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The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*

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SUMMARY

The homeobox-containing gene *tinman* (*msh-2*, Bodmer et al., 1990 *Development* 110, 661-669) is expressed in the mesoderm primordium, and this expression requires the function of the mesoderm determinant *twist*. Later in development, as the first mesodermal subdivisions are occurring, expression becomes limited to the visceral mesoderm and the heart. Here, I show that the function of *tinman* is required for visceral muscle and heart development. Embryos that are mutant for the *tinman* gene lack the appearance of visceral mesoderm and of heart primordia, and the fusion of the anterior and posterior endoderm is impaired. Even though *tinman* mutant embryos do not have a heart or visceral muscles, many of the somatic body wall muscles appear to develop although abnormally. When the *tinman* cDNA is ubiquitously expressed in *tinman* mutant embryos, via a heat-shock promoter, formation of heart cells and visceral mesoderm is partially restored. *tinman* seems to be one of the earliest genes required for heart development and the first gene reported for which a crucial function in the early mesodermal subdivisions has been implicated.

Key words: mesoderm, cell fate, heart, viscera, homeobox, *Drosophila* muscle, *tinman*

INTRODUCTION

The generation of the dorsal-ventral polarity in the early *Drosophila* embryo is under the control of a cascade of maternally active genes. This process culminates in a graded transport of the *dorsal* morphogen from the cytoplasm to the nucleus (for review, see Govind and Steward, 1991; St Johnston and Nüsslein-Volhard, 1992). As a consequence of high nuclear concentration of Dorsal protein in the ventral region of the early embryo, the two zygotic genes, *twist* (*twi*) and *snail* (*sna*), are activated to direct mesoderm formation (Alberga et al., 1991; Thisse et al., 1991; Jiang et al., 1991; Ip et al., 1992). Both *twi* and *sna* are required for the initial steps of mesoderm differentiation. In the absence of *twi* or *sna* function, the presumptive mesoderm does not differentiate and normal initiation of gastrulation, such as ventral furrow formation, does not occur (Simpson, 1983; Nusslein-Volhard et al., 1984; Thisse et al., 1987, Leptin and Grunewald, 1990). It has been suggested that *twi*, which codes for a basic helix-loop-helix protein (Thisse et al., 1988), functions as a positive activator of mesoderm differentiation, whereas *sna* (Boulay et al., 1987), a zinc finger protein, has a repressor function (Leptin, 1991; Kosman et al., 1991). However, the molecular basis for subsequent events of mesoderm development that are involved in the subdivision of the mesodermal mass has not been explored and functional requirements of genes that may act at this level have not been identified. In recent years, several genes have been cloned that are expressed in the developing mesoderm, and the expression patterns of these genes point to possible functions in the specification or differentiation of mesoderm derivatives: expression in a subset of precursors for somatic muscles (Michelson et al., 1990; Dohrmann et al., 1990; Nose et al., 1992; Bourgouin et al., 1992), in visceral muscles (Bodmer et al., 1990; Barad et al., 1988) and in the heart (Frasch et al., 1987, Bodmer et al., 1990; Lai et al., 1991; Wolfgang et al., 1991; Lee et al., 1991). In addition, a number of enhancer trap lines show reporter gene expression in mesodermal tissues (Bier et al., 1989; Hartenstein and Jan, 1992a) suggesting that many genes with a function during mesoderm development have yet to be identified. A recent study showed that a gene expressed in a subset of somatic muscle precursors was required for the formation of these muscles (Bourgouin et al., 1992). The neurogenic genes, apart from their well known function during neurogenesis (for review see Campos-Ortega and Jan, 1991; Artavanis-Tsakonas and Simpson, 1991) have also been implicated in playing a role in determining the number of certain somatic muscle precursors (Corbin et al., 1991) and cadioblasts (Hartenstein et al., 1992).

The present report describes the first gene to play a role in mesodermal subdivisions subsequent to the initial determination by *twist* and *snail*. The presented data show that the homeobox-containing gene *tinman* (*tin*), which is expressed very early in mesoderm development (Bodmer et al., 1990), is required for the formation of the heart and visceral muscle primordia indicating a function in specifying mesodermal cell fates.

MATERIALS AND METHODS

Drosophila stocks and genetic crosses

All fly strains were kept on standard corn meal medium at 18°C or 25°C. tin^{EC40}, Df(3R)GC14 were generously provided by M. L. Pardue and J. Mohler. Df(3R)eBS2, rsd/TM3 (deficient for 93C-F, E. Stevenson and M. Binder, personal communication; Bodmer et al., 1990) and Df(3R)eGp4/TM6B (Mohler and Pardue, 1984) were kindly provided by the Indiana Stock Center). The mutant strains were crossed to a different TM3 balancer chromosome (Lindsley and Grell, 1968) containing an enhancer trap insertion that expresses the reporter strongly in the trunk region of the embryo (TM3-Pw^+-*lacZ* was obtained from E. Grell). This permitted identification of homozygous mutant embryos when double stained with a tissue-specific antibody (or a nucleotide probe) and anti- -galactosidase antibodies (or with a *lacZ* antisense probe). Homozygous mutant embryos (i.e. lacking the balancer chromosome) were negative for *lacZ* expression.

Several second chromosome enhancer trap lines (Bier et al., 1989) with reporter gene expression in the heart or visceral mesoderm were crossed into a *tin* mutant background (similar to Bodmer et al., 1990). Male and female F_1 progeny of the genotype *yw*;P*lacZ*/+;*tin*/+ were then mated and the embryos of the appropriate stage were collected. Since the mutants did not abolish *lacZ* reporter expression in all tissues, the absence of expression in heart or visceral muscles of homozygous *tin* mutants was clearly identifiable.

Tissue in situ hybridization

Embryos were stained with digoxigenin (Boehringer)-labelled DNA probes (Tautz and Pfeifle, 1989) as described previously (Bodmer et al., 1990). Embryos were double-labelled with a *lacZ* probe and a *tin* cDNA probe (Fig. 1A,B), or with a *tin* probe only (Fig. 1C).

Immunocytochemistry

Procedures for antibody staining were carried out as described (Bodmer et al., 1990). Antibody dilutions were as follows: Anti-Zfh-1 (Lai et al., 1991) 1:1000, anti-Eve (Frasch et al., 1987) 1:5000, anti-DMM (*Drosophila* muscle myosin, Kiehart and Feghali, 1986) 1:1000, anti-Disco (Lee et al., 1991) 1:10, anti-FasIII (2D5, Patel et al., 1987) 1:10 and anti-FasII (1D4, G. Helt and C. Goodman, unpublished) 1:10. Anti- -galactosidase (Vector Labs) was preabsorbed with wild-type embryos (Bodmer et al., 1990) and used at a dilution of 1:4000.

