Drosophila MEF2, a transcription factor that is essential for myogenesis

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mef2 encodes the only apparent *Drosophila* homolog of the vertebrate myocyte-specific enhancer factor 2 (MEF2). We show herein that the *Drosophila* MEF2 protein is expressed throughout the mesoderm following gastrulation. Later in embryogenesis, its expression is maintained in precursors and differentiated cells of the somatic and visceral musculature, as well as the heart. We have characterized genetic deficiencies and EMS-induced point mutations that result in complete loss of MEF2 protein in homozygous mutant embryos. These embryos exhibit a dramatic absence of myosin heavy chain (MHC)-expressing myoblasts and lack differentiated muscle fibers. Examination of earlier events of muscle development indicates that the specification and early differentiation of somatic muscle precursors are not affected because *even-skipped*-, *nautilus*-, and β 3-tubulin-expressing myoblasts are present. However, these partially differentiated cells are unable to undergo further differentiation to form muscle fibers in the absence of *mef2*. The later aspects of differentiation of the visceral mesoderm and the heart are also disrupted in *mef2* mutant embryos, although the specification and early development of these tissues appear unaffected. Midgut morphogenesis is disrupted in the mutant embryos, presumably as a consequence of abnormal development of the visceral mesoderm. In the heart, the cardiac cells do not express MHC. These results indicate that MEF2 is required for later aspects of differentiation of the three major types of musculature, which include body wall muscles, gut musculature, and the heart, in the *Drosophila* embryo.

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Genes regulating the processes of determination and differentiation control cell-fate decisions that culminate in the progression of multipotential cells to distinct cell types. During *Drosophila* embryogenesis, the larval body wall musculature arises from morphologically indistinct mesodermal cells to an elaborate pattern of segmentally repeated muscle fibers over a period of ~10 hr. Genetic and molecular studies have identified components of the myogenic pathway, which begins with specification of the mesoderm. The mesoderm is established by a morphogenetic gradient, in which the maternal Dorsal protein is selectively localized to nuclei on the ventral side of the embryo and activates subsequently the zygotic genes *twist* and *snail* (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). These two genes are required for specification of the mesoderm and appear to regulate, either directly or indirectly, genes that are expressed ubiquitously in this germ layer (Kosman et al. 1991; Lep- tin 1991; see also references therein). The *Drosophila*

mef2 gene is one of the first genes to be activated shortly after *twist* expression in the presumptive mesoderm (Lilly et al. 1994; Nguyen et al. 1994). Other genes include the homeo box-containing genes *tinman* and *zfh-1*, as well as *DFR1*, the *Drosophila* homolog of fibroblast growth factor, and *Drac1*, a GTPase (Lai et al., 1991; Azpiazu and Frasch 1993; Bodmer 1993; Shishido et al. 1993; Luo et al. 1994).

The mesoderm epithelium separates into two layers ~6 hr after fertilization. The inner layer, in the dorso-lateral position, contains the progenitors of the visceral musculature. *tinman*, which is initially expressed throughout the mesoderm and becomes restricted to the dorsal cells of the mesoderm at later stages, is essential for proper development of the visceral musculature and the heart (Azpiazu and Frasch 1993; Bodmer 1993). *bag-pipe*, a homeo box-containing protein that is restricted to the primordia of the visceral mesoderm, is necessary for its formation and differentiation (Azpiazu and Frasch 1993). Additional genes, including *zfh-1* and the homeo box-containing gene *H2.0*, are also expressed in the inner

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mesodermal layer but their precise role remains unclear (Barad et al. 1991; Lai et al. 1993). The somatic musculature is specified by the cells in the outer mesodermal layer. Several genes have been identified that are expressed in cells of this layer, including *apterous*, *S59*, *nautilus (nau)/Dmyd*, *even-skipped (eve)*, *DFR1*, and *vestigial* (Frasch et al. 1987; Dohrmann et al. 1990; Michelson et al. 1990; Paterson et al. 1991; Bourgouin et al. 1992; Bate et al. 1993; Shishido et al. 1993). These genes have expression profiles and, in some cases, mutant phenotypes that include distinct subsets of muscle precursors and differentiated muscle fibers.

D-mef2, hereafter designated as *mef2*, is expressed throughout the mesoderm following gastrulation (Lilly et al. 1994; Nguyen et al. 1994). The ubiquitous *mef2* expression decreases during germ-band extension and later increases in somatic muscle precursors, visceral mesoderm, and heart precursors. By the completion of germ-band retraction, *mef2* transcripts are abundantly expressed throughout the somatic musculature. The *mef2* pattern of expression is in marked contrast to those of *eve* and *nau*, which appear to mark subsets of muscle precursors and differentiated muscle fibers.

mef2 encodes the only apparent *Drosophila* homolog of a family of vertebrate myogenic factors, termed MEF2 (myocyte-specific enhancer factor 2). The encoded proteins all contain the evolutionarily conserved MADS box-containing DNA-binding domain, as well as the MEF2-specific domain. MEF2-binding sites, conforming to the consensus sequence PyT(A/T)(A/T)AAATAPu, have been found in the upstream region of a number of muscle-specific genes, including *MyoD* and *myogenin* (Cossett et al. 1989; Wentworth et al. 1991; Edmonson et al. 1992; Nakatsuji et al. 1992; Navankasattusas et al. 1992; Tapscott et al. 1992). In some cases, these sites have been shown to be functionally important (Edmonson et al. 1992; Cheng et al. 1993; Yee and Rigby 1993).

