Control of *Drosophila* Paramyosin/Miniparamyosin Gene Expression

DIFERENTIAL REGULATORY MECHANISMS FOR MUSCLE-SPECIFIC TRANSCRIPTION*

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To define the transcriptional mechanisms contributing to stage- and tissue-specific expression of muscle genes, we performed transgenic analysis of *Drosophila* paramyosin gene regulation. This gene has two promoters, one for paramyosin and one for miniparamyosin, which are active in partially overlapping domains. Regions between −0.9 and −1.7 kilobases upstream of each initiation site contribute to the temporal and spatial expression patterns. By comparing the *Drosophila* melanogaster and *Drosophila virilis* viralis promoters, conserved binding sites were found for known myogenic factors, including one MEF2 site and three E boxes. In contrast with previous data, our experiments with the paramyosin promoter indicate that the MEF2 site is essential but not sufficient for proper paramyosin gene transcription. Mutations in the three E boxes, on the other hand, do not produce any effect in embryonic/larval muscles. Thus MEF2 site- and E box-binding proteins can play different roles in the regulation of different muscle-specific genes. For the miniparamyosin promoters, several conserved sequences were shown to correspond to functionally important regions. Our data further show that the two promoters work independently. Even when both promoters are active in the same muscle fiber, the transcription driven by one of the promoters is not affected by transcription driven by the other.

The correct patterning and differentiation of muscles require the coordinate execution of regulatory programs. These include the differential expression of muscle genes and the production of specific protein isoforms (1). Muscle genes are activated coordinately. Their transcription is regulated by DNA sequences, promoters, and enhancers, which permit the interaction with unique combinations of transcription factors in each cell type. Myogenesis has a determinative stage in which mesodermal precursors become myoblasts and a differentiation stage involving the fusion of single myoblasts to form multinucleated myotubes that express the contractile protein genes. In vertebrates this occurs during embryogenesis; two families of transcriptional factors, MyoD and MEF2, are essential to the transcription of muscle structural genes and are critical for the stable determination of myoblast lineages (2, 3). In skeletal muscles, MyoD and MEF2 work cooperatively in muscle gene activation (2, 4). The MyoD family is exclusively expressed in somatic muscles, in contrast to mef2 genes that are expressed in skeletal, cardiac, and smooth muscles. This suggests that MyoD-type proteins play important roles in activating transcription within each myogenic lineage (5). The analysis of MEF2 functions has been facilitated by the isolation of the *Drosophila* mef2 gene (6, 7). This single gene is required for differentiation of skeletal, cardiac, and visceral muscles (8, 9).

The *Drosophila* paramyosin/miniparamyosin gene (PM/mPM) represents a good model system to elucidate muscle gene regulatory mechanisms. Previous studies have suggested that the molecular pathways controlling muscle formation are ancient and evolutionarily conserved in flies and vertebrates (10). Interest in studying expression of PM/mPM is enhanced by the fact that the two mRNAs arise from overlapping transcriptional units. The mPM promoter is located inside a PM intron that is 8 kb downstream of the PM promoter (11, 12). Whereas PM is expressed at the two distinct stages in all muscles, as are most other *Drosophila* muscle proteins, mPM is present only in the adult musculature. The two proteins are expressed at the same stage of adult development, suggesting that regulation of the two promoters has to be coordinated (13).

*Drosophila* develops distinct sets of muscles during its life cycle, with separate muscles at the embryonic/larval stages and in the adult (14). Myoblast determination and differentiation occur independently at each phase (15). During embryogenesis, mesodermal precursors appear at gastrulation during ventral furrow formation. Body wall muscles and some visceral muscles are derived from precursor myoblasts expressing the twist gene (16–18). The second phase of myogenesis occurs several days after hatching, when the larval muscles are replaced by embryonic muscles (16). Embryonic muscles are derived from muscle progenitors that are migratory, and are responsible for dorsalization of the embryo (19). The embryonic muscles do not express *Drosophila* myogenesis and embryonic muscles (16, 18).
days later during metamorphosis. The specialized adult muscles, including the indirect flight muscles (IFM) and the tergal depressor of the trochanter (TDT), form during pupation when most larval muscles are histolyzed (19–22).