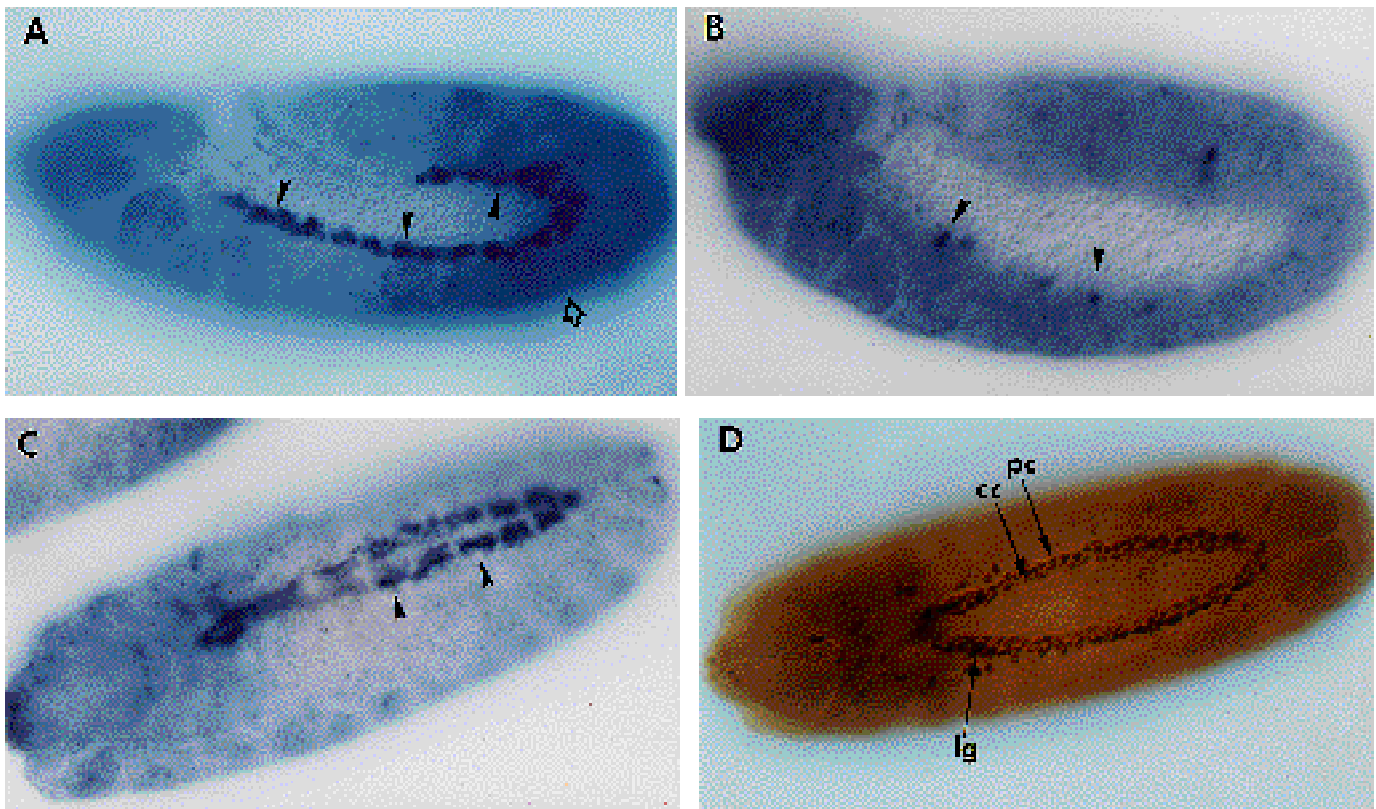

Fig. 1. Wild-type (A,C,D) and tin^{EC40} mutant (B) expression pattern of *tin* RNA. All embryos are in the dorsal up and anterior to the left orientation unless indicated otherwise. (A) $7\frac{1}{2}$ hour embryo (late stage 11, Campos-Ortega and Hartenstein, 1985) heterozygous for tin^{EC40} showing the wild-type *tin* RNA expression in segmentally repeated 'beads' of heart precursor cells (arrowheads). In order to distinguish heterozygous (A) from homozygous *tin* mutant embryos (B) the non-*tin* chromosome had been marked with P(*lacZ*) (obtained from H. Bellen); the heterozygous embryo shows *lacZ* expression in the trunk region (open arrow). (B) Homozygous tin^{EC40} of the same stage as A expresses *tin* RNA only in a few cells (arrowheads). (C) Dorsal view of 14 hour (stage 17) wild-type embryo showing *tin* expression in segmentally repeated 4-cell-wide strips (arrowheads). (D) Dorsal view of a 12 hour (stage 16) wild-type embryo just before dorsal closure, which has been stained with an antibody against Zfh-1, a protein expressed in the four rows of heart cells. cc indicates the cardial cells that will form the central heart muscle, pc indicates the pericardial cells and lg indicates the lymph glands that are associated with the heart.

Molecular biology techniques

Genomic DNA from the *tin* gene was isolated from a lambdaDash library (produced by Statagene) using the 1.7 kb *tin* cDNA (Bodmer et al., 1990) according to standard procedures (Maniatis et al., 1982). Isolated genomic *tin* clones were restriction mapped and the intron junctions were sequenced (according to Sanger et al., 1977).

For genomic Southern blots, DNA from homozygous mutant embryos was obtained as follows: Heterozygous (*tin*/+) mothers and fathers were crossed, the progeny embryos were allowed to hatch and the unhatched embryos were collected. The great majority of these unhatched embryos were homozygous for *tin* as determined by their bloated gut appearance (because of the absence of midgut constrictions). Preparation of genomic DNA for tin^{EC40}, Df(3R)GC14, Df(3R)eBS2 and Df(3R)eGp4 was performed as described by Roberts (1986). Approximately 5 μg genomic DNA was loaded in each lane (Fig. 2B) and Southern blots were prepared and hybridized with the 1.7 kb *tin* cDNA probe according to Maniatis et al. (1982).

PCR of *tin* mutant DNA

Homozygous tin^{EC40} DNA (see above) was isolated with PCR (Innis et al., 1990) using the primers 5 -CAACAGCTCCGCT-GAG and 5 -TCGGACTTCAACTTCAG targeting a 900 bp fragment which contains the homeodomain. Five independent PCR amplification were subcloned into the pCR[1000] vector (Invitrogen) and sequenced (Sanger et al., 1977). Two different plasmids from each of these PCR amplifications of tin^{EC40} were sequenced.

Heat-shock *tinman* flies

The heat-shock *tin* (hs*tin*) construct was made by inserting the full-length *tin* cDNA into the *Kpn*I site of the P-element vector pWH1 vector, a derivative of pHT4 containing the *white* (*w*) mini-gene (H. Vaessin, unpublished). The hs*tin* vector was injected into w^1 embryos in conjunction with the helper plasmid 25.7wc (Karess and Rubin, 1984). Of several transformants, the hs*tin*9A insertion on the 3rd chromosome was used in most experiments. hs*tin*9A was combined with tin^{EC40} and Df(3R)GC14 and kept over TM3-Pw^+-*lacZ* for identification of embryos that carry a homozygous *tin* mutation (see above).

