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Uncoupling gastrulation and mesoderm differentiation in the *Drosophila* embryo

Y.Tony Ip[1], Keith Maggert and Michael Levine

Department of Biology, Center for Molecular Genetics, Bonner Hall, 9500 Gilman Drive, UCSD, La Jolla, CA 92093-0322, USA
[1]Present address: Program in Molecular Medicine, University of Massachusetts, Medical Center, 373 Plantation Street, Worcester, MA 01605, USA

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In *Drosophila*, ventral furrow formation and mesoderm differentiation are initiated by two regulatory genes, *twist* (*twi*) and *snail* (*sna*). Both genes are evolutionarily conserved and have also been implicated in vertebrate gastrulation. Evidence is presented that *sna* is sufficient to initiate the invagination of the ventral-most embryonic cells in the absence of twi^+ gene activity. The invaginated cells fail to express mesoderm regulatory genes, suggesting that ventral furrow formation can be uncoupled from mesoderm differentiation. Despite the previous demonstration that sna functions as a sequence-specific transcriptional repressor, low levels of sna that fail to repress neuroectoderm determinants in the presumptive mesoderm are nonetheless able to promote invagination. Cells that possess an ambiguous developmental identity can initiate the invagination process, providing further evidence that ventral furrow formation need not be linked to mesoderm differentiation.

Key words: *Drosophila*/gastrulation/mesoderm differentiation/*snail*/*twist*

Introduction

Dorso-ventral patterning of the *Drosophila* embryo is initiated by the dorsal (dl) morphogen gradient (reviewed by Govind and Steward, 1991; Ip and Levine, 1992; St Johnston and Nüsslein-Volhard, 1992). dl is a member of the Rel family of transcription factors, which includes the mammalian regulatory factor, NF-κB (reviewed by Liou and Baltimore, 1993). A dl concentration gradient is established in precellular embryos by an elaborate maternal signal transduction pathway that is related to the mammalian interleukin 1 cytokine pathway (reviewed by Wasserman, 1993). A transmembrane receptor, Toll, is locally activated in ventral regions by the spätzle ligand (Morisato and Anderson, 1994). This triggers an intracellular signaling cascade that ultimately releases dl from a cytoplasmic inhibitor, cactus (Geisler *et al.*, 1992; Kidd, 1992). A broad dl nuclear gradient is formed, with peak levels of protein in ventral regions, low levels in lateral regions, and little or none in dorsal regions (Roth *et al.*, 1989; Rushlow *et al.*, 1989; Steward, 1989). This dl gradient initiates the differentiation of three basic embryonic tissues: mesoderm, neuroectoderm and dorsal ectoderm.

Once in the nucleus, dl promptly initiates the transcription of a key mesoderm regulatory gene, *twist* (*twi*) (Jiang *et al.*, 1991; Pan *et al.*, 1991; Thisse *et al.*, 1991). Genetic circuitry studies and promoter dissection analyses suggest that the crude dl gradient triggers a steeper pattern of *twi* expression. Subsequently, dl and twi function synergistically to activate a second regulatory gene, *snail* (*sna*) (Ip *et al.*, 1992a). The dorso-ventral limits of the *sna* expression pattern coincide with the limits of the presumptive mesoderm, which spans the ventral-most 18–20 cells of early embryos. The sharp lateral limits of the *sna* expression help establish the boundary between the ventral mesoderm and lateral neuroectoderm (Alberga *et al.*, 1991; Kosman *et al.*, 1991; Leptin, 1991). *twi* and *sna* encode unrelated regulatory proteins, containing a basic helix–loop–helix (bHLH) motif and zinc fingers, respectively (Boulay *et al.*, 1987; Thisse *et al.*, 1987). *twi* and *sna* homologs have been implicated in the gastrulation of a broad spectrum of vertebrates, including zebra fish, frogs, chicks and mice (Sargent and Bennett, 1990; Hopwood and Gurdon, 1991; Wolf *et al.*, 1991; Nieto *et al.*, 1992, 1994; Smith *et al.*, 1992; Hammerschmidt and Nüsslein-Volhard, 1993; Nieto *et al.*, 1994).

twi^- and sna^- mutants are unique among all known zygotic patterning genes in *Drosophila* in that they completely fail to form a ventral furrow and lack mesoderm derivatives such as somatic and visceral muscles (Simpson, 1983). Mutations in other zygotic genes that participate in gastrulation and mesoderm differentiation cause less severe disruptions. For example, the ventral furrow is attenuated, but not abolished, in *folded gastrulation* (*fog*) mutants (Costa *et al.*, 1994), while mutations in *tinman* (*tin*) and *bagpipe* (*bap*) (Bodmer *et al.*, 1990; Azpiazu and Frasch, 1993; Bodmer, 1993) result only in the loss of specific subsets of mesoderm derivatives.

Previous studies suggest that both twi and sna are required for ventral furrow formation and mesoderm differentiation. As mentioned above, both processes are disrupted in each mutant. However, several lines of evidence suggest that twi and sna might exert distinct effects on embryogenesis. First, twi appears to function primarily as a transcriptional activator (Ip *et al.*, 1992a; Kosman *et al.*, 1991; Leptin, 1991), while sna is a repressor (Ip *et al.*, 1992b; Gray *et al.*, 1994). In twi^- mutants there is a failure to activate mesoderm-specific genes such as *tin* and *bap* (Azpiazu and Frasch, 1993; Bodmer, 1993). In contrast, *sna* mutants display a massive derepression of mesectodermal and neuroectodermal regulatory genes in ventral regions (Kosman *et al.*, 1991; Leptin, 1991; Arora and Nüsslein-Volhard, 1992). Normally, these genes are restricted to lateral regions, but they are activated in both lateral and ventral regions in *sna* mutants. This

