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D-mef2: A *Drosophila* mesoderm-specific MADS box-containing gene with a biphasic expression profile during embryogenesis

(myocyte-specific enhancer factor 2/myogenesis/differentiation/somatic muscles/heart and visceral musculature)

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ABSTRACT **We have identified a mesoderm-specific *Drosophila* gene, designated *D-mef2*. The encoded protein contains the MADS- and MEF2-specific domains, which are characteristic of the myocyte-specific enhancer factor 2 (MEF2) family of transcription factors. *D-mef2* RNA is first detectable in the presumptive mesoderm at late cellular blastoderm stage and is expressed in all mesoderm after invagination. Following the dorsal migration of the mesodermal layer, *D-mef2* expression becomes restricted to the primordia for visceral muscle and the heart. In the second phase, *D-mef2* expression is first distinct in heart precursors and then becomes prominent sequentially in visceral and somatic muscles. *twi* activity is required for *D-mef2* expression, while *sna* function may be needed for the maintenance of *D-mef2* expression but not its initiation. *D-mef* expression is not dependent on the function of *tin*, and embryos that are deficient for the mesodermal gene *DFR1* also show normal initiation of *D-mef2* expression at blastoderm. These results suggest that *D-mef2* could have a function in early mesoderm differentiation and may be required for subsequent cell fate specifications within the somatic and visceral/heart mesodermal layers.**

The expression of the zygotic genes twist (*twi*) and snail (*sna*) is essential for normal initiation of mesoderm determination in *Drosophila melanogaster* (1, 2). The genes downstream of *twi* and the genetic interactions that govern the process of mesoderm differentiation are under investigation. A limited number of genes have been isolated whose expression profiles in the developing mesoderm suggest a role in mesoderm specification. Among these genes, tinman (*tin*), *zfh-1*, and *Drosophila* fibroblast growth factor receptor 1 (*DFR1*) are first expressed at late cellular blastoderm stage (3–5) while *s59*, nautilus (*nau*), homeobox 2.0 (*H2.0*), and apterous (*ap*) are expressed at a later stage during germband extension (6–10). Deletion of the *H2.0* gene does not result in malformation of the visceral muscle, and loss of *zfh-1* function causes aberrations in muscle patterning (11, 12). In the absence of *ap* function, however, there is a decrease in *ap*-expressing muscles (10). More recent phenotypic analyses of null mutations demonstrate that *tin* function is necessary for proper development of the heart and visceral musculature, while bagpipe (*bap*) expression is needed for proper differentiation of the visceral mesoderm (13, 14).

In mammalian systems, the understanding of myogenesis has been advanced by the analyses of a family of basic-helix–loop–helix (b-hlh) skeletal-specific myogenic factors (reviewed in refs. 15 and 16). More recently, several isoforms of a distinct family of myogenic factors, MEF2 (myocyte-specific enhancer factor 2), have been cloned (17–21). These gene products have in common a DNA-binding domain that is related to the MADS box, identified in the yeast gene *MCM1*, the homeotic plant genes agamous and deficiens, and the gene encoding the serum response factor, SRF (reviewed in refs. 22 and 23). MEF2-binding sites, conforming to the consensus sequence of $^{C}_{T}$T$^{AA}_{TT}$AAATA$^{A}_{G}$, are important for muscle-specific transcription of several genes (reviewed in ref. 24).

To explore further the role of MEF2 gene products, we have isolated and characterized the single *Drosophila mef2* gene (*D-mef2*).** The encoded protein exhibits properties that are similar to those of its vertebrate homologues. Its pattern of expression in the mesoderm during embryogenesis and in several mutants that are defective in mesoderm formation or differentiation provides an insight into the potential role of *D-mef2* in mesoderm differentiation.

MATERIALS AND METHODS

cDNA Library Screening and DNA Sequencing. A *Drosophila* 4- to 8-h embryonic cDNA library (25) was screened with a 300-bp fragment encompassing 60 bp of 5′ untranslated and 240 bp of coding sequences from the *hMF2C* cDNA clone (18). The nucleotide sequences of the *D-mef2* cDNA clones were obtained with an automated sequencing system (Applied Biosystems).

