In addition, in all three cases, the bound probe could be efficiently competed by addition of excess unlabeled probe or an unlabeled control (MCK) probe. Furthermore, each of the shifted species was ineffectively competed by addition of excess unlabeled mutant probe, Mutant 6, in which a

single base substitution within the MEF2 core binding site has been introduced (Cserjesi and Olson, 1991). Taken together, these results show that dMEF2 recognizes and binds specifically and directly to sequences derived from the *Mlp60A* and *Mlp84B* genes.

Myoblast Fusion Is Not Required for Muscle LIM Protein Expression

Fusion of myoblasts into syncytial muscle fibers is a key feature of somatic muscle development in *Drosophila* (Bate, 1990). Although the mechanism of fusion is not well understood, mutations that disrupt specific aspects of the process are beginning to be characterized. In *Drosophila*, embryos that harbor mutations in genes such as *myoblast city* (*mbc*) and *rollingstone* (*rost*) display severe defects in the process of myoblast fusion such that many single unfused myoblasts persist late into embryogenesis after they would normally be incorporated into a growing muscle fiber (Paululat *et al.*, 1995; Rushton *et al.*, 1995). Previously, we noted that myoblast fusion precedes Mlp protein accumulation by a few hours, suggesting that Mlps are not likely to be required for the fusion process (Stronach *et al.*, 1996). However, the temporal relationship of fusion events and Mlp expression raises the possibility that Mlp accumulation in the somatic lineage might depend on cell fusion. Furthermore, because fusion defects are also associated with mutations in *dMEF2*, we assessed the expression of Mlps in the *rost* mutant background in which a failure of myoblast fusion is the primary defect. In embryos mutant for the *rost* gene, both Mlp60A and Mlp84B are observed in single unfused myoblasts (Figure 5), distributed in characteristic positions where, under normal circumstances, they would provide a pool of fusion competent cells for the developing syncytial myotubes (Bate, 1990). Thus, from these observations, we conclude that myoblast fusion is not required for Mlp expression, an observation that has also been noted for myosin and $\beta 3$ tubulin (Paululat *et al.*, 1995; Rushton *et al.*, 1995).

Mlp84B Subcellular Distribution in Wild-Type and Mutant Visceral and Somatic Muscles

Knowledge of the subcellular distribution of a protein often contributes substantially to an understanding of its function. In embryonic somatic muscles, Mlp60A and Mlp84B are found in both the nuclear and cytoplasmic compartments, consistent with either a regulatory or structural role in differentiating muscle (Stronach *et al.*, 1996). When expressed in rat embryo fibroblast cells, *Drosophila* Mlps showed a specific association with the actin cytoskeleton (Stronach *et al.*, 1996). To determine the precise localization of Mlps within mature myofibrils at higher resolution, we double-labeled whole, third instar larval midguts using antibodies directed against the Mlps and α -actinin, which marks Z-bands (Figure 6).

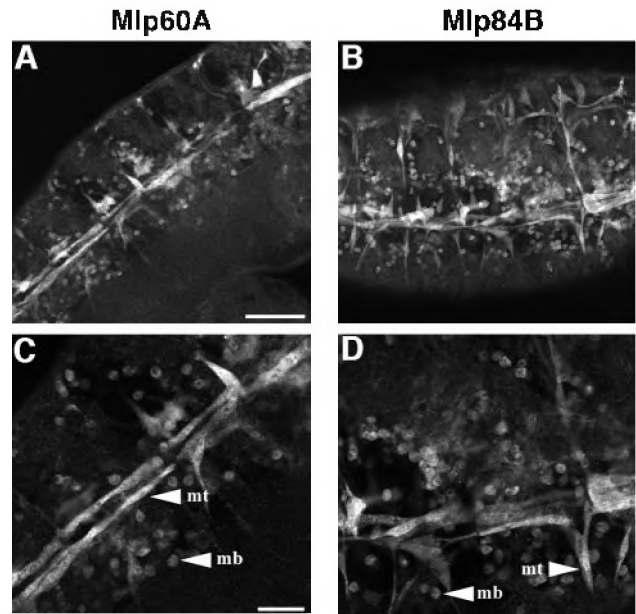


Figure 5. Myoblast fusion is not required for muscle LIM protein expression. Immunofluorescent detection of Mlp60A (A and C) or Mlp84B (B and D) in embryos mutant for the *rollingstone* gene is shown. These embryos display severe defects in myoblast fusion. Both Mlps are expressed in single unfused myoblasts (mb) as well as in myotubes (mt) that have formed (see higher magnification; C and D). All panels display ventral-lateral views of late stage embryos with anterior to the left. Bars: A and B, 50 μ m; C and D, 20 μ m.

