Development 121, 2107-2116 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995

The somatic-visceral subdivision of the embryonic mesoderm is initiated by dorsal gradient thresholds in *Drosophila*

Keith Maggert[1], Michael Levine[1] and Manfred Frasch[2]

[1]Department of Biology, Center for Molecular Genetics, Pacific Hall, 9500 Gilman Drive, UCSD, La Jolla, CA 92093-0347, USA
[2]Mount Sinai School of Medicine, Brookdale Center for Molecular Biology, One Gustave L. Levy Place, New York, NY 10029, USA

SUMMARY

The maternal dorsal regulatory gradient initiates the differentiation of the mesoderm, neuroectoderm and dorsal ectoderm in the early *Drosophila* embryo. Two primary dorsal target genes, *snail* (*sna*) and *decapentaplegic* (*dpp*), define the limits of the presumptive mesoderm and dorsal ectoderm, respectively. Normally, the sna expression pattern encompasses 18-20 cells in ventral and ventrolateral regions. Here we show that narrowing the sna pattern results in fewer invaginated cells. As a result, the mesoderm fails to extend into lateral regions so that fewer cells come into contact with dpp-expressing regions of the dorsal ectoderm. This leads to a substantial reduction in visceral and cardiac tissues, consistent with recent studies suggesting that dpp induces lateral mesoderm. These results also suggest that the dorsal regulatory gradient defines the limits of inductive interactions between germ layers after gastrulation. We discuss the parallels between the subdivision of the mesoderm and dorsal ectoderm.

Key words: *Drosophila*, mesoderm, induction, *dorsal*, *decapentaplegic*, gradient

INTRODUCTION

The dorsal (dl) regulatory gradient initiates the differentiation of the embryonic mesoderm, neuroectoderm and dorsal ectoderm (Govind and Steward, 1991; Jiang and Levine, 1993). There are peak levels of dl protein along the ventral surface of precellular embryos and progressively lower levels in lateral and dorsal regions (Rushlow et al., 1989; Steward, 1989; Roth et al., 1989). High concentrations of dl initiate the expression of mesoderm genes in ventral regions (Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991; Ip et al., 1992a), while low levels trigger the expression of neuroectodermal regulatory genes in lateral regions (Ip et al., 1992b). dl also functions as a repressor that restricts the expression of certain genes to dorsal regions where they are important for the differentiation of the dorsal ectoderm (Jiang et al., 1992, 1993; Huang et al., 1993; Kirov et al., 1993, 1994; summarized in Fig. 1).

In an effort to determine how the dl regulatory gradient initiates these three territories of tissue differentiation, previous studies have characterized target genes that are directly regulated by different concentrations of nuclear dl protein. These studies suggest that there are two classes of dl target promoters. Type 1 promoters contain low affinity dl-binding sites, so that expression is restricted to ventral regions where there are high concentrations of dl (Thisse et al., 1991; Jiang et al., 1991; Pan et al., 1991; Ip et al., 1992a). Type 2 promoters contain high affinity dl-binding sites and, consequently, they can be activated in both ventral and lateral regions, the presumptive mesoderm and neuroectoderm, in response to both high and low levels of dl (Ip et al., 1992b; Jiang and Levine, 1993).

Transcriptional repression is essential for converting these two thresholds into three territories of tissue differentiation (summarized in Fig. 1). The dl target gene, *snail* (*sna*), contains a type 1 promoter and, consequently, its expression is restricted to the ventral mesoderm (Leptin, 1991; Kosman et al., 1991; Alberga et al., 1991; Ip et al., 1992a). The sna protein functions as a sequence-specific repressor, and type 2 promoters that contain sna repressor sites are excluded from the ventral mesoderm and restricted to the lateral neuroectoderm (Ip et al., 1992b; Gray et al., 1994; summarized in Fig. 1). The third tissue territory, the dorsal ectoderm, is established by dl-mediated repression. dl activates target genes in the presumptive mesoderm and neuroectoderm, and also works as a repressor that restricts the expression of genes such as *zerknüllt* (*zen*; Jiang et al., 1992, 1993; Kirov et al., 1993; Lehming et al., 1994) and *decapentaplegic* (*dpp*; Huang et al., 1993) to the dorsal ectoderm. In principle, these latter genes can be activated throughout the early embryo, but they are excluded from the ventral mesoderm and lateral neuroectoderm by dl. The *zen* and *dpp* promoters contain dl-binding sites and closely linked 'corepressor' sites; the corepressors mediate long-range repression, or silencing (Doyle et al., 1989; Ip et al., 1991; Jiang et al., 1992, 1993; Kirov et al., 1993; Lehming et al., 1994).

After the three tissue territories are established prior to cellularization, they are subsequently subdivided into multiple

cell types during gastrulation and germ band elongation. For example, the dorsal ectoderm gives rise to dorsal epidermis and the amnioserosa (Ray et al., 1991; Rushlow and Arora, 1990). In the present study, we investigate the role of the dl regulatory gradient in the subdivision of the embryonic mesoderm. After formation of the ventral furrow and invagination of the ventralmost cells, the internal mesodermal layer appears to be 'naive' and the cells are capable of forming any particular mesodermal lineage. Normally, the ventralmost mesoderm, which is in contact with the overlying neuroectoderm, forms the somatic mesoderm (Dohrmann, 1990; Leptin et al., 1992; Azpiazu and Frasch, 1993; Bate and Rushton, 1993). In contrast, the lateral mesoderm, which abuts the dorsal ectoderm, gives rise to the gut musculature and heart tissues (Bodmer et al., 1990; Azpiazu and Frasch, 1993; Bodmer, 1993). Recent studies suggest that the initial subdivision of the mesoderm into ventral and lateral lineages involves inductive interactions with the dorsal ectoderm (Staehling-Hampton et al., 1994; Frasch, 1995). In particular, lateral mesoderm that comes into contact with dpp-expressing cells in the dorsal ectoderm is 'induced' to form the gut muscles and heart.

Here we show that a subtle alteration in the threshold response of *sna* to the dl regulatory gradient can disrupt the subdivision of the mesoderm into somatic, visceral and cardiac lineages. The type I *sna* promoter contains low affinity dl-binding sites and unlinked E boxes, which are binding sites for the twist (twi) protein and other helix-loop-helix (HLH) activators. The two proteins work synergistically to activate *sna* within the limits of the presumptive mesoderm in ventral and ventrolateral regions of precellular embryos (Ip et al., 1992a). The present study exploits a previously described fusion gene ('$Psna_g$') that contains the *sna*-coding sequence attached to a heterologous *twist* (*twi*) promoter sequence (Ip et al., 1994). The *twi* promoter includes two regulatory regions, the distal element (DE) and proximal element (PE). The 260 bp PE sequence contains low affinity dl-binding sites, but lacks E box sequences. As a result, the PE can be activated only by peak levels of dl protein in ventral regions (Jiang and Levine, 1993). The chimeric PE-sna fusion gene is expressed in just a subdomain of the normal mesoderm anlagen, spanning the ventralmost 12-16 cells (rather than 18-20 cells). Evidence is presented that these narrowed limits of *sna* expression cause a reduction in both the ventral furrow and the number of invaginated cells. Consequently, the mesoderm fails to extend into lateral regions, and fewer cells come into contact with the dpp-expressing regions of the dorsal ectoderm. This causes a severe reduction of lateral mesoderm derivatives, including both the gut muscles and heart. In contrast, the somatic musculature is still formed. We discuss these results in the context of dl thresholds and draw parallels between the subdivision of the mesoderm and dorsal ectoderm.