Heat-shock treatments were carried out as follows: Embryos were collected on grape agar plates for 2 hours at 25°C, aged for 4 hours at 25°C, then placed for 30 minutes in a 37°C water bath for heat-shock, followed by a 30 minutes incubation at room temperature. The heat-shock was repeated once or twice. Then the

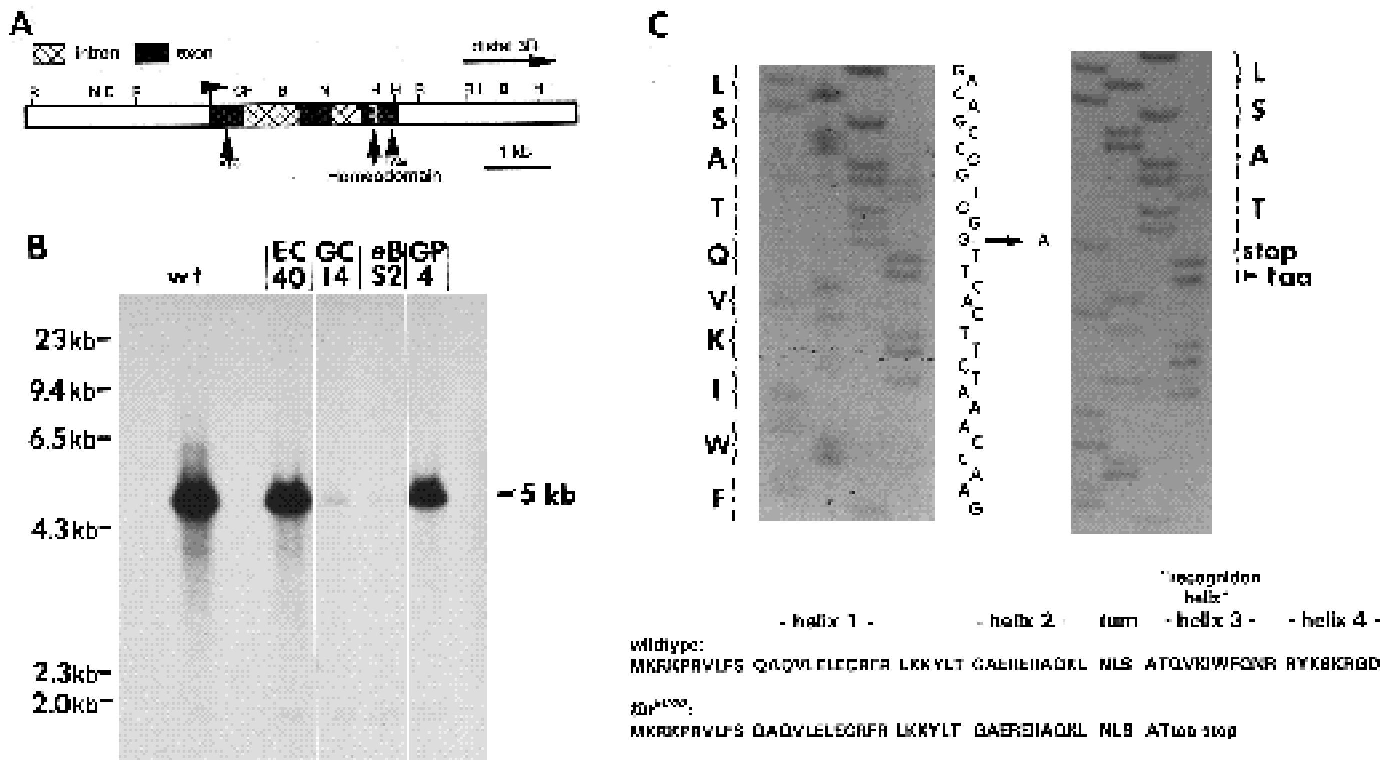

Fig. 2. (A) Map of the genomic region of *tin*. The transcribed region of *tin* (Bodmer et al., 1990) contains two introns, the first is 0.9 kb in length and starts after base pair 474 (Bodmer et al., 1990), the second one is 0.5 kb in length and starts after base pair 1087 (Bodmer et al., 1990). Restriction sites are indicated: R, *Eco*RI; B, *Bam*HI, C, *Cla*I; H, *Hin*dIII; N, *Nhe*I. ATG, TAG are the hypothetical translation start and stop sites, respectively. (B) Genomic Southern blot of wild-type and homozygous *tin* mutant DNA (see Experimental Procedures) probed with the 1.7 kb *tin* cDNA (Bodmer et al., 1990). The lanes with Df(3R)GC14 and Df(3R)eBS2 DNA show virtually no hybridization to the *tin* probe. (This also demonstrates that this procedure highly enriches for mutant DNA derived). The tin^{EC40} and Df(3R)eGp4 lanes show no polymorphism with *Eco*RI and several other restriction enzymes (data not shown). (C) In order to test if tin^{EC40} is due to a point mutation, tin^{EC40} DNA has been isolated with PCR (Innis et al., 1990). Sequencing of tin^{EC40} showed a single base pair change in the 'recognition helix' of the homeodomain. The sequencing ladder on the left is derived from wild-type genomic DNA, the one on the right from tin^{EC40} using a reverse primer (the corresponding bases are indicated in between the ladders). Conceptual translation of the reverse complement of the sequences, shown on either side of the ladders, results in a stop codon for tin^{EC40}. The amino acid sequences of wild-type and mutant homeodomains are shown at the bottom.

embryos were incubated for several more hours at 25°C before fixation and antibody staining (see above).

RESULTS

tinman expression in mesodermal tissues

The *tin* gene is first expressed at the blastoderm stage in all mesodermal primordia of the trunk region and this expression occurs immediately after the onset of expression of the mesoderm determinant, *twist* (Bodmer et al., 1990). *tin* expression persists during gastrulation as the mesoderm invaginates ventrally. After a portion of the mesoderm has migrated dorsally beneath the ectoderm (Leptin and Grunewald, 1990; Bodmer et al., 1990), two layers form. The inner layer of mesoderm contributes to the visceral muscles, while the outer layer gives rise to the somatic muscles (Poulson, 1965; Campos-Ortega and Hartenstein, 1985). At this point, *tin* RNA becomes restricted to the visceral mesoderm and the heart precursors (the latter are

