Transcription of the Myogenic Regulatory Gene Mef2 in Cardiac, Somatic, and Visceral Muscle Cell Lineages Is Regulated by a Tinman-Dependent Core Enhancer

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The MADS-box transcription factor MEF2 is expressed specifically in developing cardiac, somatic, and visceral muscle cell lineages during Drosophila embryogenesis and is required for myoblast differentiation and muscle morphogenesis. To define the mechanisms that regulate Mef2 transcription, we have analyzed the Mef2 upstream region for sequences sufficient to recapitulate the expression pattern of the gene in Drosophila embryos. Here we describe a complex enhancer located 5.8 kb upstream of the Drosophila Mef2 gene that controls transcription in cardiac cells of the dorsal vessel, a subset of somatic muscle founder cells, and the visceral muscle cells. The core of this enhancer contains two evolutionarily conserved binding sites for the homeodomain protein Tinman (Tin), expressed in developing cardiac, somatic, and visceral muscle lineages. Both Tin binding sites are required for enhancer activity in all three muscle cell lineages. Whereas the 285-bp enhancer core alone is sufficient for expression in cardiac cells, expression in somatic founder cells and visceral muscle is dependent on the core enhancer plus unique flanking sequences that include an evolutionarily conserved E box. These results reveal an essential role for Tin in activation of Mef2 transcription in multiple myogenic lineages and demonstrate that transcriptional activity of Tin is dependent on combinatorial interactions with other factors unique to different muscle cell types. © 1999 Academic Press

INTRODUCTION

Genetic studies in mice and Drosophila have revealed important components of the regulatory circuitry involved in specification and differentiation of skeletal, cardiac, and visceral muscle cells and indicate that the signaling systems and transcription factors that control development of these different muscle cell types are evolutionarily conserved (Manak and Scott, 1994; Baylies et al., 1998). Dissection of the roles of individual myogenic regulatory factors is complicated in vertebrates by functional redundancy among related factors, whereas in Drosophila, regulatory circuitry is simpler, with single genes controlling key steps in myogenic pathways.

Specification of muscle cell lineages during Drosophila embryogenesis occurs soon after gastrulation as the nascent mesoderm along the ventral midline of the embryo invaginates and becomes patterned in response to both intrinsic and extrinsic factors (Bate, 1993). The dorsalmost mesodermal precursors give rise to the cardiac cell lineage, which forms a heart-like organ, known as the dorsal vessel, along the dorsal midline. Visceral mesoderm, which ultimately gives rise to the visceral musculature of the gut, is also derived from a subpopulation of dorsal mesodermal cells, while somatic muscle originates from both dorsal and ventral mesoderm (Azpiazu et al., 1996; Riechmann et al., 1997). Somatic muscle formation occurs as somatic founder cells recruit adjacent mesodermal cells to the somatic lineage through formation of syncytia that go on to form a stereotypical pattern of 30 skeletal muscle fibers reiterated in each hemisegment along the anterior–posterior axis of the embryo (Bate, 1990; Rushton et al., 1995).

Mesoderm formation in Drosophila is controlled by the basic helix-loop-helix (bHLH) factor Twist (Leptin, 1991). Thereafter, subdivision of the mesoderm into different

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Muscle cell lineages require combinatorial interactions between several mesoderm-restricted transcription factors and growth factor-mediated signaling pathways. The homeobox gene tinman (tin) is first expressed in all mesodermal precursor cells at the blastoderm stage. As mesodermal cells invaginate and spread laterally during gastrulation (stage 7), tin expression becomes restricted to the dorsal mesoderm in response to signaling by the transforming growth factor-β-like peptide Decapentaplegic (Frasch, 1995). At this stage, tin expression marks cardiac and visceral muscle precursors, as well as a subset of dorsal somatic muscle precursors. Later, by stage 12, tin expression is restricted to cardiac cells of the dorsal vessel (Azpiazu and Frasch, 1993; Bodmer, 1993). In tin mutant embryos, the cardiac, visceral, and dorsal somatic muscle lineages do not form, indicating that one of the functions of tin is to specify these lineages (Azpiazu and Frasch, 1993; Bodmer, 1993). Within the visceral muscle lineage, tin is essential for activation of the related homeobox gene bagpipe (bap), required for specification of visceral muscle cells (Azpiazu and Frasch, 1993). Given the persistence of tin expression in the dorsal vessel, it is likely that tin plays roles in activation of cardiac muscle structural genes, in addition to its role in cardiac specification. Very few target genes for Tin have been identified and it is unknown whether Tin activates the same or entirely different genes in each myogenic developmental pathway.

