twist and *snail* as positive and negative regulators during *Drosophila* mesoderm development

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***twist* and *snail* are members of the helix–loop–helix and zinc-finger protein families, respectively, and determine the development of the mesoderm in *Drosophila*. This paper analyzes their role in mesoderm development by examining how they affect the expression of downstream genes. *twist* and *snail* act by regulating gene expression in the mesoderm and in neighboring regions, and have distinct roles in this process. *snail* prevents expression in the mesoderm of genes that are destined to be active only in more lateral or dorsal regions. *twist* is required for the activation of downstream mesodermal genes. *twist* is also required for the full expression of *snail* and for the maintenance of its own expression. Only the absence of both *twist* and *snail* results in the complete loss of all mesodermal characteristics.**

[*Key Words*: *twist*; *snail*; mesoderm; *Drosophila*; helix–loop–helix proteins; embryogenesis]

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The mechanisms by which the mesoderm is determined during early development vary in different species. For example, in vertebrates, soluble factors secreted by non-mesodermal cells induce mesoderm formation (for review, see Slack 1991), whereas in *Drosophila*, the nuclear concentration of a maternal transcription factor, the dorsal protein, determines mesodermal fate (Rushlow et al. 1989; Steward 1989; Roth et al. 1989). However, early mesoderm-specific genes, such as *twist*, *snail*, or MyoD, which are expressed after the initial mesoderm determination and probably regulate further mesoderm development, appear to be remarkably homologous even between insects and vertebrates (Davis et al. 1987; Hopwood et al. 1989; Michelson et al. 1990; Paterson et al. 1991; Wolf et al. 1991; A. Nieto and D.G. Wilkinson, pers. comm.). This suggests that once the mesoderm is determined, different species use similar regulatory molecules to govern its further development.

In *Drosophila*, the mesoderm arises from the ventral region of the embryo. A group of maternal genes, the dorsal group genes and *cactus*, define positional values along the dorsoventral axis of the embryo by the nuclear concentration of the *dorsal* gene product (Roth et al. 1989; Rushlow et al. 1989; Steward 1989). The *dorsal* nuclear protein directly or indirectly regulates the activation or repression of zygotic genes. Differences in the expression of zygotic genes along the dorsoventral axis are visible as early as 2 hr after fertilization, which is ~1 hr before the invagination of the mesoderm begins. At least four domains along the dorsoventral axis can be distinguished by their fates and their gene-expression patterns. The region next to the mesoderm is occupied by the ventral ectoderm, followed more dorsally by the dorsolateral ectoderm and, finally, by the amnioserosa.

Two zygotic genes, *twist* and *snail*, are known to be required for the formation of the mesoderm (Nüsslein-Volhard et al. 1984). Embryos mutant in these genes gastrulate abnormally, form no mesodermal germ layer or mesodermal derivatives, and die at the end of embryogenesis (Simpson 1983; Grau et al. 1984). The lateral and dorsal parts of the embryo are not affected in the mutants. The products of the *twist* and *snail* genes are members of the helix–loop–helix and zinc-finger protein families, respectively (Boulay et al. 1987; Thisse et al. 1988) and are therefore probably involved in transcriptional regulation. They are expressed in the mesoderm anlage (Thisse et al. 1988; Leptin and Grunewald 1990). Their structure and expression patterns, and the mutant phenotypes suggest that *twist* and *snail* play a role in determining the differentiation and morphogenesis of the mesoderm, probably by regulating the transcription of other genes.

Although neither *twist* nor *snail* mutant embryos develop a mesoderm, their phenotypes are different (Leptin and Grunewald 1990). In *twist* mutants, the ventral furrow (the early invagination of the mesoderm) is only half the width of the wild-type furrow, suggesting that the mesoderm anlage might be narrower than in the wild-type embryo. In *snail* mutants, which make no furrow, the ventral epithelium buckles irregularly over the normal width of the mesoderm anlage. The phenotype of embryos mutant for both genes is much stronger than that of either single mutant alone; the ventral epithelium is indistinguishable from the neighboring ecto-

derm. This indicates that *twist* and *snail* do not act exclusively in the same pathway but have at least partly independent functions. Because they act most likely as transcriptional regulators, we tested their roles in mesoderm development by examining their effects on the expression of a number of genes normally expressed in the mesoderm and at its borders. As representative examples, we used PS2α, expressed in the mesoderm (Bogaert et al. 1987); *single-minded* (*sim*), expressed in mesectodermal cells that lie at the border of the mesoderm (Crews et al. 1988) and do not contribute to the mesoderm, but become part of the nervous system (Campos-Ortega and Hartenstein 1985); and *crumbs*, expressed outside the mesoderm, in the ventral and more dorsal ectoderm (Knust et al. 1987). These genes are required for the proper development of the cells in which they are expressed, [i.e., for the differentiation of mesectodermal cells (*sim*), the attachment of mesodermal cells to the ectoderm (PS2α), and for the organization of the epidermis (*crumbs*)]. Under the control of *twist* and *snail*, respectively, these genes are activated in or their expression is confined to the areas in which they are destined to be active.

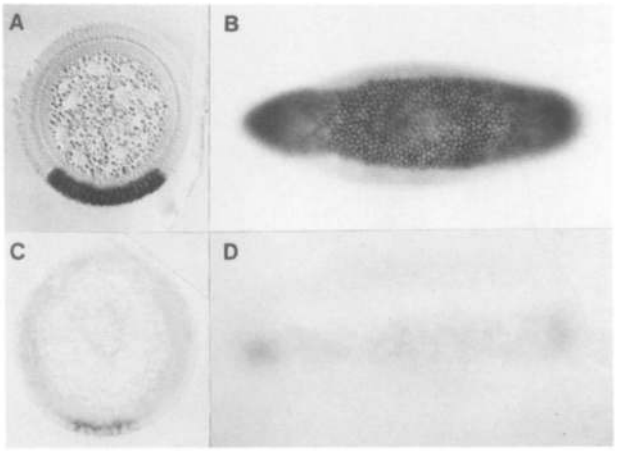

Figure 1. Cellular and early syncytial blastoderm expression patterns of *twist* and *snail*. *snail* and *twist* RNA distribution in cellular blastoderm (*A,B*) and early syncytial blastoderm (*C,D*) embryos are shown. *A* and *C* are transverse sections of embryos hybridized with a *snail* probe (dorsal side up); *B* and *D* are ventral views of embryos hybridized with a *twist* probe. Apart from the differences shown in Fig. 2, the two genes are expressed in the same patterns.