Identification of *Drosophila* muscle promoter/enhancer sequences and their associated binding factors has not been nearly as extensive as in vertebrates. The majority of identified transcription factors are required for mesoderm formation. *twist* and *snail* are involved in establishing the mesoderm germ layer; *tinman* is exclusively expressed in the dorsal vessel muscle primordia; and *bagpipe* is involved in the development of visceral muscles (23–25). *Nautilus*, the MyoD homologue, is expressed in some so-called founder cells, a subset of myoblast cells of the somatic mesoderm probably involved in formation and/or patterning of embryonic body wall muscles (26–28). However, no targets of NAU are known. DMEF2 is expressed in nearly as extensive as in vertebrates. The majority of identified transcription factors are required for mesoderm formation.

### EXPERIMENTAL PROCEDURES

**Isolation of Genomic Clones, Construction of P-transformation Plasmids, and Generation of Transformed Drosophila Lines—** *Drosophila melanogaster* and *Drosophila virilis* genomic clones (12) containing the 5′ upstream regions from the transcriptional initiation sites of the PM and mPM were subcloned and sequenced as described (32). Selected fragments from these regions were cloned into P-transformation vectors with native orientations relative to the basal promoters. +1 bp on our map refers to the mRNA initiation start of the PM and mPM (12). The P element plasmid vectors (33) were pCaspeR 4. The artificial intron was made joining the *PM* and mPM (12). The putative promoter regions (Fig. 1), three conserved regulatory features. Previous studies have been submitted to GenBank™/EBI Data Bank under accession numbers AJ243067, AJ243068, AJ243069, and AJ243070.

**RESULTS**

**Distinct Conserved Elements Are Present in the Distant 5′ Regions of the PM/mPM Genes of *D. melanogaster* and *D. virilis—** As an initial step in the identification of transcriptional enhancer sequences of the PM/mPM gene in *D. melanogaster*, we isolated the PM/mPM homologue from a distantly related species, *D. virilis*. These two Drosophilidae species diverged more than 50 million years ago, and sequence comparison is useful for detecting conserved regulatory features. Previous studies (12) revealed that regions extending 90–100 nucleotides upstream of the PM and mPM transcriptional initiation sites are over 90% conserved, indicating that they correspond to RNA polymerase complex binding domains.

The alignment of the more distant sequences allowed identification of cis elements important for muscle expression (Fig. 1). For the sequences 5′ to the PM start sites, the only homologies are the proximal region, one binding site for MEF2 at −1488, and three E boxes at −1587, −1461, and −1436 in the *D. melanogaster* PM promoter. The most common binding site for MEF2 and MyoD regulates muscle gene expression in vertebrates (2, 4). Thus, having a MEF2 site and several E boxes within 150 bp makes this region worth studying in more detail. Near this region two MEF2 sites and two PDP1 sites are present in *D. melanogaster* but not in *D. virilis*. In the upstream sequences of the mPM transcription unit, three regions are conserved in sequence and position. These are located at −477 to −740 (X element), −1173 to −1207 (BP2 element), and −1432 to −1469 (AB element).
mPM Expression—To determine the regions regulating the expression of PM and mPM, based on the comparative analysis described above, constructs were made and analyzed by P element-mediated transformation. The transgenic lines transformed with constructs containing 4-kb (PM4) or 2.7-kb (mP2.7) fragments upstream from the transcription start sites of the PM or mPM, respectively, express \( \text{LacZ} \) at high levels with similar patterns as the endogenous proteins, except for the TDT and IFM (Figs. 2 and 3). The PM4 lines do not express the transgene in IFM muscles (Fig. 2 and Table I). In contrast, the mP 2.7 lines showed \( \beta \)-galactosidase staining in IFM but not in TDT muscles (Fig. 3 and Table II). In fact, the \( \text{LacZ} \) expression patterns in the thoracic muscles of these lines reflect an inverse situation to the levels of endogenous protein accumulation. These results indicate that all the regulatory elements are located in the regions cloned in these constructs, except for those controlling the expression of PM in IFM.