Members of the MEF2 family of MADS-box transcription factors bind the control regions of the majority of muscle-specific genes and play central roles in myogenesis and morphogenesis of all muscle cell types (reviewed in Black and Olson, 1998). In vertebrates, there are four mef2 genes, mef2A, -B, -C, and -D, expressed throughout development of each myogenic lineage. Similarly, in Drosophila, the single Mef2 gene, D-mef2, is also expressed in all developing muscle cell lineages and their differentiated descendants (Lilly et al., 1994; Nguyen et al., 1994; Taylor et al., 1995). Loss-of-function mutations in D-Mef2 prevent differentiation of cardiac, skeletal, and visceral muscle cells, but do not affect myoblast specification or positioning in the embryo (Lilly et al., 1995; Bour et al., 1995; Ranganayakulu et al., 1995), revealing a shared dependence of all muscle cell types on Mef2 for late steps in development. The highly conserved expression patterns, structure, and transcriptional activity of MEF2 factors from vertebrates and fruit flies imply a conserved role in muscle development. Indeed, mice lacking MEF2C show severe abnormalities in cardiac and smooth muscle development (Lin et al., 1997, 1998; Bi et al., 1999). However, because of functional redundancy among the different mef2 genes, it has not yet been possible to generate mice completely lacking MEF2 activity.

While numerous transcriptional targets of MEF2 factors have been identified (Black and Olson, 1998), little is known of the regulatory factors that act upstream of Mef2 genes in developing muscle cell lineages. This problem has been difficult to address in vertebrates, in which the 5′ untranslated regions of the four Mef2 genes contain multiple alternatively spliced exons separated by large introns and the precise transcriptional initiation sites have not been determined (B. Black and E. Olson, unpublished). In contrast, the complete intron/exon organization of the Drosophila Mef2 gene has been defined and the 13 kb of DNA immediately upstream of the gene has been shown to be sufficient to direct the entire muscle-specific expression pattern during embryogenesis. Within this upstream region, at least a dozen independent transcriptional enhancers have been identified, each of which regulates transcription of the gene in a subset of developing muscle cells in the embryo (Lilly et al., 1995; Schulz et al., 1996; Gajewski et al., 1997, 1998; Cripps et al., 1998; Nguyen and Xu, 1998).

The early expression of D-Mef2 in the mesoderm is similar to that of tin. Both genes are coexpressed in the nascent ventral mesoderm and are activated by Twist (Taylor et al., 1995; Yin et al., 1997; Lee et al., 1997; Cripps et al., 1998). Subsequently, as the mesoderm becomes subdivided, ultimately giving rise to cardiac, visceral, and somatic muscle lineages, Mef2 expression is maintained in all myogenic cells, whereas tinman becomes restricted to dorsal mesodermal cells before becoming completely restricted to the dorsal vessel.

Previously, we and others have identified an enhancer, located about 5.8 kb upstream of D-Mef2, that controls transcription in cardiac cells of the dorsal vessel (Schulz et al., 1996; Gajewski et al., 1997, 1998; Nguyen and Xu, 1998). This enhancer contains two binding sites for Tin, both of which are required for transcriptional activity in the cardiac lineage. Since Tin also is required for differentiation of visceral and somatic muscle lineages, we sought to determine whether the Tin binding sites in the cardiac cell enhancer might also regulate Mef2 expression in these lineages. Here we show that the Tin-dependent cardiac cell enhancer is the central module of a more complex regulatory region that utilizes combinations of adjacent sequences to activate Mef2 transcription in visceral muscle and somatic founder cells. Our results reveal a combinatorial mechanism for control of Mef2 transcription and demonstrate a shared dependence of cardiac, visceral, and somatic muscle cell lineages on Tin for activation of Mef2 transcription.