Results

Expression patterns of twist *and* snail

Because the temporal and spatial limits of *twist* and *snail* expression are important for this study, they were analyzed and compared in detail.

As reported previously (Thisse et al. 1988; Leptin and Grunewald 1990), both *twist* and *snail* RNA are expressed in the prospective mesoderm at the cellular blastoderm stage (Fig. 1A,B). However, when first detectable (during nuclear cycle 12), they appear in a diffuse band only half the width of the prospective mesoderm (Fig. 1C,D).

The expression patterns of *twist* and *snail* differ in a number of aspects. Along the anterior–posterior axis, *twist* RNA and protein extend farther anterior and posterior than *snail* RNA and protein (Fig. 2A–D). *snail* RNA and protein form a sharp posterior boundary (Fig. 2B,D). [*snail* protein like *twist* (Thisse et al. 1988), is nuclear (Fig. 2J), consistent with the role suggested by the zinc-finger motifs in its sequence.] Although *twist* continues to be expressed in the mesoderm throughout germ-band extension (Fig. 2E), *snail* products completely disappear from the mesoderm halfway through germ-band extension and appear in a subset of cells in the neural ectoderm, which by their distribution and shape are probably neuroblasts (Figs. 2F and 6E).

The lateral boundaries of *twist* and *snail* expression are not identical. Both *snail* RNA and protein have a discrete lateral boundary, whereas *twist* RNA and protein form a gradient at the edge of the prospective mesoderm (Fig. 2G–J). The fainter part of this gradient extends beyond the sharp *snail* boundary into the territory of the prospective ectoderm. Figure 2 shows this difference at its most extreme point, in mid-cellularization. Much earlier, the *snail* boundary is slightly more diffuse than shown here, and later, during gastrulation, the *twist* boundary sharpens.

Expression domains of other genes along the dorsoventral axis

The expression of a number of genes normally expressed in and around the mesoderm in embryos lacking *twist* and *snail* function was examined. The localization of the expression domains of some of these genes relative to each other and relative to *twist* and *snail* was established in double-labeling experiments (in cases where the subcellular localization of the RNAs differ; e.g., Fig. 4B) and by comparisons on serial sections. Their expression patterns are summarized in Figure 3. *sim* is expressed in mesectodermal cells at the border of the mesoderm (Fig. 4A,B; Crews et al. 1988); *crumbs* is expressed outside the mesoderm, in the ventral and more dorsal ectoderm (Knust et al. 1987; Fig. 4B). Its expression domain includes the mesectodermal cells. PS2α is expressed in the mesoderm (Fig. 4C; Bogaert et al. 1987); like *snail*, it is expressed only in the region between the two lines of mesectodermal cells. The *snail* and PS2 expression domains do not overlap the *sim* expression domain. In contrast, *twist* expression is not confined to this region at the blastoderm stage. The *sim*-expressing cells lie within the region of the graded distribution of *twist* protein. Thus, at this stage, low levels of *twist* protein are found in mesectodermal and some ectodermal cells. During late germ-band extension, however, *twist* products disappear from nonmesodermal cells; and when the mesoderm has invaginated fully, the mesectodermal cells no longer express *twist* (Leptin and

Grunewald 1990). Other genes expressed in the prospective mesoderm or ectoderm are listed in the legend to Figure 3.

Expression patterns in twist *and* snail *mutants*

In *snail* embryos, PS2 and *twist* are activated in the same pattern as in the wild type (Fig. 4C,F; Leptin and Grunewald 1990). This is also the case of other early mesodermal genes (see Fig. 4 legend). In contrast, the expression patterns of *sim* and of ventrolateral genes are altered. *crumbs* and *sim* are expressed in the mutant mesoderm (Fig. 4G,H; for *sim*, see also Leptin and Grunewald (1990); Nambu et al. (1990); Rushlow and