MATERIALS AND METHODS

Embryo collections and fly strains

The *sna*$^{-}$;2xPE-sna mutant strain was generated by crossing the 2xPE-sna P-transposon into a *sna*$^{-}$ genetic background. The *sna*^{IIG05} allele was used for this purpose since previous studies have shown that it probably corresponds to a null allele (Grau et al., 1984), despite the

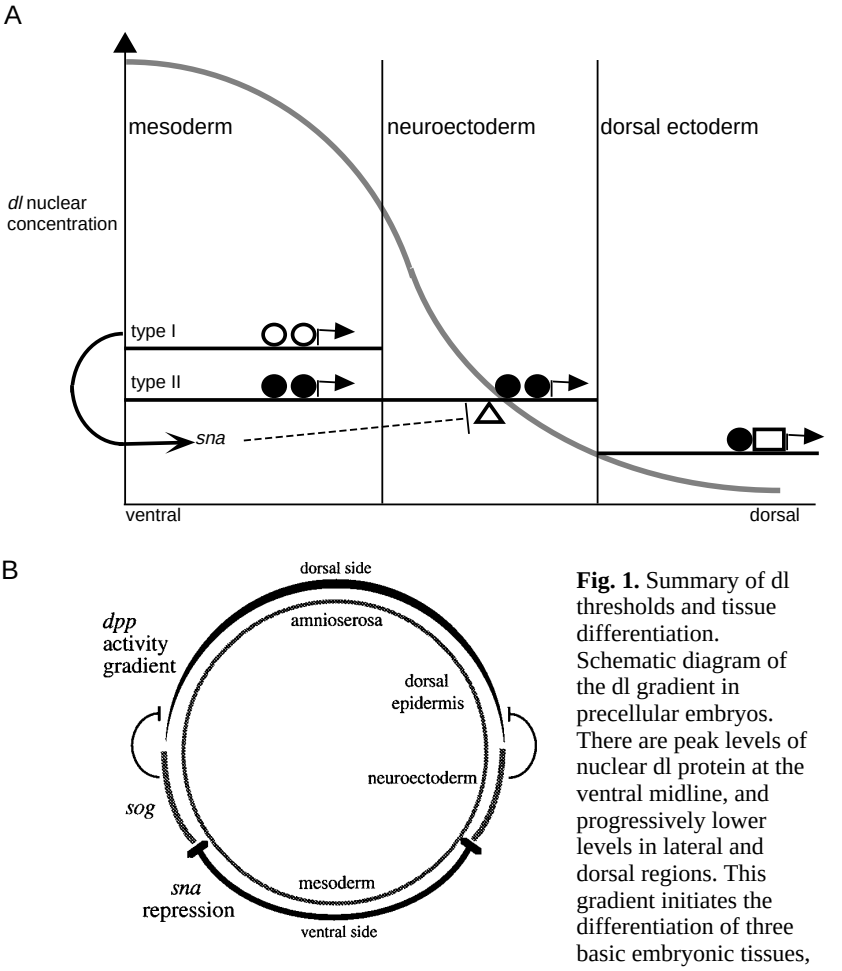

Fig. 1. Summary of dl thresholds and tissue differentiation. Schematic diagram of the dl gradient in precellular embryos. There are peak levels of nuclear dl protein at the ventral midline, and progressively lower levels in lateral and dorsal regions. This gradient initiates the differentiation of three basic embryonic tissues, the mesoderm, neuroectoderm and dorsal ectoderm. The characterization of 4 different dl target genes suggests that there are two essential threshold responses to this regulatory gradient. Type 1 promoters (i.e., those driving *twi* and *sna* expression) contain low affinity dl-binding sites (indicated by unfilled circles). As a result, these sites are occupied only in those regions containing high concentrations of dl protein (the presumptive mesoderm). In contrast, type 2 promoters contain high affinity dl-binding sites (indicated by the filled circles) and can be activated in regions where there are both high and low levels of dl (the presumptive mesoderm and neuroectoderm). Certain type 2 promoters (i.e., *rhomboid*) also contain sna protein repressor sites (indicated by the unfilled triangle). *sna* contains a type 1 promoter, so expression of sna protein is restricted to the mesoderm, thereby excluding the expression of these type 2 promoters from the mesoderm and restricting them to the neuroectoderm. Finally, the third territory of tissue differentiation, the dorsal ectoderm, requires that dl functions as a repressor. There are promoters that can be activated throughout the early embryo, but they are excluded from the presumptive mesoderm and neuroectoderm by high and low levels of dl, which now functions as a repressor due to its interactions with neighboring 'corepressors' (indicated by unfilled rectangle) also present in these promoters. These latter promoters respond to the same dl thresholds as type 2 promoters.

presence of RNA expression (unpublished observation). The following *sna* mutant stock was used for the initial matings: *y w*; *bw* sna^{IIG05} *cn*/CyO, P{ftz-lacZ, ry^{+}}. Homozygous sna^{-} embryos were identified by the lack of ftz-lacZ stripes generated by the CyO balancer chromosome. Mutants also possess an expanded cephalic furrow. The details of the 2xPE-sna fusion gene are described by Ip et al. (1994). It includes the entire *sna* genomic coding region and 1.6 kb of the 5′ flanking sequences. This genomic fragment was placed downstream of two tandem copies of the 260 bp *twi* PE regulatory sequence, which spans the interval from −440 to −180 bp of the *twi* promoter (Jiang et al., 1991; Pan et al., 1991). The heterologous promoter contains *twi* sequences that direct expression only in the ventralmost regions where there are peak levels of nuclear dl protein. Various wild-type strains were used for control stainings, including Canton S and y^{1} w^{1118}. Wild-type and mutant embryos were collected for 3 hours at room temperature, and subsequently aged for either 3 or 6 hours. They were dechorinated and fixed as described in previous reports (e.g., Ip et al., 1994).

In situ hybridization and histochemical staining

In situ hybridization assays involved the use of digoxigenin-labeled antisense RNA probes (digU), exactly as described previously (Tautz and Pfeifle, 1989; Jiang et al., 1991). Hybridization signals were visualized via histochemical staining with alkaline phosphatase. The eve protein was visualized as a marker for heart morphogenesis using rabbit polyclonal anti-eve antibodies, as described previously (Small et al., 1992). In several instances (Figs 3, 4, 6), embryos were subjected to a double-staining procedures, whereby RNA and protein patterns were simultaneously visualized. Double stainings were done by first visualizing the protein pattern (using biotin-conjugated secondary antibodies and the horseradish peroxidase enzyme) and then performing in situ hybridization to detect the RNA pattern. This double staining procedure was done exactly as described by Azpiazu and Frasch (1993). Figs 3, 4 and 6 involved the use of a rabbit anti-twi antibody, a rabbit anti-Cf1a antibody (Anderson et al., 1995), and a mouse anti-fasciclin III antibody, respectively.

Tissue sections

Sections were prepared by embedding stained embryos in either Araldite or Spurr's resin, as described by Ip et al. (1994). 5 or 10 μm sections were cut with a Sorvall MT2-B Ultra Microtome. Whole-mount preparations and sectioned embryos were photographed using Nomarski DIC optics on either a Zeiss Axiophot or a Nikon Microphot-FXA with a Nikon 20× PlanApo objective.

RESULTS

The regulation of the *sna* expression pattern is summarized in Fig. 2A. Genetic studies and promoter analyses suggest that the broad dl gradient triggers a steeper pattern of *twist* (*twi*) expression. Subsequently, dl and twi function synergistically to activate *sna* exactly within the limits of the presumptive mesoderm (Kosman et al., 1991; Ip et al., 1992a). The sharp lateral borders of the *sna* expression pattern (see Fig. 2B) coincide with the boundary between the presumptive mesoderm and neuroectoderm. A narrower pattern of *sna* expression was obtained through the use of a heterologous promoter, 2xPE. This synthetic promoter contains two tandem copies of the proximal PE region from the *twi* promoter. The PE sequence contains just a few low affinity dl-binding sites, thereby limiting the expression of the 2xPE promoter to the ventralmost regions of the embryo in response to peak levels of the dl gradient (Jiang and Levine, 1993; Ip et al., 1994; summarized in Fig. 2A).

The 2xPE-sna fusion gene was expressed in transgenic embryos via P-transformation. It was subsequently crossed into a sna^{-} genetic background, so that the only source of sna protein corresponds to the 2xPE-sna fusion gene (Fig. 2C). The distribution of sna mRNAs was visualized by in situ hybridization using a digoxigenin-labeled sna antisense RNA probe. As predicted from previous analyses of the PE promoter sequence (Jiang et al., 1991; Jiang and Levine, 1993), the synthetic *sna* fusion gene is expressed within narrower limits as compared with the wild-type endogenous gene (compare Fig. 2B and C). In the remainder of this study, we describe the consequences of the reduced *sna* expression pattern with regard to mesoderm differentiation.

Narrowed limits of *sna* expression reduce the number of invaginating cells

Previous studies suggest a correlation between the limits of the *sna* expression pattern and the extent of the ventral furrow (Ip et al., 1994). To obtain more rigorous evidence that the narrowed *sna* pattern results in a reduction in mesoderm invagination, embryos were double stained to reveal the distribution of twi protein and dpp RNA; cross sections of these embryos are presented in Fig. 3. Transgenic sna^{-} embryos carrying the 2xPE-sna fusion gene (sna^{-}; 2xPE-sna) exhibit narrowed limits of *twi* expression in ventral regions (Fig. 3B; compare with A). Thus, narrowing the limits of the *sna* pattern cause a corresponding narrowing of the *twi* pattern. This observation is consistent with the finding that *twi* expression is initially normal in sna^{-} mutants, but the pattern prematurely disappears (Leptin, 1991; Ray et al., 1991). Altered patterns of *sna* and *twi* expression do not influence the dorsolateral limits of the *dpp* pattern (Fig. 3 A and B).