**MATERIALS AND METHODS**

**Generation and analysis of enhancer constructs.** Mef2 genomic fragments for enhancer analysis were generated either by restriction digestion (constructs 1–3, Fig. 1) or by polymerase chain reaction (PCR) using specific oligonucleotide primers, as follows: construct 4, bin 98 (5′-AAACATGTGTTCTTATCAAAAT-3′) and bin 79 (5′-CCACGTTGCTAACTGACGCACTG-3′); construct 5, bin 105 (5′-CGCTTCCGTTTGGCTTTAGGTA-3′) and bin 107 (5′-GAAGCCGAACAGACGCACTGAT-3′); construct 6, bin 105 and bin 86 (5′-CTACGCCCGTCCATTAAGAATATG-3′); construct 7, bin 105 and bin 104 (5′-ATCTTTACACCCCTTACCAAT-3′); construct 8, bin 105 and bin 103 (5′-AGATACCTCA-
GAATCCCAAGCC-3'); construct 9, bin 115 (5' - CTCTTAAACAAGATAAGATCTACACAGAAAAGACAGAC-3') and bin 107; construct 10, bin 115 and bin 112 (5' -GGCATTTCCGGTCTGGATGAG-3') and bin 107; construct 12, bin 162 (5' -TAGATATGCTATTGGATGGGAG-3') and bin 107; construct 13, bin 170 (5' -ATGTGGCTATTGGATGGGAG-3') and bin 107.

Products were cloned into the CaSpeR-hsp43-AUG-gal (CHAB) P-element vector, which contains a lacZ reporter gene downstream of a minimal hs43 heat-shock promoter (Thummel and Pirrotta, 1992). P-element-mediated germ-line transformation was performed essentially as described by Rubin and Spradling (1982). Embryos of the genotype y w were cojected with the CHAB constructs and a D2-3 helper plasmid (Robertson et al., 1988), and transgenic adults were identified in the G1. Independent lines were maintained by backcrossing to y w and selecting for the darker eye color of homozygotes in subsequent generations. For each enhancer sequence tested, a minimum of three independent lines were analyzed. Flies were raised at 25°C on standard cornmeal–glucose–yeast agar medium. Enhancer–reporter gene downstream of a minimal hs43 heat-shock promoter was harbored using a 5'–3' grape juice. Immunohistochemistry of whole-mount embryos was carried out as described (Patel et al., 1987). The primary antibody was an anti-β-galactosidase monoclonal antibody from Promega (1:400 dilution); localized primary antibody was detected using the Vectastain Elite Staining Kit (Vector Laboratories, CA). Stained embryos were cleared and mounted in 80% (v/v) glycerol for imaging.

Cloning of the Drosophila virilis Mef2 gene and its flanking sequences. Initial cloning of the D. virilis Mef2 gene was accomplished in Cripps et al. (1998). Bacteriophage clones containing additional upstream sequences were isolated from a D. virilis genomic library made by J. Tamkun and provided by S. Wasserman, using a 5' probe made from existing D. virilis genomic clones (Cripps et al., 1998). Sequencing of the 5' flanking region of the D. virilis Mef2 gene and of PCR-generated mutant constructs was carried out with an ABI 373 automated sequencer.

Immunohistochemistry of Drosophila embryos. Embryos at specific time points were collected from agar plates containing grape juice. Immunohistochemistry of whole-mount embryos was carried out as described (Patel et al., 1987). The primary antibody was an anti-β-galactosidase monoclonal antibody from Promega (1:400 dilution); localized primary antibody was detected using the Vectastain Elite Staining Kit (Vector Laboratories, CA). Stained embryos were cleared and mounted in 80% (v/v) glycerol for imaging.

PCR-based site-directed mutagenesis. Mutagenesis of the Mef2 enhancer was carried out by PCR-based site-directed mutagenesis (Horton, 1993). Oligonucleotides with mutated Tin binding sites were used in a 30-cycle standard PCR to generate primary products. Purified primary products were then annealed and used as templates for secondary PCR using either TaKaRa LA Taq from Boehringer Mannheim. The distal, Tin1, sequence was mutated using the oligonucleotides Bin-tin-1-5-3 (5'-TAAGGGGCGCCCCGCTTGGGTGGTCCCTTCCGCG-3') and Bin-tin-1-3-5 (5'-CGGAAAGGGCGCACCCCAAGCGCCCGGCGCCCTTA-3') (underlined sequences indicate changes from the wild-type sequence). The proximal, Tin2, site was mutated using the oligonucleotides Bin-tin-2-5-3 (5'-GACGCTAACAGGATCGCCGGCCGGAGCCTGCTCTG-3') and Bin-tin-2-3-5 (5'-AGCAGGGCCGCTCCGGCGGCGCATCCTTATAGCTG-3') (underlined sequences indicate changes from the wild-type sequence). Oligonucleotides containing these sequences were incapable of competing with the wild-type sequence for Tin binding in an electrophoretic gel mobility shift assay, indicating that they were incapable of binding the Tin protein (data not shown). The E box was mutated using the oligonucleotides Bin 159 (5'-GGGAAATGTGCCCTCCGAGATTGGGAG-3') and Bin 160 (5'-GGGAATCCCAAGGCTCAGGACATT-3') (underlined sequences indicate changes from the wild-type sequence).