DNA-Binding and Transient Transfection Assays. DNA-binding assays were performed as described (17). The core nucleotide sequences of the double-stranded oligonucleotides used are shown below.

MEF2	5′-CGCTCTAAAAATAACCCT-3′
MEF2 mt1	5′-CGCTCTAAGGCTAACCCT-3′
MEF2 mt4	5′-CGCTCTATAAATAACCCT-3′
CArG	5′-GGGGACCAAATAAGGCAA-3′

The *D-mef2* cDNA was subcloned in the eukaryotic expression vector pcDNA I/Amp (Invitrogen) to generate pcDNA/D-MEF2. Reporter constructs, pE102CAT and p8TKCAT, and those with two copies of the MEF2-binding site, 2xMEF2/pE102CAT and 2xMEF2/p8TKCAT, have been described (17). The MEF2 mt1 core sequence is in 2xMEF2mt/pE102CAT and 2xMEF2mt/p8TKCAT. Tran-

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Abbreviations: SRF, serum response factor; CAT, chloramphenicol acetyltransferase.

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**The sequence reported in this paper has been deposited in the GenBank data base (accession no. U07422).

sient transfection of HeLa cells and chloramphenicol acetyltransferase (CAT) assays were done as described (17).

RNA Isolation and Northern Blot Analysis. Flies at appropriate developmental stages were collected, frozen and pulverized. Total RNA and poly(A)$^+$ RNA were obtained and Northern blot analysis was done using standard conditions (26). The DNA probes included a 1370-kb *Hin*dIII–*Bam*HI fragment of the *D-mef2* cDNA, a 1.6-kb *nau* cDNA, a 2069-bp *Hin*dIII fragment spanning exons 2, 3a, and 3b from the myosin heavy chain (*Mhc*) gene (27), and a 400-bp *Xba* I–*Sal* I genomic fragment of the α-tubulin gene (28).

Whole Mount *in Situ* Hybridization. Embryos were collected, fixed, and hybridized essentially as described (29). Digoxigenin-labeled *D-mef2* RNA probes were synthesized by using the RNA Genius labeling mixture (Boehringer Mannheim).

***Drosophila* Stocks.** Fly stocks were maintained on standard yeast/cornmeal/agar medium at 25°C. The *twi* allele twi^{ID96}, tin^{EC40} (13), and $Df(3R)sr^{16}$ (5) were provided by N. Perrimon, J. Mohler, and H. Bellen, respectively. The *sna* alleles sna^{HG31} and sna^{IIG05} were obtained from the Indiana Stock Center (Bloomington).

RESULTS

***D-mef2* Contains the Conserved MADS- and MEF2-Specific Domains.** A *Drosophila* homologue of the human *MEF2* gene was isolated from a 4- to 8-hr embryonic cDNA library (25). The *D-mef2* cDNA contains an open reading frame which encodes a 515-amino acid polypeptide, with a calculated molecular mass of 54.2 kDa and isoelectric point of 7.86 (data not shown). Fig. 1 shows a comparison of the MADS- and MEF2-specific domains between D-MEF2 and other members of the MADS supergene family. The high degree of sequence conservation in the MADS- and the MEF2-specific domains indicates that D-MEF2 is the *Drosophila* homologue of the *MEF2* gene products.

DNA-Binding and Trans-Activation Properties of Cloned D-MEF2 Are Similar to Those of Its Mammalian Homologues. *In vitro*-translated D-MEF2 products were analyzed in binding assays (Fig. 2), using as probes double-stranded oligonucleotides corresponding to a consensus MEF2-binding site (17). A single slower-migrating DNA–protein complex is observed; an excess of unlabeled wild-type MEF2 or mutated MEF2mt4 binding site DNA specifically competes for binding to the protein. The mutated MEF2mt1 binding site and the binding site for human SRF (30) do not serve as efficient competitors. These results demonstrate that the cloned D-MEF2 product can distinguish DNA-binding sites in a manner similar to that of its mammalian MEF2 counterparts (17–21, 31).