To address the role of *mef2* in vivo, we have characterized genetic mutations that result in the loss of *mef2*-encoded protein. We show that these *mef2* mutant embryos exhibit a dramatic absence of myosin heavy chain (MHC) expression in cells in positions of the somatic musculature and lack differentiated muscle fibers. Somatic muscle precursors that are present in the *mef2* mutant embryos are specified but do not undergo full differentiation to multinucleate muscle fibers in the absence of *mef2*. We observe further that the early development of the visceral mesoderm and heart appears to be unaffected. However, midgut morphogenesis is disrupted and MHC expression is not detected in the heart at later stages of development.

Results

MEF2 protein is nuclear and present in the progenitors of all three muscle types

Previous studies have established that *mef2* RNA transcripts are detectable throughout the mesoderm during germ-band extension and later become restricted to the

somatic and visceral muscles, as well as heart precursor cells in germ band-retracted embryos (Lilly et al. 1994; Nguyen et al. 1994). Here, we present immunocytochemical studies showing that MEF2 protein expression is also detected throughout the mesoderm during germ-band extension (Fig. 1A). After subdivision of the mesoderm, MEF2 expression is observed in the cephalic mesoderm and the primordia for visceral and somatic musculature (Fig. 1B). Throughout germ-band retraction and in older embryos, MEF2 expression persists in the visceral and pharyngeal musculature, as well as in cardiac cells, the heart precursor cells that will form the heart muscle (Fig. 1C–E). In addition, its somatic expression is detected in segmentally repeating clusters of mesodermal cells that are in positions corresponding to groups of forming somatic muscles (Fig. 1E,F). Notably, MEF2 expression is restricted to muscle progenitors and it is not detected in fat body and pericardial cells. Moreover, MEF2 protein expression at all stages is localized to the nucleus, which is consistent with the fact that it is a transcription factor.

Molecular characterization of mef2 null mutations

To determine the function of *mef2*, we have focused on identifying and analyzing *mef2* mutants. The cytological region 46C–D, the location of both *FMRFamide* and *mef2* genes, was mutagenized previously (O'Brien et al. 1994). Two X-ray induced deletions, *Df(2R)X1* and *Df(2R)X3*, have been mapped to this region. As shown in Figure 2, the proximal breakpoints of *Df(2R)X1* and *Df(2R)X3* are located distal and proximal to the *FMRFamide* gene, respectively. In addition, *Df(2R)P520*, which was generated by mobilization of a P-element in this region, deletes ~70 kb of the region surrounding the *FMRFamide* gene.

Southern blot analysis with DNA that was enriched for the mutant chromosomes (Bodmer 1993), indicated that *Df(2R)X3* and *Df(2R)P520* lack the *mef2* gene and that *Df(2R)X1* breaks within the *mef2* gene itself (data not shown). To map the breakpoints of these deficiencies, we determined the structural organization of the *mef2* gene. Detailed Southern blot analyses were performed with restriction fragments derived from cosmid and λ genomic clones spanning this region (see Fig. 2). The intron–exon boundaries were then identified by targeted sequencing. As depicted in Figure 2, the *mef2* gene spans >14 kb and contains eight introns, the largest of which is ~6 kb in length. Taken together, the information indicates that the proximal breakpoint of *Df(2R)X3* is downstream of the *mef2* gene, whereas that of *Df(2R)X1* is within the large intron that interrupts its 5'-untranslated region. On the basis of the location of the breakpoints of *Df(2R)P520*, we conclude that *trans*-heterozygotes of *Df(2R)X1* and *Df(2R)P520* would carry a deletion of ~12 kb that includes the *mef2* gene locus.

Because the long stretch of DNA deleted in these *trans*-heterozygotes could include another gene, it was important to obtain point mutants that map to this region. A previous screen had identified several lethal

Figure 1. MEF2 protein is nuclear and present in the precursors of all three muscle types. Wild-type embryos were stained with a rabbit polyclonal antibody against *Drosophila* MEF2 protein. The embryos are oriented with the anterior to the left [stages are according to Campos-Ortega and Hartenstein 1985]. (A) Lateral view, stage 11 germ-band-extended embryo with MEF2 protein expression throughout the mesoderm (m). (B) Lateral view, late stage 12 germ-band-retracting embryo. MEF2 expression is restricted to the visceral (vm) and somatic (sm) mesodermal layers, and the cephalic mesoderm (cm). (C) Dorsal view, late stage 13 germ-band-retracted embryo showing MEF2 expression in the somatic (sm) and visceral (vm) mesoderm, and the pharyngeal musculature (phm). (D) High magnification of an embryo at a slightly later stage than in C. Focus is on MEF2 expression in the somatic (sm) and visceral (vm) musculatures. (E) Dorsal view, stage 15 embryo. MEF2 expression is observed in repeating nuclear clusters of fused somatic mesodermal (sm) cells and two rows of heart precursor (hp) cells. (F) Lateral view, stage 16 embryo. MEF2-positive clusters of nuclei in locations corresponding to syncytial fibers of differentiating somatic muscles.

complementation groups in this region (see Materials and methods). On the basis of the molecular characterization of *mef2* sequences in the deficiencies described above, we anticipated that the desired mutations would be identified by lack of complementation to *Df(2R)X1*, *Df(2R)X3*, and *Df(2R)P520*. Ten lethal complementation groups were uncovered by *Df(2R)X1* and *Df(2R)X3* (see Table 1). Of these, only one, designated as group C, was unable to complement *Df(2R)P520*. A representative ethyl methane sulfonate (EMS)-induced allele from this group, designated as 22-21, was analyzed further for alterations in the *mef2* gene sequence.