RESULTS

Localization of a cardiac, somatic, and visceral muscle regulatory region upstream of Mef2. Previous studies showed that a region from −9120 to −3560 bp upstream of Mef2 contains elements sufficient to direct transcription in cardiac and visceral muscle lineages, as well as in a subset of somatic founder cells, during embryogenesis (Schulz et al., 1996) and that a 237 bp region from −5907 to −5670 is active only in cardiac cells of the dorsal vessel (Gajewski et al., 1997). This cardiac cell enhancer contains two Tin binding sites essential for transcriptional activity.

In an effort to segregate the regulatory regions required for cardiac, somatic, and visceral muscle expression, we subdivided the −9120/-3560 region (construct 1, Fig. 1) in half and tested for enhancer activity in vivo in transgenic flies, using a lacZ reporter construct. Although both halves of construct 1 were able to direct expression of the lacZ transgene in the dorsal vessel, only the downstream region (−5942/-3560), containing the Tin-dependent enhancer, showed activity in cardiac precursor cells (constructs 2 and 3, Fig. 1). Furthermore, the downstream half also was capable of driving lacZ expression in somatic founder and visceral muscle precursor cells.

Further deletions showed that cardiac, as well as visceral and somatic, muscle expression were controlled by nucleotides −5990 to −5180 (construct 5, Fig. 1), containing the previously characterized cardiac cell enhancer (Gajewski et al., 1997, 1998). Transcriptional activity of this region was first detected at stage 10 in dorsal and ventral clusters of cells coincident with the positions of precursors of the dorsal vessel and a subset of two to three somatic muscle founder cells, respectively (Fig. 2A). Shortly thereafter, reporter gene expression was also detected in precursor cells of the visceral musculature (Fig. 2B). Expression in somatic and visceral muscle cells was maintained through stage 12 (Fig. 2C). Subsequently, as differentiation proceeded, expression was lost from somatic and visceral muscle cells, but high levels of enhancer activity persisted in a segmentally repeating pattern within cardiac cells of dorsal vessel (Fig. 2D).

The dorsal vessel contains six pairs of cardiac cells within each hemisegment. The 810 bp cardiac enhancer (construct 5) was active in four of these six pairs of cells, corresponding to the expression pattern of Tin (Jagla et al., 1997). A larger enhancer region encompassing construct 5 was also recently shown to direct expression in precisely the same cells that express Tin (Nguyen and Xu, 1998). In contrast, the more distal upstream region (construct 2) directed lacZ expression in the other two cardiac cell pairs within each hemisegment.
hemisegment, but not in the four Tin-expressing cells (not shown).

Although enhancer activity in the somatic and visceral muscle lineages was transient, mirroring the expression pattern of Tin in these cells, we were able to use the perdurance of β-galactosidase to identify the muscles that formed from cells in which the enhancer was active. In the somatic lineage, these corresponded to muscles V04, V05, and V06 (Bate, 1993).

**FIG. 1.** Deletion mutations define a region sufficient for cardiac, visceral, and somatic muscle expression of Mef2. The Drosophila Mef2 gene and upstream region are schematized and regions of 5' DNA tested for enhancer activity are indicated. The location of the minimal cardial cell enhancer (~5907–5670) is indicated in red. Cell types in which lacZ transgene expression was observed are shown. P, PstI site; R, EcoRI site.