Reduced limits of *sna* and *twi* expression result in a narrowing of the ventral furrow (data not shown), and the invagination of fewer cells (Fig. 3, compare C and D). The ventral furrow encompasses about 18 to 20 cells in wild-type embryos (Leptin and Grunewald, 1990). All of these cells are fully invaginated during the rapid phase of germ band elongation (Fig. 3C); note that invaginated, twi-expressing cells can be seen in both bottom and top portions of this section due to germ band elongation. The original lateral limits of the *dpp* expression pattern are maintained in both the wild-type and mutant embryos (Fig. 3C,D; blue RNA staining).

More definitive evidence that fewer cells invaginate in mutant embryos as compared with wild-type was obtained by analyzing slightly older embryos. After one round of mitotic divisions, the invaginated cells migrate into lateral regions immediately after the completion of the rapid phase of germ band elongation (Leptin and Grunewald, 1990). This migration converts the presumptive mesoderm into a single internal layer of cells that is in tight contact with the overlying ectoderm (Fig. 3E,F). At this time, the width of the mesoderm spans about 40 cells in wild-type embryos, but only about 24-30 cells in the mutant embryos. Normally, the mesodermal cells migrate dorsally until they reach the dorsal ectodermal cells that contact the amnioserosa. In contrast, in mutant embryos, the mesodermal cells fail to reach the dorsalmost ectoderm due to the reduced number of invaginated cells. Although individual mesodermal cells undergo more extensive spreading in order to compensate for the reduced numbers, the mesodermal layer

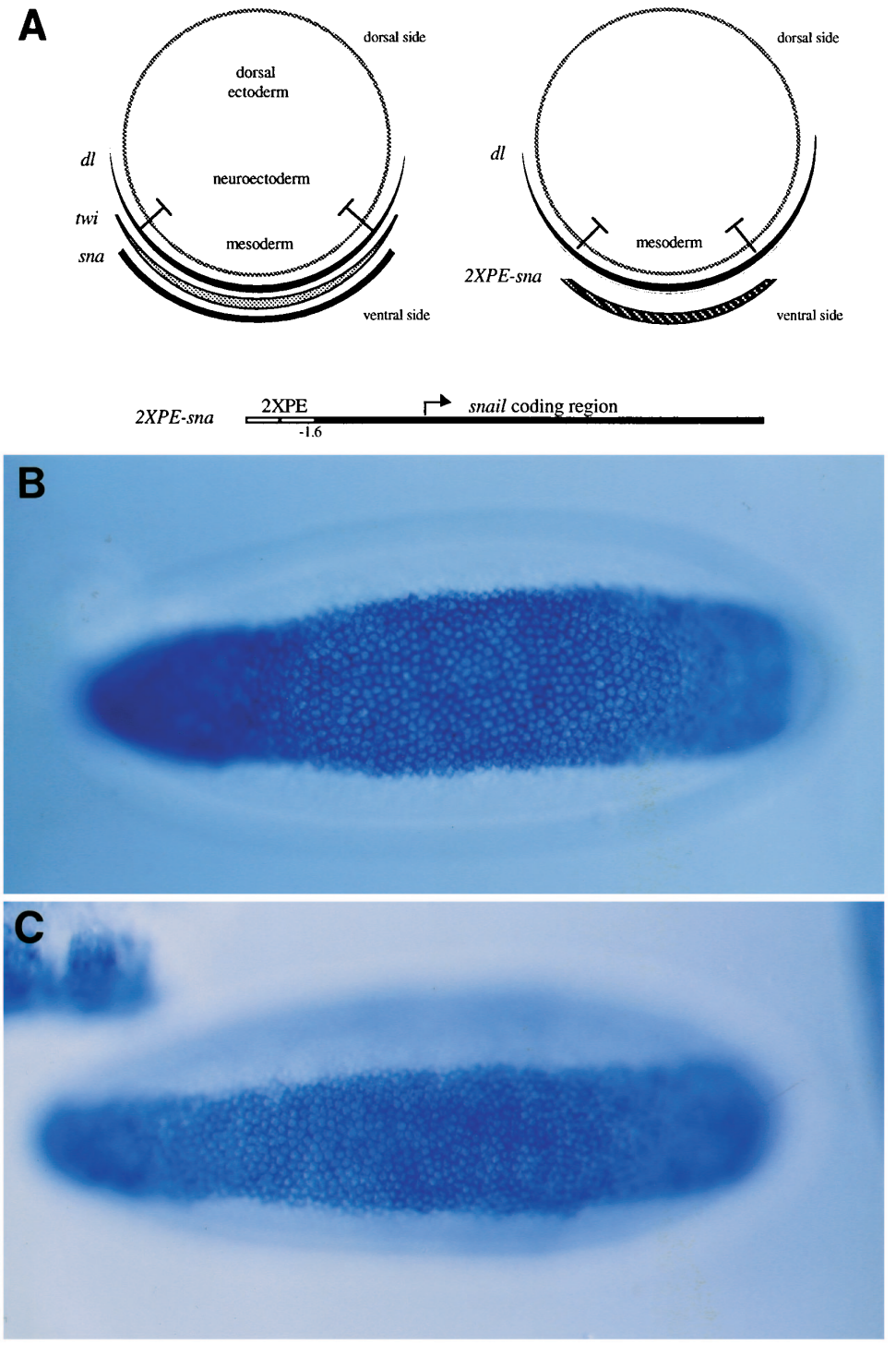

Fig. 2. Reducing the limits of the mesoderm. (A) The circles represent cross sections through blastoderm stage embryos. The left circle summarizes the regulation of the *sna* expression pattern. *dl* and *twi* synergistically activate *sna* in the entire presumptive mesoderm (indicated by the brackets), which spans about 18 to 20 cells in ventral and ventrolateral regions. The right circle depicts the expression pattern generated by the 2xPE-sna fusion gene, whereby the *sna*-coding sequence was placed under the control of the heterologous *twi* PE regulatory sequence. This *sna* fusion gene is regulated solely by dl and is expressed in narrower limits than the wild-type gene (12-16 cells). The horizontal line below the circles represents the *sna* fusion gene used in this study. A *sna* genomic DNA fragment including the first 1.6 kb of the 5′ flanking region was placed downstream of two tandem copies of the *twi* PE. (B) Ventral view of a wild-type, cellularizing embryo that was hybridized with a digU-labeled sna antisense RNA probe. sna RNAs are restricted to the presumptive mesoderm and include about 18-20 cells. (C) Same as B except that a *sna*$^{-}$; 2xPE-sna mutant embryo is shown. The only source of sna RNA corresponds to the transgene since the *sna* mutant background does not specify detectable RNAs at this stage. Staining is somewhat narrower than normal (compare with A) and includes just 12 to 16 cells, depending on the exact location along the anteroposterior axis. For example, the expression pattern is narrowest in the region where the cephalic furrow will form. The embryos in A and B are oriented with anterior to the left.

remains intact and narrower than normal. It should be noted that the dorsoventral limits of the 2xPE-sna expression pattern, and ventral furrow, are somewhat variable along the anteroposterior axis (e.g., Fig. 2C); there is also variation among different mutant embryos.

In wild-type embryos, the mesodermal layer extends virtually throughout the region of the dorsal ectoderm containing dpp-expressing cells (Frasch, 1995; Fig. 3E). In contrast, in mutant embryos, the mesoderm usually extends just 1 or 2 cells beyond the ventral limit of the *dpp* expression pattern in the dorsal ectoderm (Fig. 3F); in some instances, they fail to reach the dpp-expressing cells at all (the exact limits of lateral migration depend on the plane of sectioning and is variable among different embryos).