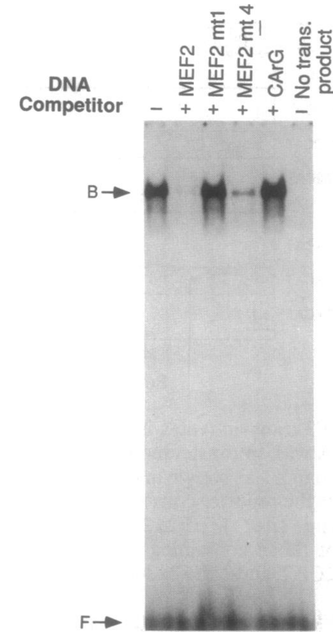

FIG. 2. DNA-binding specificity of D-MEF2 is similar to that of its vertebrate homologues. *In vitro*-synthesized D-MEF2 products were tested in binding assays using labeled double-stranded oligonucleotides corresponding to the consensus MEF2-binding site. The positions of bound (B) and free (F) oligonucleotides are indicated. Unlabeled specific competitors, MEF2 and MEF2mt4 oligonucleotides, or noncompetitors, mutated MEF2mt1 and the SRF binding site CArG, were used.

To explore the trans-activation potential of D-MEF2, a *D-mef2* expression construct was cotransfected with specific reporter constructs into HeLa cells, which have been shown to be devoid of endogenous MEF2 activity (17). Fig. 3 shows that D-MEF2 is capable of trans-activating the reporter constructs in a MEF2-binding site-dependent manner. Trans-activation is not observed with the mutant MEF2mtx2-pE102CAT and MEF2mtx2-pTKCAT constructs, thereby demonstrating that D-MEF2 can interact with mammalian cellular factors to form a transcriptionally active complex with a specific DNA-binding activity.

***Drosophila* MEF2 Is Encoded by a Single Gene.** Southern blot analysis and *in situ* chromosomal hybridization have established that there is a single *D-mef2* gene in *Drosophila*, which is mapped on the right arm of chromosome 2 at band position 46C (data not shown).

Northern blot analysis was done to determine the *D-mef2* expression profile during development. RNA transcripts of

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                                        MADS                                                          MEF2

D-MEF2  GRKKIQISRI TDERNRQVTF NKRKFGVMKK AYELSVLCDC EIALIIFSSS NKLYQY          ASTDMDRVLL KYTEYNEPHE SLTNKNIIE
hMEF2C  -------T-- M--------- T-----L--- ---------- -------N-T ---F--  (88%)  ------K--- ---------- -R--SD-V-  (83%)
hMEF2A  -------T-- M--------- T-----L--- ---------- -------N-- ---F--  (89%)  ------K--- ---------- -R--SD-V-  (83%)
hMEF2B  ---------- L-Q------- T-----L--- ---------- -------N-A -R-F--  (86%)  ---------- -----S---- -R--TD-L-  (83%)
hMEF2D  -------Q-- ---------- T-----L--- ---------- -------NH- ---F--  (89%)  ------K--- ---------- -R--AD-V-  (83%)
  SL-1  -------Q-- ---------- T-----L--- ---------- -------NH- ---F--  (89%)  ------K--- ---------- -R--AD---  (86%)
  SL-2  --R----T-- M-D--K---- T-----L--- ---------- -------N-- ---F--  (86%)  ------K--- ---------- -R--SD-V-  (83%)