**TABLE 1.**

<table>
<thead>
<tr>
<th>Construct</th>
<th>1 -9120-3560</th>
<th>2 -8765-5942</th>
<th>3 -5942-3560</th>
<th>4 -6389-4640</th>
<th>5 -5990-5180</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardiac precursor</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Visceral muscle</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Somatic founder cells</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Somatic muscle</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

**FIG. 2.** Expression pattern directed by construct 5. Embryos bearing construct 5 (see Fig. 1) were stained for lacZ expression at stages 10 (A), 11 (B), 12 (C), and 14 (D). Anterior is to the left in all cases. Each panel shows a lateral view, except D, which is a dorsal view. LacZ expression is observed in somatic founder cells (sf), cardial precursor cells (cp), visceral precursor cells (vp), ventral somatic and visceral muscle (vsm and vm, respectively), and dorsal vessel (dv).
Identification of cardiac, visceral, and somatic regulatory elements. The consensus DNA sequence for Tin binding is T(C/T)AAGTG (Chen and Schwartz, 1995). The 237-bp cardiac cell enhancer (5907/-5670), encompassed by construct 5, contains two Tin binding sites, required for enhancer activity in cardiac cells (Gajewski et al., 1997). This minimal enhancer shows the same temporospatial expression pattern in the cardiac lineage as construct 5, but it is inactive in visceral and somatic muscle cell lineages. This indicates that sequences in addition to the core cardiac enhancer are required for expression in the somatic and visceral musculature.

To further define the boundaries of the cardiac, visceral, and somatic muscle regulatory elements within the -5990/-5180 region (construct 5), we created a further series of deletion mutations. Deletions from the 3' side to -5322, -5517, or -5627 (constructs 6, 7, and 8, Fig. 3) had no effect on enhancer activity in any myogenic lineage. Deletion from the 5' end to -5940 (construct 9) also did not affect cardiac, visceral, or somatic expression. However, combined 5' and 3' deletions to create a 285-bp enhancer (-5940/-5656; construct 10) eliminated activity in visceral and somatic founder cells, without affecting expression in the cardiac lineage (Fig. 3). Since 5' deletion to -5940 within the context of the larger region had no effect on enhancer activity (construct 9, -5940/-5180), we conclude that the region between -5656 and -5627 is essential for somatic and visceral muscle expression and not for expression in cardiac muscles. Interestingly, this region contains an E box, conserved in D. melanogaster and D. virilis (see below).

Deletion from the 5' end to -5898 eliminated expression in visceral muscle without affecting expression in cardiac or somatic founder cells (Fig. 3, construct 11). These results indicated that sequences between -5940 and -5898 were essential for enhancer activity in visceral, but not cardiac or somatic, muscle lineages. Further 5' deletion to -5808 (construct 12), which eliminated one Tin binding site, abolished expression in all muscle cell types. An additional construct deleted from the 5' end to -5662 (construct 13) was also inactive.

These deletion mutations indicated that sequences within and surrounding the minimal Tin-dependent enhancer region (-5907/-5670) were required for expression in cardiac, somatic, and visceral muscle lineages. While cardiac expression was achieved with the enhancer core alone, somatic expression was dependent on sequences downstream of the core enhancer and visceral expression on sequences surrounding the core enhancer. Thus, the region between -5940 and -5627 functioned as a composite enhancer utilizing overlapping, but distinct, sequences for expression in different muscle cell types.

Homology between D. melanogaster and D. virilis enhancers. To guide us in identifying regulatory elements within the Mef2 upstream region that might be essential for enhancer activity, we sequenced the 13-kb region immedi-
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ately preceding the Mef2 gene from D. virilis and searched for cis-acting sequences sufficient to direct expression in cardiac, visceral, and somatic muscle cell lineages. Using an overlapping series of DNA fragments spanning the upstream region, we identified a D. virilis genomic region from −9894 to −9094 that directed muscle-specific expression of lacZ in a pattern similar to that of the minimal 810-bp composite enhancer from D. melanogaster (Fig. 4A). The D. virilis fragment also controlled expression in additional regions of the mesoderm (Fig. 4A and data not shown), and therefore likely contains additional enhancers.

By comparing the sequence of the minimal D. melanogaster composite enhancer (construct 8, Fig. 3) with that of D. virilis, we identified five blocks of conserved sequence (termed A–E, Fig. 4B). Of note, both Tin binding sites (regions B and D) were conserved in the two species. The region immediately 5′ of the Tin1 site (region A), corresponding to the region shown to be essential for enhancer activity in visceral muscle, was also conserved. There was only a short region of homology between the Tin binding sites (region C). Sequence similarity between D. melanogaster and D. virilis was also striking in a region 38 nucleotides downstream of the Tin2 site (region E). Although this region is not required for cardiac enhancer activity, this segment of homology corresponds with the region we found to be required for expression in visceral muscle and somatic founder cells. This conserved region contained an E box (CANNTG), the consensus binding site for BHLH proteins.