The presence of *mef2*-encoded protein was analyzed in EMS allele 22-21 by use of the antibody directed against MEF2 (see Materials and methods). As shown in Figure 3, no MEF2 is observed in embryos bearing this mutation. To characterize the lesion molecularly, genomic DNA from homozygous mutant embryos was amplified by the polymerase chain reaction (PCR). The second and third exons, which include the MADS and MEF2-specific regions, were amplified from mutant genomic DNA, subcloned, and sequenced. The results from this analysis identified a base change that converts the sixth codon into a nonsense mutation (Fig. 3C). Thus, we designate this allele as *mef2*²²⁻²¹ and conclude that it is a protein null lesion in *mef2*.

Embryos lacking mef2 exhibit severe defects in myogenesis

To determine the role of *mef2* in *Drosophila* myogenesis, we examined the mutant phenotype of embryos that

are lacking detectable MEF2 protein. Included in this study were embryos *trans*-heterozygous for the deficiencies *Df(2R)X1/Df(2R)P520*, which is a MEF2 protein null combination (data not shown), and embryos *trans*-heterozygous for *mef2*²²⁻²¹/*Df(2R)P520*, the EMS-induced protein null *mef2* allele and a deficiency. Both genetic combinations produce embryonic lethality. Somatic muscle development was initially visualized with a monoclonal antibody to *Drosophila* muscle-specific MHC. A comparison of wild-type and mutant embryos is shown in Figure 4. Whereas the wild-type embryo (Fig. 4A) exhibits an ordered array of muscle fibers, both *trans*-heterozygous combinations of *mef2* mutant embryos (Fig. 4B–D) show an almost complete absence of MHC-expressing cells and lack muscle fibers. In addition, MHC expression is not observed in the pharyngeal musculature. Of note, a very limited number of elongated MHC-expressing cells are observed (see arrows in Fig. 4B,C). Although the exact nature of these cells is not clear, their presence suggests that a very small number of myoblasts are able to differentiate to some extent in the absence of MEF2.

mef2 sequences rescue aspects of the mutant phenotype

To demonstrate that the loss of the *mef2* gene is responsible for the mutant phenotype described above, we utilized the GAL4 system of Brand and Perrimon (1993) to provide restricted *mef2* function in the mutant embryos. The *mef2* cDNA was placed under the control of UAS GAL4-binding sites. The resulting construct, upstream

Figure 2. Molecular map of the 46C region surrounding the *Drosophila mef2* and *FMRFamide* genes. Cosmid (Cos) and λ genomic clones are shown above the *HindIII* and *EcoRI* restrictions maps. Deficiency breakpoints are denoted by a wavy line, and the direction of the deletion by an arrow. The region spanning the *mef2* gene has been expanded to illustrate its structural organization. The solid boxes correspond to exons; the hatched boxes correspond to untranslated regions. The start of translation is indicated by an arrow.

activating sequence (UAS)—*mef2*, was transformed into *Drosophila*, as described in Materials and methods. The presence of the original *mef2* mutation in the rescue strain was confirmed by lack of complementation of the *Df(2R)P520* deficiency, as well as by the presence of *mef2* mutant embryos in the progeny (data not shown). Furthermore, the presence of the UAS—*mef2* construct was confirmed by Southern analysis of DNA from a representative number of individual flies (data not shown).

To direct the expression of the UAS—*mef2* construct, we used 24B, a line in which GAL4 expression is directed to muscle cells and a small number of cells in positions above the ventral ganglion of late stage embryos (Brand and Perrimon 1993). The 24B insertion was also crossed into a *mef2* mutant background and balanced over CyO

that is marked with β -galactosidase (see Materials and methods). The presence of a *mef2* mutation in the final stock and the presence of the GAL4 construct were confirmed, as above (data not shown).

The appropriate *Drosophila* lines described above were mated to express MEF2 in a *mef2* mutant background. The progeny of this cross exhibited GAL4-directed *mef2* expression in the muscle cells, as well as in cells over the ventral ganglion of late stage embryos, where endogenous *mef2* expression is not normally observed. Included in the progeny of this cross are *trans*-heterozygous rescued mutant embryos that are identifiable by the absence of β -galactosidase staining (see Materials and methods). As shown in Figure 5A, these embryos exhibit significant levels of MEF2 protein expression.

We visualized the muscle pattern of the rescued mutant embryos with an antibody that recognizes *Drosophila* muscle-specific MHC protein. A representative rescued embryo is shown in Figure 5B–C. By comparison to the embryos shown in Figure 4B–D, these embryos now exhibit significant muscle fiber formation. Moreover, these muscles appear to be remarkably normal with respect to location and attachment. *mef2* expression under the control of the 24B–GAL4 line is, however, not sufficient to rescue viability because these embryos do not hatch into larvae. In addition, a significant number of unfused myoblasts are found among the MHC-expressing cells in the rescued embryos (see arrows in Fig. 5C). These observations suggest that regulatory sequences present in the *mef2* gene are needed to obtain full rescue. Nevertheless, this artificial means of providing exogenous MEF2 protein does result in the recovery of muscle fibers in embryos *trans*-heterozygous for *mef2*²²⁻²¹/*Df(2R)P520*;24B-GAL4/UAS—*mef2* (Fig. 5B,C). In summary, our data demonstrate that muscle fibers do not form in the absence of *mef2*. This defect can be rescued by providing exogenous MEF2 in the somatic

Table 1. Complementation analysis between *Df(2R)P520* and lethal allele mapped to the 46C-E region

Group	Allele no.	Trans-heterozygotes ^a /total progeny	Percent trans-heterozygotes ^b
C ^c	22-21	0/106	0
E	5-3	20/89	22
F	10-9	38/95	40
F	14-10	41/157	26
I	7-12	32/107	30
M	7-5	31/109	28
O	20-10	41/89	46
Q	26-19	30/100	30
T	68-11	27/87	32
U	78-10	32/121	26
X	97-16	28/76	37

^aMutant/*Df(2R)P520*.