To more precisely define elements necessary for PM expression, constructs containing fragments of 1.7 (PM 1.7), 1.39 (PM 1.4), 0.92 (PM 0.9), 0.55 (PM 0.5), 0.34 (PM 0.3), and 0.15 (PM 0.15) kb from the PM initiation site were generated (Fig. 2).

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**Fig. 2. Comparison of \( \beta \)-galactosidase gene expression driven by selected sequences upstream from the PM transcription start site.**

A, constructs inserted in the pCasper \( \beta \)-gal plasmid, identified by name and size. B, X-gal staining of third instar larvae, dissected abdomens, and thin sections of thoraces transformed with distinct constructs containing 5' sequences of 4 kb (PM 4), 1.69 kb (PM 1.7), 1.38 kb (PM 1.4), and 0.87 kb (PM 0.9). White asterisk, hypodermic ventral muscles and white arrowhead, hypodermic dorsal muscles; black asterisk, IFM; black arrow, TDT.

**Fig. 3. Comparison of \( \beta \)-galactosidase gene expression driven by selected sequences upstream from the mPM transcription start site.**

A, constructs inserted in the pCasper \( \beta \)-gal plasmid, identified by name and size. B, X-gal staining of third instar larvae, dissected abdomens, and thin sections of thoraces transformed with constructs containing 5' sequences of 2.7 kb (mP 2.7), 1.68 kb (mP 1.7), 1.2 kb (mP 1.2), 0.89 kb (mP 0.9), and 0.57 kb (mP 0.5). asterisk, IFM; arrow, TDT. The strong staining in dorsal abdomen of mP 1.7 line is nonspecific. The specific staining is indicated with the arrowhead.
Analysis of the transgenic lines revealed that 5′ upstream sequences of less than 0.9 kb (PM 0.9, PM 0.5, PM 0.3, and PM 0.15) do not express significant levels of β-galactosidase, as measured by enzyme staining in embryos, larvae, or adults (Fig. 2; data not shown). Reverse transcriptase-PCR assays on the PM 0.3 and PM 0.15 lines revealed very low levels of LacZ transcription (data not shown). The region implicated (from 20.9 to 21.7 kb) contains the conserved MEF2 site and E boxes and also the PDP1 sites and one of the CF2 sites described above (Figs. 1 and 2). 

In vitro transcribed-translated DMEF2, NAU, PDP1, and CF2 products bind specifically to these sequences (data not shown). No binding was detected with TWIST.

Detailed analysis of transgenic lines with constructs containing intermediate length fragments (PM 1.7 and PM 1.4) established the importance of the putative regulatory sites in these constructs. In the PM 1.7 lines, containing the MEF-E region, flies express significant levels of LacZ in all muscles including leg and visceral muscles, but not IFMs. Except for a slightly lower level of expression, the pattern is the same as the one obtained with the PM4 construct. Surprisingly, the absence of the MEF-E region in the PM 1.4 lines yields LacZ expression in all muscles. Although the absence of this region markedly diminished the LacZ expression (Fig. 2 and Table I), it does not abolish it completely. In fact, β-galactosidase staining appears in all muscles including IFM.

### Table I

**Comparison of LacZ gene expression driven by selected sequences upstream from the start site of the PM transcription unit**

The highest expressing lines, PM4, achieved the maximal blue intensity for most muscles and are referred to as "+++." The level of intensity of the other lines was determined by comparison to these lines. nd, none detected.

| Analyzed Lines | TDT | IFM | Dorsal | Ventral | Visceral Muscles | Larvae | Embryo |
|----------------|-----|-----|--------|---------|-----------------|--------|--------|
| PM 4 LacZ      | +++ | --- | +++    | +++     | +++             | +++    | +++    |
| PM 1.7 LacZ    | ++  | +   | ++     | ++      | ++              | ++     | ++     |
| PM 1.4 LacZ    | +   | +/− | +/−    | +/−     | +/−             | +/−    | +/−    |
| PM 0.9 LacZ    | +   | −   | −      | −       | −               | −      | −      |
| PM 0.5 LacZ    |       |       |       |         |                 |        |        |
| PM 0.3 LacZ    | +   | −   | −      | −       | −               | −      | −      |
| PM 0.15 LacZ   | +   | −   | −      | −       | −               | −      | −      |

*1/8 lines do not give any detectable LacZ expression.