Recently, it was reported that mutation of a GATA site in the D. melanogaster Mef2 enhancer located immediately 3′ of the Tin2 site changed specificity of the enhancer from cardinal cells of the dorsal vessel to pericardial cells (Gajewski et al., 1998). This GATA site is not conserved in D. virilis, nor are there other GATA sites nearby, making its functional significance unclear.

Requirement of the Tin sites for cardiac muscle, visceral muscle, and somatic founder cell enhancer activity. To investigate the potential roles of conserved sequences in the composite cardiac, visceral, and somatic regulatory region, we mutated each Tin site singly and in combination within the context of the 810-bp enhancer (construct 5). Mutation of the Tin1 site resulted in dramatic reduction in lacZ expression in all muscle lineages (Fig. 5). LacZ expression was undetectable in visceral muscle precursors and only weakly detectable in a few cardiac and somatic muscle cells with this mutant enhancer. Mutation of the Tin2 site also severely reduced enhancer activity, with only very low levels of β-galactosidase detectable in some regions of the midgut (Fig. 5). The enhancer with both Tin sites mutated showed the same expression pattern as the Tin2 mutant (Fig. 5). These results demonstrate a similar dependence of all three myogenic lineages on occupancy of both Tin sites in the enhancer core for transcriptional activity.

Requirement of the E box for visceral muscle and somatic founder cell enhancer activity. The deletion analyses indicated that the evolutionarily conserved region immediately 3′ of the Tin-dependent enhancer core, containing the E box, was required for enhancer activity in visceral muscle and somatic founder cells. To address the functional significance of this E box, we mutated it within construct 5 and tested enhancer activity in vivo (Fig. 5). Strikingly, this mutant enhancer showed no activity in visceral or somatic muscles at any stage of embryogenesis, revealing an essential role for the conserved E box in Mef2 expression in those muscle lineages. However, this mutant enhancer was fully active in the cardiac muscle lineage, indicating a specific requirement for the E box in visceral and somatic muscle transcription of Mef2. These findings are consistent with our deletion analysis in which we found that 3′ deletion to −5656 (Fig. 3, construct 10), which removes this conserved region, resulted in a loss of enhancer activity in the somatic and visceral lineages, but not in the cardiac lineage.

The composite enhancer requires Tin and is independent of Bap. The mutational analyses demonstrated that enhancer activity in all three muscle cell lineages was dependent on the two Tin sites, making it likely that Tin or a related homeodomain protein was the activator of Mef2 transcription in these lineages. To further address this, we examined expression of the enhancer (−5990/−5627, construct 8) in tin mutant embryos (Fig. 6). Absence of tin function resulted in a loss of enhancer activity in all cells throughout embryogenesis, reflecting the essential role of Tin in enhancer activation (Fig. 6).

In the visceral muscle lineage, Tin is required for expression of the related homeodomain protein, Bap (Azipiazu and Frasch, 1993). Therefore, we also tested enhancer activity in bap mutant embryos to determine if the effect of loss of Tin function reflected a direct role of Tin as an activator of the enhancer or an indirect role mediated by Bap. Although the visceral mesoderm barely forms in Bap mutants, lacZ expression was clearly detected at high levels in all three muscle lineages in bap mutant embryos (Fig. 6). We conclude that the composite enhancer is specifically activated by Tin in cardiac, visceral, and somatic muscle cell lineages.