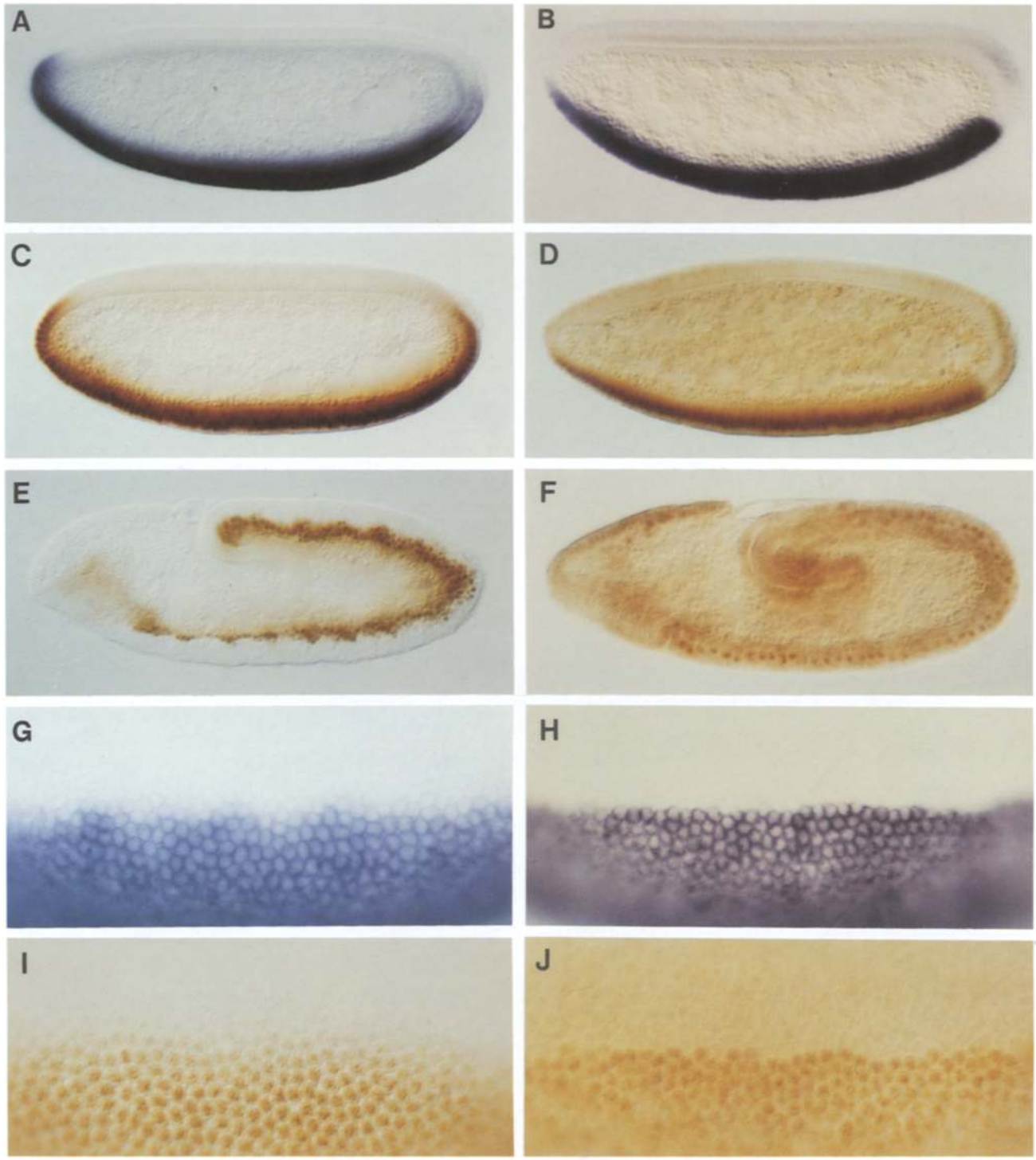

Figure 2. (*See facing page for legend.*)

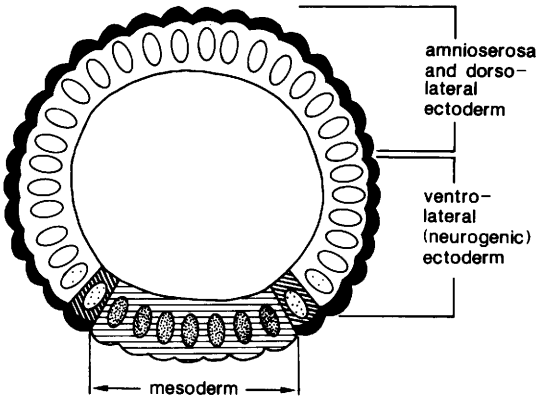

Figure 3. Diagram of zygotic gene expression patterns along the dorsoventral axis. *sim* (▨) is expressed in the mesectodermal cells at the edge of the mesoderm. PS2 and *snail* (▤) are expressed in the prospective mesoderm, i.e., the region between the mesectodermal cells. *twist* (▩) is expressed at high levels in the prospective mesoderm and in lower amounts in mesectodermal and the neighboring ectodermal cells. *crumbs* (■) is expressed in the ventrolateral and dorsolateral ectoderm and the prospective amnioserosa. Other genes expressed in the mesoderm at this stage are *msh-2* (Bodmer et al. 1990), the early expression of transcript 48 (t48; Gould et al. 1990), and *string* (Edgar and O'Farrell 1989). Other genes expressed in the ectoderm are *Delta* (Vässin et al. 1987) and the later t48 (Gould et al. 1990).

Arora (1990)], as are other genes that are normally restricted to more lateral regions [t48 (data not shown); *Delta* (M. Haenlin and J. Campos-Ortega, pers. comm.); mesectodermal genes (Nambu et al. 1990)]. This indicates that *snail* is not required to activate mesodermal genes, but to repress ventrolateral genes in the mesoderm territory. However, during germ-band extension, *twist* and PS2 disappear earlier than during wild-type development, suggesting a direct or indirect late role of *snail* in the maintenance of the expression of these genes (see Discussion).

In *twist* mutants, the two *sim* lines lie closer together than in the wild type, at the edge of the region that forms the mutant ventral furrow (Fig. 4D). The *crumbs* expression domain also extends farther ventrally in *twist* mutant embryos (Fig. 4B,E), as do those of two other genes expressed in the lateral and dorsal regions at this stage (t48, not shown; *Delta*, M. Haenlin and J. Campos-Ortega, pers. comm.). Thus, the mutant mesoderm anlage in *twist* embryos is narrower than in the wild type, consistent with the finding that the ventral furrow in *twist* embryos is narrower. PS2 is not expressed in these embryos, nor are other genes that are expressed in wild-type mesoderm at this stage (*string*, B. Edgar, pers. comm.; *msh-2*, Bodmer et al. 1990; t48, data not shown). Thus, *twist* is required for the activation of early mesodermal genes but not for the repression of lateral genes.

When both *twist* and *snail* function are removed (in *twist snail* double mutants), no mesodermal or mesectodermal genes are expressed, and the genes of the ventral ectoderm are expressed over the whole ventral surface [data not shown, as *crumbs* expression in the double mutants looks like Fig. 4H, and PS2 expression looks like Fig. 4F. Double-mutant embryos do not express the *sim* gene (data not shown; Rushlow and Arora 1990)].

snail *and* twist *expression in* twist *mutants*

The normal expression pattern of *twist* protein in *snail* mutants (Leptin and Grunewald 1990) shows that *snail* function is neither required for turning on *twist* expression nor for its early maintenance. Specifically, the width of the mesodermal territory can be defined in the absence of *snail*.