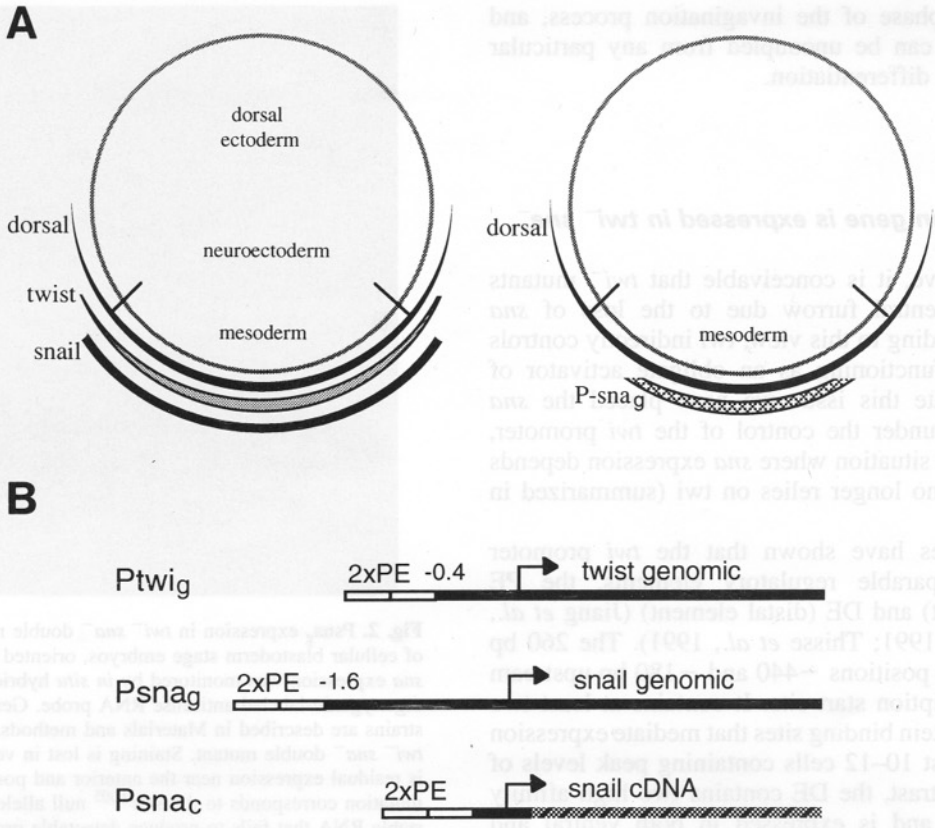

Fig. 1. Summary of twi and sna regulation. (**A**) The circles represent transverse sections through early embryos, with dorsal up and ventral down. Regulated nuclear transport generates a dorsal (dl) protein gradient, with peak levels in ventral regions and progressively lower levels in lateral and dorsal regions. This broad dl gradient triggers a steeper twi pattern that extends just beyond the presumptive mesoderm, into the neuroectoderm. dl and twi act together to initiate *sna* expression within the limits of the presumptive mesoderm. The circle on the right shows that $Psna_g$ expression is activated solely by dl and does not require twi. Expression is restricted to the ventral-most 12–14 cells and does not include the entire presumptive mesoderm. (**B**) *twi* and *sna* fusion genes used for genetic complementation assays. Coding sequences were placed under the control of the PE region of the *twi* promoter, which maps between positions −440 and −180 bp upstream of the *twi* transcription start site. It contains a series of low affinity dl binding sites that mediate expression in the ventral-most 12–14 cells in response to peak levels of dl protein. The $Ptwi_g$ transgene was prepared by placing two tandem copies of the PE upstream of a *twi* genomic DNA fragment containing the entire *twi* coding sequence and 440 bp of the 5′ flanking region. The $Psna_g$ transgene contains two copies of the PE upstream of a *sna* genomic DNA that includes the entire coding sequence and the first 1.6 kb of the *sna* promoter. The $Psna_c$ transgene contains a full-length *sna* cDNA, 340 bp of the *twi* promoter (from position −180 to +160 relative to the start site), and two copies of the PE. The unfilled rectangles correspond to the 260 bp PE sequence, while the filled horizontal bars represent genomic DNA. The stippled bar corresponds to the *sna* cDNA, and the arrows indicate the location of the transcription start site.

derepression causes an expansion of the mesectoderm at the expense of mesodermal derivatives (Rao *et al.*, 1991). These studies suggest that twi initiates mesoderm differentiation by activating target genes such as *tin*, while sna functions only indirectly in this process, by excluding the neuroectoderm fate. Another indication that twi and sna function differently stems from detailed analyses of the gastrulation process (Leptin and Grunewald, 1990). *sna* mutants cause a more complete loss of the ventral furrow than *twi* mutants. twi^- embryos display small, transient pockets of partially ingressing cells, while *sna* mutants are virtually devoid of such cells. Several models have been proposed to account for the loss of both the ventral furrow and mesoderm derivatives in *sna* mutants (e.g. Kosman *et al.*, 1991; Leptin, 1991). However, the interpretation of these earlier studies is compromised by the tight linkage of the *twi* and *sna* expression patterns. In particular, *sna* expression depends on twi^+ gene activity, and consequently, twi^- embryos are nearly devoid of *sna* (Kosman *et al.*, 1991; Leptin, 1991; Arora and Nüsslein-Volhard, 1992). In the present study we have uncoupled *twi* and *sna* activity by creating a *sna* transgene that is active in twi^- embryos. This involved placing the sna protein coding region under the control of *twi* promoter sequences, thereby creating a situation where *sna* is activated in direct response to dl.

Here we show that sna is sufficient to induce the formation of an attenuated ventral furrow in the absence of twi^+ gene activity. The resulting furrow is interrupted by non-invaginating cells, similar to the situation seen in fog^- mutants (Costa *et al.*, 1994). Expression of *sna* in the absence of twi^+ gene activity uncouples ventral furrow formation and mesoderm differentiation, in that the invaginating cells fail to express various mesoderm marker genes. Evidence is presented that sna need not function as a transcriptional repressor to promote invagination. For example, low levels of sna that are insufficient to repress the ventral expression of *single-minded* (*sim*; Crews *et al.*, 1988; Thomas *et al.*, 1988) are nonetheless adequate for furrow formation. When these low levels are supplied to a twi^+ embryo the invaginating cells express both mesoderm and mesectoderm markers, suggesting that they possess an intermediate developmental identity. These results suggest that sna is essential for initiating the

stochastic, slow phase of the invagination process, and that invagination can be uncoupled from any particular program of tissue differentiation.