^bExpected frequency of *trans*-heterozygotes is 33%.

^cThis C designation does not correspond to the C group in O'Brien et al. (1994).

Figure 3. *mef2*²²⁻²¹ is a protein null allele. Heterozygous and homozygous *mef2*²²⁻²¹ mutant embryos were doubly stained with a rabbit polyclonal antibody against MEF2 and a mouse monoclonal antibody against β -gal. The embryos are oriented with the anterior to the left and the ventral facing downward. (A) The heterozygous embryo shows MEF2 expression in the somatic (arrow) and visceral (solid arrowhead) mesoderm. Because the nonmutant chromosome was marked with a P[lacZ] (see Materials and methods), the heterozygous embryo also shows LacZ expression (open arrowhead). (B) There is no detectable MEF2 protein in the somatic (arrow) or visceral (arrowhead) mesoderm in the homozygous embryo. (C) The exons spanning the MADS-MEF2 region were amplified from genomic mutant DNA by use of the PCR, subcloned, and sequenced. The mutation was identified as being a base change that converted the sixth codon into a nonsense codon. This is depicted in an expanded form below the schematic diagram of the MEF2 protein. G/S, S, and Q denote amino acid repeats (Nguyen et al. 1994).

mesoderm, thereby indicating that *mef2* function is essential for myogenesis.

Partially differentiated myoblasts are present in mef2 mutant embryos

MHC expression and multinucleate muscle fiber formation are relatively late aspects of myogenesis. Therefore, it was of interest to assess the extent of myogenesis in the *mef2* mutant embryos at earlier developmental stages. To study these events, we examined the expres-

sion of three additional markers, *eve*, *nau*, and β 3-tubulin.

eve encodes a homeo box-containing factor that has been detected during germ-band retraction in precursor cells of one dorsal somatic muscle, which appears beneath the dorsal ectoderm in each hemisegment (Frasch et al. 1987; Bodmer 1993). *eve* also marks a small subset of pericardial cells of the dorsal vessel (discussed below) (Azpiazu and Frasch 1993; Bodmer 1993). *nau* encodes a basic-helix-loop-helix (b-HLH) domain-containing factor that is expressed in the nucleus in a subset of muscle precursor cells beginning at late germ-band extension

Figure 4. MHC expression in wild-type and *mef2* mutant embryos. Embryos were analyzed with a monoclonal antibody against MHC (see Materials and methods). The embryos are oriented with the anterior to the left. (A) Wild-type, stage 16, lateral view. (B) *mef2*²²⁻²¹/*Df*(2R)P520 *trans*-heterozygote, stage 15, lateral view. (C) *Df*(2R)X1/*Df*(2R)P520 *trans*-heterozygote, stage 15, dorsal view. (D) *mef2*²²⁻²¹/*Df*(2R)P520 *trans*-heterozygote, stage 15, ventral view. Arrows denote MHC-expressing cells in positions corresponding to that of the somatic musculature (sm). Arrowheads show MHC-expressing cells in the visceral musculature (vm).

Figure 5. Rescued embryos exhibit MHC and MEF2 expression. Embryos were analyzed as in Fig. 4, with either a rabbit polyclonal antibody against MEF2 (A) or a mouse monoclonal antibody against MHC (B,C). The embryos are oriented with the anterior to the left. (A) *mef2²²⁻²¹/Df(2R)P520;24B/UAS-mef2* trans-heterozygote, stage 15, lateral view. (B) *mef2²²⁻²¹/Df(2R)P520;24B/UAS-mef2* trans-heterozygote, stage 15, lateral view. (C) High magnification view of embryo shown in B. Arrowheads in C indicate unfused myoblasts alongside and just underneath the somatic musculature.

and can be followed into distinct differentiated muscle fibers (Michelson et al. 1990; Paterson et al. 1991). Therefore, the products of these two genes serve as markers for subsets of the differentiating somatic mesoderm and can be followed through the transition from muscle precursors into muscle fibers.

As shown in Figure 6, both EVE- and NAU-expressing cells are readily apparent in the mutant embryos, indicating that *mef2* is not necessary for the initial cell fate determination of these populations of myoblasts. In wild-type embryos, the EVE-positive dorsal nuclei become more numerous as muscle fiber formation progresses (Fig. 6A). In *mef2* mutant embryos, however, only one mononucleate EVE-positive cell is detected per segment instead of several EVE-positive nuclei (Fig. 6B). Similar to EVE, NAU-expressing cells are observed in

appropriate positions in *mef2* mutant embryos (Fig. 6D). However, these mutant embryos do not exhibit NAU-expressing multinucleate syncytia, which are detected in wild-type embryos at the same developmental stage (Fig. 6C). These observations indicate that although *mef2* is not necessary for the initial specification of these muscle precursors, it is essential for the continued differentiation of the EVE- and NAU-positive somatic muscle precursors to form typical muscle fibers.