Surrounding the midgut, elongated visceral mesodermal cells form a lattice of transverse and longitudinal fibers. Although these cells do not undergo myoblast fusion, they appear striated and display sarcomeric repeats (Saide *et al.*, 1989; Tepass and Hartenstein, 1994). Within the midgut visceral mesoderm, α -actinin prominently localizes to Z-bands (Figure 6A). Z-bands demarcate the ends of individual sarcomeres (Figure 6D), where the barbed ends of actin thin filaments terminate. In the same tissue, Mlp84B distributes as a doublet that flanks each Z-band (Figure 6B). As seen in merged images (Figure 6C), α -actinin and Mlp84B are localized in adjacent regions. Mlp84B extends away from the periphery of the Z-band, whereas α -actinin is clearly more restricted (Figure 6, C and D). The localization of Mlp84B to discrete sites within the muscle sarcomere provides evidence for a specific association of Mlp84B with the microfilament cytoskeleton *in vivo*. No nuclear staining for Mlp84B in the visceral muscles was observed at this time in development. Although Western immunoblot analysis revealed that Mlp60A is present in isolated midgut preparations, we were unable to detect the protein using immunofluorescent methods. It is unclear why Mlp60A protein was not observed *in situ*, but perhaps within the mature myofibril, Mlp60A is complexed with protein partners such that the epitopes recognized by our antibodies are masked.

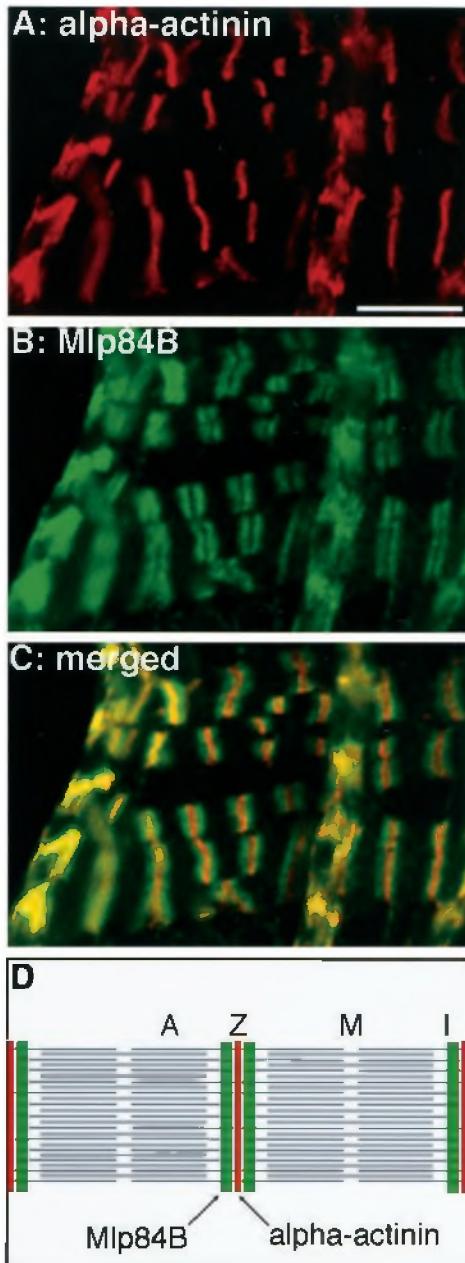


Figure 6. Mlp84B localizes to discrete sites within sarcomeres of larval midgut visceral muscles. The mesoderm surrounding a third instar larval midgut has been double labeled for α -actinin (red), to visualize Z-bands, and Mlp84B (green). Most of the visceral muscles are positioned horizontally, but muscle cells can be seen on top of these positioned vertically in the figure. Mlp84B localizes as doublets within the muscles (B). In the merged image (C), Mlp84B is observed to flank α -actinin in a region adjacent to the Z-bands (see diagram of two adjacent sarcomeres with bands indicated in D). Bar, 10 μ m.