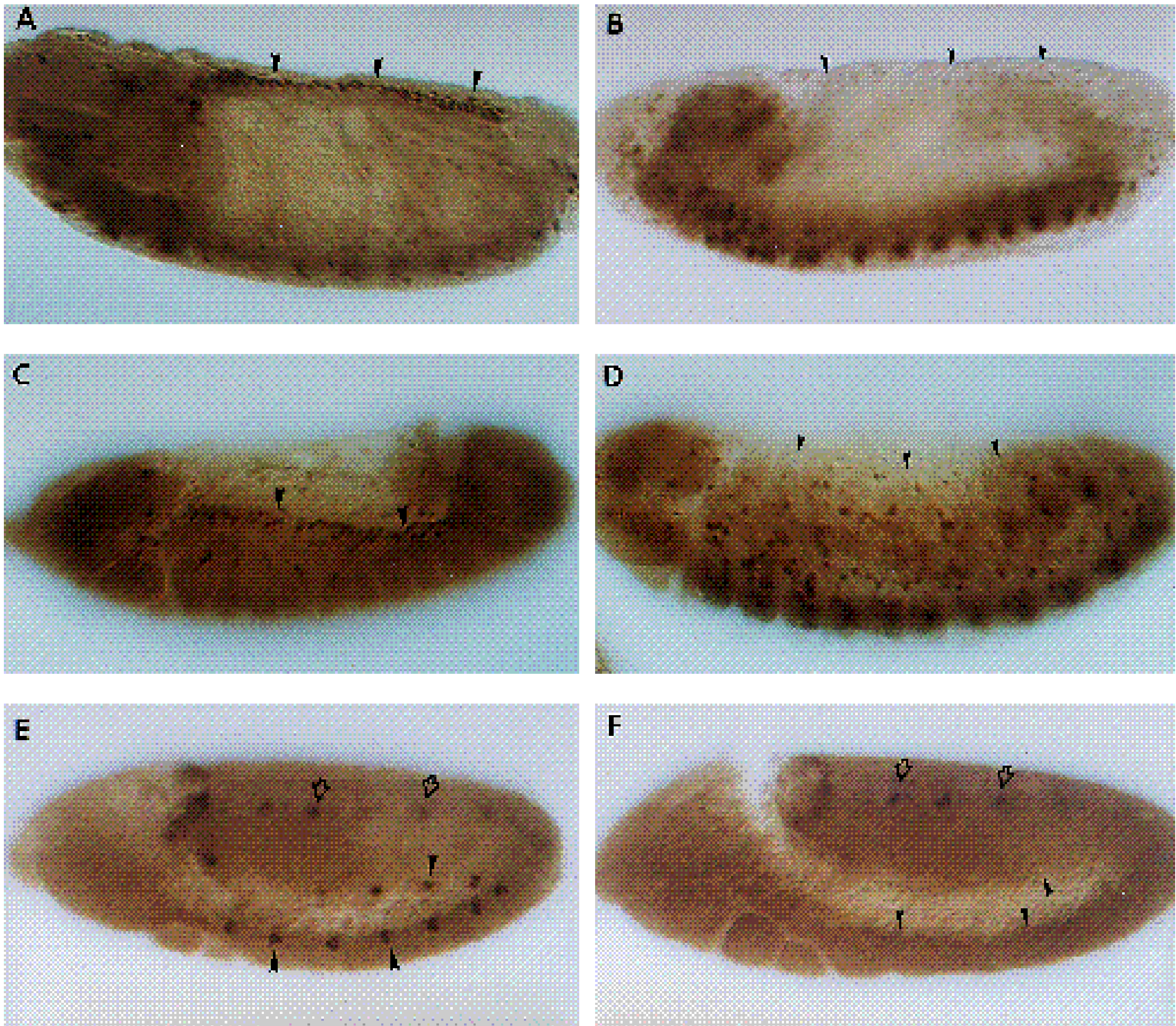

Fig. 3. Wild-type (A,C,E) and *tin*^{EC40} (B,D,F) embryos have been stained with anti-Zfh-1 (A-D) and anti-Eve (E,F) antibodies to illustrate the development of the heart. (A) Side view of a 14-hour-old (stage 17) embryo showing Zfh-1 immunoreactivity in the morphologically differentiated heart (arrowheads). (B) Same stage *tin* mutant embryo shows no immunoreactivity and no morphological differentiation of the heart. (C,D) 8- to 9-hour-old (stage 12/13) embryos showing the presence (C) and the absence (D) of heart precursors (arrowheads). The embryo in D is a slightly more ventral view as compared with the embryo in C. (E,F) 7-hour-old (late stage 11) embryos showing the presence (arrowheads in E) and the absence (arrowheads in F) of early heart precursors (during the germ band extended stage, embryos are folded over). Note, in wild-type embryos, only a subset of the heart precursors are labelled with anti-Eve (E) as compared with the *tin* expression pattern at this stage (Fig. 1A). Note that Eve expression in the CNS appears unaffected (open arrows).

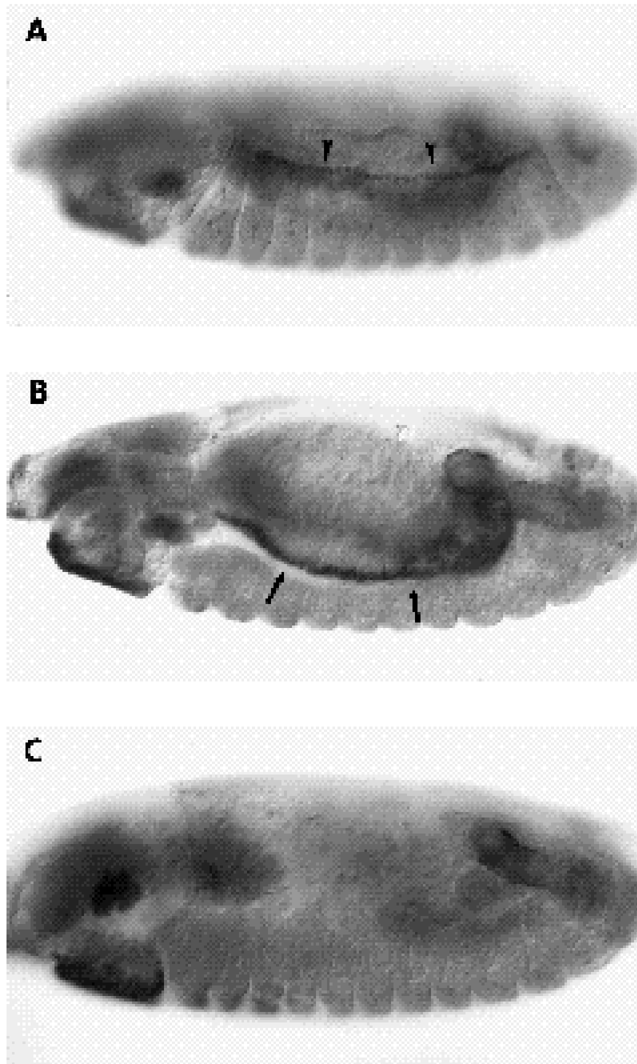

Fig. 4. Wild-type (A,B) and *tin*^{EC40} (C) embryos have been stained with anti-Disco antibodies (Lee et al., 1991). (A) Side view of a 10-hour-old (stage 13) embryo showing Disco immunoreactivity in the cardiac precursor cells of the developing heart (arrowheads). (B) Same embryo shown at a deeper focal plane showing anti-Disco staining of the visceral mesoderm (arrows). (C) Same stage *tin* mutant embryo shows no anti-Disco staining in either the cardiogenic region or in the visceral region of the midgut, but the staining of the visceral mesoderm of the hindgut is unaffected (see also legend to Fig. 5).

located most dorsally between the somatic and visceral mesoderm, Bodmer et al., 1990). Shortly thereafter, *tin* RNA is exclusively localized in the developing heart and this expression persists throughout embryogenesis. The heart is assembled as an elongated dorsal vessel by segmentally repeated rows of mesodermal cells originating from both sides of the embryo (Fig. 1A,C,D; see also Bodmer et al., 1990; Hartenstein and Jan, 1992).

Identification of *tinman* null mutations

In order to determine if the dynamics of *tin* expression reflect an involvement of *tin* in specifying mesodermal subdivisions, I have analyzed the phenotype of mutations in the *tin* gene. The 93D,E region, the location of the *tin* gene on the third chromosome (Kim and Nirenberg, 1989), has previously been mutagenized (Mohler and Pardue, 1984), and two of the mutations that are still available show abnormalities in mesoderm differentiation. Both mutations lack visceral muscles and heart formation suggesting that a defect in the *tin* gene may be responsible for the observed mutant phenotype (shown below). In order to find out whether or not the *tin* gene is, in fact, defective in these putative *tin* mutants, genomic DNA from these mutants was analyzed. It has been reported that one mutation, Df(3R)GC14, covers four lethal complementation groups and the other, *tin*^{EC40}, is a putative point mutant generated with the chemical mutagen ethyl-methanesulfonate (Mohler and Pardue, 1984). The two mutations exhibit identical phenotypes with respect to mesodermal derivatives and they do not complement each other, indicating they affect the same gene function.