Narrowing the limits of the presumptive mesoderm results in loss of visceral and heart lineages

Recent studies suggest that dpp-expressing cells in the dorsal ectoderm induce the lateral mesoderm to form the visceral mesoderm and heart lineages. *dpp*$^{-}$ embryos form only somatic

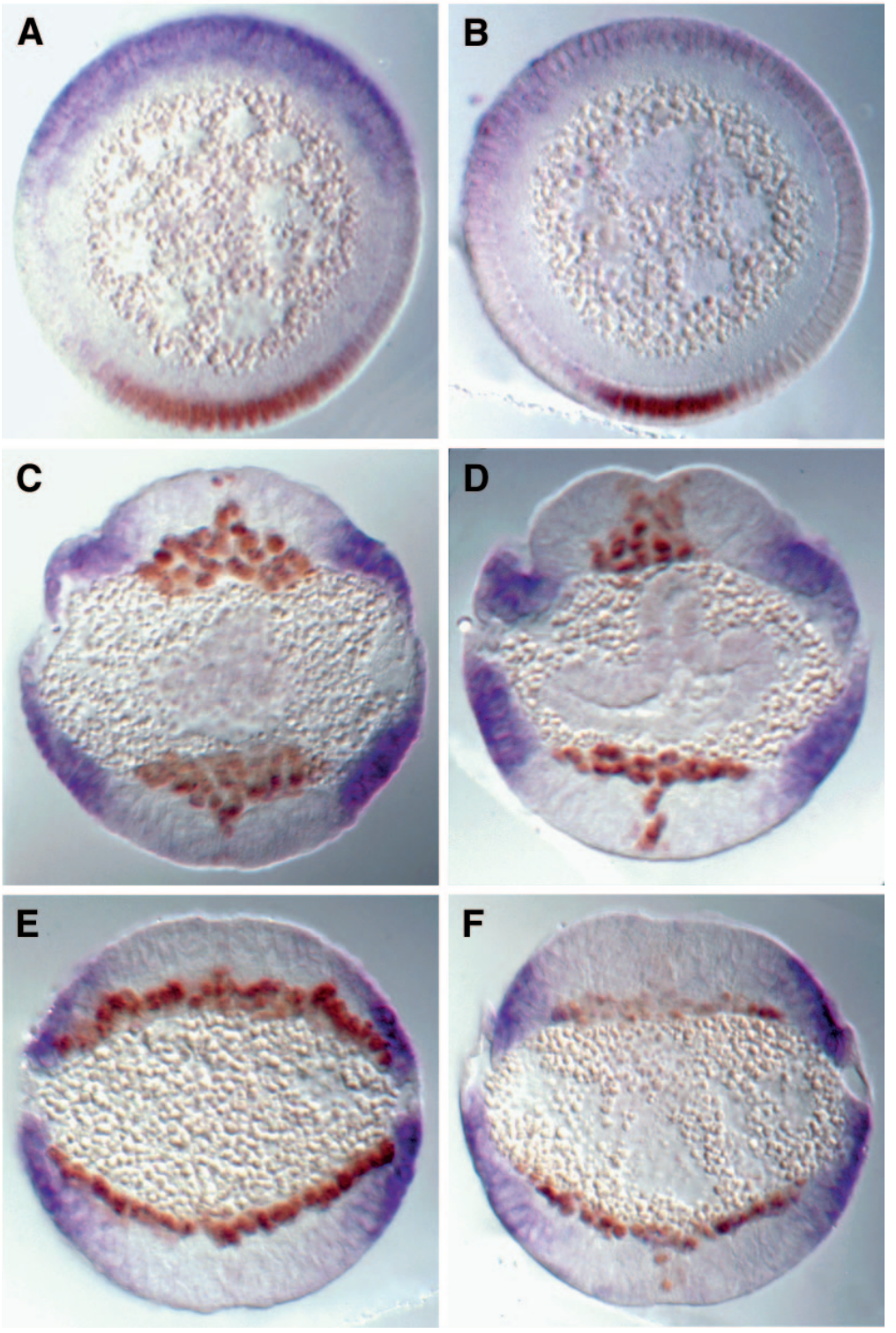

Fig. 3. twi and dpp expression in gastrulating embryos. Transverse sections are oriented with the dorsal surface up. Embryos were double stained to visualize the twi protein (brown) and dpp RNA (blue). Embryos in A, C and E are wild-type; B, D and F are mutant (*sna*$^{-}$; 2xPE-sna). (A,B) Comparison of wild-type and mutant embryos during cellularization. In both cases, dpp RNAs are distributed in the dorsalmost 40% of the circumference. However, the twi pattern indicates a narrowing of the presumptive mesoderm (B). This pattern is similar to the reduced *sna* limits shown in Fig. 1C. (C,D) Embryos that have completed invagination of the ventral furrow and the resealing of the ventral midline. Fewer cells invaginate in the mutant (D) as compared with wild-type. Invaginated cells are seen in both the top and bottom of the embryos due to germ band elongation. The central gut structures appear distinct in the two sections due to slightly different planes of sectioning along the anteroposterior axis. The section in D is somewhat more anterior than that in A and, consequently, it includes more tissues of the posterior midgut invagination after germ band elongation. (E,F) Embryos that have completed lateral migration of the invaginated mesoderm. In wild-type embryos (E), the 2-3 lateralmost mesodermal cells come into contact with dpp-expressing cells in the dorsal ectoderm. These cells are 'induced' to give rise to cardiac and visceral derivatives. Fewer cells reach the dpp-expressing cells in mutant embryos (F).

derivatives (M. F., unpublished observation). Moreover, ectopic expression of *dpp* transforms the presumptive somatic regions so that it forms visceral derivatives (Frasch, 1995). To determine whether reducing the mesodermal layer disrupts this subdivision of the mesoderm into somatic, visceral and cardiac tissues, we examined the expression of *tinman* (*tin*) (Bodmer et al., 1990; Bodmer, 1993; Azpiazu and Frasch, 1993) and *bagpipe* (*bap*) (Azpiazu and Frasch, 1993) in wild-type and mutant embryos. Previous studies have shown that *tin* is initially expressed throughout the entire presumptive mesoderm, beginning with the formation of the ventral furrow. After invagination and lateral migration, *tin* expression is lost in ventral regions of the mesoderm (presumptive somatic regions), but maintained in lateral regions that will form the visceral and heart lineages. This maintenance of the late *tin* pattern depends on induction by *dpp*-expressing cells (Frasch, 1995).

As shown previously, *tin* expression persists throughout the entire mesoderm after germ band elongation and lateral