Effects of α -Actinin Mutations on Mlp84B Distribution. It has been reported that vertebrate CRP1 interacts with α -actinin (Pomies *et al.*, 1997). This interaction may underlie the association of CRPs with the

microfilament cytoskeleton. By inference, *Drosophila* Mlps, relatives of the vertebrate CRPs, may associate with the cytoskeleton via interactions with α -actinin. Although α -actinin and Mlp84B are not extensively colocalized, the distribution in adjacent domains may indicate that a subset of the molecules associate at the Z-band periphery. To evaluate whether Mlp84B localization within sarcomeres depends on the presence of α -actinin, we analyzed the distribution of Mlp84B in α -actinin mutant larval midguts. Mutant larvae become progressively paralyzed and flaccid between hatching and the second instar molt. These larvae were dissected, and midguts were double labeled for Mlp84B and α -actinin in parallel with similar staged wild-type larval midguts. Figure 7 illustrates comparable Mlp84B localization in α -actinin mutant versus wild-type midgut visceral muscles (Figure 7, compare B and D). Although mutant myofibers appear generally more disorganized than wild type, doublets of Mlp84B protein were observed in repeated arrays, reflecting some residual sarcomeric organization. Identical results were seen in four other α -actinin mutant backgrounds. Thus, in the absence of functional α -actinin protein, Mlp84B is still capable of localizing to discrete sites within the developing sarcomeres. Therefore, α -actinin is not absolutely essential for recruiting Mlp84B to its normal subcellular location within muscle tissue. We do observe some inappropriate localization of Mlp84B in α -actinin-deficient muscles (Figure 7D). This may result from the decaying muscle cytoarchitecture in the mutant or may reflect some contribution of α -actinin in restricting Mlp84B localization.

Localization of Mlp84B to Muscle Attachment Sites (MASs) Does Not Require PS2 Integrins. The identification of components required for normal Mlp84B localization may provide insight into its role in muscle differentiation. We noted previously that, in embryos, Mlp84B is enriched at the ends of somatic muscle fibers where they make attachments to the body wall. These attachment sites are thought to be analogous to the prominent focal adhesions of cultured fibroblast cells. Indeed, both focal adhesions and MASs are areas where actin filaments terminate at the membrane and become linked to extracellular matrix through a series of protein interactions culminating with the transmembrane integrin receptors (BurrIDGE *et al.*, 1988; Reedy and Beall, 1993; Tepass and Hartenstein, 1994). To confirm that the enrichment of Mlp84B at the ends of the somatic muscle fibers coincides with the location of the MAS, we double labeled embryos with antibodies directed against Mlp84B and the β_{PS} integrin subunit (Figure 8). β_{PS} forms a dimer with α_{PS2} in the muscle cell and with α_{PS1} in the neighboring tendon cell membrane (Bogaert *et al.*, 1987; Leptin *et al.*, 1989). As revealed in the merged image (Figure 8C), significant colocalization of Mlp84B and β_{PS} was observed at the MASs. Thus, we conclude that Mlp84B colo-