Molecular cloning of genomic DNA from the wild-type *tin* gene revealed that the *tin* coding region is contained within a 5 kb *Eco*RI fragment and that the gene has two introns (Fig. 2A). Analysis of genomic DNA from wild-type and *tin* mutants indicates that the *tin* mutations are due to a molecular defect in the *tin* gene itself (Fig. 2B,C). Southern blots derived from genomic DNA of wild-type and homozygous *tin* mutants (see Materials and methods) were hybridized with the full-length *tin* cDNA (Bodmer et al., 1990). The *tin* probe does not hybridize to genomic DNA from Df(3R)GC14 or to genomic DNA containing the larger deletion Df(3R)eBS2. This indicates that these two mutants are deficient for the *tin* coding region (Fig. 2B). However, the *tin* probe does hybridize to *tin*^{EC40} genomic DNA and there appears to be no restriction fragment polymorphism (Fig. 2B). The breakpoint for Df(3R)GC14 lies between the *tin* gene and another gene, NK3 (Kim and Nirenberg, 1989), which is about 7 kb centromere-distal to *tin* on the right arm of the third chromosome (data not shown). The deficiency Df(3R)eGp4 (Mohler and Pardue, 1984) partially overlaps with Df(3R)GC14 on the centromere-proximal side. These two deficiencies in *trans* from each other uncover the heat-shock gene *hsr* (Mohler and Pardue, 1984), the lack of which appears to have no apparent effect with respect to mesoderm development (data not shown). Although Df(3R)GC14 lacks *tin* coding sequences, the possibility that a nearby gene is also involved in generating the mutant phenotype could not be ruled out by these data.

In order to determine whether *tin*^{EC40} contains a mutation in *tin* itself rather than in a nearby gene, *tin*^{EC40} genomic DNA was isolated by PCR. Five independent PCR amplifications were sequenced. All of them showed a nonsense codon due to a single base pair change in the homeobox region (Fig. 2C). This region is known to be important for DNA binding (Desplan et al., 1985; for review see Gehring, 1987; Treisman et al., 1992). The stop codon of *tin*^{EC40} is at the beginning of the ‘recognition helix’ (Fig. 2C) suggesting that a non-functional, truncated protein is generated. This strongly indicates that the observed mutant phenotype of *tin*^{EC40} is caused by the loss of function of the *tin* gene itself.

Consistent with the mutant sequence data are tissue in situ

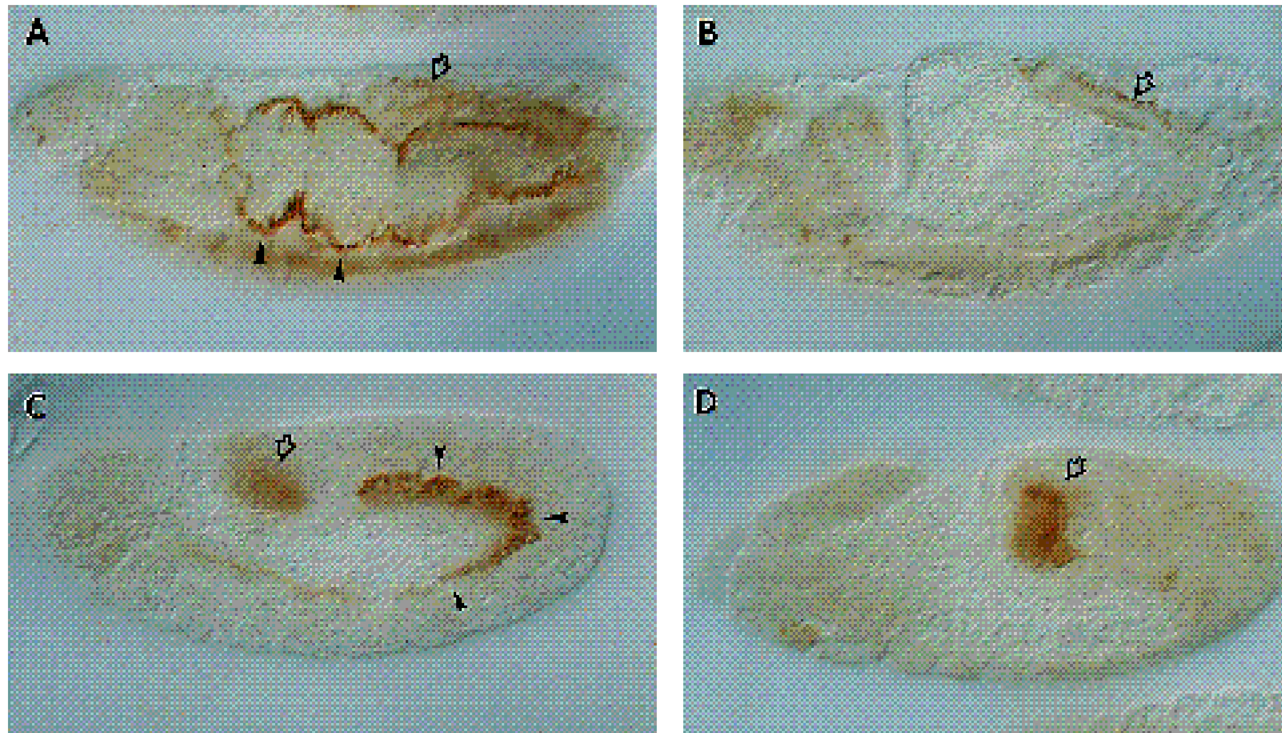

Fig. 5. Wild-type (A,C) and *tin*^{EC40} (B,D) embryos have been stained with anti- -galactosidase antibodies to illustrate the development of the visceral muscles. (A) Side view of a 14-hour-old (stage 17) embryo of the enhancer trap line B6-2-30 (Bier et al., 1989; Hartenstein and Jan, 1992) showing anti- -galactosidase immunoreactivity in the morphologically differentiated visceral muscles of the midgut (arrowheads) and the hindgut (open arrow). Note the constrictions of the midgut (between arrowheads). (B) Same stage mutant embryo lacks visceral muscle formation of the midgut. Note also the absence of midgut constrictions. Visceral muscles of the hindgut apparently develop normally (open arrow). (C,D) 7-hour-old (late stage 11) embryos showing the presence (arrowheads in C) and the absence (D) of visceral mesoderm in the midgut region. The primordium for the hindgut visceral muscles seems unaffected.

hybridizations of mutant embryos with the *tin* cDNA probe. Whereas Df(3R)GC14 embryos show no *tin* expression, homozygous *tin*^{EC40} embryos show normal *tin* RNA levels at early gastrula stages (data not shown). However, *tin* expression in *tin*^{EC40} mutants drops off rapidly at the time when wild-type *tin* RNA becomes restricted to the visceral mesoderm. Only a few cells in the heart primordial region stay positive long enough to be detected (Fig. 1B). This finding is consistent with the hypothesis that a functional Tin protein is necessary for maintaining its own expression in the heart, possibly through some autoregulatory mechanism. Alternatively, the reduction of *tin* mRNA in *tin*^{EC40} could also be due to an increased instability of the mutant transcripts caused by the nonsense mutation. From this analysis, I conclude that the Df(3R)GC14 and *tin*^{EC40} mutants are null mutants of the *tin* gene (referred to as *tin* mutants in the following analysis of the muscle phenotypes).