### Table II

**Comparison of β-galactosidase gene expression driven by selected sequences upstream from the start site of the mPM transcription unit**

The highest expressing construct, mP 2.7, achieved the maximal blue intensity for most muscles and is referred to as "+++." The level of intensity of the other lines was determined by comparison to these lines. nd, none detected.

| Analyzed lines | TDT | IFM | Dorsal | Ventral | Visceral Muscles | Larvae | Embryo |
|----------------|-----|-----|--------|---------|-----------------|--------|--------|
| mP 2.7 LacZ    | ++  | ++  | ++     | ++      | ++              | ++     | ++     |
| mP 1.7 LacZ    | ++  | +   | ++     | ++      | ++              | ++     | ++     |
| mP 1.2 LacZ    | +   | −   | −      | −       | −               | −      | −      |
| mP 0.9 LacZ    | +   | −   | −      | −       | −               | −      | −      |
| mP 0.5 LacZ    | +   | −   | −      | −       | −               | −      | −      |
| mP 0.3 LacZ    | +   | −   | −      | −       | −               | −      | −      |
| mP 0.15 LacZ   | +   | −   | −      | −       | −               | −      | −      |

Drosophila Paramyosin/Miniparamyosin Gene Expression
A similar study was done with the sequences 5’ to the mPM start site (Fig. 3 and Table II). Transgenic lines transformed with constructs containing fragments of 1.68 (mP 1.7), 1.2 (mP 1.2), 0.89 (mP 0.9), 0.57 (mP 0.5), 0.27 (mP 0.3), and 0.143 (mP 0.15) kb were generated. As in the case of PM, no LacZ expression was detected in the lines containing 5’ upstream fragments of less than 0.89 kb. Reverse transcriptase-PCRs of the mP 0.3 and mP 0.15 lines were performed (data not shown). The results suggest that these regions, as in the PM promoter, contain elements involved in basal transcription. The two intermediate lines (mP 1.7 and mP 1.2) showed muscle β-galactosidase staining with a more complex pattern than that for the PM constructs. The mP 1.7 lines present a pattern of staining similar to that of the endogenous gene, except that expression is not detected in larvae. Expression in dorsal hypodermal adult muscles was consistently very low and heterogeneous among the three lines when compared with the levels observed in the mP 2.7 line (Fig. 3). Curiously, mP 1.7 lines show staining in TDT muscles, in contrast to the mP 2.7 lines. Sequences upstream of −1.6 kb, which are part of other exons and introns (Fig. 1), increase expression and seem to deregulate it. Thus, in mP 2.7 lines we did not detect staining in TDT muscles, whereas the IFM staining was very strong (Fig. 3). The mP 1.2 lines gave no activity at all in the hypodermal abdominal muscles. The region involved in the control of mPM expression appears to be located between −0.9 and −1.6 kb upstream of the start site and is part of intron 7. Interestingly, the region from −0.89 to −1.6 contains the AB and BF2 conserved elements (Fig. 3). Our results suggest that the AB element may be implicated in the expression in abdominal adult muscles and that the BF2 element may be implicated in the other muscle types including TDT and IFMs. Band shift analysis with overlapping oligonucleotides corresponding to these regions and adult nuclear extracts revealed several specific binding sites (data not shown).

The Role of MEF2 and the E Boxes in Regulating the Expression of Drosophila PM—Drosophila MEF2 is expressed in the precursors of all muscle lineages early in development, and expression persists as the descendants of these cells differentiate (5–7). During the larval stages the mef2 gene is expressed in cells that give rise to the adult somatic muscles (20, 42). Moreover, ectopic expression of MEF2 in the epidermis induces epidermal expression of muscle genes and abnormal muscle development (43). Our in vivo analysis revealed that the region carrying the E boxes and the MEF2 site of the PM promoter plays an important role in regulation of PM expression.