Results

A twi–sna fusion gene is expressed in twi⁻ sna⁻ double mutants

As discussed above, it is conceivable that *twi*⁻ mutants fail to form a ventral furrow due to the loss of *sna* expression. According to this view, twi indirectly controls invagination by functioning as an obligate activator of *sna*. To investigate this issue we have placed the *sna* coding sequence under the control of the *twi* promoter, thereby creating a situation where *sna* expression depends solely on dl and no longer relies on twi (summarized in Figure 1A).

Previous studies have shown that the *twi* promoter contains two separable regulatory elements, the PE (proximal element) and DE (distal element) (Jiang *et al.*, 1991; Pan *et al.*, 1991; Thisse *et al.*, 1991). The 260 bp PE maps between positions −440 and −180 bp upstream of the *twi* transcription start site. It contains at least two low affinity dl protein binding sites that mediate expression in the ventral-most 10–12 cells containing peak levels of dl protein. In contrast, the DE contains two high affinity dl binding sites, and is expressed in both ventral and ventrolateral regions where there are high and intermediate levels of the dl protein. In addition, there is reason to believe that the DE also mediates twi autoregulation (Jiang *et al.*, 1991). The PE region of the *twi* promoter was used to express sna protein coding sequences since it is primarily activated by dl, and has only a minimal dependence on *twi*⁺ or *sna*⁺ activity.

Previous studies have shown that one, two or four copies of the PE sequence drive progressively more intense expression of a *lacZ* reporter gene in response to the dl gradient (Jiang and Levine, 1993). Consequently, two copies of the PE were placed upstream of a genomic DNA fragment encompassing the entire *sna* transcription unit, including the first 1.6 kb of the *sna* promoter (Figure 1B). It has been shown that the truncated, 1.6 kb *sna* promoter directs an expression pattern quite similar to the *twi* PE, so that its expression is restricted to the ventral-most 12–14 cells of the early embryo (Ip *et al.*, 1992a). The resulting $Psna_g$ fusion gene was introduced into the *Drosophila* germline via P-transformation (Spradling and Rubin, 1982).

twi⁻ *sna*⁻ double mutants are virtually devoid of *sna* expression (Figure 2A); during the completion of cellularization there is only residual expression of endogenous *sna* RNAs at the anterior and posterior poles. In contrast, comparable mutants that contain the $Psna_g$ transgene exhibit strong expression in the ventral-most 12–14 cells (Figure 2B). The expression pattern exhibits crude pair-rule modulations, reminiscent of the *twi* expression pattern in *twi*⁻ embyros (Jiang *et al.*, 1991). This observation suggests that twi might exert a weak autoregulatory effect on the PE. Nonetheless, the staining pattern shown in Figure 2B clearly demonstrates that the $Psna_g$ fusion gene is efficiently expressed in the absence of endogenous *twi*⁺ and *sna*⁺ products.

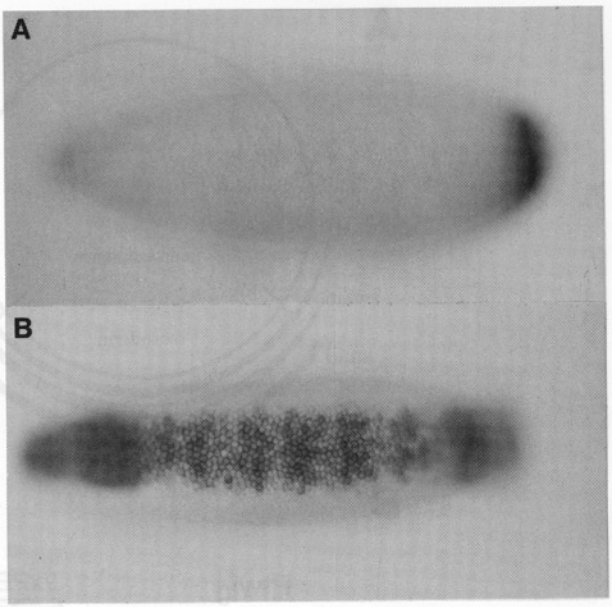

Fig. 2. $Psna_g$ expression in *twi*⁻ *sna*⁻ double mutants. Ventral views of cellular blastoderm stage embryos, oriented with anterior to the left. *sna* expression was monitored by *in situ* hybridization using a digoxygenin-labeled antisense RNA probe. Genetic crosses and mutant strains are described in Materials and methods. (**A**) *sna* expression in a *twi*⁻ *sna*⁻ double mutant. Staining is lost in ventral regions, but there is residual expression near the anterior and posterior poles. The *sna* mutation corresponds to the sna^{IIG05} null allele, which encodes a stable RNA that fails to produce detectable protein. The loss of this RNA in ventral regions is due to the absence of *twi*⁺ and *sna*⁺ gene activity. (**B**) Same as (A) except that the *twi*⁻ *sna*⁻ double mutant contains the $Psna_g$ transgene. Strong expression is detected in the ventral-most 12–14 cells. This pattern is narrower and less uniform than wild-type *sna* expression, but is quite similar to the *twi* pattern seen in *twi*⁻ embryos (Jiang *et al.*, 1991).