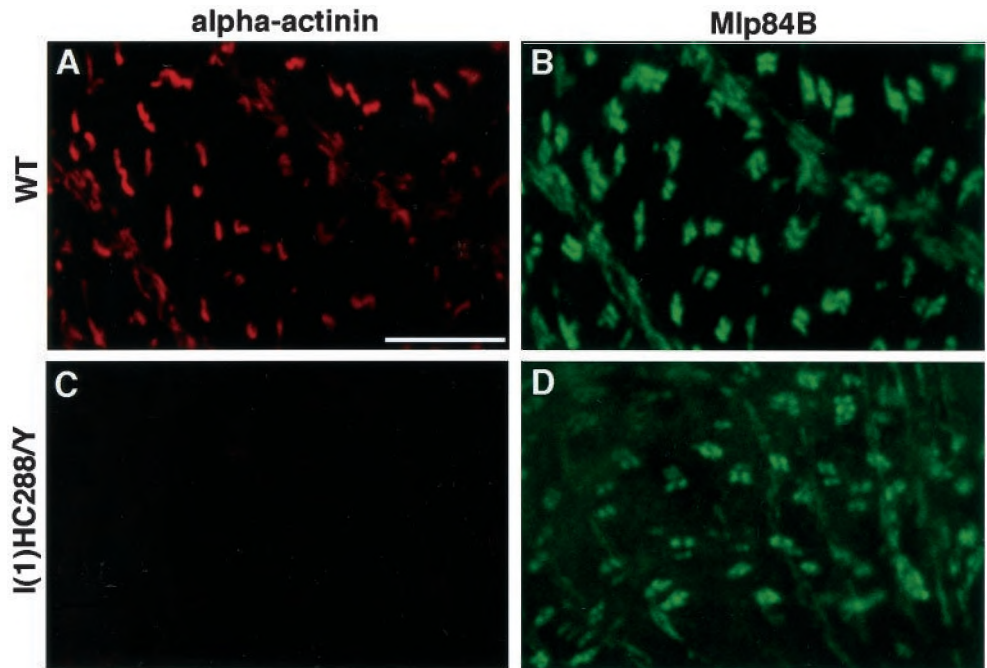


Figure 7. Loss of α -actinin does not affect Mlp84B localization within visceral muscles. α -Actinin null mutant *l(1)HC288/Y* and wild-type midgut muscles have been double labeled for α -actinin (A and C) and Mlp84B (B and D). The null mutant lacks staining for α -actinin protein (C), and wild type is shown for comparison (A). Note that Mlp84B distribution is similar in the wild-type (B) and mutant (D) visceral muscles showing doublets flanking the Z-bands. In the mutant, Mlp84B still localizes to discrete sites within sarcomeres. Bar, 10 μ m.

calizes with muscle integrin complexes. Integrin staining that was not coincident with Mlp84B was also observed in between the neighboring longitudinal muscles (Figure 8C, arrow), which may reflect the presence of β_{PS} complexes in the tendon cell membranes.

Colocalization of Mlp84B and β_{PS} integrin at MASs raises the possibility that integrin receptors, through interactions with extracellular ligands, recruit and stabilize the association of Mlp84B with the junction and that this association is required for muscle development or attachment. We sought to address the role of integrin receptor engagement in the recruitment of Mlp84B by analyzing the distribution of Mlp84B in an integrin mutant background. PS2 integrin mutations are lethal in part because of detachment of the muscles late in embryogenesis and subsequent failure of larvae to hatch from the eggshell (Wright, 1960). Using null alleles for both the β_{PS} subunit encoded by the *mysospheroid* (*mys*) gene and the α_{PS2} subunit encoded by *inflated* (*if*) (Leptin *et al.*, 1989; Wilcox *et al.*, 1989; Brown, 1994), we looked specifically for the presence of Mlp84B at the MASs of muscles in the mutant embryos (Figure 9). In both *mys* and *if* mutants, Mlp84B was observed at the junctional attachment sites in muscles before complete detachment or at remnant sites after detachment (Figure 9, C and E). Often we detected Mlp84B enriched at the membrane of muscles that appeared to be adhering end-to-end with one another (Figure 9, D and F). Therefore, Mlp84B localizes to MASs independent of PS2 integrin-ligand interactions. By staining mutant embryos with the anti- β_{PS} antibody, we detected no residual

integrin complexes containing the β_{PS} subunit that may have been contributed maternally (our unpublished results).

DISCUSSION

Key to understanding the process of muscle differentiation is the characterization of myogenic regulatory factors and their molecular targets. Presumably, it is the unique combination of these target proteins that ultimately defines the differentiated morphology and function of distinct muscle types. In this study, we have described the results of molecular epistasis analysis designed to unravel mechanisms responsible for regulating *Mlp* gene expression during the process of myogenic differentiation and for establishing the subcellular distributions of Mlps within muscles. This analysis confirms that Mlps lie downstream of events involving mesoderm specification and patterning. Our findings indicate that Mlps are likely targets of transcriptional regulation by a member of the MEF family of MADS (named for MCM-1, agamous, deficiens, and serum response factor) box myogenic transcription factors that regulate muscle differentiation. In addition, the process of myoblast fusion in the somatic muscle lineage is not required for Mlp expression. Furthermore, we demonstrate that Mlp84B associates specifically with the microfilament-based cytoskeleton within sarcomeres and that Mlp84B is enriched at the MASs in embryonic somatic muscles. The discrete localization of Mlp84B is not affected by loss of the