However, if *twist* is required for the expression of all early mesodermal genes, expression of *snail* and possibly even of *twist* itself should depend on *twist* activity. This is indeed the case. *snail* transcription is never established over the whole mesodermal territory in *twist* mutant embryos. Its early expression in a narrow band is the same as in the wild type, but it does not become expressed in a wider band (Fig. 5C). Instead, it disappears at the cellular blastoderm, usually by first breaking up into patches (Fig. 5E). Thus, the initial transcription of *snail*

Figure 2. Differences between *twist* and *snail* expression patterns. (*A,C,E,G,I*) *twist* expression patterns (*B,D,F,H,J*); *snail* expression patterns. (*A,B*) *twist* and *snail* RNA distribution at the cellular blastoderm. (*C,D*) *twist* and *snail* protein distribution at the cellular blastoderm. The proteins are found in ventral nuclei. *snail* has a sharp posterior boundary and does not extend around the anterior tip of the embryo. Along the anterior—posterior axis neither *twist* nor *snail* is confined to the mesoderm anlage. (*E,F*) Protein distribution at the fully extended germ-band stage. The mesoderm has invaginated completely and forms a layer of cells on the inside of the ectoderm, clearly visible in *E*, which shows that *twist* is still expressed in the mesoderm. In contrast, *snail* is no longer present in the mesoderm but is expressed in neuroblast nuclei (individual brown dots within the ectodermal layer). (*G–J*) Views of whole-mount embryos from a ventrolateral angle, focusing on the lateral edge of the mesoderm. (*G,H*) *twist* and *snail* RNA distribution at the mesoderm–ectoderm boundary (during early cellularization). *twist* (*G*) is expressed in a gradient, whereas *snail* has a sharp boundary (*H*). (*I,J*) *twist* (*I*) protein, expressed in a gradient and *snail* (*J*) protein, with a sharp boundary at the edge of the mesoderm. Note that the *snail* expression patterns observed with our antibody differ in some details from those published recently by Alberga et al. (1991). We find that (1) the protein appears well before, and not after, gastrulation begins—consistent with the finding that the *snail* mutant phenotype is visible from the very onset of gastrulation; (2) the protein is never seen only in individual mesodermal cells, but the antibody stains all mesodermal cells homogeneously, as expected, as *snail* mutations affect all mesodermal cells; (3) the protein is seen only in regions of the embryo that also express the RNA; (4) also in neuroblasts, the protein appears to be nuclear and not cytoplasmic. These observations are in agreement with those of Kosman et al. (1991).

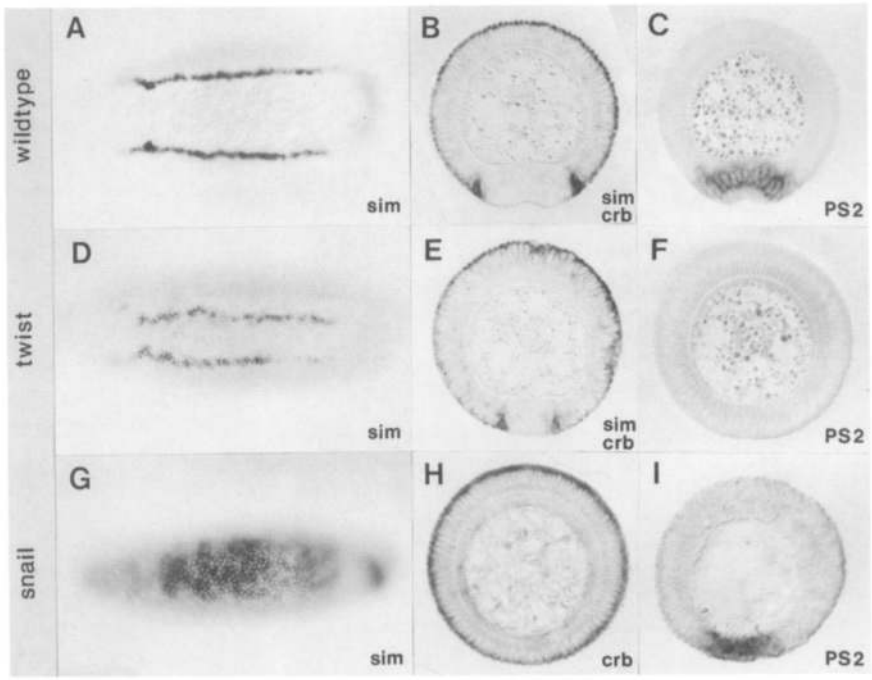

Figure 4. Expression of mesodermal, mesectodermal, and ectodermal genes in wild-type embryos and in *twist* and *snail* mutants. (*A,D,G*) Ventral views of in situ hybridizations of whole-mount embryos at cellular blastoderm; all other panels are transverse sections of embryos during early gastrulation, dorsal side up. In wild-type embryos (*B,C*), the ventral furrow has begun to form. (*A,D,G*) *sim* is expressed in two lines, each one cell wide, at the edge of the mesoderm. In *twist* mutants, these lines lie closer together (*D*); in *snail* mutants, the gene is expressed over the whole width of the mesodermal territory (*G*). (*B,E*) Embryos hybridized simultaneously with probes for *sim* and *crumbs*. Because *crumbs* RNA is localized only in the apical part of each cell and *sim* is distributed over the whole cytoplasm, their expression domains can be distinguished in sectioned embryos (*B,E*). In *twist* embryos, both the *sim* cells and the edges of the *crumbs* domain lie farther ventrally and closer to each other than in the wild type. (*H*) *snail* mutant embryo hybridized with a *crumbs* probe. *crumbs* is expressed around the whole periphery of the embryo. Two other genes expressed in the ectoderm, t48 (Gould et al. 1990) and *Delta*, show the same changes of expression patterns in the mutants as *crumbs* (results not shown; M. Haenlin and J. Campos-Ortega, pers. comm.). (*C,F,I*) Embryos hybridized with a PS2 probe. PS2 is not expressed in *twist* mutants (*F*) but is expressed normally in *snail* mutants (*I*). Other early mesodermal genes that behave in this way are *twist*, the early ventral expression of t48 (Gould et al. 1990; results not shown), and *msh-2* (Bodmer et al. 1990). In embryos mutant for both *twist* and *snail*, the *crumbs* expression pattern looks like that in *H*, and PS2 looks like that in *F*.

in a narrow band is independent of *twist*, probably directly activated by the maternal genes, but its maintenance and later expression across the whole width of the mesoderm require *twist* function. The late expression of *snail* in neuroblasts is entirely independent of *twist* (Fig. 6F).

twist function appears also to be required for the maintenance of expression of its own RNA. The effects of four strong *twist* alleles on *twist* expression were tested. In embryos mutant for any of them, *twist* RNA is initially transcribed normally, first in a narrow band, then over the whole width of the mesoderm. During cellularization, however, the RNA begins to fade (see Fig. 5D,F) and finally disappears at the beginning of gastrulation (see Fig 5H). Thus, the early *twist* transcription pattern is established, but its expression cannot be maintained in the absence of detectable *twist* function.