sna is sufficient to induce an attenuated ventral furrow

The impact of $Psna_g$ expression was examined by *in situ* hybridization and tissue sectioning. Particular efforts centered on the expression pattern of the mesectodermal regulatory gene, *sim*. *sim* is expressed in two lateral lines that extend along the length of the embryo (Crews *et al.*, 1988; Thomas *et al.*, 1988). Each *sim* line encompasses just a single cell in width and is located just beyond the lateral limits of the presumptive mesoderm (Figure 3A). After the completion of ventral furrow formation the two *sim* lines are brought together at the ventral midline, which gives rise to various neuronal and non-neuronal cell types of the ventral nerve cord in older embryos (Nambu *et al.*, 1990). As shown previously, the *sim* expression pattern is disrupted in either *twi*⁻ (Figure 3B) or *sna*⁻ (Figure 3C) embryos (Nambu *et al.*, 1990; Kosman *et al.*, 1991; Leptin, 1991; Arora and Nüsslein-Volhard, 1992). In *twi*⁻ mutants the *sim* lines are shifted to more ventral positions, so that they are separated by just 10–12 cells, rather than 18–20 cells (compare Figure 3B with A). *sna* mutants show a severe derepression of the *sim* pattern, so that staining encompasses the ventral-most 8–10 cells. As shown previously (Arora and Nüsslein-Volhard, 1992), *sim* expression is completely lost in *twi*⁻ *sna*⁻ double mutants (Figure 3D).

Each of the mutants shown in Figure 3B, C and D lacks a ventral furrow although, as noted previously, *twi* mutants exhibit transient pockets of partially invaginating cells

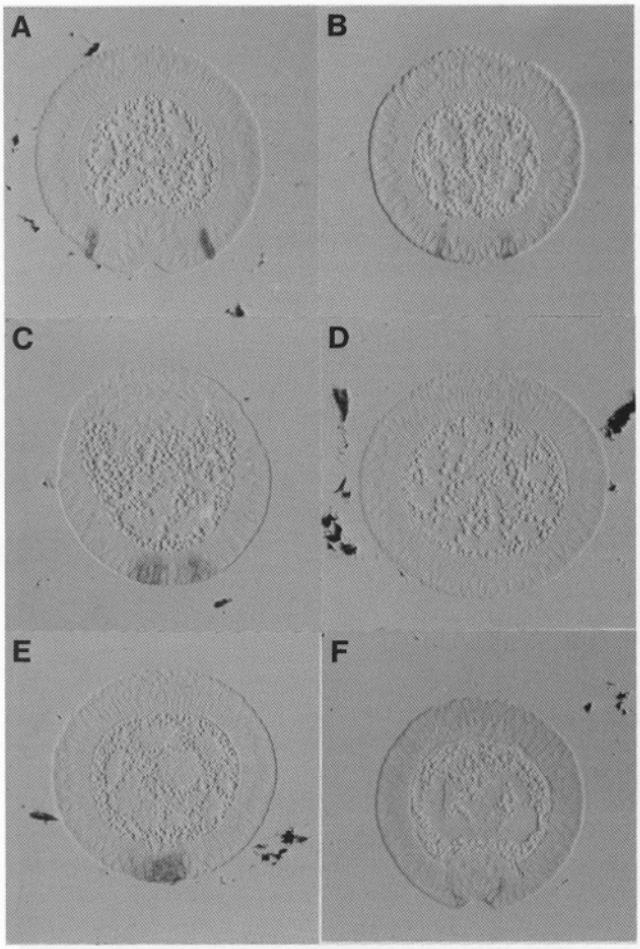

Fig. 3. sna is sufficient to induce invagination. Embryos were stained with a *sim* hybridization probe, embedded in plastic, and sectioned. They are oriented with dorsal up. (**A**) Wild-type gastrulating embryo. The *sim* lines bracket the ventral furrow, which includes the ventral-most 18–20 cells. (**B**) twi^- embryo. The *sim* lines are shifted to more ventral positions, and there is no ventral furrow. (**C**) sna^- embryo. There is a severe derepression of the *sim* staining pattern, so that expression encompasses the ventral-most 8–10 cells. There is no ventral furrow. (**D**) twi^- sna^- double mutant. *sim* expression is abolished and there is no ventral furrow. (**E**) twi^- sna^- double mutant containing the $Ptwi_g$ transgene. *sim* expression is restored in the ventral-most regions, similar to the pattern observed in *sna* mutants (C). However, there is no ventral furrow. (**F**) twi^- sna^- double mutant containing the $Psna_g$ transgene. A ventral furrow is restored that includes invagination of the ventral-most 8–10 cells. The *sim* lines bracket the furrow.

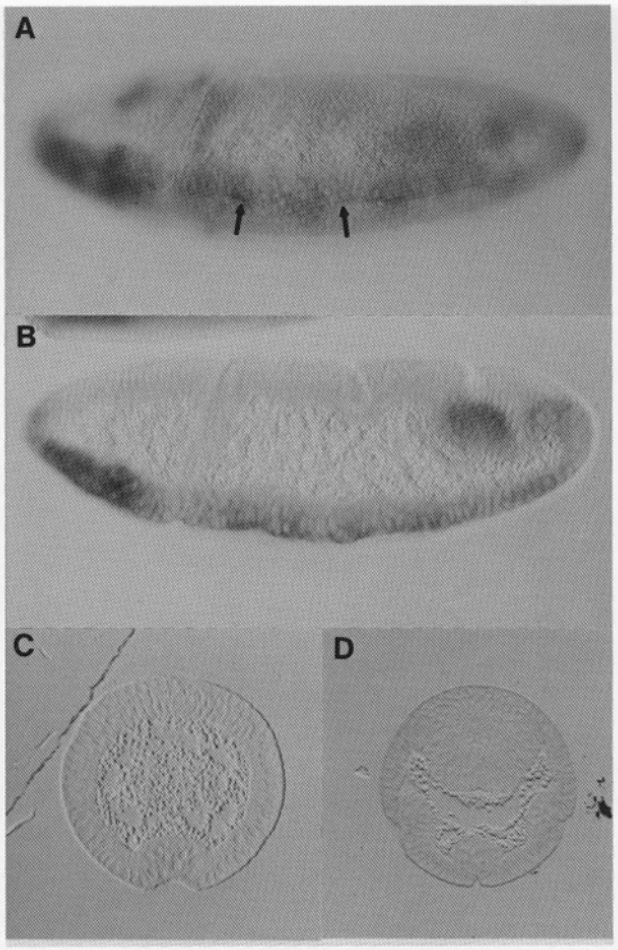

Fig. 4. *sna* expression in gastrulating embryos. twi^- sna^- double mutants carrying the $Psna_g$ transgene. Embryos were hybridized with a *sna* antisense RNA probe. (**A** and **B**) Whole mount preparations of stained embryos with anterior to the left and dorsal up. (**C** and **D**) Cross-sections with dorsal up. The ventral furrow is not continuous along the length of the embryo, and is interrupted by patches (between arrows, A) of uninvaginated cells. *sna* expression is prematurely lost in these embryos (as compared with wild-type) so that staining is barely detected after invagination (C and D). The ventrolateral invaginations in (D) might correspond to the cephalic furrows.