In contrast to EVE and NAU, which mark specific subsets of muscle precursors, β 3-Tubulin serves as a marker for the entire myoblast population. In wild-type embryos, it is expressed in all somatic mesoderm during germ-band extension (Leiss et al. 1988), several hours before MHC expression at late germ-band retraction (Bernstein et al. 1993), and can be followed into all somatic muscle fibers (Fig. 6E). A large number of β 3-Tubulin-expressing myoblasts are evident in *mef2* mutant embryos (Fig. 6F), indicating that the bulk of the myoblast population has been specified and exhibits early aspects of myogenesis. However, as compared with similarly staged wild-type embryos (Fig. 6E), the vast majority of *mef2* mutant β 3-Tubulin-expressing myoblasts are unfused and are unable to activate the expression of MHC. Therefore, *mef2* is not required for the initial cell fate determination of the general myoblast population but it is essential for its continued differentiation.

We explored the possibility that the partially differentiated mononucleate myoblasts present in the *mef2* mutant embryos might undergo programmed cell death. We used the vital stain acridine orange to assay for cells undergoing programmed cell death (Abrams et al. 1993; White et al. 1994). There are moderate levels of cell death in various parts of wild-type embryos before the muscle fibers are fully formed (Abrams et al. 1993). After dorsal closure, when the muscle fibers have formed, significant levels of cell death occur only in the CNS (Fig. 6G). In contrast, there is an increase in cell death in the cell layer of the mesoderm in *mef2* mutant embryos of the corresponding developmental stages (Fig. 6H). We do not see an obvious increase in cell death in the region of the heart or visceral mesoderm (data not shown).

Taken together, the data indicate that the somatic mesoderm is formed normally and that somatic muscle precursor cells express markers of all (β 3-Tubulin) or subsets (NAU and EVE) of somatic muscles in the absence of *mef2* function. However, the continuing differentiation of these somatic muscle precursors into multinucleate muscle fibers requires *mef2* function.

Mutant embryos lacking mef2 also exhibit abnormalities in the visceral musculature and the heart

In contrast to the somatic musculature, a significant number of MHC-expressing cells surround the foregut and hindgut of *mef2* mutant embryos (see Fig. 4B–D). Lowered, but detectable levels of MHC are also observed in a thin layer around the midgut. Because it has been established that morphogenesis of the midgut requires

Figure 6. *mef2* mutant embryos express mesodermal markers and exhibit increased cell death in the mesoderm at later stages. Embryos were analyzed with a rabbit polyclonal antibody against EVE (A,B), a rat polyclonal against NAU (C,D), or a rabbit polyclonal against $\beta 3$ -Tubulin (E,F). In G and H, the embryos were analyzed by use of the vital dye acridine orange. In all views, the anterior is to the left. (A) Wild-type, stage 13, dorsolateral view. (B) *Df(2R)X1/Df(2R)P520* trans-heterozygote, stage 13, dorsolateral view. EVE-positive pericardial cells (pc) are denoted by arrowheads. EVE-positive nuclei in dorsal muscle precursors (sm) are highlighted by arrows. (C) Wild-type, stage 13, lateral view. (D) *mef2²²⁻²¹/Df(2R)P520* trans-heterozygote, stage 13, lateral view. Arrows highlight fused (C) and unfused (D) NAU-expressing cells. (E) Wild-type, stage 14, lateral view. (F) *mef2²²⁻²¹/Df(2R)P520* trans-heterozygote, stage 14, lateral view. (G) Wild-type, stage 15, lateral view. (H) *mef2²²⁻²¹/Df(2R)P520* trans-heterozygote, stage 15, lateral view.

the presence of visceral mesoderm (Reuter and Scott 1990; Azpiazu and Frasch 1993; Bodmer 1993), we determined whether *mef2* could have a function in this process. In embryos lacking *mef2*, the morphology of the visceral mesoderm appears normal and midgut formation appears to proceed correctly in the early stages. We monitored visceral mesoderm formation by first examining the expression of fasciclin III (FASIII) (Patel et al. 1989). During germ-band retraction, FASIII expression in the visceral mesoderm of *mef2* mutant embryos appears indistinguishable from wild-type embryos (data not shown). $\beta 3$ -Tubulin expression in the visceral mesoderm also appears normal in mutant embryos (data not shown). However, the formation of constrictions and looping of the midgut at later stages are abnormal (Fig. 7A,B). Partial rescue of the gut constriction abnormality is observed in response to *mef2* expression under the

control of the 24B-GAL4 line (data not shown). These observations support the possibility that midgut morphogenesis, a late-occurring aspect of visceral mesoderm differentiation, is affected in *mef2* mutants.

Because *mef2* is expressed in cardiac cells, we examined a possible requirement of *mef2* function for heart formation. In *mef2* mutant embryos, cardiac cells are present and express high levels of $\beta 3$ -Tubulin (Fig. 7C); however, MHC expression is not detectable in these cells (data not shown). The formation of the cardiac cells is, therefore, not affected by the absence of *mef2*. We next examined the pericardial cells that are also mesodermally derived heart precursors. As mentioned previously, EVE expression in the pericardial cells of *mef2* mutant embryos remains unchanged (see Fig. 6A,B). For further assessment, we used a monoclonal antibody that recognizes a cell-surface protein on pericardial cells of

Figure 7. Visceral mesoderm and heart formation in *mef2* mutants. Embryos were analyzed with a mouse monoclonal antibody against MHC (A,B), a rabbit antibody against β 3-Tubulin (C) or a mouse monoclonal antibody against an epitope on pericardial cells (see Material and methods). (D) In all views, the anterior is to the left. (A) Wild-type, stage 16, dorsal view. (B) *mef2*²²⁻²¹/*Df*(2R)P520 *trans*-heterozygote, stage 16, dorsal view. Arrows highlight the abnormal constriction observed in the mutant embryo. (C) *mef2*²²⁻²¹/*Df*(2R)P520 *trans*-heterozygote, stage 16, dorsal view. The cardiac cells are indicated by arrows. (D) *mef2*²²⁻²¹/*Df*(2R)P520 *trans*-heterozygote, stage 16, dorsolateral view. The pericardial cells are indicated by arrowheads.

the heart (T. Volk, unpubl.). As observed with EVE, the pericardial cells that are stained with this monoclonal antibody appear normal (Fig. 7D). On the basis of this analysis, it appears that *mef2* is not essential for early heart formation. However, it is required for later differentiation of the heart cells because these cells do not express MHC in the absence of *mef2*. Notably, MHC expression is detected in the cardiac cells of rescued embryos, in response to *mef2* expression under the control of the 24B-GAL4 line (data not shown).