To assess the functional role of the MEF2 site and the three E boxes, we generated transgenic lines in which the MEF2 site or the E boxes were altered (Fig. 4 and Table III). EMSA had previously revealed that oligonucleotides containing the mutated E boxes were unable to compete the protein binding. We made a 4-bp mutation in the MEF2 binding site or alternatively a 2-bp mutation in one, two, or three of the E boxes in the context of the entire distal promoter. Band shift assays confirmed that the Drosophila MEF2 protein is unable to bind the mutated MEF2 binding site (data not shown). Lines carrying the 1.7-kb fragment with the mutated MEF2 binding site (MM) were checked for β-galactosidase activity. Embryos and larvae show either low level expression or no expression. In the adults, muscle staining showed a decrease of the LacZ expression compared with lines that do not carry the mutation (Fig. 4B). These lines, in adults, gave a similar pattern of expression as PM 1.7 lines, except that in this case, IFM were stained. However, TDT, visceral, and abdominal muscles were stained at slightly lower levels than in PM 1.7 lines. Curiously, the distinct lines that selectively carried one, two, or three mutated E boxes (E2M, 1/3EM, and 3EM) have similar transgene activity. There is no effect in larval muscles, whereas adult muscles show a slight decrease compared with lines that do not contain the mutation. These lines show IFM staining. The reduction is present in six of the seven generated lines, whereas IFM are stained in all the altered lines (Fig. 4 and Table III). In summary, the MEF2 site seems to be essential for expression of PM in embryonic muscles but not in adult muscles. Mutations in
the E boxes do not produce any effect in embryonic/larval muscles. In adult muscles, although none of these sites seem to be essential for expression, they appear to be required for the proper regulation of PM expression, because mutations in these sites produce IFM staining.

**Paramyosin and Miniparamyosin Expression in Adult Muscles Is Not Coordinated**—Although the endogenous PM is expressed in IFM, our data show that the element controlling expression of PM in IFMs is not present in regions analyzed at the 5' end of PM. Because the PM/mPM gene contains two overlapping transcriptional units, it is possible that the IFM-controlling element(s) of mPM also drives PM expression in IFMs in a coordinated fashion.

To investigate whether PM and mPM transcription is coordinated, we studied the coexpression of the LacZ and GFP genes under the control of the PM and mPM promoters, respectively. The main limitation of the previous approach is the possible influence of enhancers located close to the insertion region in the chromosome. If the two transcriptional units are situated in the same construct, then positional effects could be ruled out because they would basically influence the two transcriptional units at the same time. Three constructs were made and analyzed by P element-mediated transformation (Fig. 5). The first construct, LG, was designed to reproduce the situation of the endogenous PM/mPM gene. The 5' upstream region controlling PM (4 kb) drives the expression of LacZ, and the 5' upstream region controlling mPM (2.7 kb) drives the GFP gene. The distance between LacZ and GFP initiation sites is similar to the PM and mPM initiation sites in the endogenous gene. Moreover, to allow correct splicing of the two possible transcripts, an artificial intron (basically intron 8 without the middle region; see “Experimental Procedures”), followed by the SV40 polyadenylation signal, was placed downstream of the LacZ expression. Two more constructs were made as a control, PM4i (PM) and mPGFP (mPM) in which PM and mPM promoters were independently fused upstream of the respective reporter genes. They also contained the fragment with the artificial intron and the polyadenylation signal. Several transgenic lines were obtained (Fig. 5 and Table IV).

Analysis of LacZ and GFP expression showed that both temporal and spatial expression patterns were similar to those obtained previously in the lines transformed with constructs containing 4-kb (PM4 and PM4i) or 2.7-kb (mP 2.7 and mPGFP) fragments. Thus, LG, LGD, and LGI lines express β-galactosidase at high levels with the same pattern as the PM endogenous protein, except in the IFM. On the other hand, they express GFP at high levels in all muscles as the mPM endogenous protein, except for the TDT.