(Leptin and Grunewald, 1990; Figure 3B). Both *sim* expression and a ventral furrow are restored in twi^- sna^- double mutants that contain the $Psna_g$ fusion gene (Figure 3F). The ultimate fate of the invaginated cells is unclear since they fail to express a number of mesoderm markers, including tin (Azpiazu and Frasch, 1993; Bodmer, 1993), nautilus (Michelson *et al.*, 1990) and zfh1 (Fortini *et al.*, 1991; Lai *et al.*, 1991) (data not shown). The absence of overt mesoderm derivatives in these embryos suggests that ventral furrow formation is not sufficient to induce mesoderm differentiation.

The ventral furrow that is formed in twi^- sna^- double mutants containing the $Psna_g$ fusion gene is abnormal in several important aspects. First, the furrow includes only 10–12 cells, while the normal furrow contains 18–20 cells (compare Figure 3F with A). Second, the sna-directed furrow does not invaginate as deeply as the normal furrow, and the invaginated cells fail to make tight contact with the overlying ectoderm (Figure 4D). Third, there are discontinuities in the furrow, so that coherent clusters of cells remain at the ventral midline (Figure 4A). Thus, in many ways, the furrow that is observed is only a bit more robust than the transient pockets of invaginating cells observed in twi^- mutants (Leptin and Grunewald, 1990). This is consistent with the notion that sna is sufficient to induce at least some aspects of invagination in the absence of twi^+ gene activity.

There are several possible explanations for the defective furrows seen in twi^- sna^- embryos carrying the $Psna_g$ fusion gene. Foremost among these is the observation that the dorso-ventral limits of $Psna_g$ fusion gene are narrower (12–14 cells) than that observed for the normal, endogenous gene (18–20 cells). Furthermore, direct comparison of the transgenic and endogenous expression patterns suggests that the levels of $Psna_g$ expression are about half that of wild-type (data not shown). In addition, the maintenance of the sna pattern depends on both twi^+ and