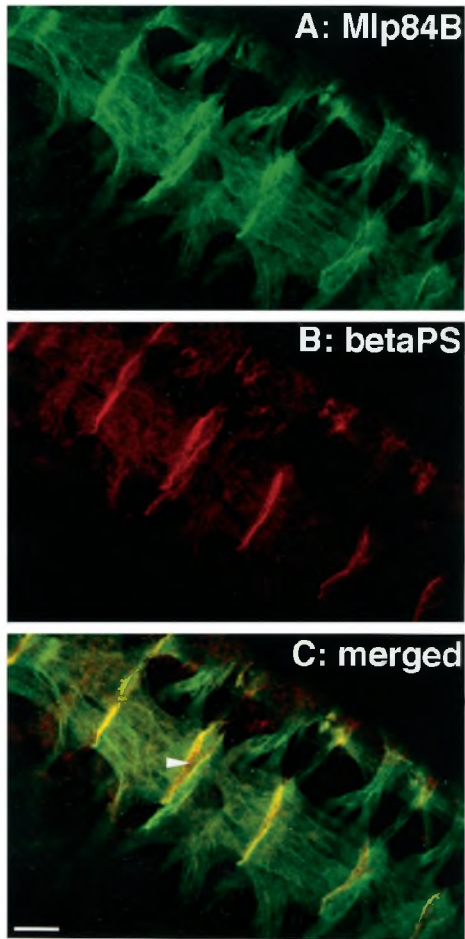


Figure 8. Mlp84B colocalizes with β_{PS} integrin at the MASs. Ventral-lateral longitudinal muscles of a stage 16 embryo are double labeled for Mlp84B (green) and the β_{PS} subunit of integrin (red). The merged image (C) reveals significant colocalization of the two proteins (yellow) at the MASs. The arrow in C indicates β_{PS} complexes in the tendon cells. Bar, 20 μ m.

prominent Z-band protein α -actinin or the transmembrane PS2 integrin receptor.

Mlps Require dMEF2 Activity during Myogenesis

Many lines of evidence point to a role for CRP family members in muscle differentiation. To understand at what point in the differentiation program Mlps may function, we examined the interplay between dMEF2 transcription factor function and Mlp expression. dMEF2 is an invertebrate member of a family of vertebrate myocyte enhancer binding factors that display a MADS box and MEF2 domain, which promote DNA binding and dimerization (Nguyen *et al.*, 1994; Olson *et al.*, 1995). Cell biological and biochemical studies using vertebrate systems have implicated the MEF family of transcription factors in myogenesis, specifi-

cally in regulating muscle differentiation (Olson *et al.*, 1995). During *Drosophila* development, dMEF2 accumulates in all muscle subtypes and displays a biphasic expression pattern suggesting a requirement for the protein in larval and adult myogenesis (Nguyen *et al.*, 1994; Bour *et al.*, 1995; Ranganayakulu *et al.*, 1995). Despite early expression of dMEF2 during gastrulation, genetic analysis revealed a role for the protein relatively late in the myogenic pathway. *dMEF2* mutant embryos display defective myoblast fusion and a failure of muscle differentiation (Bour *et al.*, 1995; Lilly *et al.*, 1995), consistent with the postulated role of vertebrate MEF2 proteins. Identification of the targets of dMEF2 has provided substantial insight into why differentiation fails in mutant embryos. dMEF2 activity regulates expression of several late markers that contribute to muscle structure and function. These include myosin, tropomyosin I, and the α_{PS2} integrin (Ranganayakulu *et al.*, 1995; Lin *et al.*, 1996). Our findings demonstrate clearly that dMEF2 is also essential for the expression of both Mlp60A and Mlp84B in all of the muscle tissues in which they are normally expressed, because we observe no Mlp-positive cells in *dMEF2* null mutant embryos. Indeed, in embryos homozygous for a severe hypomorphic allele of *dMEF2* (*mef¹¹³*) in which some nuclear dMEF2 protein can be detected (Ranganayakulu *et al.*, 1995), we observed very weak expression of Mlp60A and Mlp84B (our unpublished observations). This result supports the notion that *Mlp* genes may be sensitive to changes in the levels of dMEF2 transcriptional activity.