tinman **is required for heart development**

In order to determine the function of *tin* in heart development *tin* mutant embryos were incubated with an antibody against Zfh-1, a gene product that contains several zinc fingers and a homeodomain (Fortini et al., 1991) and which is expressed in the heart (Lai et al., 1991). At mid-embryonic stages (stage 16-17, Campos-Ortega and Hartenstein, 1985) when the heart is morphologically differentiated, Zfh-1 protein is localized in all four rows of heart cells as well as the lymph glands (Figs 1D, 3A), which are the larval blood-forming organs (Rizki, 1978). In *tin* mutant embryos, no Zfh-1 immunoreactivity can be detected in the region where the heart normally forms (Fig. 3B) indicating that *tin* mutants have no heart. Inspection of younger embryos (stage 12/13) marked with anti-Zfh-1 antibodies indicate that not only is there a lack of heart differentiation but that the precursor cells for the heart, the cardioblasts, are also absent (Fig. 3C,D). The Even-skipped protein (Eve, Frasch et al., 1987) is the product of another homeodomain-containing gene which is normally expressed in a subset of heart precursors shortly after *tin* expression is observed in these cells (Fig. 3E). Similar to *zfh-1* expression, no Eve-positive heart precursors staining can be detected in *tin* mutant embryos with anti-Eve antibodies (Fig. 3F). In addition, the gene *disconnected* (*disco*, Lee et al., 1991) is expressed in the developing cardial cells (Fig. 4A), and this expression is absent in *tin* mutants in the presumptive cardiogenic region (Fig. 4C). These results indicate that *zfh-1*, *eve* and *disco* expression in cardioblasts depends on *tin* function. Several other markers of developing heart cells, including a number of enhancer trap lines (Bier et al., 1989), terminal differentiation markers such as anti-muscle myosin (Fig. 7B,D), as

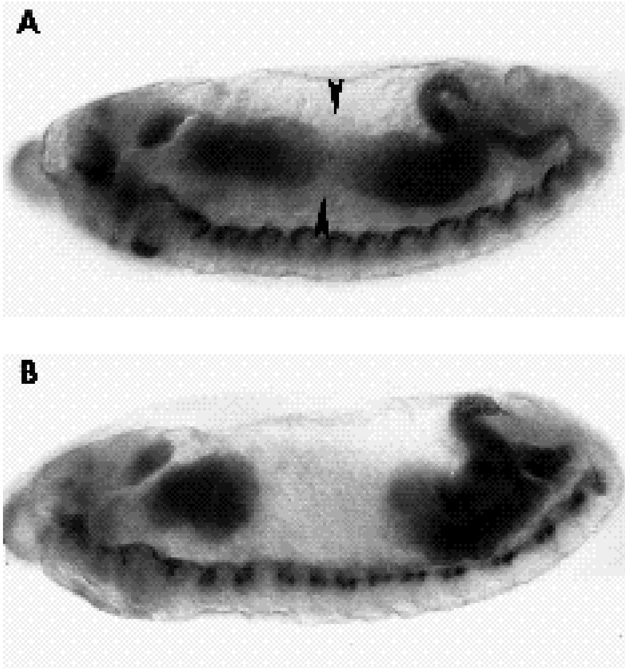

Fig. 6. Wild-type (A) and tin^{EC40} (B) embryos (10 hours old) have been stained with anti-FasII antibodies to illustrate the endodermal development of the midgut. (A) A sheath of fasII-positive endodermal cells of the anterior and posterior midgut primordium are joining each other in the middle region of the embryo at this stage (arrowheads). (B) FasII-stained endodermal cells in *tin* embryos remain in the anterior and posterior regions and will not form a contiguous sheath around the yolk.

well as morphological observations (using Nomarski optics, Figs 3B,7D), show no evidence of heart formation in *tin* mutant embryos (data not shown). Therefore, it is likely that, in *tin* mutants, cardioblasts are not formed, either because the cells that normally give rise to these cells are not specified appropriately or because they die before showing any sign of differentiation. Interestingly, in wild-type embryos, both Zfh-1 and Eve proteins are initially expressed in a subset of the heart precursor cells (Fig. 3E) as compared to the *tin* expression pattern (Fig. 1A). Later Zfh-1 protein is present in all heart cells that form the dorsal vessel, cardial cells as well as pericardial cells (Fig. 1D). In contrast, Eve protein remains in a segmentally repeated subset of heart-associated cells (as well as in precursor cells for the dorsal-most somatic muscles, Frasch et al., 1987, see also Fig. 8A). These observations imply that not all the heart cells are equivalent along the anterior-posterior axis. Taken together, these results indicate that *tin* mutants lack appropriate specification of the heart precursor cells and consequently have no heart.

tinman is required for visceral muscle development

In order to determine a possible role of *tin* in visceral muscle formation, P*lacZ* enhancer trap lines with reporter gene expression in the early visceral mesoderm were crossed into a *tin* mutant background. Compared to the wild-type, *tin* mutants exhibit no reporter gene expression in visceral mesoderm or differentiated visceral muscles of the trunk region (Fig. 5). On the other hand, visceral muscles around the hindgut form normally (open arrow in Fig. 5A,B). Therefore, the *tin* gene appears to be involved in the specification of the visceral muscles associated with the midgut but not the hindgut. This observation is consistent with the expression pattern of *tin*: the extent of *tin* expression shortly after gastrulation probably does not include the mesodermal primordia for the hindgut muscles (see Bodmer et al., 1990). This would explain why differentiation of hindgut mesoderm is apparently not affected in *tin* mutants. Similar observations on the development of visceral muscles in *tin* mutants have been obtained with other enhancer trap lines as well as with antibodies such as against Disco (Fig. 4B,D) and against Fasciclin III (Fig. 8D,E, Patel et al., 1987). These findings suggest that *tin* is required not only for specification of the heart but also of the visceral muscles.

In addition to the lack of visceral muscles, *tin* mutant embryos also do not form midgut constrictions, a process that appears to involve signal transduction from the visceral mesoderm to the underlying endoderm (Panganiban et al., 1990; Immerglueck et al., 1990). Normally, the midgut is formed by a fusion of the anterior midgut primordium with the posterior midgut primordium on either side of the embryo (Campos-Ortega and Hartenstein, 1985). In order to investigate the formation of the midgut in *tin* mutants, embryos were stained with an antibody against Fasciclin II (FasII, G. Helt and C. Goodman, unpublished) which marks anterior and posterior midgut cells prior to their fusion (Fig. 6A). In *tin* mutants, FasII-positive cells of the anterior and posterior midgut do not fuse normally with one another, but rather stay as separate clumps in anterior and posterior regions, respectively (Fig. 6B). This suggests that midgut formation does not occur normally in the absence of visceral mesoderm. Since the formation of visceral mesoderm precedes the formation of the midgut (Fig. 5C, 8D), it appears that the visceral mesoderm mediates midgut morphogenesis.

tinman requirements for formation of somatic muscles

In order to determine if *tin* has also a function in the formation of somatic muscles of the trunk body wall, *tin* mutant embryos were stained with an antibody against muscle myosin (Kiehart and Feghali, 1986). In contrast to heart and visceral muscle development, the formation of the somatic body wall muscles appears only moderately unaffected (Fig. 7). However, the segmentally stereotyped muscle pattern of wild-type embryos is somewhat disorganized in *tin* mutants and the number of muscles that can be identified in each segment is usually lower than normal (Fig. 7C,D). Interestingly, anti-Eve antibodies not only stain heart-associated cells but also the dorsal-most somatic muscles (Figs 8A, 7B). In *tin* mutants, *eve* expression is not only absent in the heart, but also in these dorsal somatic muscles (Fig. 8B). Some dorsal somatic muscles, when inspected with anti-DMM antibodies, are present in *tin* mutant embryos although they show considerable pattern defects (arrows in Fig. 7D). This is in contrast to heart formation which does not occur (Fig. 7B,D). To test a