To establish whether transcription was correctly carried out, Northern blot analyses (Fig. 6) were performed with total RNAs from late pupae of the LG, LGD, and LGI lines. LG lines basically present two bands, corresponding to the LacZ and GFP transcripts with the expected size. Thus, the LacZ transcript appears as a band of higher size, LacZ plus exons 5, 6, and 7. In the LGD lines, besides the expected LacZ and GFP transcripts, we detect an additional higher band, a consequence of incorrect functioning of the polyadenylation signal as a terminator. In LGI lines, besides the expected LacZ and GFP transcripts, an additional band of unknown composition appears. In any case, we have never detected cross-hybridization between the different bands, demonstrating that transcription and intron processing were carried out correctly.

Transcript accumulation in these lines was carried out comparing the relative expression of LacZ and GFP in each one of the LG, LGD, and LGI lines via densitometric analysis of Northern blots (Fig. 6). The introduction of a polyadenylation signal to make both units independent in the LGD lines signif-

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**TABLE III**

| Analyzed Lines | PM 1.7 | MM | E2M | 3EM | 1/3EM |
|---------------|--------|----|-----|-----|------|
| PM 1.7        | ++     | -- | ++  | +   | ++   |
| MM            | ++/+   | ++ | ++/+| ++/+| ++/+ |
| E2M           | ++/+   | ++/+| ++/+| ++/+| ++/+ |
| 3EM           | ++/+   | ++/+| ++/+| ++/+| ++/+ |
| 1/3EM         | ++/+   | ++/+| ++/+| ++/+| ++/+ |

# 1/5 lines do not give any detectable LacZ expression.
* 1/3 lines give very high LacZ expression.

**Abdomen**

| TDT | IFM | Ventral | Muscles | Larvae | Embryo |
|-----|-----|---------|---------|--------|--------|
|     | ++  | ++/+    | ++/+    | ++/+   | ++/+   |
|     | ++  | ++/+    | ++/+    | ++/+   | ++/+   |
|     | ++  | ++/+    | ++/+    | ++/+   | ++/+   |
|     | ++  | ++/+    | ++/+    | ++/+   | ++/+   |
|     | ++  | ++/+    | ++/+    | ++/+   | ++/+   |

**Notes**

- PM 1.7: LacZ expression driven by sequences upstream from the start site of the PM transcription unit with and without mutations in the MEF-2 site or the E boxes.
- PM 4 and mP 2.7 lines: Fragments with the artificial intron and the polyadenylation signal, was placed downstream of the LacZ expression.
- Two more constructs were made as a control, PM4i (PM) and mPGFP (mPM) in which PM and mPM promoters were independently fused upstream of the respective reporter genes.
- LacZ and GFP expression showed that both temporal and spatial expression patterns were similar to those obtained previously in the lines transformed with constructs containing 4-kb (PM4 and PM4i) or 2.7-kb (mP 2.7 and mPGFP) fragments.
- Northern blot analyses (Fig. 6) were performed with total RNAs from late pupae of the LG, LGD, and LGI lines.
analyses, have determined the location of elements that regulate PM and mPM gene expression. The 150-bp sequences proximal to the PM and mPM start sites drive very low levels of transgene expression. Because no β-galactosidase staining was detected, it was not possible to verify that the transcription is muscle-specific. However, the expression detected by reverse transcriptase-PCR, along with the evolutionarily conserved sequences present in these regions (12), suggests that they contain binding sites for the basal transcriptional machinery. Temporal and spatial transgene expression patterns in both promoters depend on regions located between ~0.9 and ~1.7 kb of the PM and mPM initiation sites, except for IFMs in the case of PM and larval muscles in the case of mPM.

For PM, a group of E boxes and MEF2, PDP1, and CF2 sites are present in a region important for muscle-specific expression. The E boxes and the MEF2 site are conserved between the two Drosophilidae species. Transgenic flies containing this region express β-galactosidase with patterns similar to endogenous PM, indicating that these sites are important for muscle transgene activity. Moreover, transgene expression is not completely abolished until the region containing the PDP1 sites is eliminated (compare PM 1.4 and PM 0.9 lines). Our results indicate that these and/or other sites present in this region may play a role in muscle transgene activity.