DISCUSSION

Through analysis of the Mef2 upstream region, we have defined a Tin-dependent enhancer responsible for Mef2 transcription in cardiac, somatic, and visceral muscle cell lineages during Drosophila embryogenesis. This is, to our knowledge, the only example of a specific target enhancer for Tin involved in gene activation within all three muscle cell lineages. The requirement of this enhancer for surrounding sequences that differ for each muscle cell type suggests that Tin acts combinatorially with other regulatory factors to control muscle-lineage-restricted patterns of transcription. Consistent with this conclusion, previous studies have suggested that the N-terminus of Tin interacts
FIG. 4. Enhancer activity of D. virilis upstream region and sequence homology with D. melanogaster. (A) The expression pattern of lacZ in D. melanogaster embryos directed by a region upstream of the Mef2 gene from D. virilis. Left, stage 11 embryo, lateral view. Right, stage 15 embryo, dorsal view. Expression is observed in cardial precursors (cp), visceral muscle (vm), somatic founder cells (sf), and dorsal vessel (dv), as well as in somatic muscle cells (marked in right). (B) Nucleotide sequence homology between the enhancer region for cardiac, somatic, and visceral expression from D. melanogaster and D. virilis. A schematic of the enhancer is shown at the top and regions of homology are shown below. Region A is essential for visceral expression and region E for visceral and somatic.
with a cofactor involved in cardiac gene activation (Rangana-
yakulu et al., 1998).

**Combinatorial control of Mef2 transcription by Tin.** The Tin-dependent Mef2 enhancer is a composite of overlapping cis-regulatory elements, which rely on the centrally located Tin binding sites for activation in different muscle cell lineages (Fig. 7). Enhancer activity in all muscle cell types requires both Tin sites in the enhancer core. However, each muscle cell type differs with respect to other enhancer sequences required for transcriptional activity. In

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**FIG. 5.** Mutational analysis of Tin sites and an E box in the composite enhancer. (A) The Tin sites and E box in the composite enhancer within construct 5 were mutated and tested for activity in vivo. Cell types in which enhancer activity was detected are indicated. (B) Lateral views of stage 12 embryos bearing the transgene constructs depicted in A and stained for lacZ are shown. cp, cardiac precursors; dv, dorsal vessel; sf, somatic founders; vm, visceral musculature.

**FIG. 6.** Expression of the composite enhancer in tin and bap mutant embryos. Expression of construct 5 in wild-type (A, B), tin mutant (C, D), and bap mutant (E, F) embryos is shown at stages 12 (A, C, E) and 13 (B, D, F). The enhancer is completely inactive in tin mutants but retains activity in bap mutants. cp, cardiac precursors; sf, somatic founders; vm, visceral musculature, dv, dorsal vessel.

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somatic founder cells, sequences 3' of the enhancer core that include an evolutionarily conserved E box are required for activity. In contrast, visceral expression requires sequences immediately 5' of the enhancer core, in addition to the 3' E box necessary for somatic muscle expression. Cardiac expression requires only the enhancer core, with no apparent role of surrounding sequences.

Although Bap is expressed in visceral mesoderm and is structurally related to Tin, it is not required for activation of the Tin-dependent enhancer in the visceral mesoderm, as demonstrated by the normal level of enhancer activity in bap mutant embryos. While we favor the conclusion that Tin is the true activator of the Mef2 enhancer in cardiac, somatic, and visceral muscle cells, it is formally possible that another factor that binds the Tin sites in the enhancer core is the actual activator of the enhancer. However, if this was the case, such a factor would have to act downstream of Tin in each myogenic lineage since enhancer activity in all muscle cell types was abolished in tin mutant embryos. It is also interesting to note that while Tin is required for activation of the enhancer described here in cardiac, somatic, and visceral muscle cell lineages, there are also multiple Mef2 enhancers active in each of these lineages that do not contain Tin binding sites.

During this work, another study showed that mutations of both Tin sites together in the Mef2 enhancer abolished expression in cardiac and somatic muscle and that the E box was also required for somatic expression (Gajewski et al., 1998). However, the potential roles of individual Tin sites were not investigated and the ability of the enhancer to direct expression in visceral muscle was not demonstrated, nor was the homology to D. virilis identified. Taken together, both our studies and those of Gajewski et al. define a novel, evolutionarily conserved enhancer required for Tin-dependent transcriptional activation in multiple muscle cell lineages.

**E-box-dependent regulation of somatic and visceral muscle transcription.** We do not currently know the identity of the factor that binds the evolutionarily conserved E box required for somatic and visceral expression, but a bHLH protein would be a likely candidate. The MyoD-like factor nautilus is expressed in a subset of somatic founder cells (Michelson et al., 1990; Paterson et al., 1991), but it is not expressed in visceral muscle. The bHLH factor lethal-of-scute (L'sc) is also expressed in somatic founder cells (Carmena et al., 1995), but the Tin-dependent enhancer is active in L'sc mutant embryos (unpublished results), ruling out a role for this bHLH factor in enhancer activation. The factor bHLH54 is also expressed in a subset of somatic and visceral muscle precursors (Georgias et al., 1998). However, neither bHLH54 nor any of the other bHLH factors mentioned above bind the E box from the Mef2 composite enhancer (J. Lu and E. Olson, unpublished results). Twist is also expressed in early mesodermal precursors of visceral and somatic muscle, but recent studies showed that it represses activity of this Mef2 upstream region (Gajewski et al., 1998). Thus, no known bHLH factor appears likely to act through the essential E box in the enhancer.