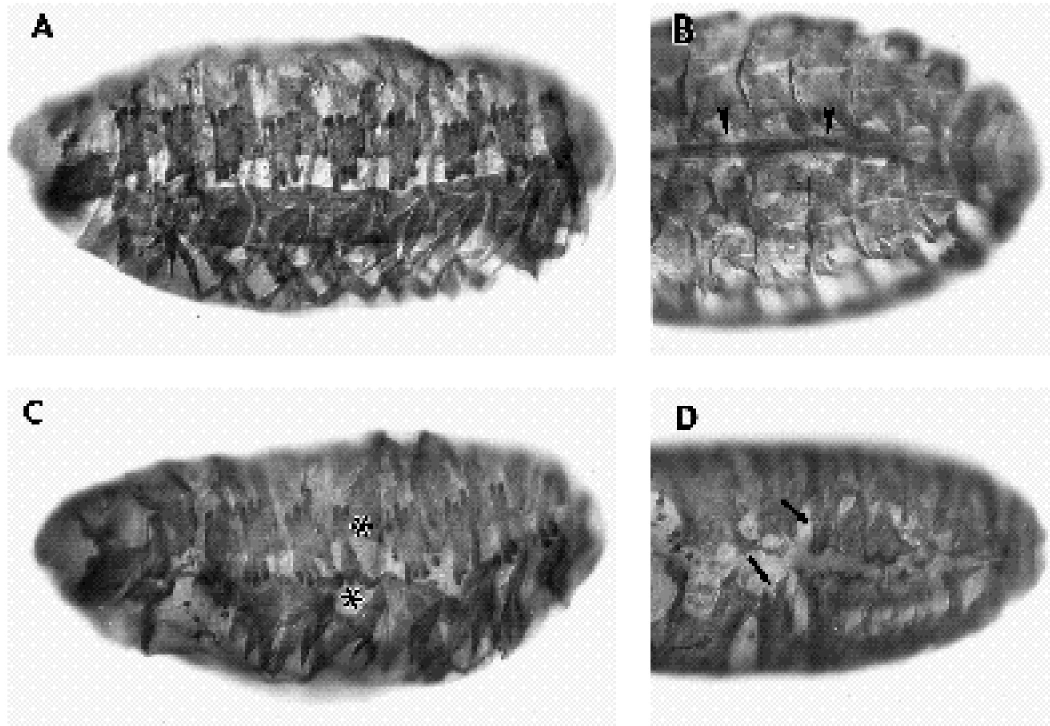

Fig. 7. Wild-type (A,B) and *tin*^{EC40} (C,D) embryos (14 hours, stage 17) have been stained with anti-DMM antibodies to illustrate the development of the somatic muscles. (A,C) Side view of the segmentally stereotyped pattern of somatic muscles. The pattern in the mutant (C) is not as regular as in the wild-type (A). Mutant embryos also appear to have a number of muscles missing in varying locations (asterisks). Most consistently reduced are the longitudinal muscle bands in the ventral regions (lower asterisk). (B,D) Dorsal view of the muscle pattern. The dorsal somatic muscle pattern (arrows) in the mutant (D) seems quite abnormal as compared with the wild-type (B). Note that most of these muscles are oriented dorsoventrally instead of longitudinally (arrows). Interestingly, *eve* is expressed in some of these muscles and this expression is absent in *tin* mutants. Anti-DMM also stains the cardial cells (see Fig. 1D) of the heart (arrowheads in B) and they are missing in *tin* mutants (D) indicating that absence of terminal differentiation markers of the heart in *tin* mutants.

possible function of *tin* in other mesodermal derivatives, such as the fat body, reporter gene expression of additional enhancer trap lines was analyzed in a *tin* mutant background, but no further requirements for *tin* were identified (data not shown).

Heat-shock *tin* transgene restores absence of heart and visceral muscle precursor cells

In order to associate the defect in the *tin* mutant embryos unequivocally with a lesion in the *tin* gene itself, a rescue of the mutant phenotype was attempted by expressing a *tin* transgene in a *tin* mutant background. The full-length *tin* cDNA was inserted into a transformation vector containing a heat-shock promoter (see Materials and methods). Transgenic (hs*tin*) flies were obtained by germline transformation (Roberts, 1986). Hs*tin* flies were crossed into a *tin* mutant background (*tin*$^{-}$) and subjected to repeated heat-shocks between 3 and 8 hours of development (see Materials and methods). Transgenic hs*tin*,*tin*$^{-}$ flies that were maintained at 25°C showed the *tin* mutant phenotype (Fig. 8B,E). However, if hs*tin*,*tin*$^{-}$ flies were heat-shocked by elevating the temperature to 37°C, they showed expression of Eve protein in cells in the same dorsal location beneath the epidermis as wild-type embryos (Fig. 8A,C). Similar results was obtained with anti-Zfh-1 antibodies (data not shown). Similarly, staining of heat-shocked embryos with anti-FasIII antibodies also showed a partial rescue of visceral mesoderm formation (Fig. 8D,F). This indicates that the lack of cardioblasts and visceral mesoderm in *tin* mutant embryos can be restored by supplying *tin* gene function ubiquitously via a transgene. Therefore, it is concluded that the normal *tin* gene function is required for the formation of the heart and visceral muscles.

Hs*tin*,*tin*$^{-}$ mutant embryos were also tested for an alteration in the somatic body wall muscle pattern. Upon heat-shock, the somatic muscles developed as without heat-shock (data not shown), but a detailed analysis of the somatic muscle pattern was difficult to obtain due to the retarded development after heat-shock. In *tin* mutant embryos, Eve protein is absent not only in heart-associated cells but also in some of the dorsal somatic muscles (Fig. 8B). However, hs*tin*,*tin*$^{-}$ mutant embryos that were subjected to heat-shock treatment showed restoration of both heart-associated *eve* expression (Fig. 8C, arrowheads) and dorsal muscle-associated *eve* expression (Fig. 8C, arrows). This suggests that *tin* may also have a function in the development and/or pattern formation of some of the somatic muscles.