Late fate changes

Many mesodermal and ventral-ectodermal genes are only expressed during or after germ-band extension, at least 3 hr later than the genes analyzed above. Late mesodermal genes, such as β_3-tubulin (Leiss et al. 1988; Kimble et al. 1990), *pox meso* (Bopp et al. 1989), and the embryonic muscle actin Act57A (Tobin et al. 1990) are neither expressed in *twist* nor in *snail* mutants (Fig. 6A–D; Leiss et al. 1988). Although this suggests that *snail* may have a role in the activation of these genes, it should be noted that in *snail* mutants, *twist* also disappears early. Furthermore, even in the wild type, *snail* disappears from the mesoderm before β3-tubulin, *pox meso*, and Act57A are expressed. Therefore, the failure of *snail* mutants to express late mesodermal genes is consistent with a direct requirement for *twist* for the activation of these genes and an indirect role for *snail*.

The transient expression of *snail* in *twist* mutant embryos is not sufficient to repress late ventrolateral genes. The mutant mesodermal region in *twist* embryos begins to show aspects of ventrolateral behavior after germband extension, in that cells delaminate from it. These cells have the typical shape of delaminating neuroblasts and express genes normally expressed by neuroblasts at this stage (as with *hunchback* and the late, neuroblast-specific *snail*; Fig. 6C–E). This suggests that the presence of *snail* is required continually to repress all lateral characteristics in the mesodermal region.

Discussion

Together, the results shown here suggest the following early functions for *twist* and *snail* (Fig. 7). *twist* responds to maternal positional information defining the boundaries of the mesoderm. Within this territory, it is respon-

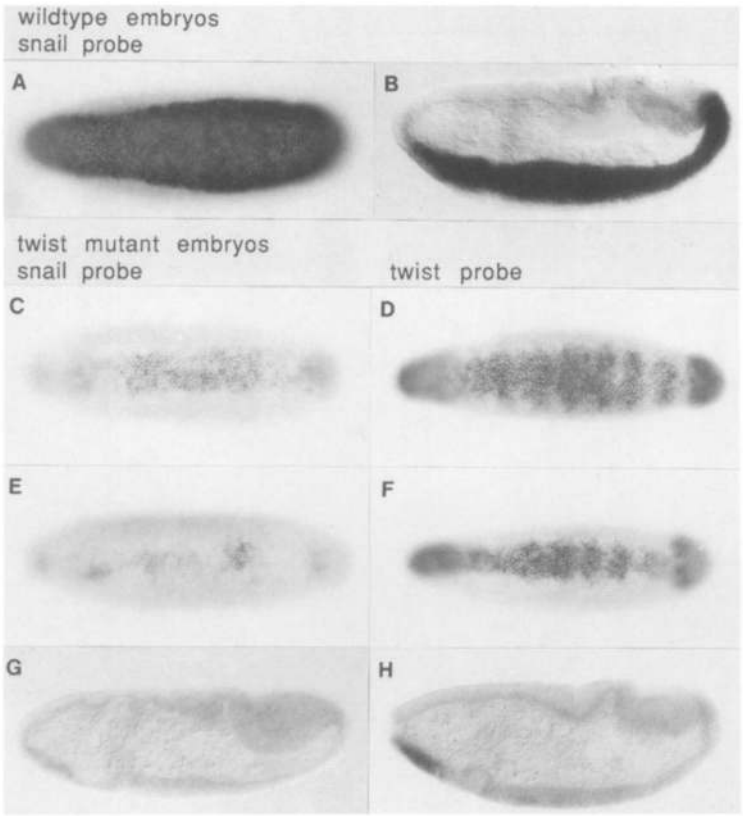

Figure 5. Expression of twist and snail RNA in twist mutants. (*A,B*) Expression pattern of *snail* RNA in wild-type embryos at cellular blastoderm (*A*, ventral view) and early gastrulation (*B*, lateral view). Apart from the differences described in Fig. 1, *twist* is expressed in the same patterns. (*C–H*) Expression patterns of *snail* and *twist* RNA in *twist* mutant embryos. (*C,G,E*) *twist* mutant embryos [from the null allele Df(2R)twi^{S60}] hybridized with a *snail* probe. (*D,F,H*) *twist* mutant embryos (allele twi^{IIE}) hybridized with a *twist* probe. (*C,D*) Syncytial blastoderm at the beginning of cellularization (ventral views); (*E,F*) Cellular blastoderm (ventral views); (*G,H*) early gastrulation (lateral views).

sible for activating and maintaining the expression of early mesoderm-specific genes, including itself and *snail*. The expression patterns of mesodermal genes in *snail* mutants show that *twist* can perform this function in the absence of *snail*. *snail* represses genes in the mesodermal territory that are destined to be active only in more lateral or dorsal regions. *snail*, too, can perform its function alone, as in *twist* mutants lateral genes are off in the region where *snail* is expressed early. The comparison of *twist* and *snail twist* double mutants shows that the early *snail* expression has an effect on downstream genes. Because a narrow ventral band of cells differs from lateral cells in behavior and gene expression patterns in *twist*, but not in the double mutant, and as the only difference between *twist* and the double mutant is the early presence of *snail* RNA, it must be the function of this early *snail* RNA that makes the *twist* phenotype different from the double-mutant phenotype. Therefore, the narrow band of *snail*, itself, represses lateral genes in the mutant mesodermal region in *twist* embryos.

The combined presence of both *twist* and *snail* is required to achieve both activation of mesodermal and repression of lateral genes, and proper development of the mesoderm. Thus, neither gene is sufficient to regulate the whole repertoire of gene expression required to allow mesoderm development, but each alone can regulate the expression patterns of some genes. Only in the absence of both *twist* and *snail* do all mesodermal qualities disappear from the ventral region of the embryo. This situation also resembles that of dorsal fate determination, where the function of zygotic dorsal-specific genes appears to be to repress lateral fates and allow the expression of dorsal fates (Irish and Gelbart 1987). Thus, in the absence of any region-specific dorsoventral zygotic gene activity the ground state of the blastoderm appears to be the expression of neural ectoderm fates.