   SRF  --V--KMEF- DNKIR-YT-- S---T-I--- -----T-TGT QVL-LVA-ET GHV-TF  (43%)  -TRKLQPMIT SE-GKALIQT C-WSPDSPP  (10%)
  MCM1  --R--E-KF- ENKTR-H--- S---H-I--- -EP----TGT QVL-LVV-ET GLV-TF  (45%)  STPKFEPIVT QQEGR-LIQA C-NAPDDE-  (10%)
    AG  --G--E-K-- ENTT------ S--RN-IL-- ---------A -V---V---R GR--E-  (66%)  SNNSVKGTIE R-KKAISDNS NTGSVAE-N  (7%)
   DEF  A-G----K-- ENQT-----Y S--RN-LF-- -H-------A KVSI-MI--T Q--HE-  (57%)  I-PTTATKQ- FDQYQKAVGV D-WSSHYEK  (10%)
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FIG. 1. Comparison of the MADS- and MEF2-specific domains. The degree of amino acid identity of each member of the MADS gene family versus D-MEF2 is indicated on the right. The MEF2 family includes the human (hMEF2A, hMEF2B, hMEF2C, and hMEF2D) and *Xenopus* (SL-1 and SL-2) proteins (17–21, 30). Other MADS members include the human SRF, yeast MCM1, and plant (AG, DEF) gene products (23, 24).

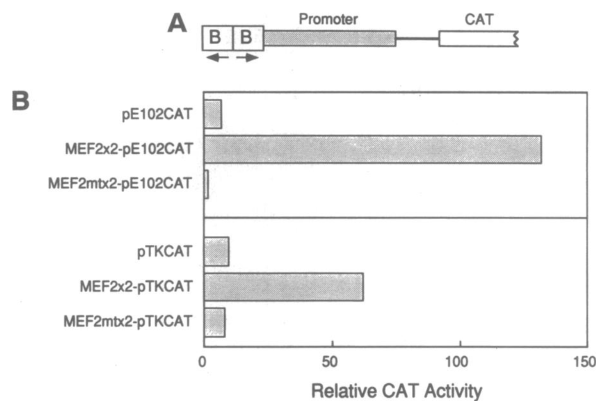

FIG. 3. D-MEF2 trans-activates in a sequence-specific manner. The *D-mef2* cDNA was cotransfected into HeLa cells with reporter constructs depicted in *A*. As shown in *B*, D-MEF2 can trans-activate to significant levels the reporter constructs with duplicated copies of the MEF2-binding site, MEF2x2-pE102CAT and MEF2x2-pTKCAT, and not those containing mutated MEF2-binding sites, MEF2x2mt-pE102CAT and MEF2x2mt-pTKCAT.

approximately 4 kb are first detected in 0- to 4-h embryos and remain at significant levels throughout embryogenesis (Fig. 4). The appearance of *D-mef2* RNA transcripts precedes that of *nau* and *Mhc* RNA transcripts. *D-mef2* RNA levels decrease during the larval stages, at which time the muscle structure is unchanged, and increase during the pupal stages, when the adult muscle pattern is being generated.

Biphasic Expression of *D-mef2* in the Mesoderm During Embryogenesis. Whole-mount *in situ* hybridizations were performed to define the *D-mef2* expression pattern during embryogenesis. *D-mef2* transcripts are first detected at late cellular blastoderm stage in the mesodermal primordia (Fig. 5*A*). Throughout mesodermal invagination, extension of the germband and spreading dorsally of the mesodermal mass, *D-mef2* is expressed in all mesoderm (Fig. 5 *B* and *C*). At mid-germband extension, there is a change from the general mesodermal to a more restricted pattern. *D-mef2* expression in the ventrolateral mesodermal layer is reduced while it remains undiminished in the dorsal region (in bracket) (Fig. 5*D*). *D-mef2* expression then decreases in the dorsal region and a new pattern of *D-mef2* expression emerges, first in distinct heart precursor cells at the dorsal-most margin (Fig. 5*E*). During germband retraction, *D-mef2* expression is increased in the visceral mesoderm and the position of its somatic mesodermal expression coincides with that of somatic muscle precursors (Fig. 5 *F* and *G*; refs. 7 and 33). Thereafter, *D-mef2* expression increases dramatically in all somatic mesoderm (Fig. 5*H*). Throughout the germband retraction stage, expression is high in segmentally repeated clusters of cells that are in positions corresponding to the ventral, pleural, and dorsal groups of forming somatic muscles (33) and a row of cardioblasts at the dorsal margin (Fig. 5*I*). In older embryos, *D-mef2* expression persists in both somatic and visceral musculature (Fig. 5*J*) and cardial cells of the heart (data not shown).