To investigate whether dMEF2 is capable of stimulating Mlp expression, we used the GAL4-UAS system to express dMEF2 ectopically in the epidermis, a tissue in which it is not normally found. As a consequence of ectopic dMEF2 expression, we noted a robust up-regulation of myosin protein, a target of dMEF2. Similarly, Mlp84B was induced in the epidermis. We did not observe Mlp60A protein accumulation in epidermal cells that were programmed to express dMEF2. It is possible that either transcription from the *Mlp60A* gene is not initiated in the context of epidermal cells expressing dMEF2 or that protein is produced but then rapidly degraded. Consistent with the former hypothesis, Lin and colleagues (1997), using a similar dMEF2 overexpression system, failed to detect *Mlp60A* transcripts by *in situ* hybridization in the embryonic epidermis, with the exception of several cells at the ventral midline. Perhaps the *Mlp60A* gene is subject to different regulatory constraints than *myosin* and *Mlp84B*. For instance, *Mlp60A* expression may be specifically repressed by an epidermal transcriptional regulator or may lack a required coactivator present in mesoderm but not ectoderm. Collectively, these results indicate that Mlps may be members of a group of dMEF2 target proteins that contribute to differentiated muscle cytoarchitecture and function.

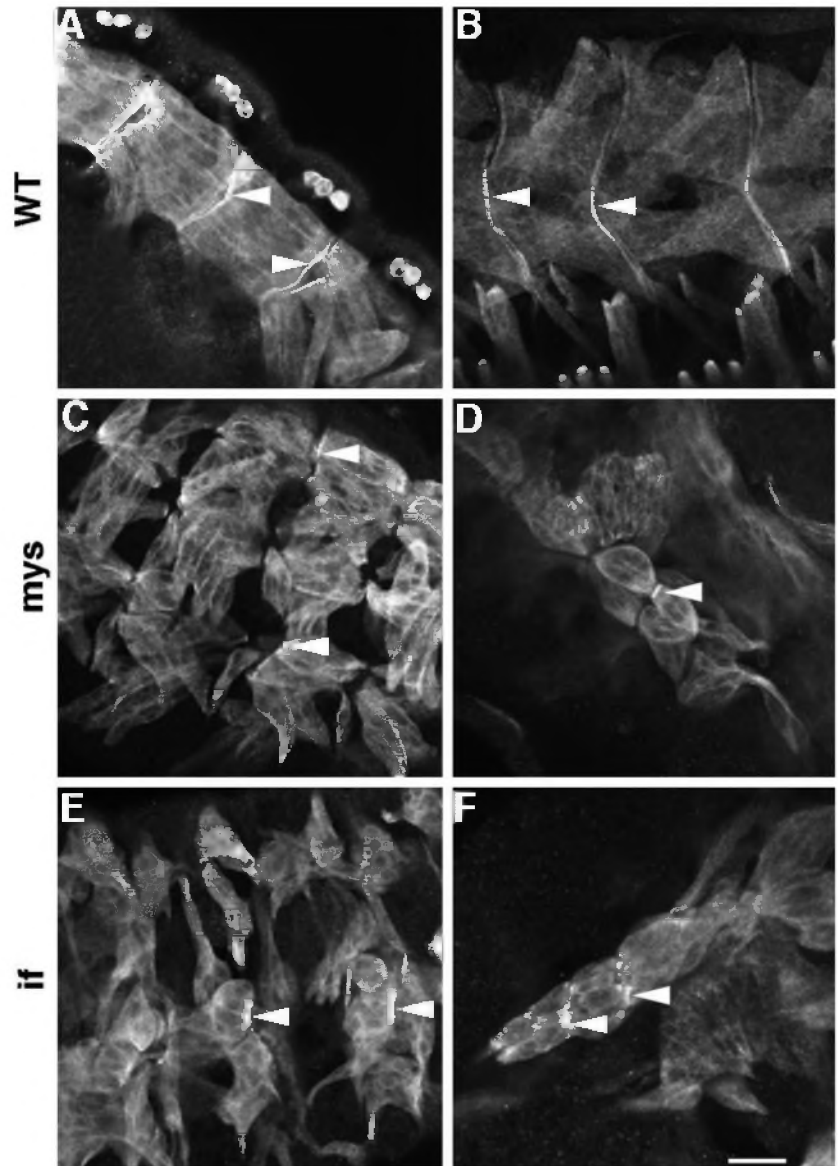


Figure 9. Mlp84B is capable of associating with MASs in the absence of integrin–ligand interactions. Immunofluorescent detection of Mlp84B in somatic muscles of stage 16 wild-type (A and B), *mys* (C and D), or *if* (E and F) embryos is shown. Note the characteristic rounded muscles observed in embryos mutant for either integrin subunit. Arrows indicate Mlp84B enrichment at the MASs of wild-type embryos (A and B) or at remnant junctions in mutant embryos (C–F). Bar, 20 μ m.