**Modular regulation of Mef2 transcription.** The Drosophila Mef2 gene is regulated by an extraordinary number of independent transcriptional enhancers that demarcate specific myogenic lineages during embryogenesis. To date, at least 12 independent Mef2 enhancers have been identified, but only a few have been characterized with respect to their upstream activators. We showed that transcriptional activation of Mef2 in the embryonic mesoderm and in adult somatic muscle precursors requires binding of Twist to an evolutionarily conserved E box in a 175-bp enhancer located

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**FIG. 7.** Schematic diagram of regions of the composite enhancer required for expression in different myogenic cell types. Regions required for transcription in each muscle cell lineage are shown beneath the schematic of the overall genomic region.
2.2 kb upstream of the gene (Cripps et al., 1998). An additional Mef2 enhancer has also been shown to be a target for activation by Dpp signaling (Nguyen and Xu, 1999). This enhancer is activated by Medea, a Smad protein that acts downstream of the Dpp receptor.

Modular regulation of muscle genes, as exemplified by the complex arrangement of independent cis-regulatory modules upstream of Mef2, is emerging as a common theme in muscle gene regulation (Firulli and Olson, 1997). This type of regulation enables a regulatory gene, such as Mef2, to integrate a diverse array of inputs throughout development and may also provide a foundation for evolution of specialized programs of gene expression through addition of independent transcriptional regulatory elements to a more primitive control region.

In this regard, while it has been well documented that the hearts of vertebrates are patterned along the anteroposterior axis into populations of precursor cells that may use different transcriptional regulatory programs (reviewed in Olson and Srivastava, 1996), it is not generally appreciated that the dorsal vessel in Drosophila may also be segmentally patterned. Such patterning is revealed by the expression of Tin and the core cardiac cell enhancer in six of eight pairs of cardiac cells in each hemisegment along the anteroposterior axis. Activation of Mef2 transcription in the remaining two cardiac cell pairs within each hemisegment is dependent on more distal 5' flanking sequences (contained in construct 2, Fig. 1). It will be of particular interest to identify the factors that bind this Tin-independent regulatory region to confer cardiac cell expression.

Evolutionary conservation of myogenic regulatory pathways. Our results raise interesting questions about evolutionary conservation of myogenic regulatory mechanisms. In Drosophila, tin is the only NK-type homeobox gene known to be required for cardiac differentiation. Like the closely related gene, ceh-22, from Caenorhabditis elegans, is required for development of pharyngeal muscle, which is functionally related to cardiac (O’kema and Fire, 1994). Moreover, tin can substitute for ceh-22 to support pharyngeal muscle development in C. elegans, consistent with an evolutionarily conserved role of these homeobox genes in muscle formation (Huan et al., 1998). Mouse Nkx2-5 can also substitute for tin to support visceral but not cardiac muscle development in Drosophila (Ranganayakulu et al., 1998). In vertebrates, there are multiple NK-2-class homeobox genes (reviewed in Harvey, 1996). Nkx2-5, the most extensively studied (Komuro and Izumo, 1993; Lints et al., 1993), is required for morphogenesis of the heart tube and activation of a subset of cardiac contractile protein genes (Lyons et al., 1995). However, it is not required for specification of cardiac identity, suggesting that other related genes expressed in the heart can also perform this function.

Considering the similarity in expression patterns of Mef2 genes in fruit flies and vertebrates, it is tempting to speculate that the regulatory factors responsible for Mef2 expression will also be evolutionarily conserved and that NK-2-class homeodomain proteins may also activate Mef2 gene expression in multiple muscle cell lineages in vertebrates. By taking a cross-species approach to define the functions and regulation of Mef2 genes, we hope to uncover fundamental regulatory circuitry responsible for development of different muscle cell types during embryogenesis.

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REFERENCES


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