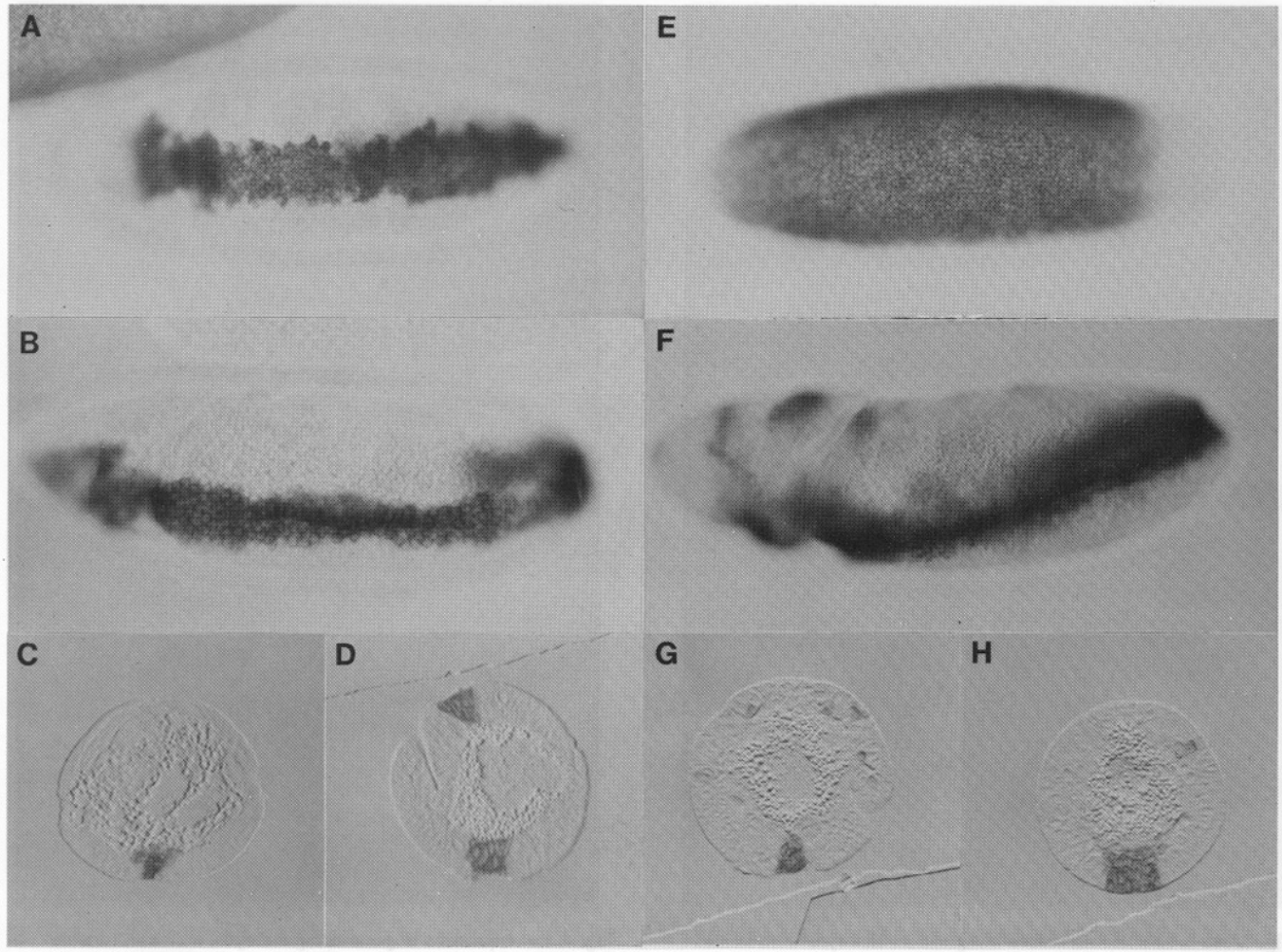

Fig. 5. Derepression of sim and rho does not preclude invagination. *sna*$^-$ embryos were stained following hybridization with either a *sim* (A–D) or *rho* (E–H) antisense RNA probe. Whole mount embryos (A, B, E and F) are presented with anterior to the left. Sections (C, D, G and H) are oriented with dorsal up. (**A**) Gastrula-stage *sna*$^-$ mutant stained to show the distribution of *sim* RNAs. There is a derepression of the pattern, so that staining includes the ventral-most 8–10 cells. The stained cells fail to form a ventral furrow. (**B**) An elongating *sna*$^-$ mutant carrying the $Psna_c$ transgene that was stained with a *sim* hybridization probe. Some of the *sim*-expressing cells invaginate through a shallow ventral furrow. The invaginated cells fail to make intimate contact with the overlying ectoderm after elongation (**C** and **D**). The two patches of staining on the top and bottom of the embryo shown in D results from germ band elongation. (**E**) Gastrula-stage *sna*$^-$ mutant that was stained to show the distribution of *rho* RNAs. There is a complete derepression of the pattern in ventral regions. None of the stained cells form a ventral furrow. (**F**) An elongating *sna*$^-$ mutant carrying the $Psna_c$ transgene that was stained with a *rho* hybridization probe. Some of the *rho*-expressing cells invaginate through a shallow ventral furrow. As mentioned above, the stained cells do not deeply invaginate, and they fail to make tight contact with the overlying ectoderm. (**G** and **H**) Elongating embryos were sectioned after hybridization with the *rho* probe. Some of the stained cells manage to enter through a shallow furrow.

sna$^+$ activities, and consequently, the $Psna_g$ transgene directs a discontinuous pattern (Figure 2B) and is markedly reduced in older *twi*$^-$ *sna*$^-$ double mutants (see Figure 4A–D). Despite these disruptions in furrow formation, it would appear that sna is sufficient to drive at least some aspects of invagination in the absence of *twi*$^+$ gene activity. However, we cannot exclude the possibility that *twi* independently controls aspects of cytoskeletal reorganization and some of the cell adhesion changes associated with invagination.

twi is unable to induce ventral furrow formation in the absence of *sna*$^+$ gene activity. To compare the activities of *twi* and *sna* in gastrulation, a P-transposon containing the *twi* coding region ($Ptwi_g$; Figure 1B) was expressed in *twi*$^-$ *sna*$^-$ double mutants. The $Ptwi_g$ transposon contains two tandem copies of the *twi* PE sequence placed upstream of a *twi* genomic DNA fragment that contains the entire *twi* coding region and the first 400 bp of the 5′ flanking sequence. $Ptwi_g$ is strongly expressed in the ventral-most 12–14 cells in early embryos (data not shown), consistent with previous promoter studies (Jiang and Levine, 1993). When expressed in *twi*$^-$ *sna*$^-$ double mutants it restores *sim* expression but fails to induce a ventral furrow (Figure 3E), similar to the situation observed in *sna*$^-$ mutants containing the endogenous *twi* gene (Figure 3C). These results suggest that *sna* is sufficient to induce a ventral furrow, while *twi* is not. They are consistent with the previous observation that *sna* mutants cause a more severe loss of the ventral furrow than comparable *twi* mutants (Leptin and Grunewald, 1990).

Ventral furrow formation without repression of neuroectoderm-specific genes

It has been proposed that sna promotes ventral furrow formation indirectly, by excluding the expression of mesectodermal and neuroectodermal regulatory genes

from the mesoderm and restricting them to lateral regions that form the neuroectoderm (Kosman *et al.*, 1991; Leptin, 1991; Arora and Nüsslein-Volhard, 1992). According to this view, regulatory genes normally restricted to lateral regions become derepressed in ventral regions of *sna*$^-$ mutants, and inhibit ventral furrow formation and mesoderm differentiation. In an effort to determine whether sna repression activity is essential for furrow formation, the dose of *sna*$^+$ gene activity was reduced by expressing low levels of sna protein. For this purpose, a *sna* cDNA was placed under the control of the *twi* PE promoter sequence (Psna$_c$, see Figure 1B). The Psna$_c$ fusion gene lacks *sna* promoter sequences, and is only weakly expressed in early embryos (at least a 3- to 4-fold reduction in expression as compared with Psna$_g$; data not shown).

The *sim* and *rhomboid* (*rho*) expression patterns normally bracket the limits of the ventral furrow, and are restricted to lateral stripes in the presumptive mesectoderm and neuroectoderm (Crews *et al.*, 1988; Thomas *et al.*, 1988; Bier *et al.*, 1990; Ip *et al.*, 1992b). In *sna*$^-$ mutants the *sim* and *rho* patterns are derepressed in ventral regions (Figure 5A and E; compare with Figure 3A). Previous promoter fusion studies have demonstrated that the sna protein binds to the *rho* promoter region and directly represses its transcription in the presumptive mesoderm (Ip *et al.*, 1992b). *twi*$^+$ *sna*$^-$ embryos carrying the Psna$_c$ transgene fail to repress *sim* and *rho* expression in ventral regions (Figure 5B and F). Thus, it would appear that the weak expression provided by the Psna$_c$ transposon is not sufficient to repress either *sim* or *rho*, so that both genes are completely derepressed in ventral regions (Figure 5B and F).