Interestingly, forced premature overexpression of dMEF2 in the mesoderm did not result in premature accumulation of Mlps in muscle tissue (our unpublished observations). This observation implies that there are likely to be additional transcriptional regulatory inputs that converge at the *Mlp* gene promoters during myogenic development.

To explore whether the regulation of *Mlp* gene expression by dMEF2 could be direct, we searched the noncoding regions of *Mlp* genomic DNA for potential dMEF2 binding sites. Indeed, we identified several such sequences in both *Mlp* genes that matched exactly the reported consensus binding sites for MEF2 transcription factors (Olson *et al.*, 1995). These putative binding sites are located in regions of the genes where regulatory

information, such as enhancer binding sites, are characteristically found. We chose one putative site from each gene to test for dMEF2 binding in an *in vitro* binding assay. Labeled oligonucleotides derived from the *Mlp60A* and *Mlp84B* genes were directly and specifically bound by dMEF2 protein. Demonstration of candidate dMEF2 sites in the regulatory regions of the *Mlp* genes, coupled with the ability of a site from each gene to support specific binding of dMEF2 protein *in vitro*, suggests that Mlps could be direct targets of dMEF2 activity *in vivo*. Definitive proof that Mlps are direct targets of dMEF2 will require further *in vivo* analysis of the MEF2 sites present in the *Mlp* genes.

Our analysis of the genomic organization of the *Mlps* genes revealed an interesting finding. Although

the *Mlp60A* gene encodes a protein with a single LIM domain, sequences 3' to the coding region appear to contain information that may have, at one time, encoded an additional four LIM domains. Perhaps an ancestral gene coding for a protein with five LIM domains gave rise to two *Mlp* genes, with the result that the *Mlp84B* gene was maintained to encode five domains, whereas the *Mlp60A* gene diverged and was corrupted to produce a truncated reading frame encoding a single LIM domain. It is also noteworthy that each of the two *Mlp* genes has a putative dMEF2 binding site located just downstream of the gene and that these two sites appear to be related to each other. Otherwise, the genomic structure of these two genes appears to have significantly diverged.

Myoblast Fusion Is Not Required for Mlp Expression

In addition to a failure of muscle differentiation, the process of myoblast fusion is also affected in *dMEF2* mutant embryos. To address whether the failure to accumulate Mlps is a result of defects in fusion, we analyzed Mlp expression in *rost* mutant embryos. In these embryos, unfused myoblasts persist well beyond the normal period of fusion and eventually express several differentiation markers including myosin (Paululat *et al.*, 1995). We demonstrate that Mlps can also be expressed in single myoblasts in *rost* mutants. Thus, the defects in myoblast fusion resulting from mutations in *dMEF2* do not appear to be the primary cause of the observed failure of Mlp expression in *dMEF2* mutant embryos. Moreover, myoblast fusion does not appear to be a crucial checkpoint for ensuing muscle differentiation. Late in embryogenesis, these myoblast levels decline, presumably through an apoptotic mechanism coupled with macrophage-mediated phagocytosis (Rushton *et al.*, 1995). We have never detected Mlp expression in macrophages under normal conditions. However, it is possible that some Mlp-positive cells observed in the *rost* mutant embryos represent phagocytic cells that have engulfed Mlp-expressing myoblasts.

Mlps Are Components of the Contractile Apparatus in Myofibrils

Vertebrate CRPs have been localized to the actin cytoskeleton in certain cell types; *CRP3/MLP* gene disruption in the mouse leads to defects in cardiac muscle cytoarchitecture; and *Drosophila* Mlps are able to colocalize with actin fibers in fibroblast cells (Arber *et al.*, 1994, 1997; Stronach *et al.*, 1996; Louis *et al.*, 1997). These observations point to a potential role for CRP family members in establishing muscle structure. In previous studies we noted the appearance of a linear staining pattern for Mlps in somatic myotubes in the embryo; however, it was not possible to assess clearly