Discussion

mef2 functions

We have presented evidence demonstrating that *mef2* function is essential for myogenesis. Embryos lacking *mef2* exhibit a striking absence of MHC-expressing cells in the position of the somatic musculature and do not develop differentiated muscle fibers. This role in mesodermal differentiation is consistent with the spatial and temporal profiles of *mef2* mRNA and protein expression (Lilly et al. 1994; Nguyen et al. 1994; also Fig. 1). Aspects of the observed mutant phenotype can be rescued by providing exogenous *mef2* gene activity in the mesoderm, by use of the GAL4 system of Brand and Perrimon (1993).

To define the *mef2* mutant phenotype more precisely, we have examined the expression of markers for specific

aspects of mesoderm differentiation. We show that NAU-, EVE-, and β 3-Tubulin-expressing myoblasts are present in the somatic musculature. These observations indicate that *mef2* gene activity is not needed for the initial cell fate specification of these myoblasts. Rather, *mef2* affects the continuing development of cells that have already undergone some aspects of myogenic specification. More specifically, *mef2* is essential for subsequent activation of muscle-specific structural genes such as MHC, and the progression to multinucleate muscle syncytia.

The first morphological signs of myogenesis in the *Drosophila* embryo are binucleate and trinucleate cells, termed muscle precursors, that form in close proximity to the ectoderm (Bate 1990). These muscle precursors occur in reproducible positions that prefigure the final muscle pattern. Such morphological observations have lead to the idea that muscles are formed by the fusion of a single mononucleate founder cell, which is responsible for the unique identity of each muscle fiber, with unspecified fusion-competent cells (Bate 1990). It has been postulated further that the identity of individual founder cells is determined by genes, such as *S59*, which are expressed in a distinct subset of muscle precursors and differentiated fibers. As fusion proceeds, the identity of the founder cells would be conveyed to the cells that are recruited by fusion, and they, in turn, would activate expression of genes such as *S59* (Dohrmann et al. 1990).

By analogy, one might anticipate that NAU and EVE proteins, which are also expressed in subsets of muscle precursors and differentiated muscle fibers, may specify some of the hypothetical founder cells. Within the context of this model, our results would suggest that the initial specification of founder cells does not require *mef2*. By comparison, β 3-Tubulin is expressed in many, if not all of the presumptive myoblasts, and would therefore be expressed in the hypothetical fusion-competent cells. Our results would also suggest that the initial specification of these cells does not require *mef2*. We do not know whether *mef2* is expressed in all cells, including those that express NAU and EVE or whether it is restricted to the hypothetical fusion-competent cells. Whether *mef2* is needed for the differentiation of just one, or both, of these cell types remains to be determined.

Increased numbers of dying cells were observed in the mesodermal layer that corresponds to the somatic musculature in *mef2* mutant embryos. Interestingly, previous studies have shown that mutations disrupting cellular differentiation result in the death of those cells that are unable to complete the differentiation process (Smouse and Perrimon 1990; Tepass et al. 1990; Abrams et al. 1993; Bonini et al. 1993). Thus, our findings suggest that the partially differentiated somatic precursor cells present in these mutant embryos are subject to programmed cell death as a consequence of their failure to undergo complete differentiation.

In contrast to *tinman* and *bagpipe* (Azpiazu and Frasch 1993; Bodmer 1993), *mef2* activity is required neither for the formation nor for the specification of the visceral mesoderm. On the basis of the observation that the midgut constrictions are abnormal in the absence of *mef2*, we suggest that it is required for later events in the differentiation of this tissue. Likewise, the formation of the cardiac and pericardial cells do not appear to be affected in *mef2* mutant embryos. However, the cardiac cells in the mutant embryos express β 3-Tubulin but not MHC. This observation raises the possibility that *mef2* function is needed in later aspects of differentiation, including activation of genes that are necessary for generating a functional heart. It is not known whether this function could involve interactions with *tinman*, which has been shown to be required for the formation of the heart, and like MEF2, is expressed in cardiac cells of late stage embryos (Azpiazu and Frasch 1993; Bodmer 1993).

Comparisons between mef2/MEF2 and nau/MyoD gene families

It has been established that activation of skeletal muscle gene expression in vertebrates is regulated by the MyoD gene family, which encodes muscle-specific transcription factors sharing a common b-HLH motif (for review, see Weintraub 1993; Lassar and Munsterberg 1994; Olson and Klein 1994). Recent gene knockout studies have shown that unique, as well as overlapping functions, can be ascribed to these myogenic b-HLH factors (Braun et al. 1992; Rudnicki et al. 1992, 1993; Hasty et al. 1993;

Nabeshima et al. 1993). More recently, the myogenic MEF2 gene products have been implicated in the regulation of gene expression in both skeletal and cardiac muscles. DNA-binding activities that are attributable to the MEF2 transcription factors are restricted to all muscles (skeletal, cardiac, smooth muscle) and the brain (for review, see Lassar and Munsterberg 1994; see also references therein). The expression profiles of the MEF2 gene products during murine embryogenesis (Edmonson et al. 1994) and the functional importance of MEF2-binding sites have given further support for the proposed regulatory function of MEF2 (Edmonson et al. 1992; Cheng et al. 1993; Yee and Rigby 1993). However, confirmation must await the analysis of MEF2 mutant mice.