Despite the ventral derepression of the *sim* and *rho* expression patterns, *sna*$^-$ mutants carrying the Psna$_c$ transposon exhibit a shallow ventral furrow spanning six to eight cells (Figure 5B and F). The invaginating cells express both *sim* and *rho* (Figure 5C, D, G and H), although in wild-type embryos, cells that express these genes never enter the ventral furrow, but instead form portions of the ventral midline and ventral neuroectoderm. These results suggest that the expression of *sim* and *rho* do not preclude invagination.

The invaginating cells seen in *sna*$^-$;Psna$_c$ embryos appear to possess a mixed developmental identity, in that they express both mesectoderm/neuroectodermal genes and mesodermal genes. For example, *tin* is normally activated in the invaginating cells of the ventral furrow, and ultimately becomes restricted to the heart progenitors of the internal mesoderm in lateral regions (Bodmer *et al.*, 1990). *tin* expression is absent in either *twi*$^-$ or *sna*$^-$ mutants (Bodmer *et al.*, 1990; Figure 6A). Expression is restored in *sna*$^-$ mutants carrying the Psna$_c$ transposon (Figure 6B). The *tin*-expressing cells invaginate through the shallow ventral furrow and enter internal regions (Figure 6C and D). As shown above (Figure 5), these invaginated cells also express *sim* and *rho*. The apparent differences in the extent of invagination seen in Figures 5 and 6 probably result from the exact plane of sectioning along the anteroposterior axis since the furrows are discontinuous (see Figures 5B and 6B).

Although low levels of *sna* are sufficient to induce a ventral furrow in a *twi*$^+$ background, these levels are insufficient for furrow formation in a *twi*$^-$ *sna*$^-$ background (data not shown). These observations suggest that *twi* might influence ventral furrow formation beyond its role in regulating *sna* expression (see Discussion). In addition, it is somewhat surprising that the Psna$_c$ transgene fails to repress *sim* and *rho* expression in *twi*$^+$ embryos since previous genetic studies suggest that residual levels of *sna* are sufficient to repress both *sim* and *rho* in *twi*$^-$ mutants (Kosman *et al.*, 1991; Leptin, 1991). It would appear that the presence of *twi*$^+$ gene products somehow attenuates sna repression activity (see Discussion).

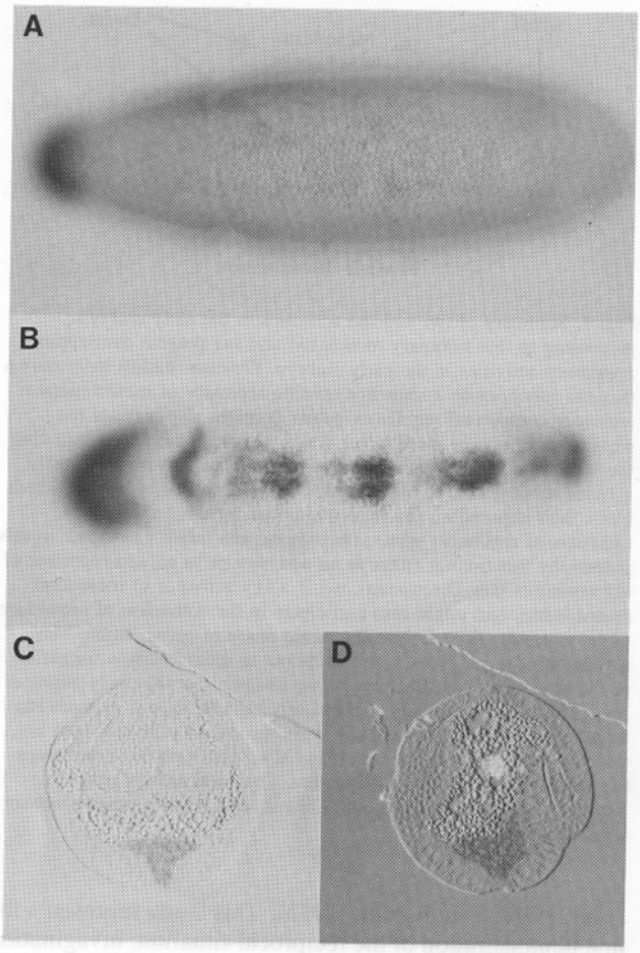

Fig. 6. Cells with an ambiguous developmental identity can invaginate. *sna*$^-$ mutants were stained following hybridization with a *tin* antisense RNA probe. Both whole mount preparations (**A** and **B**) and cross-sections (**C** and **D**) are presented as described in the legend to Figure 5. (A) *tin* expression in a *sna*$^-$ mutant. Residual staining is observed in anterior regions, but is lost in the presumptive mesoderm. (B) A *sna*$^-$ mutant carrying the Psna$_c$ transgene. *tin* expression is restored in ventral regions. Some of the stained cells invaginate through a shallow ventral furrow. The sections in C and D indicate that the stained cells are fully invaginated in specific regions along the anteroposterior axis.

Discussion

We have presented evidence that invagination and mesoderm differentiation can be partially uncoupled during *Drosophila* gastrulation. Previous studies in sea urchins and frogs have shown that endoderm and mesoderm differentiation can occur in the absence of invagination, through the use of experimental manipulations that produce 'exogastrulae' (e.g. Ransick and Davidson, 1993; Ransick