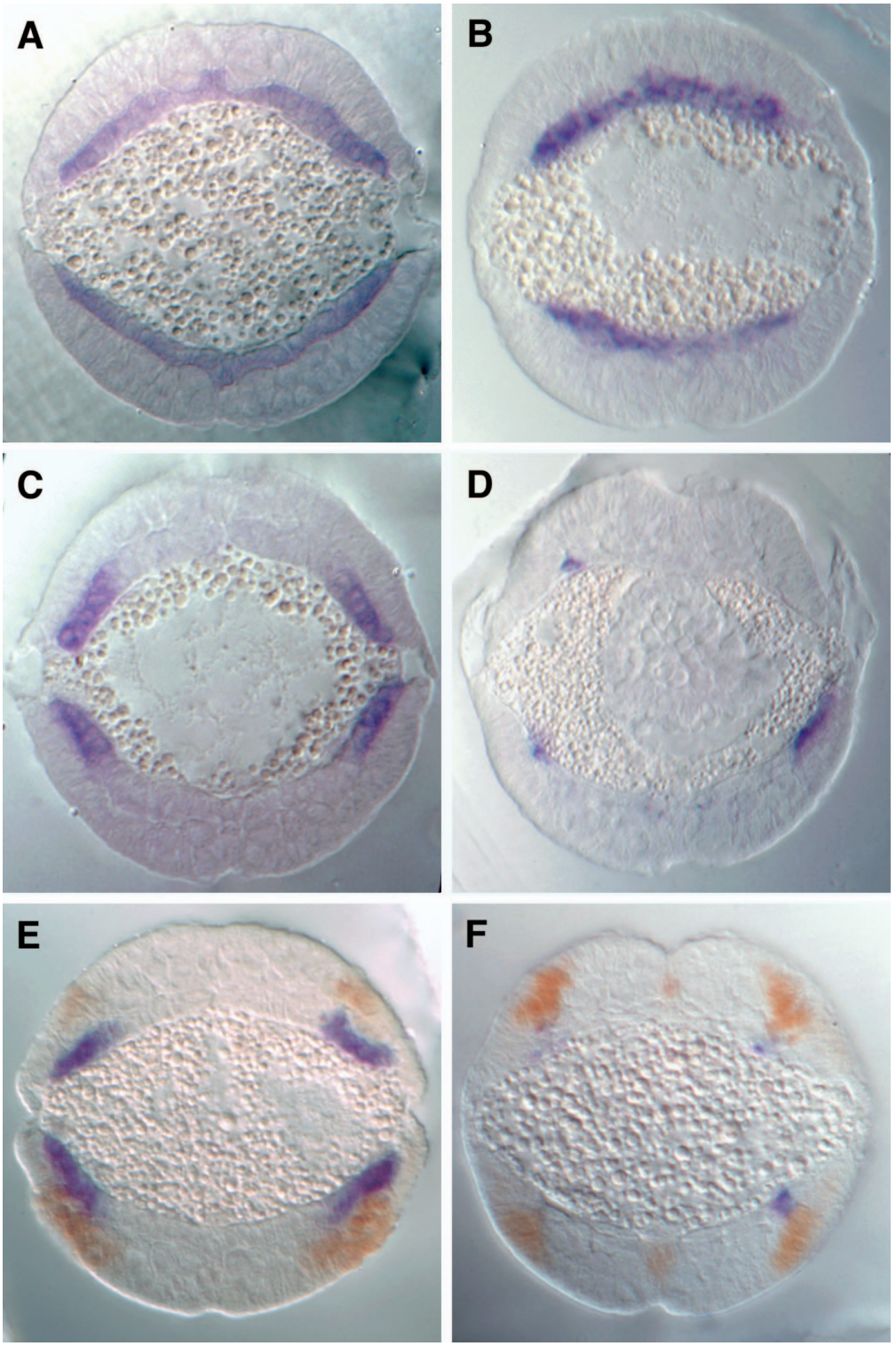

Fig. 4. Altered expression of tin and bap in mutant embryos. Tissue sections are presented as in Fig. 3. (A-D) The distribution of tin RNAs; (E,F) double stained to reveal the distribution of bap RNAs (blue) and protein encoded by the Cf1a marker gene (brown), which is expressed in the dorsolateral ectoderm. (A,B) *tin* expression patterns in wild-type (A) and mutant (B) embryos just after lateral migration of the invaginated mesoderm. (C,D) *tin* expression patterns in slightly older embryos. Normally, the *tin* pattern is maintained in lateral cells that reside near dpp-expressing cells in the dorsal ectoderm (C). In contrast, mutant embryos possess fewer lateral mesoderm cells, and consequently, fewer cells exhibit the late *tin* expression pattern (D). As in Fig 3, the differences seen in the gut morphology are due to different planes of sectioning of the embryos and are not phenotypes caused by the transgene. (E,F) *bap* is normally (E) activated in lateral mesoderm cells that are in contact with the dorsal ectoderm (stained brown). In mutant embryos, there is a reduction in the number of bap-expressing cells, which correlates with the general reduction in lateral mesoderm (F).

migration (Bodmer, 1993; Azpiazu and Frasch, 1993; Fig. 4A). In slightly older embryos, *tin* expression is maintained only in lateral regions (Fig. 4C), corresponding to the cells that contact the dorsal ectoderm. Mutant embryos (*sna*$^{-}$; 2xPE-sna) also exhibit early *tin* expression immediately after lateral migration of the mesoderm (Fig. 4B), although the staining is less extensive due to restricted lateral migration. In older embryos, *tin* expression is virtually lost, although staining is sometimes seen to be retained in just one or two of the lateralmost cells (Fig. 4D; compare with C). This reduction in the late *tin* pattern correlates with the general failure of the mesoderm to reach dpp-expressing regions of the dorsal ectoderm (see Fig. 3 and Discussion). There is a correlation between the extent of migration and proportion of cells that express *tin* (Fig. 4D).

Previous studies suggest that the late *tin* expression pattern is important for the activation of the homeobox gene, *bap*,

which subsequently controls the differentiation of the visceral mesoderm (Azpiazu and Frasch, 1993). In wild-type embryos, the dorsoventral limits of *bap* expression appear to coincide with those of the late *tin* pattern in the lateral mesoderm (Fig. 4E). This embryo was double stained to show bap RNAs in the normal lateral mesoderm (blue), as well as the expression of an ectodermal gene, Cf1a (brown). The Cf1a pattern serves as a marker for the extent of mesoderm lateral expansion. In both wild-type and mutant embryos, the ventral limits of the *bap* expression pattern are positioned below the center of the Cf1a domains. However, mutant embryos exhibit a reduced *bap* pattern that correlates with the reduction in *tin* expression (Fig. 4F). These results suggest that reducing the invaginated mesoderm restricts lateral migration, so that fewer cells come into contact with the dorsal ectoderm and receive the inductive signal from *dpp*. Consequently, there is a selective reduction in the expression of regulatory genes required for differentiation of the heart and gut musculature (see below; Discussion).

Reduced heart in mutant embryos

The consequences of restricting mesoderm-dorsal ectoderm interactions were investigated by analyzing the expression of a number of marker genes in advanced-staged mutant embryos. These studies suggest that tissues arising from the lateralmost regions of the mesoderm are the most severely disrupted, while those arising from ventral regions are less affected.

The heart, or dorsal vessel, is derived from the lateralmost regions of the mesoderm (Bate and Martinez-Arias, 1993; Bodmer, 1993; Azpiazu and Frasch, 1993). The expression of two 'marker genes', *tin* and *even-skipped* (*eve*), suggests that mutant embryos possess severely reduced hearts. Fig. 5 shows dorsal views of wild-type and mutant embryos undergoing dorsal closure, just after germ band shortening. *tin* and *eve* are expressed in different tissues of the heart, corresponding to cardioblasts and pericardial cells, respectively (Fig. 5A,C). Normally, the developing heart extends from the labium/prothorax through the seventh abdominal segment. Mutant embryos exhibit a severe reduction in heart tissues, particularly in abdominal regions (Fig. 5B,D).

Additional marker genes were analyzed in order to assess the differentiation of other mesodermal derivatives, including the visceral mesoderm and somatic musculature. A fasciclin III probe was used to examine the early differentiation of the visceral mesoderm (Strong et al., 1994; Fig. 6). Wild-type and mutant embryos were double stained to visualize fas III protein and eve RNA. Both expression patterns are reduced in mutant embryos (Fig. 6B; compare with A), indicating a loss of both visceral and heart tissues. Analysis of markers for the somatic musculature, such as the myosin heavy chain (MHC) gene, suggests that derivatives of the ventral mesoderm differentiate and are not as severely disrupted (data not shown). In addition, the use of a *nautilus* (Michelson et al., 1990) hybridization probe suggests that the mutant embryos possess essentially a normal number of founder somatic myoblasts. These results suggest that limiting the scope of the *dpp*-mesoderm interaction causes a selective loss in the derivatives of the lateral mesoderm, including the heart and gut musculature.