***D-mef2* RNA Expression Pattern in Mutants Defective in Mesoderm Formation or Differentiation.** *D-mef2* expression was examined in *twi* and *sna* mutant embryos. In twi^- embryos, *D-mef2* is not expressed at any stage during embryogenesis (Fig. 6 *A* and *B*). *D-mef2* is expressed at low levels during early germband extension in sna^- embryos (Fig. 6*C*); however, this expression is not maintained in older mutant embryos (data not shown). These results suggest that *twi* positively regulates *D-mef2*, while *sna* is needed mainly for the maintenance of *D-mef2* expression.

We also examined *D-mef2* expression in tin^- embryos. Initial *D-mef2* expression at late cellular blastoderm is nor-

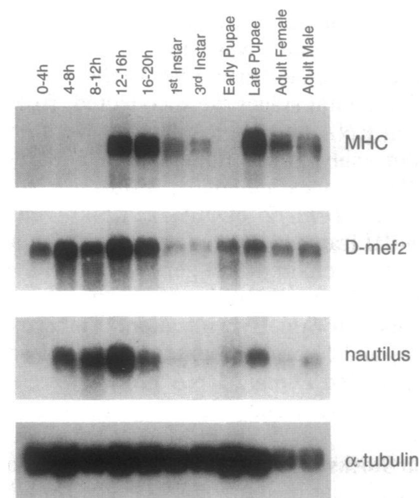

FIG. 4. *D-mef2* RNA expression profile during development. The developmental northern blot was hybridized to *D-mef2*, nautilus, and myosin heavy chain (MHC) probes. The α-tubulin probe served as control for the amount of RNA loaded in each lane.

mal in tin^- embryos (Fig. 6*D*). In later stages, *D-mef2* expression is detectable in the somatic mesoderm but not in the region of heart precursor cells (Fig. 6 *F* and *H*) and visceral muscles that are absent in tin^- embryos (data not shown), thus suggesting that *D-mef2* and *tin* could function in parallel during their early phase of expression in the developing mesoderm.

In embryos that carry a deficiency of *DFR1* (5), mesodermal invagination and initial *D-mef2* expression are normal (Fig. 6*E*). However, the number of *D-mef2*-positive cells in late germband-extended and -retracted mutant embryos is greatly reduced and disorganized (Fig. 6*G*).

DISCUSSION

Comparisons Between D-MEF2 and Vertebrate MEF2 Gene Products. The evidence provided in this report establishes that D-MEF is the *Drosophila* homologue of the vertebrate MEF2 proteins. D-MEF2 protein contains the MADS- and MEF2-specific domains and exhibits *in vitro* DNA binding and trans-activation properties that are similar to those of its vertebrate counterparts. *D-mef2* is expressed in the somatic and visceral musculature as well as in heart cells, which is analogous to the expression of its vertebrate counterparts in skeletal, smooth, and cardiac muscles (17–21, 30). It is, however, not expressed in the neuroectoderm, while the vertebrate MEF2 genes are expressed in the brain.

In contrast to vertebrate MEF2 and myogenic basic-helix–loop–helix gene products, which are encoded by multigene families, both D-MEF2 and nautilus are encoded by single genes (this report; refs. 7 and 8). It will be interesting to determine whether *D-mef2* and *nau* have unique functions or whether some of their functions could be overlapping with and compensated by other mesodermal genes.

Results from cell culture systems suggest that MEF2 is downstream of the basic-helix–loop–helix gene products; however, the MEF2-binding site found within the myogenin gene promoter is important for its expression *in vivo* (17, 24). Our present study shows that *D-mef2* cannot be downstream of *nau* because *D-mef2* expression starts at late cellular blastoderm stage and thus precedes the expression of *nau* by several hours of development. Whether the regulation of *D-mef2* expression, during its second phase of expression, could involve autoregulation or crossregulation by other mesodermal genes remains to be clarified.