For the mPM promoter region, we found no conserved binding sites for known muscle transcriptional factors. Instead, three conserved regions were detected. Based on our in vivo analysis, the AB element (126 nucleotides) may be involved in the regulation of mPM expression in abdominal hypodermal muscles. The BF2 (34 nucleotides) element contributes to the regulation of mPM expression in other adult muscles. Its deletion abolishes mPM expression in the thorax. The absence of staining in the mP 0.9 lines carrying only the X conserved element was surprising. This element may need flanking regions that are absent in this construct to activate the transgene, such as the conserved BF2 region.

The inclusion of sequences upstream of ~1.6 kb deregulates mPM expression in the thorax. In mP 2.7 lines we did not detect staining in TDT muscles, but IFM staining was very strong (Fig. 3). The fact that these sequences contain other exons and introns of the gene may yield an artificial effect in the transgene.

**The Role of MEF2 and the E Boxes in the PM Promoter—Cooperative activation of muscle gene expression by MEF2 and myogenic bHLH factors occurs in vertebrates. This requires direct interactions between the DNA binding domains of MEF2 and the bHLH factors, but only one of the factors needs to be bound to DNA. These interactions allow either factor to activate transcription through the binding site of the other factor (2).**

Our findings with the paramyosin promoter define an important role for the whole MEF2-E region, located ~1400 bp upstream of the start site, for proper paramyosin expression. The MEF2-E region seems to act as a distal muscle activator enhancer that differentially regulates PM expression in embryonic/larval and adult muscles. The region is essential for the high expression in larval muscles. In adults, however, deletion results in misexpression in the IFM and a decrease of PM expression in all muscles.

Substitution mutations within the MEF2-E region of the conserved elements, the MEF2 site and the three E boxes, revealed a different role for these sites at distinct muscle stages. This indicates that MEF2 may carry out tissue-specific roles in myogenesis. The MEF2 site is the main requirement for maintaining the high PM levels of transcription in larval muscles, but it is not really important for high expression in adult muscles (Fig. 4). When this site is mutated, the decrease
in expression is minor. No effect in the larval musculature was observed when all E boxes were mutated (Fig. 4C). It is also clear that no synergism involving MEF2 working through the E boxes is seen in larval muscles (see larvae in Fig. 4, B and C).

In adults, IFM misexpression (regarding PM 4 and PM 1.7 lines) appears in the lines that have either the MEF2 site or the distinct E boxes mutated, indicating that the distinct sites are important for a proper PM expression in the adult muscles. The direct interaction of an MEF regulatory complex with these sites may be needed to give specificity to PM expression in individual muscle types. Moreover, in adults, the reduction of transgene activity is minor in the lines carrying mutations either in the MEF2 site or in the E boxes (MM, E2M, 1/3M, and 3EM) when compared with the lines carrying the MEF2-E region deleted (PM 1.4). These results suggest that other cis elements are located in the distal muscle activator enhancer and are important to maintaining the levels of transgene expression. It is possible that cooperative activation involving the MEF2 site and the E boxes present in the region is important for PM gene activation in adult musculature. If the E boxes located in this region exert a role in regulation of PM expression in adult muscles, NAU or another bHLH factor could be involved. In vitro transcribed-translated NAU, the homologue of MyoD in Drosophila (26), binds to these E boxes. Another explanation could be that these E boxes do not play the same role as in vertebrate muscle genes.

With respect to the exact role of the MEF2 site and the E boxes in the regulation of the PM expression, our results lead us to hypothesize that the absence of the MEF2 site in larvae transforms the promoter into a weak tissue-specific promoter. Instead, in adults, the direct interaction of an MEF2 regulatory complex with this region may be needed not only to reach high levels of expression but also to give specificity to the PM expression in individual muscle types. Furthermore, the PM misexpression in IFM may be due to an incorrect binding of the whole MEF2 regulatory complex when the MEF2 site or the E boxes are altered. On the other hand, these findings may reveal the presence of other proteins different from MEF2 and bHLH factors participating in the MEF2 regulatory complex (Fig. 7B), mediating a repressive effect in some muscles, as may happen with IFM. Supporting this idea, an MEF2 binding repressor in Xenopus has been identified recently (44).