This model for the roles of *twist* and *snail* as mesoderm gene activators and lateral gene repressors, respectively, is necessarily preliminary. It is possible that mesodermal or lateral genes might be discovered that do not obey the rules described for the genes analyzed here. Furthermore, interactions between genes might complicate the interpretation of mutant expression patterns. For example, a gene under negative control of lateral genes (which are repressed ventrally by *snail*) would be expressed in the mesoderm but depend on *snail* for its expression. It is also not possible to distinguish whether the disappearance of *twist* in *snail* mutants indicates that *snail* is normally directly reponsible for the maintenance of *twist* expression, or whether the disappearance is due to effects of ventrolateral genes normally repressed by *snail*. The latter seems more likely. First, *snail* protein itself disappears from the mesoderm during germ-band extension in the wild type, and it is therefore difficult to see how it could be directly responsible for the late maintenance of *twist* expression. Second, even during wild-type development, *twist* disappears from

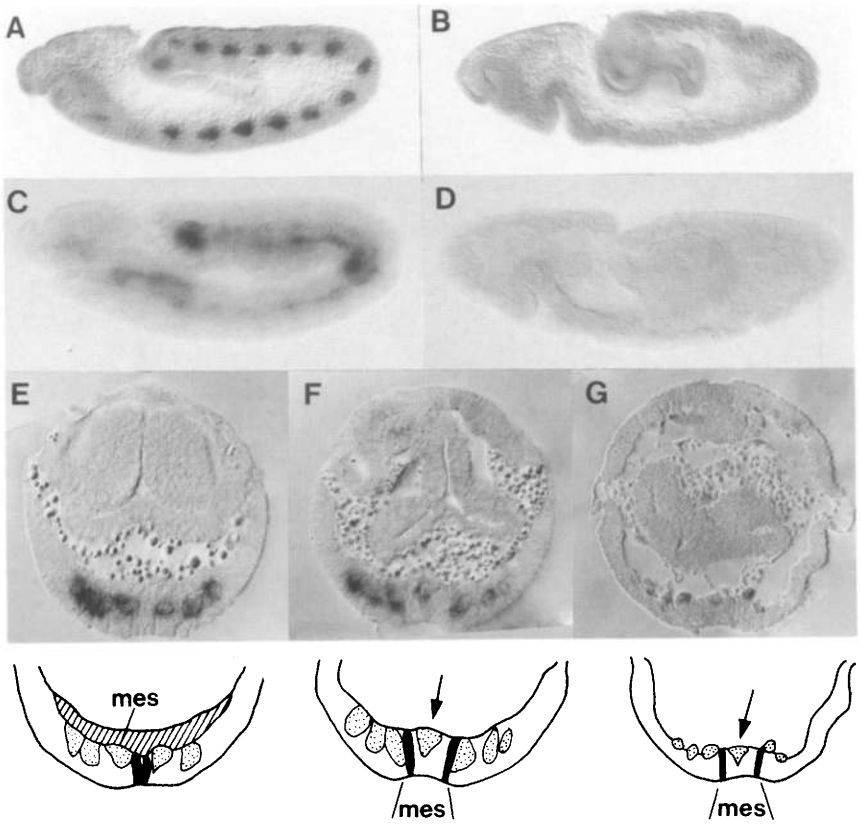

Figure 6. Expression of late mesodermal and neuroblast markers in mutants. (*A,B*) Lateral views of extended germ-band wild-type (*A*) and *snail* embryo (*B*) stained with antibodies against the *pox meso* product (Bopp et al. 1989). (*C,D*) Lateral views of extended germ-band wild-type (*C*) and *snail* (*D*) embryo hybridized with a probe for the muscle actin gene Act57A. (*E–G*) Sections through extended germ-band wild-type (*E*) and *twist* (*F,G*) embryos. *E* and *F* were hybridized with a *snail* probe; *G* was stained with anti-*hunchback* antibodies. The tracings beneath the sections highlight the mesoderm in the wild type (hatched), the stained neuroblasts (stippled), and the mesectoderm cells at the edge of the mesoderm [(solid) inferred from previous experiments (Leptin and Grunewald 1990)]. (mes) Mesoderm. Arrows indicate neuroblasts delaminating from the mutant mesodermal region.

cells expressing *sim* and ventrolateral genes (i.e., from mesectoderm cells) during germ-band extension (Leptin and Grunewald 1990). At this time, therefore, it is possible that genes active in this region repress or destabilize *twist*, which they would also do over the whole mesodermal region in *snail* mutants.

The transformation of the *twist* mutant mesoderm into something resembling neural ectoderm is interesting with respect to neuroblast differentiation. The mutant mesodermal region does not express ventrolateral genes early. This shows that neuroblasts do not require a wild-type ectodermal environment to differentiate and delaminate. Furthermore, as neuroblasts can delaminate in *twist* mutants, an underlying mesoderm is not required for their differentiation.

Definition of the mesoderm boundaries

The differences in the lateral boundaries of *twist* and *snail* expression domains might reflect the differences in the way the domains are set up. Although the boundary of *twist* initially appears to be determined by the maternal gradient of nuclear dorsal concentration alone, *snail* clearly requires *twist* activity to be expressed in the