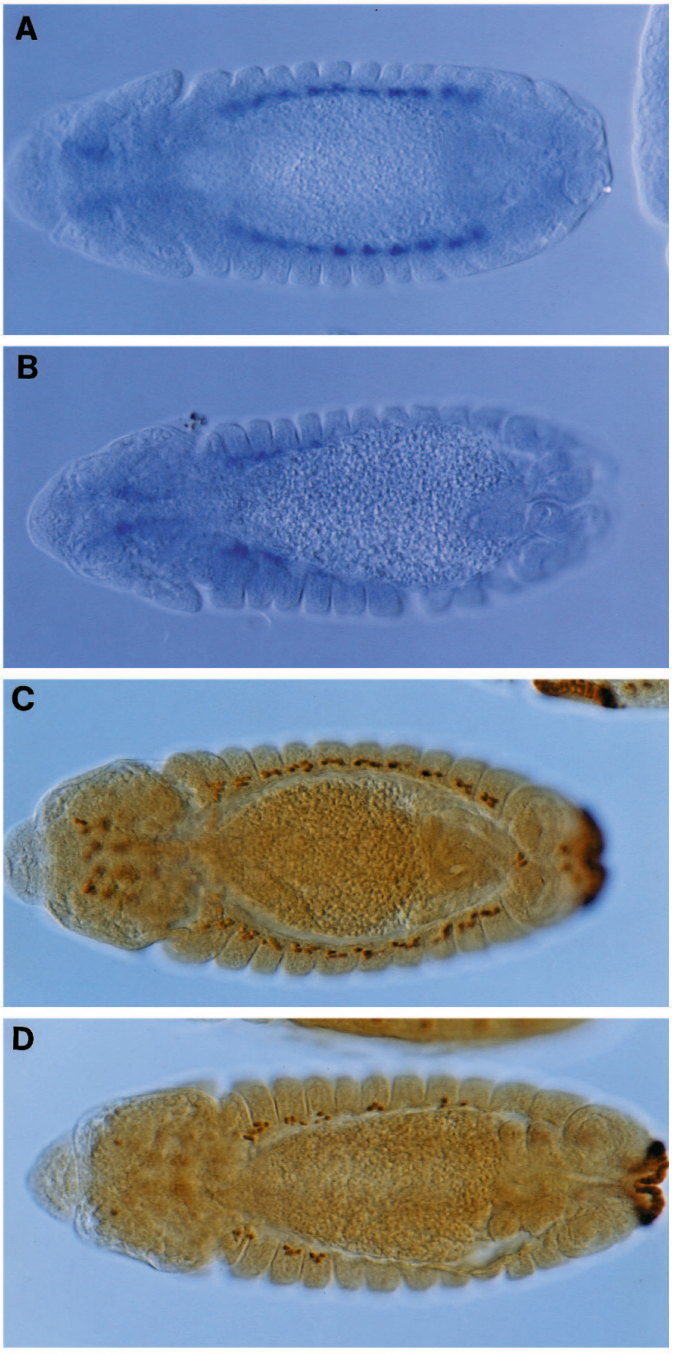

Fig. 5. Reduction of cardiac mesoderm in mutant embryos. Dorsal views of retracting embryos; anterior is to the left. Embryos were stained to show the distribution of tin RNAs (A,B) or eve protein (C,D). (A,B) *tin* is normally expressed in all of the cardioblasts (A). Dorsal closure will bring the bilaterally positioned groups of cells together in a single dorsal vessel spanning the dorsal midline. Mutant embryos exhibit a severe reduction in the number of cardioblasts, particularly in abdominal regions. (C,D) eve is normally expressed in pericardial cells of the presumptive dorsal vessel (C). These are also reduced in the mutant (D).

DISCUSSION

We have presented evidence that differential thresholds established by the dl gradient are directly responsible for the subdivision of the embryonic mesoderm into visceral and somatic lineages. Classical ablation experiments in short germ band insects demonstrated that the neurogenic ectoderm and the

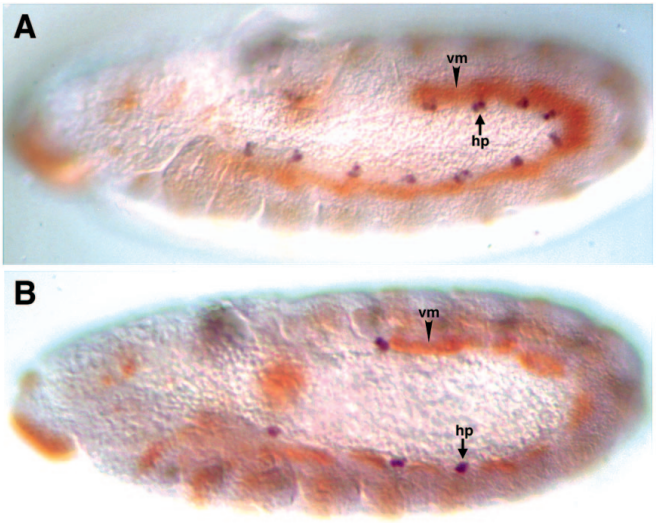

Fig. 6. Reduction of visceral mesoderm in mutant embryos. Lateral views of late extended embryos that were double stained to visualize the fasciclin III protein (brown) and eve protein (blue). Wild-type (A) and mutant (B) embryos. At this stage, fas III stains all of the cells of the presumptive visceral musculature, which can be seen as a continuous lateral band of brown stain. The presumptive pericardial cells (blue) are located just dorsally of the visceral mesoderm. Mutant embryos show a severe reduction in both the visceral and cardiac lineages. vm, visceral mesoderm; hp, heart progenitors.

dorsal ectoderm possess distinctive properties with regard to mesoderm induction (Bock, 1939, 1941; Seidel et al., 1940; Haget, 1953). In *Drosophila*, it would appear that this asymmetry is a direct consequence of the dl gradient, which defines the lateral limit of *dpp* expression in the dorsal ectoderm. Recent studies suggest that dpp-expressing cells induce the lateralmost mesoderm to form both heart and visceral derivatives (Staehling-Hampton et al., 1994; Frasch, 1995). The mechanisms underlying the subsequent subdivision of the lateral mesoderm into these two distinct lineages are currently unknown. This study provides evidence that the subdivision of the mesoderm is surprisingly nonplastic. A slight reduction in the limits of the presumptive mesoderm leads to a severe loss of specific mesodermal lineages, particularly those arising from the lateral mesoderm such as the heart and gut muscles.

dl thresholds and the subdivision of embryonic tissues

Gastrulation is essential for the juxtapositioning of diverse embryonic tissues, which subsequently interact to define cell fate. It has become increasingly clear in a variety of embryonic systems that the interacting tissues are not naive, but instead possess an intrinsic developmental bias. In insects, the type of mesodermal derivatives that are obtained depend on the source of the ectoderm. Dorsal ectoderm induces the differentiation of 'lateral' mesoderm, including both visceral and cardiac mesoderm derivatives (Seidel et al., 1940). In contrast, neurogenic ectoderm (or neuroectoderm) appears to be required for the differentiation of somatic derivatives (Bock, 1939, 1941; Seidel et al., 1940; Haget, 1953). This study provides evidence that the parameters of mesoderm-ectoderm inductive interactions are stringently set by differential threshold responses to the dl gradient, as summarized in Fig. 7.

sna is activated precisely within the limits of the presumptive mesoderm through synergistic interactions between dl and one of its target genes, *twi* (Kosman et al., 1991; Leptin, 1991; Ip et al., 1992a). Previous studies suggested a link between the *sna* expression pattern and the limits of the ventral furrow and invaginated mesoderm. Mutant embryos with an altered *sna* pattern show a corresponding disruption in mesoderm differentiation. For example, there are gaps in the *sna* expression pattern in *dl*/+, *twi*/+ double heterozygotes, and these regions fail to invaginate (Kosman et al., 1991). There is reason to believe that the bHLH twi activator must form a heterodimer with one or more ubiquitously expressed bHLH proteins, such as daughterless (da), which is maternally expressed (González-Crespo and Levine, 1993). *sna* expression is virtually eliminated in *da*/+, *dl*/+, *twi*/+ triple heterozygotes and, consequently, no ventral furrow forms and there is a severe loss of mesodermal derivatives. It was not possible to establish a causal link between the altered *sna* pattern and invagination, since these mutants impair dl activity and, as a result, a number of target genes are disrupted in addition to *sna*.

The present study provides evidence for a direct link between the *sna* expression pattern and mesoderm invagination. The use of the PE heterologous promoter introduces a rather subtle perturbation in essentially normal embryos. The only discernible difference between wild-type embryos and the mutant embryo (sna^{-}; 2xPE-sna) is the narrowing of the *sna* expression pattern, from approximately 18-20 cells to about 12-16 cells (see Fig. 2). The dl gradient and its interacting partners such as bHLH activators and corepressors, are not altered. The initial expression of primary dl target genes, including *dpp*, *zen*, *rho* and *twi* appear normal (data not shown).

Drosophila dorsoventral patterning is inflexible

It would appear that there are no compensatory cell divisions to re-establish the normal number of invaginated cells in sna^{-}; 2xPE-sna mutants. In principle, an additional division cycle would be sufficient to permit expansion of the mesodermal layer into proximity with the dorsal ectoderm. The consequences of a slightly narrowed *sna* expression pattern provide perhaps the most striking example of the inflexibility of the dorsoventral (DV) patterning process. Other examples include the dorsoventral patterning defects observed in dl-bHLH double heterozygotes (González-Crespo and Levine, 1993). Moreover, *dl*/+ heterozygotes, containing just a 2-fold reduction in the normal levels of dl protein, exhibit a high incidence of lethality (Simpson, 1983). *dl*/+ heterozygotes (more accurately, embryos derived from heterozygous females) show a slight narrowing of the *sna* expression pattern, so it is conceivable that this lethality stems, at least in part, from disruptions in mesoderm-dorsal ectoderm inductive interactions.