The absence of an element that enhances PM expression in

| Analyzed lines | TDT | IFM | Abdomen | Larvae | Embryo |
|---------------|-----|-----|---------|--------|--------|
| 6             | +   |     |         |        |        |
| 1             |     |     |         |        |        |
| 3             | +   |     |         |        |        |
| 2             |     |     |         |        |        |
| 2             | +   |     |         |        |        |

**Table IV**

Comparison of \(\beta\)-galactosidase and GFP expression patterns under the control of the PM (4 kb) and mPM (2.7 kb) promoters

+ and − indicate the presence or absence of staining.
IFM and the lack of effect of the IFM element in the mPM promoter upon PM expression leave the issue of how PM is expressed in IFM unresolved. In the tropomyosin (37), myosin heavy chain (47), and troponin T2 genes in Drosophila, the IFM-controlling elements are localized in intron 1. The element responsible for IFM expression of PM is not located in intron 1 of the PM/mPM gene (data not shown).

In Fig. 7A, we present two models of how the MEF2-E region may participate in the regulation of PM expression. Both models call for the presence elsewhere of an enhancer element that mediates increased levels of PM expression in IFM. The element controlling PM expression in IFM may act either through its interaction with the MEF-E regulatory complex or in an independent manner. Our results do not distinguish between the two possibilities. In the latter case, as suggested above, interaction of a regulatory protein complex with the whole MEF2-E region would be required for proper expression in the other specialized muscles (Fig. 7A). In larvae, only the requirement of the MEF2 site seems to be essential for proper expression.

Interestingly, the intron 7 region that controls mPM expression does not contain MEF2 sites or E boxes. Our in vivo studies show that the MEF2 site in the PM promoter is not involved in spatial and temporal control of mPM expression. Our studies clearly reveal that expression of the adult-specific mPM protein is regulated differently from most of the Drosophila muscle proteins that are expressed in both embryonic and adult muscles. The absence of an MEF2 site has also been seen in the Act 88F promoter region, which drives protein expression exclusively in IFMs (49). If the MEF2 factor is needed to control the expression of mPM, regulation has to occur indirectly through another transcriptional complex. An important overall conclusion of our work is that the regulation of some Drosophila muscle genes may not follow the same rules as in vertebrates.

**The Two Transcriptional Units of the PM/mPM Gene Act Independently**—The promoters of the gene separately regulate the expression of two transcripts. These transcripts share two exons and are expressed in the same fibers during pupal myogenesis. This type of genomic organization is also present in the Drosophila tropomyosin and myosin heavy chain genes (48, 50–52). Internal promoters in these genes produce transcripts encoding cytoplasmic tropomyosin and the myosin rod protein, respectively (48).

The PM/mPM gene is a good model for studying the interaction, if any, of dual promoters. Steric impediments may exist if RNA polymerases transcribe PM and mPM RNAs at the same time during pupal myogenesis. It is unclear how RNA polymerase solves the problem of read-through and whether this solution provides a mechanism for regulating the level of expression of both proteins in adults. We did investigate whether enhancer/s required for PM expression might be located in the mPM regulatory region or vice versa. Because the element controlling the expression of PM in IFM is not present in its upstream regulatory region, we investigated whether the IFM-controlling element of mPM also drives PM expression in IFM. Our results showed that each promoter regulates the expression of each transcript independently and that the element controlling the expression of mPM in the IFM is not able to drive the expression of PM in these muscles. Likewise, the PM elements did not enhance mPM expression in the TDT muscles that lacked this transcript in the mP 2.7 lines. Thus, although they are encoded by overlapping units and share two exons, PM and mPM are transcriptionally regulated as if they were in different loci.

A possible explanation of how the two promoters of the gene can function with no influence of one on the other may be that both promoters, in fact, do not function in the same nucleus. Muscle fibers are a syncytium. Recently Newlands et al. (38) demonstrated in mice that individual nuclei present in the

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2 J. A. Mas, P. Benoist, and M. Cervera, unpublished data.
same muscle cell do not transcribe the same genes at the same time. Genes can be transcribed or not in a particular nucleus, but the number of nuclei that transcribe a specific gene is constant. This may occur for the PM/mPM gene. If so, both PM and mPM transcripts would never be transcribed in the same nucleus, and the two transcriptional machineries would work independently. Another possible explanation may be the presence of an insulator separating the two promoters.

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