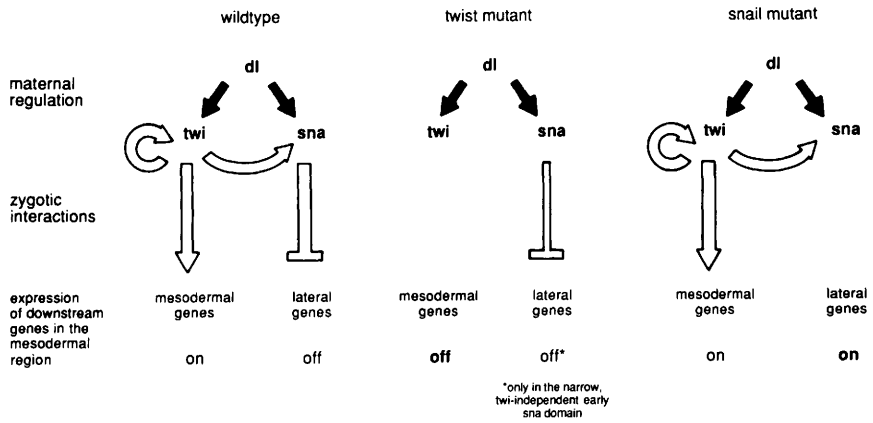

Figure 7. Diagram summarizing early gene interactions in the mesoderm anlage. Black arrows represent maternal regulation of the early *twist* and *snail* transcription; white arrows represent the effects of zygotic genes on each other. Arrowheads denote activation; bars denote repression. (For further description and discussion of the model, see text.)

whole mesoderm. It is possible that *twist* responds to *dorsal* activity in a more or less linear way, whereas *snail* responds to a combination of *dorsal* and *twist* protein in a cooperative way, thus creating a sharper boundary. The late sharpening of the *twist* boundary might depend on a similar mechanism. Because *twist* is required for its own maintenance and *dorsal* protein persists well into germ-band extension, *twist* and *dorsal* too might interact to sharpen the *twist* boundary.

These data do not suggest any simple model to explain how the *sim* lines are made. In the absence of both *twist* and *snail*, *sim* is not expressed (Leptin and Grunewald 1990; Rushlow and Arora 1990), but the presence of either *twist* or *snail* alone allows the activation of *sim*. The ability of *snail* to activate *sim* expression (in *twist* mutants) seems to contradict its function as a repressor of *sim* in the mesoderm. Even in *twist* mutants, however, *sim* is off in the ventral-most band of cells and is only activated at the edge of the *snail* domain. Clearly, the precise position and width of the line is determined by *twist* and *snail* together.

Both *twist* and *snail* are initially activated in a narrow stripe. Assume that this expression is directly regulated by the maternal genes, because a zygotic gene required for the activation of *twist* and *snail* would have to be expressed (and its RNA translated into protein) before *twist* and *snail*. It is unlikely that such a gene exists, given the very early expression of *twist* and *snail* (only two nuclear cycles or 20 min after syncytial blastoderm formation, when *dorsal* protein becomes differentially distributed). Furthermore, no other zygotic genes with phenotypes similar to *twist* and *snail* are known. This suggests that there are no zygotic genes acting upstream of *twist* and *snail* and that any other gene that might act in parallel to *twist* and *snail* cannot be absolutely required for the formation of the mesoderm. Thus, *twist* and *snail* might be the only genes at the top of the hierarchy of zygotic genes involved in mesoderm development. In this case, they would be sufficient for zygotic mesoderm determination, a hypothesis that might be tested by expressing them ectopically.

Material and methods

Fly stocks

Unless mentioned otherwise, the *twist* allele used was Df(2R)S60 (Simpson 1983) and *snail* was Df(2L)TE116GW11 (Ashburner et al. 1990). Both are complete null alleles, and no *twist* or *snail* RNA is produced, respectively. The *twist snail* double mutant was Df(2R)S60 sna^{IIG}, provided by K. Arora. The *twist* alleles used in the experiment in Figure 5 were twi^{ID}, twi^{IIE}, twi^{IIH}, and twi^{D5}.

Antibody staining and in situ hybridization

Embryos from timed collections were dechorionated with bleach, fixed in 4% formaldehyde in PBS, and stained or hybridized (Tautz and Pfeifle 1989) as decribed previously (Leptin and Grunewald 1990). The *snail* antiserum (to be described in more detail elsewhere) was produced by immunizing rats with protein purified from bacteria expressing the full-length *snail* RNA (a gift from N. Brown) in the pET-3c expression vector. The serum was used at a dilution of 1 : 30.

In situ hybridizations on frozen sections

For hybridizations on frozen sections we used a protocol provided by Sommer and Tautz (1991), with slight modifications. Fixed and dechorionated embryos were oriented for transverse sectioning in OCT embedding medium, frozen, sectioned on a cryostat, and mounted on gelatin-coated microscope slides. The sections were refixed for 20 min in 4% formaldehyde in PBS, washed, in PBS and 0.1% Tween 20, digested for 10 min in a drop of proteinase K (50 μg/ml), washed, and fixed again for 20 min in 4% formaldehyde in PBS. They were hybridized overnight under a coverslip under the same conditions as for whole-mount embryos and were also washed and stained in the same way as whole-mount embryos.

Sections of stained embryos

Stained embryos were dehydrated thoroughly, transferred to araldite, prepared for sectioning, and sectioned on a microtome (10-μm sections) as described previously (Leptin and Grunewald 1990).

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Note added in proof

In contrast to the early mesodermal genes analyzed in this work, a recently described gene, zfh-1, requires *snail* for its mesodermal expression (Lai et al. 1991. *Mech. Dev.* **34:** 123–134). It will be interesting to see whether this is due to a direct positive regulatory effect of *snail*, or to repression of negative regulatory lateral genes, as discussed above.

References

Alberga, A., J-L. Boulay, E. Kempe, C. Dennefeld, and M. Haenlin. 1991. The *snail* gene required for mesoderm formation in *Drosophila* is expressed dynamically in derivatives of all three germ layers. *Development* **111:** 983–992.

Ashburner, M., P. Thompson, J. Roote, P.F. Lasko, Y. Grau, M. El Messal, S. Roth, and P. Simpson. 1990. The genetics of a small autosomal region of *Drosophila melanogaster* containing the structural gene for alcohol dehydrogenase. VII. Characterisation of the region around the *snail* and *cactus* loci. *Genetics* **126:** 679–694

Bodmer, R., L.Y. Jan, and Y.H. Jan. 1990. A new homeobox-containing gene, *msh-2*, is transiently expressed early during

mesoderm formation of *Drosophila*. *Development* **110:** 661–669.

Bogaert, T., N. Brown, and M. Wilcox. 1987. The Drosophila PS2 antigen is an invertebrate integrin that, like the fibronectin receptor, becomes localized to muscle attachments. *Cell* **51:** 929–940.