The inflexibility of dorsoventral patterning is also observed for the subdivision of the dorsal ectoderm. An apparent dl target gene, *short gastrulation* (*sog*), is expressed in lateral regions of precellular embryos (François et al., 1994). It has been proposed that lateral stripes of *sog* expression correspond to a 'sink' that helps establish a dpp activity gradient in the dorsal half of gastrulating embryos (François et al., 1994). Peak *dpp* activity is restricted to the dorsalmost regions, while lower levels extend into dorsolateral and lateral regions. This *dpp* activity gradient is thought to be responsible for subdividing the dorsal ectoderm into the amnioserosa and dorsal

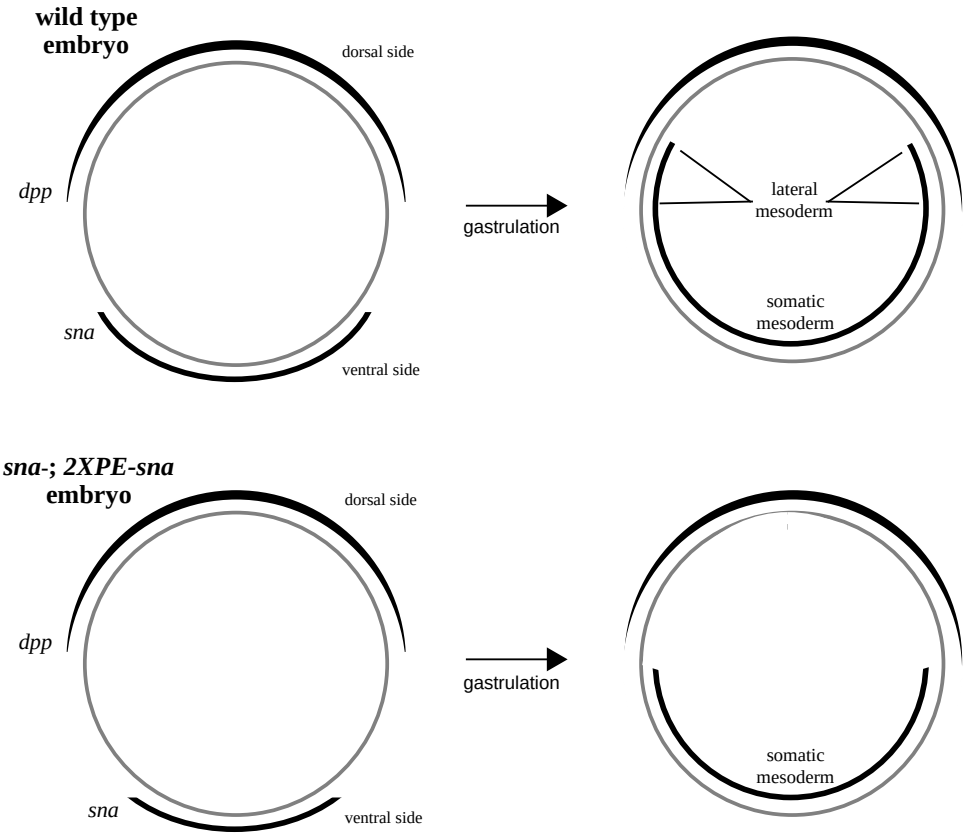

Fig. 7. The sna and dpp expression patterns set the limits of mesoderm-dorsal ectoderm interactions. The circles represent cross sections through precellular (left) and postgastrula (right) embryos. In wild-type embryos, sna is expressed in 18-20 ventral and ventrolateral cells. After invagination and migration, the lateralmost regions of the mesoderm come into contact with dpp-expressing cells in the dorsal ectoderm. These cells are 'induced' to form lateral derivatives, including both the visceral and cardiac lineages. In mutant embryos (*sna*$^{-}$; 2xPE-sna), there is a reduction in the ventrolateral limits of the *sna* pattern, but the *dpp* pattern is unchanged. Consequently, lateral migration is less extensive and fewer cells come into contact with the dorsal ectoderm. This results in a substantial reduction in both visceral and cardiac derivatives.

epidermis (Ferguson and Anderson, 1992; François et al., 1994). Peak levels of *dpp* work synergistically with another TGF-β homologue, *screw* (*scw*), to initiate the differentiation of amnioserosa (Arora et al., 1994). *dpp* and *scw* are required for the maintenance and refinement of the *zen* expression pattern. Initially, *zen*, like *dpp*, is expressed in a broad dorsal on/ventral off pattern due to repression by the dl gradient. During the completion of cellularization, *dpp* and *scw* maintain the *zen* pattern only in the dorsalmost 4-5 cells (the presumptive amnioserosa) where *dpp* is at peak activity. This refinement of the *zen* pattern appears similar to the restriction of the late *tin* pattern within the lateral mesoderm. Both processes require 'induction' by peak levels of *dpp* activity. Previous studies have shown that *dpp*/+ heterozygotes fail to maintain and refine the *zen* expression pattern and, consequently, there is a loss of the amnioserosa resulting in embryonic lethality.

These examples of dosage-sensitive embryonic lethality (*dl*/+ and *dpp*/+) contrast with the high degree of plasticity seen for tissue differentiation in other embryonic systems. For example, in sea urchins, the primary mesenchyme arises through the ingression of the micromeres (Cameron et al., 1991). Loss of micromeres through ablation causes the growing tip of the archenteron (the future foregut) to undergo additional, 'unscheduled' divisions. The resulting cells form the mesenchyme in response to the loss of the micromeres.

The inflexibility of dorsoventral patterning also contrasts with the plasticity of anteroposterior (AP) patterning in *Drosophila*. Embryos containing between 1 and 6 copies of the *bicoid* (*bcd*) gene develop properly and ultimately give rise to normal adults (Struhl et al., 1989; Driever and Nüsslein-Volhard, 1988). *bcd* is essential for head differentiation and the initiation of the segmentation cascade. Normal embryos containing two copies of *bcd* show 7 pair-rule stripes of *eve* and *fushi tarazu* (*ftz*) expression, which extend from the cephalic furrow to subterminal regions at the posterior pole, at about 10% egg length. Heterozygotes containing one-half the normal levels of *bcd* show *eve* and *ftz* stripes that extend over a broader region of the AP axis. Remarkably, all 7 pair-rule stripes are compressed within a narrow, central region of embryos containing 6 copies of *bcd*, but they develop normally presumably due to changes in programmed cell death and division cycles.

In summary, evidence was presented that the maternal dl gradient sets the limits of inductive interactions between germ layers. Altering the threshold response of just one primary target gene, *sna*, resulted in the narrowing of the presumptive mesoderm. Consequently, there is a general failure of the invaginated mesoderm to expand into lateral regions and come into contact with dpp-expressing cells in the dorsal ectoderm. Future studies will investigate the subsequent elaboration of diverse lineages (i.e., visceral and cardiac) from the lateral mesoderm.

We thank Susan Gray and Jannette Rusch for advice. We also thank Gerold Schubiger for many stimulating discussions. K. M. is a recipient of the Lucille P. Markey Charitable Trust Fellowship. M. F. is a recipient of the Pew Award. This work was funded by grants from the NIH (GM 46638 and HD 30832).

REFERENCES

Alberga, A., Boulay, J. L., Kempe, E., Dennefeld, C. and Haenlin, M. (1991). The *snail* gene required for mesoderm formation in *Drosophila* is expressed dynamically in derivatives of all three germ layers. *Development* **111**, 983-92.

Anderson, M. G., Perkins, G. L., Chittack, P., Shrigley, R. J. and Johnson, W. A. (1995). *drifter*, a *Drosophila* POU-domain transcription factor, is required for correct differentiation and migration of tracheal cells and midline glia. *Genes Dev.* **9**, 123-127.

Arora, K., Levine, M. S. and O'Connor, M. B. (1994). The *screw* gene encodes a ubiquitously expressed member of the TGF-β family required for specification of dorsal cell fates in the *Drosophila* embryo. *Genes Dev.* **8**, 2588-601.

Azpiazu, N. and Frasch, M. (1993). *tinman* and *bagpipe*: two homeo box genes that determine cell fates in the dorsal mesoderm of *Drosophila*. *Genes Dev.* **7**, 1325-40.

Bate, M. and Martinez-Arias, A. (1993). eds. *The Development of Drosophila melanogaster*. pp. 1013-1090. Plainview, NY: Cold Spring Harbor Laboratory Press.

Bate, M. and Rushton, E. (1993). Myogenesis and muscle patterning in *Drosophila*. *Comptes Rendus de L'Academie des Sciences. Serie III, Sciences de la Vie* **316**, 1047-61.

Bock, E. (1939). Bildung und Differenzierung der Keimblätter bei *Chrysopa perla*. *Z. Morphol. Okol. Tiere* **35**, 615-702.

Bock, E. (1941). Wechselbeziehungen zwischen den Keimblättern bei der Organbildung von *Chrysopa perla*. *Wilhelm Roux' Arch. EntwMech. Org.* **141**, 159-247.

Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118**, 719-29.

Bodmer, R., Jan, L. Y. and Jan, Y. N. (1990). A new homeobox-containing gene, msh-2, is transiently expressed early during mesoderm formation of *Drosophila*. *Development* **110**, 661-9.

Cameron, R. A., Fraser, S. E., Britten, R. J. and Davidson, E. H. (1991). Macromere cell fates during sea urchin development. *Development* **113**, 1085-91.

Dohrmann, C., Azpiazu, N. and Frasch, M. (1990). A new *Drosophila* homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. *Genes Dev.* **4**, 2098-111.