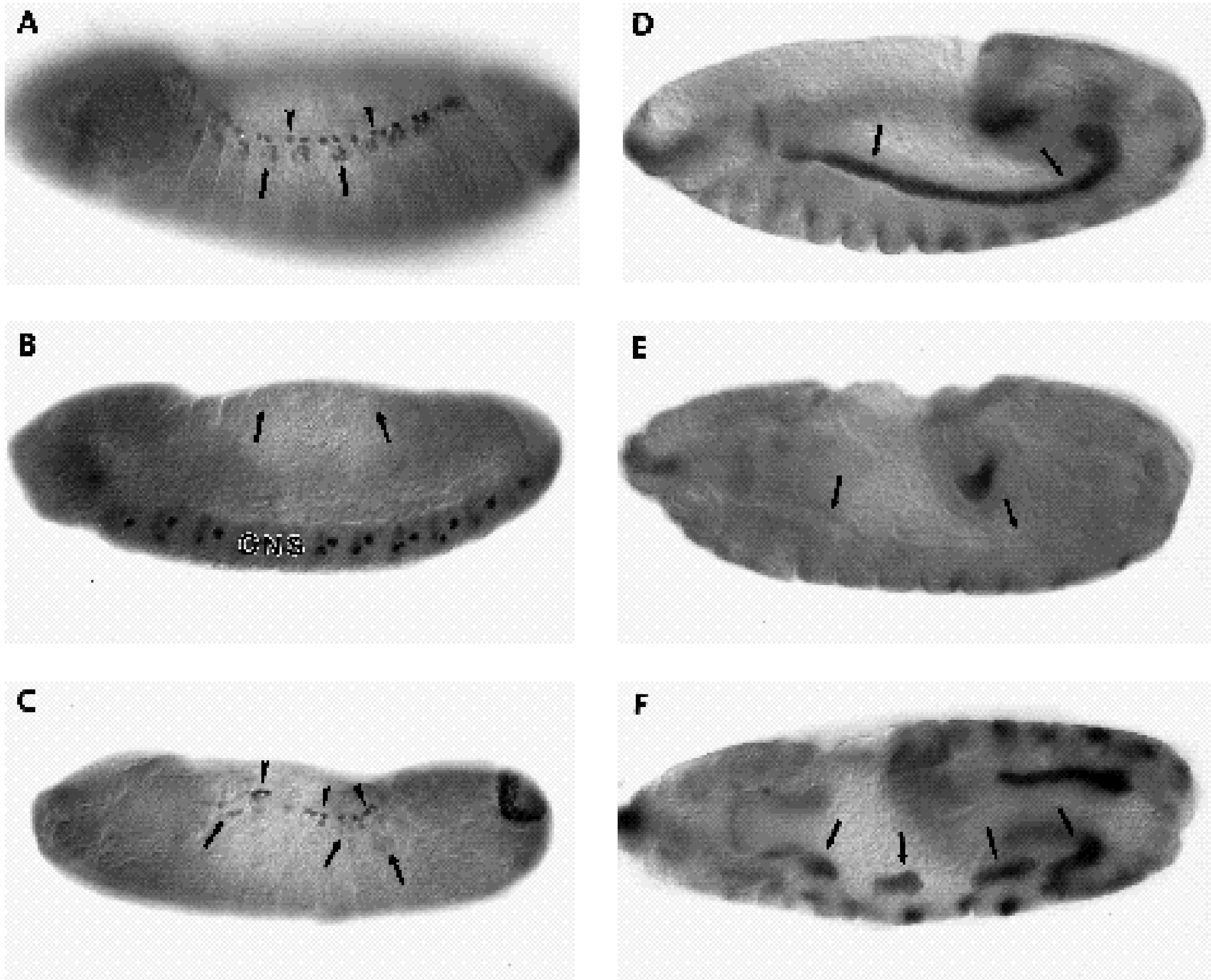

Fig. 8. Wild-type (A,D) and embryos of the genotype hs*tin*9A, Df(3R)GC14 (hs*tin*,*tin*$^-$) (B,C,E,F) were stained with anti-Eve (A-C, 10-12 hour embryos) and anti-FasIII (D-F, 8-9 hour embryos) antibodies to illustrate rescue of the *tin* mutant phenotype by ectopic *tin* expression. (A) Strong anti-Eve staining of cells associated with the developing heart (arrowheads) and weaker staining cells associated with developing dorsal somatic muscles (arrows). (B) hs*tin*,*tin*$^-$ embryo raised at 25°C showing no dorsal *eve* expression (arrows) that is a typical *tin* mutant phenotype. (C) hs*tin*,*tin*$^-$ embryo that was heat-shocked for three times 30 minutes at 37°C between 4 and 7 hours of development shows restoration of strongly anti-Eve-staining cells associated with the heart (arrowheads) and weakly staining cells associated with the dorsal muscles (arrows). (D) Anti-FasIII-positive visceral mesoderm (arrows). (E) hs*tin*,*tin*$^-$ embryo raised at 25°C showing no *fasIII* expression in the visceral mesoderm region (arrows). (F) hs*tin*,*tin*$^-$ embryo that was heat-shocked for three times 30 minutes at 37°C between 4 and 7 hours of development shows restoration of FasIII-positive visceral mesoderm (arrows). Similar results were obtained with hs*tin*9A,Df(3R)GC14 embryos.

DISCUSSION

The results of the restriction fragment polymorphism of Df(3R)GC14 and the sequencing of tin^{EC40} demonstrate that the (identical) phenotype observed in these two mutants is most likely due to a defect in the *tin* gene. The loss of *tin* gene function results in a failure of heart and visceral muscle development without severely affecting somatic muscle formation. In addition, expression of the full-length *tin* cDNA via a heat-shock promoter leads to rescue of the *tin* mutant phenotype, evidenced by the induction of *eve* and *fasIII* expression in heart and visceral mesoderm-associated cells, respectively. The pattern of *tin* expression, first in all the mesoderm and then only in the visceral mesoderm and the heart primordia, suggests that *tin* may play a role in the initial mesodermal subdivisions. The *tin* mutant phenotype strongly supports this hypothesis in that it is primarily the heart and visceral muscle development that is abolished with only moderate effects on somatic muscles. It is possible that the somatic muscle pattern is also indirectly affected by *tin* due to gross malformations of the gut. The dorsal-most somatic muscles express *eve* and this expression is abolished in *tin* mutants. This suggests that *tin* may be required for the normal pattern of a subset of somatic muscles.

The fact that *tin* contains a homeodomain (Bodmer et al., 1990) suggests that *tin* may control the expression of other

genes such as *zfh-1* (Fortini et al., 1991; Lai et al., 1991), *eve* (Frasch et al., 1987) and *disco* (Lee et al., 1991), all of which are nuclear genes with potential transcription factor functions themselves. It is tempting to speculate that a genetic cascade regulates the different events and specifies the different cell types during heart development. Since *tin* has a divergent homeodomain (Bodmer et al., 1990), it is possible that *tin* may serve as a prototype for finding genes involved in vertebrate heart development, analogous to the discovery of the *hox* genes and their emerging function in axial determination in vertebrates (for review see McGinnis and Krumlauf, 1992). At this point, however, it is not known if *tin* is required autonomously in heart precursor cells. In addition, the data do not rule out a requirement for *tin* in the early mesoderm as a prerequisite for normal heart formation.

Even though *tin* function is necessary for heart and visceral muscle development, it is not known whether *tin* is also sufficient to direct other mesodermal primordia, such as somatic mesodermal cells, to become heart or visceral muscles. Ubiquitous expression of *tin* via a heat-shock promoter in wild-type embryos did not result in significant abnormalities of somatic mesoderm or any other mesodermal derivatives that have been analyzed so far.

A role for *tinman* in mesodermal subdivisions

Taken together, these data indicate that there may be a network of genes that is involved in recruiting cardioblasts and visceral muscles primordia from the mesodermal mass, and later in specifying different cell fates in the developing heart and visceral muscles. It is likely that *tin* functions at the top of such a genetic hierarchy in specifying the heart and the visceral mesoderm, because *tin* is first expressed in all mesoderm and then becomes restricted to a large mesodermal subdomain, which does not form in the absence of *tin* function. In addition, the early onset of *tin* expression in the presumptive mesoderm at the blastoderm stage (Bodmer et al., 1990) suggests that *tin* may act immediately after *twist* to direct the developmental split of two major mesodermal layers, i.e. the somatic mesoderm and the visceral/heart mesoderm. It will be interesting to find out if Twist protein directly binds to the *tinman* promoter or if *tin* expression depends only indirectly on *twist*. A similar scenario has been proposed for the role of the *achaete-scute* genes in the selection of individual sensory organ precursor cells from larger groups of competent ectodermal cells (for review see Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1990; Campuzano and Modellel, 1992).

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