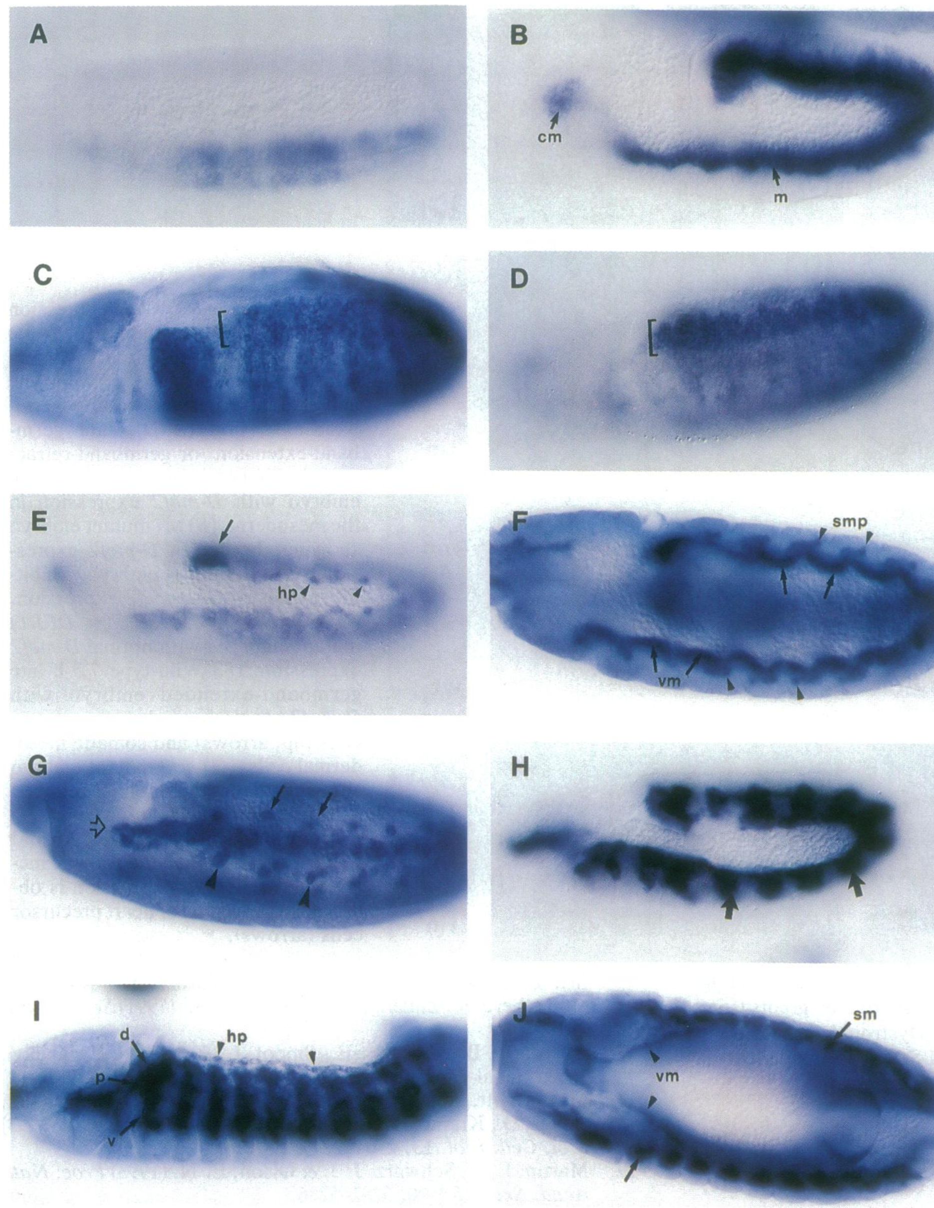

FIG. 5. Biphasic *D-mef2* RNA expression in the mesoderm during embryogenesis. Whole mount *in situ* hybridization with a digoxygenin-labeled *D-mef2* probe. The embryos are oriented with the anterior to the left and the ventral side facing downward. Stages are according to Campos-Ortega and Hartenstein (32). (All ×160.) (*A*) Lateral view of a 3-h late cellular blastoderm embryo. *D-mef2* RNA expression is detectable in the ventral region. (*B* and *C*) Lateral and ventrolateral views of germband-extending embryos. *D-mef2* transcripts are localized throughout the mesoderm (m) and the cephalic mesoderm (cm). (*D*) Ventrolateral view of a 6- to 6.5-h-old embryo. *D-mef2* expression in the ventral and lateral regions of the mesoderm is reduced while remaining undiminished in the dorsal region (in bracket). (*E*) Dorsolateral view of a 6.5- to 7-h-old embryo. General expression in the dorsal region becomes reduced but it is prominent in heart precursor cells (hp; arrowheads) and the primordium for hindgut visceral muscles (arrow). (*F*) Lateral view of a 7- to 7.5-h-old embryo. Expression is high in the visceral mesoderm (vm; arrows) and somatic muscle precursors (smp; arrowheads). (*G*) Dorsolateral view of a late germband-extended embryo. Expression is in somatic muscle precursors (arrowheads), visceral mesoderm (open arrowhead), and heart precursor cells (arrows). (*H*) Lateral view of an early germband-retracting embryo. *D-mef2* expression is high in the somatic mesoderm. (*I* and *J*) Lateral and dorsal views of a late germband-retracting embryo. *D-mef2* expression is in heart cells (arrowheads), somatic mesodermal cells (sm; arrow), in locations corresponding to ventral (v), pleural (p), and dorsal (d) muscle groups, and visceral musculature (vm; arrowheads).