Previous and current studies on the *Drosophila mef2* gene are providing insight into the potential role of the MEF2 gene family. Interestingly, the phenotype of the *mef2* mutants is very reminiscent of that of mice lacking myogenin. Myogenin-deficient mice die perinatally and exhibit severe skeletal muscle defects (Hasty et al. 1993; Nabeshima et al. 1993). Notably, severely disorganized myotubes or mononucleated myoblasts are found in the limbs of the mutant mice (Hasty et al. 1993; Nabeshima et al. 1993). As we have interpreted the *mef2* phenotype, the myogenin phenotype suggests that it is also needed for the terminal differentiation of myoblasts to myotubes and not for the determination of skeletal myoblasts. Roles in myoblast determination have been ascribed to MyoD and Myf-5 (Rudnicki et al. 1994).

The hierarchical relationship between the vertebrate MEF2 and myogenic b-HLH gene members is not clear. Cell culture studies have suggested that the myogenic b-HLH genes are upstream of the MEF2 genes (Cserjesi et al. 1991; Martin et al. 1993), whereas in vivo studies indicate that MEF2 is required for proper regulation of myogenin gene expression (Cheng et al. 1993; Yee and Rigby 1993). More recent studies show that MEF2 induces the expression of MyoD and myogenin (Kaushal et al. 1994). In *Drosophila*, the relationship is more clear. *mef2* expression is detected prior to the onset of *nau* expression and is observed in genetic deficiency lines that remove several genes, including *nau* (M. Erickson and S. Abmayr, unpubl.). Thus, *nau* does not appear to play a role in the activation of *mef2*. Likewise, our present data indicate that the initiation of NAU expression does not require *mef2*-encoded protein, because NAU-expressing cells are observed in appropriate positions in its absence. Because the temporal appearance of NAU-expressing cells is comparable to that observed in a wild-type embryo, and these cells continue to express significant levels of NAU for several hours, it appears that *mef2* does not play a major role in the maintenance of NAU expression.

Initial *mef2* expression is detected soon after *twist* expression and also depends on *twist* activity (Lilly et al. 1994; Nguyen et al. 1994). Although the observed *mef2* mutant phenotype does not suggest an obvious function for the early *mef2* expression in the undifferentiated mesodermal mass, our present data cannot rule out the possibility that this early function could be compensated by

other mesodermal gene(s), including *tinman* and *zfh-1*. To further our understanding of the role of *mef2* in mesoderm differentiation, it will be important to identify the target genes of *mef2*, some of which could be muscle-specific genes that are common between *Drosophila* and vertebrates. It will also be interesting to determine whether the activation of some of these targets would require interactions with one or more mesodermal gene products. Recent studies have shown that mammalian MEF2 gene products can interact with b-HLH factors, such as myogenin and MyoD (Funk and Wright 1992; Kaushal et al. 1994).

Material and methods

Drosophila stocks

Fly stocks were raised at 18°C or 25°C on standard media. The following three deficiency stocks were used for identifying *mef2* mutants: *Df(2R)X1/CyO*; *Adh^{nb}*, *Df(2R)X3/CyO*; *Adh^{nb}*, and *Df(2R)P520/SM6* (O'Brien et al. 1994). In a screen for specific mutations in the 46C–F region, flies were mutagenized with EMS, diepoxybutane (DEB) or γ -rays, and tested for lethality that is uncovered by *Df(2R)X1* in a standard F₂ screen. EMS mutagenesis yielded 74 lethals from ~15,000 progeny, DEB yielded 21 lethals from ~18,000 progeny, and γ -rays yielded 31 lethals from ~24,000 progeny. Lethal alleles were then tested for lethality that is uncovered by *Df(2R)X3*. Flies with mutations mapping to the overlapping region of *Df(2R)X1-Df(2R)X3* were tested further by crossing to *Df(2R)P520*.

For phenotypic studies, mutant stocks were balanced over *CyO* marked with *P[w⁺lacZ]* (obtained from H. Bellen, Baylor College of Medicine, Houston, TX), resulting in the detection of β -gal expression in the anterior and posterior ends of the embryo, or *wg^{en11}* (obtained from N. Perrimon, Harvard Medical School, Boston, MA), which yields β -gal expression in the pattern of *wg* (Kassis et al. 1992). Balancer stocks used for generating the genetic combinations in the rescue experiments included *TM3*, *Ser/TM6B*, *Hu P[w⁺lacZ]* and *yw*; *Pin/CyO*, *P[w⁺lacZ]*.

Generation of UAS-*mef2* strains and *mef2* rescue stocks

To create the UAS-*mef2* rescue construct, a 3.0-kb *EcoRI*–*HindIII* fragment of the *D-mef2* cDNA (Nguyen et al. 1994), which includes the entire coding sequence, was subcloned into pBSKII (Stratagene). This plasmid was digested with *XbaI* and *XhoI* and the resulting fragment was subcloned into the vector pUAST (Brand and Perrimon 1993). Transgenic lines containing the UAS-*mef2* construct were generated by injection of *yw⁻* embryos, according to published procedures (Rubin and Spradling 1982; Spradling and Rubin 1982). Eight independent transformant lines were obtained and expanded into homogeneous stocks (either homozygous or balanced, as appropriate).