Doyle, H. J., Kraut, R. and Levine, M. (1989). Spatial regulation of *zerknüllt*: A dorsal-ventral patterning gene in *Drosophila*. *Genes Dev.* **3**, 1515-1533.

Driever, W. and Nüsslein-Volhard, C. (1988). The *bicoid* protein determines position in the *Drosophila* embryo in a concentration-dependent manner. *Cell* **54**, 95-104.

Ferguson, E. L. and Anderson, K. V. (1992). *Decapentaplegic* acts as a morphogen to organize dorsal-ventral pattern in the *Drosophila* embryo. *Cell* **71**, 451-61.

François, V., Solloway, M., O'Neill, J. W., Emery, J. and Bier, E. (1994). Dorsal-ventral patterning of the *Drosophila* embryo depends on a putative negative growth factor encoded by the *short gastrulation* gene. *Genes Dev.* **8**, 2602-16.

Frasch, M. (1995). Induction of visceral mesoderm by ectodermal Dpp in the early *Drosophila* embryo. *Nature* (in press).

González-Crespo, S. and Levine, M. (1993). Interactions between *dorsal* and helix-loop-helix proteins initiate the differentiation of the embryonic mesoderm and neuroectoderm in *Drosophila*. *Genes Dev.* **7**, 1703-13.

Govind, S. and Steward, R. (1991). Dorsoventral pattern formation in *Drosophila*: signal transduction and nuclear targeting. *Trends in Genetics* **7**, 119-25.

Grau, Y., Carteret, C. and Simpson, P. (1984). Mutations and chromosomal rearrangements affecting the expression of *sna*, a gene involved in embryonic patterning in *Drosophila melanogaster*. *Genetics* 108, 347-360.

Gray, S., Szymanski, P. and Levine, M. (1994). Short-range repression permits multiple enhancers to function autonomously within a complex promoter. *Genes Dev.* **8**, 1829-38.

Haget, A. (1953). Analyse experimentale des facteurs de la morphogenese embryonnaire chez le Coleoptere *Leptinotarsa*. *Bull. Biol. Fr. Belg.* **87**, 123-217.

Huang, J. D., Schwyter, J. M., Shirokawa, J. M. and Courey, A. J. (1993). The interplay between multiple enhancer and silencer elements defines the pattern of *decapentaplegic* expression. *Genes Dev.* **7**, 694-704.

Ip, Y. T., Kraut, R., Levine, M. and Rushlow, C. A. (1991). The *dorsal* morphogen is a sequence-specific DNA-binding protein that interacts with a long-range repression element in *Drosophila*. *Cell* **64**, 439-46.

Ip, Y. T., Maggert, K. and Levine, M. (1994). Uncoupling gastrulation and mesoderm differentiation in the *Drosophila* embryo. *EMBO J.* **13**, 5826-34.

Ip, Y. T., Park, R. E., Kosman, D., Bier, E. and Levine, M. (1992b). The *dorsal* gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo. *Genes Dev.* **6**, 1728-39.

Ip, Y. T., Park, R. E., Kosman, D., Yazdanbakhsh, K. and Levine M. (1992a). *dorsal-twist* interactions establish *snail* expression in the presumptive mesoderm of the *Drosophila* embryo. *Genes Dev.***6**, 1518-30.

Jiang, J., Cai, H., Zhou, Q. and Levine, M. (1993). Conversion of a dorsal-dependent silencer into an enhancer: evidence for *dorsal* corepressors. *EMBO J.* **12**, 3201-9.

Jiang, J., Kosman, D., Ip, Y. T. and Levine, M. (1991). The *dorsal* morphogen gradient regulates the mesoderm determinant *twist* in early *Drosophila* embryos. *Genes Dev.* **5**, 1881-91.

Jiang, J. and Levine, M. (1993). Binding affinities and cooperative interactions with bHLH activators delimit threshold responses to the *dorsal* gradient morphogen. *Cell* **72**, 741-52.

Jiang, J., Rushlow, C. A., Zhou, Q., Small, S. and Levine, M. (1992). Individual *dorsal* morphogen binding sites mediate activation and repression in the *Drosophila* embryo. *EMBO J.* **11**, 3147-54.

Kirov, N., Childs, S., O'Connor, M. and Rushlow, C. (1994). The *Drosophila dorsal* morphogen represses the *tolloid* gene by interacting with a silencer element. *Mol. Cell. Biol.* **14**, 713-722.

Kirov, N., Zhelnin, L., Shah, J. and Rushlow, C. (1993). Conversion of a silencer into an enhancer: evidence for a co-repressor in *dorsal*-mediated repression in *Drosophila*. *EMBO J.* **12**, 3193-3199.

Kosman, D., Ip, Y. T., Levine, M. and Arora, K. (1991). Establishment of the mesoderm-neuroectoderm boundary in the *Drosophila* embryo. *Science* **254**, 118-22.

Lehming, N., Thanos, D., Brickman, J. M., Ma, J., Maniatis, T. and Ptashne, M. (1994). An HMG-like protein that can switch a transcriptional activator to a repressor. *Nature* **371**, 175-9.

Leptin, M. (1991). *twist* and *snail* as positive and negative regulators during *Drosophila* mesoderm development. *Genes Dev.* **5**, 1568-1576.

Leptin, M., Casal, J., Grunewald, B. and Reuter, R. (1992). Mechanisms of early Drosophila mesoderm formation. *Development 1992 Supplement*, 23-31.

Leptin, M. and Grunewald, B. (1990). Cell shape changes during gastrulation in Drosophila. *Development* **110**, 73-84.

Michelson, A. M., Abmayr, S. M., Bate, M., Martinez-Arias, A. and Maniatis, T. (1990). Expression of a MyoD family member prefigures muscle pattern in *Drosophila* embryos. *Genes Dev.* **4**, 2086-97.

Pan, D. J., Huang, JD. and Courey, A. J. (1991). Functional analysis of the *Drosophila twist* promoter reveals a *dorsal*-binding ventral activator region. *Genes Dev.* **5**, 1892-901.

Ray, R. P., Arora, K., Nüsslein-Volhard, C. and Gelbart, W. M. (1991). The control of cell fate along the dorsal-ventral axis of the *Drosophila* embryo. *Development* **113**, 35-54.

Roth, S., Stein, D. and Nüsslein-Volhard, C. (1989). A gradient of nuclear localization of the *dorsal* protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59**, 1189-202.

Rushlow, C. A., Han, K., Manley, J. L. and Levine, M. (1989). The graded distribution of the *dorsal* morphogen is initiated by selective nuclear transport in *Drosophila*. *Cell* **59**, 1165-77.

Rushlow, C. and Arora, K. (1990). Dorsal ventral polarity and pattern formation in the *Drosophila* embryo. *Seminars in Cell Biology* **1**, 137-49.

Seidel, F., Bock, E. and Krause, G. (1940). Die Organisation des Insekteneies. *Naturwiss* **28**, 433-446.

Simpson, P. (1983). Maternal-zygotic gene interactions during formation of the dorsoventral pattern in *Drosophila* embryos. *Genetics* **105**, 615-632.

Small, S., Blair, A. and Levine, M. (1992). Regulation of even-skipped stripe 2 in the Drosophila embryo. *EMBO J.* **11**, 4047-57.

Staehling-Hampton, K., Hoffmann, F. M., Baylies, M. K., Rushton, E. and Bate, M. (1994). *dpp* induces mesodermal gene expression in *Drosophila*. *Nature* **372**, 783-6.

Steward, R. (1989). Relocalization of the *dorsal* protein from the cytoplasm to the nucleus correlates with its function. *Cell* **59**, 1179-88.

Strong, R. K., Vaughn, D. E., Bjorkman, P. J. and Snow, P. M. (1994). Expression and crystallization of a soluble form of *Drosophila fasciclin III*. *J. Molec. Biol.* **241**, 483-7.

Struhl, G., Struhl, K. and Macdonald, P. M. (1989). The gradient morphogen *bicoid* is a concentration-dependent transcriptional activator. *Cell* **57**, 1259-73.

Tautz, D. and Pfeifle, C. (1989). A non-radioactive in situ hybridization method for the localization of specific RNAs in *Drosophila* embryos reveals translational control of the segmentation gene *hunchback*. *Chromosoma* **98**, 81-5.

Thisse, C., Perrin-Schmitt, F., Stoetzel, C. and Thisse, B. (1991). Sequence-specific transactivation of the *Drosophila twist* gene by the *dorsal* gene product. *Cell* **65**, 1191-201.

(Accepted 14 April 1995)