Bopp, D., E. Jamet, S. Baumgartner, M. Burri, and M. Noll. 1989. Isolation of two tissue-specific *Drosophila* paired box genes, *Pox meso* and *Pox neuro*. *EMBO J.* **8:** 3447–3457.

Boulay, J.L., C. Dennefeld, and A. Alberga. 1987. The Drosophila developmental gene *snail* encodes a protein with nucleic acid binding fingers. *Nature* **330:** 395–398.

Campos-Ortega, J.A. and V. Hartenstein. 1985 . The embryonic development of *Drosophila melanogaster*. Springer-Verlag. Berlin/Heidelberg.

Crews, S.T., J.B. Thomas, and C.S. Goodman. 1988. The *Drosophila* single-minded gene encodes a nuclear protein with sequence similarity to the *per* gene product. *Cell* **52:** 143–151.

Davis, R.L., H. Weintraub, and A.B. Lassar. 1987. Expression of a single transfected cDNA converts fibroblasts to myoblasts. *Cell* **60:** 987–1000.

Edgar, B.A. and P.H. O'Farrell. 1989. Genetic control of cell division patterns in the Drosophila embryo. *Cell* **57:** 177–187.

Gould, A.P., J.J. Brookman, D.I. Strutt, and R.A.H. White. 1990. Targets of homeotic gene control in Drosophila. *Nature* **348:** 308–312.

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

Hopwood, N.D., A. Pluck, and J.B. Gurdon. 1989. A *Xenopus* mRNA related to *Drosophila* twist is expressed in response to induction in the mesoderm and the neural crest. *Cell* **59:** 893–903.

Irish, V.F. and W.M. Gelbart. 1987. The *decapentaplegic* gene is required for dorsal-ventral patterning of the *Drosophila* embryo. *Genes & Dev* **1:** 868–879.

Kimble, M., R.W. Dettman, and E.C. Raff. 1990. The β3-tubulin gene of Drosophila melanogaster is essential for viability and fertility. *Genetics* **126:** 991–1005.

Knust, E., U. Dietrich, U. Tepass, K.A. Bremer, D. Weigel, H. Vässin, and J.A. Campos-Ortega. 1987. EGF homologous sequences encoded in the genome of *Drosophila melanogaster*, and their relation to neurogenic genes. *EMBO J.* **6:** 761–766.

Kosman, D., Y.T. Ip, M. Levine, and K. Arora. 1991. The establishment of the mesoderm–neuroectoderm boundary in the Drosophila embryo. *Science* (in press).

Leiss, D., U. Hinz, A. Gasch, R. Mertz, and R. Renkawitz-Pohl. 1988. β3 tubulin expression characterizes the differentiating mesodermal germ layer during *Drosophila* embryogenesis. *Development* **104:** 525–531.

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

Michelson, A.M., S.M. Abmayr, M. Bate, A. Martinez Arias, and T. Maniatis. 1990. Expression of a MyoD family member prefigures muscle pattern in *Drosophila* embryos. *Genes & Dev.* **4:** 2086–2097.

Nambu, J.R., R.G. Franks, S. Hu, and S.T. Crews. 1990. The single-minded gene of Drosophila is required for the expression of genes important for the development of CNS midline cells. *Cell* **63:** 63–75.

Nüsslein-Volhard, C., E. Wieschaus, and H. Kluding. 1984. Mutations affecting the pattern of the larval cuticle in *Drosophila melanogaster*. I. Zygotic loci on the second chromosome. *Wilhelm Roux's Arch. Dev. Biol.* **193:** 267–282.

Paterson, B.M., U. Walldorf, J. Eldridge, A. Dübendorfer, M. Frasch, and W.J. Gehring. 1991. The Drosophila homologue of vertebrate myogenic determination genes encodes a transiently expressed nuclear protein marking primary myogenic cells. *Proc. Nat. Acad. Sci.* **88:** 3782–3786.

Roth, S., D. Stein, and C. Nüsslein-Volhard. 1989. A gradient of nuclear localization of the dorsal protein determines dorsoventral pattern in the *Drosophila* embryo. *Cell* **59:** 1189–1202.

Rushlow, C. and K. Arora. 1990. Dorsal ventral polarity and pattern formation in the *Drosophila* embryo. *Sem. Cell Biol.* **1:** 137–149.

Rushlow, C.A., K. Han, J.L. Manley, and M. Levine. 1989. The graded distribution of the dorsal morphogen is initiatied by selective nuclear transport in *Drosophila*. *Cell* **59:** 1165–1177.

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

Slack, J. 1991. Embryology: Molecule of the moment. *Nature 349:* 16.

Sommer, R. and D. Tautz. 1991. Non-radioactive in situ hybridization to sectioned tissues. *Trends Genet.* **7:** 110.

Steward, R. 1989. Relocalization of the dorsal protein from the cytoplasm to the nucleus correlates with its function. *Cell* **59:** 1179–1188.

Tautz, D. and D. Pfeifle. 1989. A nonradioactive in situ hybridization method for the localization of specific RNAs in Drosophila embryos reveals a translational control of the segmentation gene hunchback. *Chromosoma* **98:** 81–85.

Thisse, B., C. Stoetzel, T.C. Gorostiza, and F. Perrin-Schmitt. 1988. Sequence of the twist gene and nuclear localization of its protein in endomesodermal cells of early *Drosophila* embryos. *EMBO J.* **7:** 2175–2183.

Tobin, S.J., P.J. Cook, and T.C. Burn. 1990. Transcripts of individual actin genes are differentially distributed during embryogenesis. *Dev. Genet.* **11:** 15–26.

Vässin, H., K.A. Bremer, E. Knust, and J.A. Campos-Ortega. 1987. The neurogenic locus *Delta* of *Drosophila melanogaster* is expressed in neurogenic territories and encodes a putative transmembrane protein with EGF-like repeats. *EMBO J.* **6:** 3431–3440.

Wolf, C., C. Thisse, C. Stoetzel, B. Thisse, P. Gerlinguer, and F. Perrin-Schmitt. 1991. The M-twist gene of *mus* is expressed in subsets of mesoderm cells and is closely related to the Xenopus X-twist and the Drosophila twist gene. *Dev. Biol.* **143:** 363–373.