the association of Mlps with subdomains of the microfilament cytoskeleton. Therefore, we turned to a later stage of development to examine the distribution of Mlps in relation to a mature sarcomeric pattern of striated muscle tissue. In the larva, a layer of striated visceral muscle cells encases the midgut and provides contractile activity to move food down the alimentary canal (Skaer, 1993). In these cells, we observed a highly localized distribution of Mlp84B protein in association with the sarcomeric cytoskeleton. Specifically, Mlp84B was concentrated in double stripes flanking the Z-bands, which are rich in α -actinin protein. To determine whether α -actinin influences the localization of Mlp84B, we assessed the distribution of Mlp84B in muscles that fail to express α -actinin. In α -actinin mutant larval midguts, sarcomeres appear somewhat disorganized, but Mlp84B is localized normally. These data suggest that α -actinin is not absolutely essential for the proper recruitment of Mlp84B to the sarcomere. Because Mlp84B displays five copies of a protein-binding LIM-glycine motif, each of which may direct interactions with additional proteins within the contractile apparatus, it is possible that the lack of redistribution of Mlp84B in α -actinin mutant muscles may reflect the involvement of multiple components for Mlp84B localization. Interestingly, a similar protein distribution has been noted for the actin capping protein tensin in cultured myotubes (Bockholt *et al.*, 1992), as well as for the vertebrate CRP3/MLP isoform in mouse cardiomyocytes (Arber *et al.*, 1997). The comparable subcellular distributions of Mlp84B and CRP3/MLP in muscle cells points to a potential functional conservation among vertebrate and invertebrate CRP family members. Although the mechanism underlying Mlp84B localization is still unknown, our data illustrate the specific association of Mlp84B with the microfilament cytoskeleton in vivo and lend support for a structural role for Mlp84B in mature differentiated muscles.

MAS Formation and Function

In striated muscles, Mlp84B localizes near regions enriched in the barbed, fast-growing ends of actin filaments, such as the Z-band. In the developing embryo, Mlp84B accumulates at MASs where the barbed ends of actin filaments terminate at the membrane and associate with transmembrane integrin receptors. Although Mlp84B and β_{PS} proteins are colocalized in vivo, in the absence of PS2 integrins, Mlp84B localizes appropriately. Both the normal muscle patterning and polarity seen in the integrin mutant embryos before muscle detachment and the ability of Mlp84B to localize to the attachment sites in the absence of integrins suggest that integrin-ligand interactions are not instructive for defining the ends of the muscle fiber and assembling the attachment site. In further support of

this notion, it is noteworthy that β_{PS} molecules, incapable of binding extracellular ligands, also localize appropriately to MASs, results that place further emphasis on the importance of intracellular mechanisms for the organization of muscle termini (Martin-Bermudo and Brown, 1996). β_{PS} complexes appear to be necessary only for the continued maintenance of the attachment site once it has been assembled, an observation previously noted by careful analysis of the mysospheroid phenotype (Wright, 1960).

Concluding Remarks

Using molecular epistasis methods, we have examined the relationships between Mlps and several gene products necessary for *Drosophila* myogenesis. This analysis revealed the dependence of *Mlp* gene expression on the transcriptional regulator dMEF2 and places *Mlp* function late in the terminal stages of muscle differentiation. We show that myoblast fusion per se is not necessary for expression of the *Mlp* proteins and, furthermore, that neither α -actinin nor PS2 integrin is required to direct *Mlp84B* to its proper position in muscle cells. Future work will continue to address the mode of *Mlp* gene regulation and their functional significance in the process of myogenesis. Genetic analysis of *Mlp* function is likely to provide important insights into their physiological roles in muscle. Thus far, no mutations in the *Mlp60A* gene have been identified, and deficiency analysis has been hampered by an apparent haploinsufficient locus in the region. However, we have begun to characterize phenotypes associated with loss of *Mlp84B* function. For this analysis, we have used a set of overlapping deficiencies that remove a small region of genomic DNA including the coding region of the *Mlp84B* gene. The *Mlp84B*-deficient animals die as larvae and early pupae, exhibiting locomotor and morphological defects consistent with abnormal muscle function (Clark and Beckerle, unpublished observations). Although additional work needs to be done to characterize fully the *Mlp84B* null phenotype, preliminary results suggest that gene function is essential for viability and proper muscle function.

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