Potential Role of *D-mef2* and Its Relationship to Other Mesodermal Genes. *twi* appears to be a positive regulator of *D-mef2* expression, while *sna* is needed mainly for its maintenance. It is possible that *sna* is needed for repressing genes that could have a negative effect on *D-mef2* expression. Whether these proposed negative regulatory genes could act directly on the *D-mef2* gene or indirectly by repressing and/or destabilizing twist activity is not known. Because the initial *D-mef2* RNA levels are lower in *sna* mutant embryos, snail could have an additional role as a positive regulator.

It appears that the processes occurring after the activation of *twi* and *sna* could be grouped into two main phases of activity. The first phase includes mesodermal invagination, extension of the germband, and dorsal spreading of the mesodermal layer. The start of the second phase corresponds to the third postblastodermal mitosis, leading to the separation of the mesoderm into the somatopleura and splanchnopleura. We would like to suggest that the two phases of *D-mef2* expression correspond to the two proposed phases in mesoderm development. *D-mef2* could function initially to subdivide the mesodermal primordia and then to specify cell fates within both the somatic and visceral/heart mesoderm. Confirmation of such a function awaits the analysis of *D-mef2* mutants.

Interestingly, the onset of expression of a number of mesodermal genes coincides with either the initial or later phase of *D-mef2* expression. Among those that are expressed early are the genes *tin*, *zfh-1*, and *DFR1* (3–5), all of which exhibit a more restricted pattern of expression following the initial general mesodermal expression. Other genes involved in muscle patterning, *s59*, *nau*, and *ap*, are first expressed at the stage corresponding to the second phase of *D-mef2* expression (6–8, 10).

In conclusion, the temporal and spatial patterns of *D-mef2* expression during embryogenesis suggest that it could be a key player in mesoderm differentiation. It is expressed in all presumptive mesoderm prior to the splitting process that generates the somatic and visceral/heart mesoderm. After the subdivision, new patterns of *D-mef2* expression emerge, and its continuing presence in both the somatic and visceral/heart mesoderm suggests that *D-mef2* is needed in cell fate specification within these two mesodermal layers.