The GAL4-expressing enhancer trap line *24B* (Brand and Perrimon 1993) was generously provided by N. Perrimon. The UAS-*mef2* stock is described above. Stocks homozygous for either *24B* or UAS-*mef2*, both located on the third chromosome, were crossed into various *mef2* mutant backgrounds to create the following lines: *Df(2R)P520/CyO*, *P[w⁺lacZ]*; UAS-*mef2*/UAS-*mef2* and *mef2²²⁻²¹/CyO*, *P[w⁺lacZ]*; *24B/24B*. These line were crossed to generate progeny in which 100% carried one copy each of the *24B* source of GAL4 and the UAS-*mef2* construct. Of these progeny, 75% carried at least one copy of a *CyO* balancer that directed expression of β -gal activity. The

source of MEF2 protein in the mutant progeny, which lack β -gal expression was the transgene.

Molecular biology techniques

The cosmid and λ genomic clones presented here were derived from a genomic walk in the region surrounding the *FMRFamide* gene (O'Brien et al. 1994). The structural organization of the *mef2* gene was determined by Southern blotting analysis of restriction digests of various cosmid and λ genomic clones, using labeled fragments corresponding to different regions of the *mef2* DNA under standard conditions (Sambrook et al. 1989). Targeted sequencing of the *mef2* gene was done by use of specific primers corresponding to various regions of the gene, and an automated sequencing system.

Genomic DNA was isolated from homozygous mutant embryos from the EMS allele *mef2²²⁻²¹*, as described previously (Bodmer 1993). Specific primers were used in PCR to amplify the fragments spanning the second and third exons, as well as the second intron. The primers, with nucleotide sequences of 5'-CCTGGGGACAATTATACGG-3' and 5'-TAGCCATGAATCGATTACACC-3' and the set of primers with nucleotide sequences of 5'-CCTTCAACAAGCGCAAGTTC-3' and 5'-CGATGATGTTCTTGTGGTG-3' were used to obtain the second and third exon, respectively. The primers with the sequences of 5'-CACGCATCACCGATGAACGC-3' and 5'-CTTCATCACGCCGAAGTTC-3' were used to amplify the second intron. Three independent PCR reactions were performed for each set of primers. The PCR-generated fragments were then subcloned into the pCRII vector (InVitrogen, CA) and sequenced by use of an automated sequencing system. Three different subclones from each PCR reaction of homozygous mutant DNA were analyzed.

β -Gal staining

β -Gal activity was used to distinguish phenotypic wild-type from homozygous mutant embryos, which were identified by the absence of β -gal activity.

The β -gal staining procedure was obtained from Drs. A. Singh and M. Kango (*Drosophila* Stock Center, India). Embryos were dechorionated in 50% bleach and fixed in 4% paraformaldehyde in citric phosphate buffer (CPB: 90 mM Na₂HPO₄, 5 mM citric acid at pH 7.6) and 50% (vol/vol) heptane. The fixative was removed, and the embryos were washed in CPB in the presence of heptane. The embryos were then air-dried and transferred to a solution consisting of 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, and 0.5% Triton X-100, prewarmed at 65°C. The substrate X-gal was added to 0.1%, and the embryos were incubated in this solution for 24–48 hr at 37°C. Stained embryos were refixed in 4% formaldehyde, 50 mM EGTA and 50% (vol/vol) heptane. Following washes in heptane, an equal volume of 5% TCA was added and the embryos were shaken for 2 min and then allowed to stand for 5 min. After additional washes in heptane, the embryos were devitellinized in an equal volume of 90% methanol, 5 mM EGTA. Subsequent immunocytochemistry with a specific antibody was performed, as described below.

Antibodies and immunocytochemistry

Polyclonal anti-MEF2 antibodies were generated by injecting a GEX2T–MEF2 fusion protein (amino acids 1–168) into New Zealand white rabbits by use of standard conditions. The various antibodies discussed herein were used at the following dilutions: anti-MEF2 (1:800) with preabsorption with wild-type

embryos, anti-NAU (1:25), anti- β 3-Tubulin (1:250) [Leiss et al. 1988], anti-MHC (1:1000) [D. Kiehart, unpubl.], anti-EVE (1:5000) [Frasch et al. 1987], and a monoclonal recognizing a cell-surface protein that is expressed on pericardial cells (1:10) [T. Volk, unpubl.].

Embryos were collected on agar/apple juice plates at 0- to 6-hr intervals and aged an additional 6 or 12 hr at 25°C, as appropriate. For double antibody stainings, in which a specific antibody and a β -gal antibody (Sigma) were used, the embryos were dechorionated in 50% bleach and fixed in 4% formaldehyde in 0.1 M HEPES (pH 7), 1 mM EGTA, 2 mM MgSO₄, and 50% (vol/vol) heptane. Subsequent immunocytochemistry with all specific antibodies was performed essentially as described [Ashburner 1989]. Detection of the biotinylated secondary antibody was performed by use of the Vectastain ABC kit (Vector Laboratories, CA) for MHC and β 3-Tubulin antibodies or the Vectastain Elite ABC kit (Vector Laboratories, CA) for MEF2, EVE, NAU, and pericardial cell-surface protein antibodies, according to manufacturer's instructions.

Cell death assay

Acridine orange staining was done as described in Abrams et al. (1993). Mutant embryos were identified on the basis of their abnormal gut morphology. The embryos were photographed with Normarski transillumination.

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Note added in proof

The phenotype of a D-mef2 deficiency was reported recently by Lilly et al. (1995) in *Science* 267: 688–693.

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Drosophila MEF2, a transcription factor that is essential for myogenesis.

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