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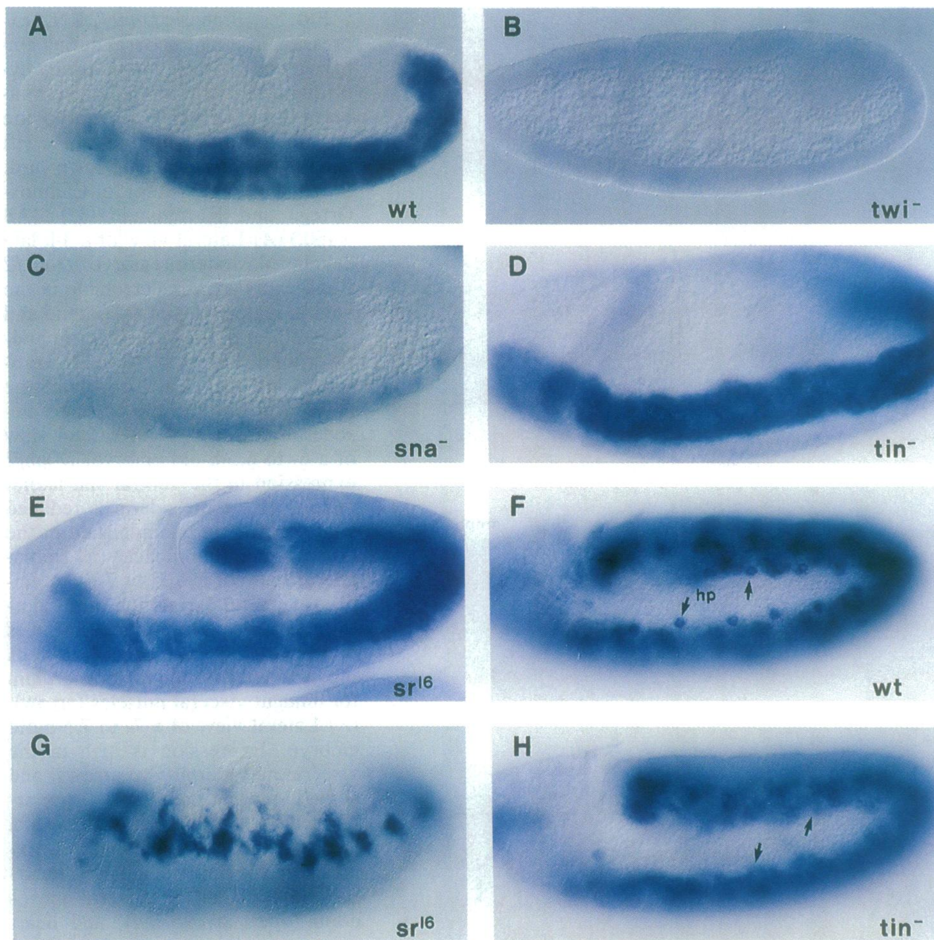

FIG. 6. *D-mef2* RNA expression in mutants that are defective in the formation of the mesoderm or its derivatives. Lateral views of wild-type and mutant embryos at early germband-extension (*A–D*), late germband-extension, or germband-retraction (*E–H*). (All ×160.) (*A*) Wild-type embryo with *D-mef2* expression in the mesoderm. (*B*) *twi* mutant embryo showing absence of *D-mef2* expression. (*C*) *sna* mutant embryo exhibiting low levels of *D-mef2* expression. (*D* and *E*) *tin* and sr^{16} (= *DFR1*) mutant embryos with normal *D-mef2* expression. (*F*) Wild-type 7.5-h late germband-extended embryo with *D-mef2* expression in heart precursor cells (hp; arrows) and somatic mesodermal cells. (*G*) sr^{16} mutant embryo showing disorganized and reduced number of *D-mef2*-positive cells. (*H*) *tin* mutant embryo with normal *D-mef2* expression in the somatic mesodermal cells. No expression is observed in the region of heart precursor cells (arrows).

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