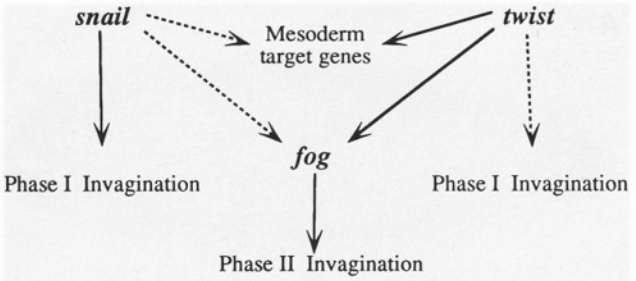

Fig. 7. Summary of twi and sna activities in the early embryo. According to this summary model, twi and sna possess overlapping regulatory activities in the early embryo. Previous studies have shown that sna functions as a sequence-specific repressor of mesectodermal and neuroectodermal regulatory genes. It might also activate the expression of one or more target genes that are required for initiating the stochastic phase of ventral furrow formation. sna might directly activate these genes, or might repress 'anti-invagination' genes which block their expression. sna is also required for the activation of mesodermal regulatory genes; once again, sna might participate in this process by functioning either as an activator or as an anti-repressor of twi inhibitors (see Discussion). twi is a key activator of mesoderm determinants, and might also participate in the activation of secondary target genes required for the stochastic phase of invagination. The activation of these genes is not sufficient to induce furrow formation in the absence of sna function, so we imagine that they help augment invagination once this process has been initiated by one or more sna target genes. Finally, twi and sna work in concert with activated fog, which is thought to trigger a cell signaling pathway that coordinates the second phase of furrow formation. The solid arrows represent 'strong' genetic interactions, while the dashed arrows indicate weaker regulatory effects.

et al., 1993; Venuti *et al.*, 1993). This study represents the first demonstration of the reciprocal situation: invagination without mesoderm differentiation. *twi* appears to coordinate both processes by activating *sna*, as well as mesoderm determinants such as *tin* and *bap*. sna might initiate invagination by activating the expression of target genes mediating changes in cell adhesion and/or reorganization of the cytoskeleton. Its failure to repress lateral neuroectodermal regulatory genes does not preclude invagination.

Relative roles of twi and sna

Evidence that ventral furrow formation and mesoderm differentiation can be uncoupled is based on the observation that sna can promote invagination in the absence of twi^+ gene activity. The invaginating cells do not appear to differentiate into mesoderm derivatives since sna^+ twi^- embryos lack expression of various mesoderm determinants such as *tin* and *bap* (Bodmer *et al.*, 1990; Azpiazu and Frasch, 1993). It would appear that twi^+ activity is required for the activation of these latter genes. However, it would be misleading to conclude that gastrulation and mesoderm differentiation can be completely uncoupled. A more conservative view is that *twi* and *sna* possess overlapping regulatory activities in the early embryo, as summarized in Figure 7. According to this model, *twi* is primarily responsible for the activation of mesoderm determinants, while *sna* regulates target genes required for the initial, stochastic phase of the invagination process. However, mesoderm differentiation also requires sna^+ gene activity, and invagination is expedited by *twi*, as discussed below.

Previous studies suggest that the invagination of the ventral furrow is a two-step process, which is initiated by the stochastic invagination of the ventral-most cells (Leptin and Grunewald, 1990; Kam *et al.*, 1991; Sweeton *et al.*, 1991). Shortly thereafter, gastrulation is synchronized by a putative cell signaling system, whereby invaginating cells signal their neighbors to enter the ventral furrow (Costa *et al.*, 1994). Signaling depends on *fog*, which appears to encode a secreted protein that might function as a ligand. It is thought to activate a cell signaling cascade that includes *concertina*, which is related to membrane associated G proteins (Parks and Wieschaus, 1991). We propose that sna initiates the stochastic phase of the invagination process by permitting the expression of one or more target genes which mediate changes in cell adhesion and/or cytoskeletal organization. *twi* and *sna* act in concert to activate *fog* expression, and thereby initiate the second phase of the invagination process. However, the furrow that is formed in sna^+ twi^- embryos (see Figures 3F and 4A) does not appear to be as robust as that observed in fog^- embryos. This would suggest that *twi* does more to promote invagination than activate *sna* and *fog*. Perhaps twi also participates in the activation of additional target genes that are important for the completion of stochastic invagination. Further support for this possibility stems from the observation that low levels of sna which are able to initiate invagination in twi^+ embryos are insufficient for invagination in the absence of twi^+ function.

Although *twi* is essential for the activation of mesoderm regulatory genes, *sna* also participates in this process. Most notably, mesoderm determinants such as *tin* and *bap* are virtually silent in twi^+ sna^- embryos. The expression of these genes is restored even with minimal sna^+ activity (Figure 6). As discussed below, it is possible that sna potentiates the twi-mediated activation of mesoderm regulatory genes by blocking the expression of *twi* inhibitors which are normally restricted to the lateral neuroectoderm.

Conservation of sna activity

sna has been shown to function as a sequence-specific repressor that excludes the expression of mesectodermal and neuroectodermal regulatory genes from the presumptive mesoderm (Ip *et al.*, 1992b; Gray *et al.*, 1994). It is conceivable that this is the basis by which sna potentiates twi-mediated activation of mesoderm target genes. For example, a number of bHLH regulatory proteins are expressed in the lateral neuroectoderm of early embryos, including those encoded by genes contained in the achaete-scute (AS-C) and Enhancer of split [E(spl)] complexes (e.g. Campuzano and Modolell, 1992; Schrons *et al.*, 1992). Several of these genes are derepressed in ventral regions of sna^- mutants (Kosman *et al.*, 1992; Leptin, 1992), and it is possible that their products block *twi* activity through the formation of inactive twi−E(spl) and/or twi−AS-C heterodimers. However, this model is potentially compromised by the observation that low levels of sna which fail to repress *sim* are nonetheless sufficient to restore twi-mediated activation of mesoderm target genes, such as *tin* (see Figure 6). It would appear that the derepression of the divergent sim bHLH protein in ventral regions does not block twi activity. We are currently determining whether these embryos also exhibit ventral derepression of AS-C and E(spl) genes.

sna might promote ventral furrow formation by repressing the expression of one or more unknown target genes that inhibit invagination. If so, it would appear that target promoters might respond to distinct thresholds of sna repressor, whereby low levels which fail to repress *sim* are nonetheless sufficient to repress 'anti-invagination' genes (see Figure 7). An alternative view is that sna functions as both an activator and a repressor. Perhaps low levels that are sufficient to activate invagination genes are unable to repress target genes such as *sim* and *rho*. Evidence that sna might function as an activator stems from the observation that *sna* expression is severely down-regulated in *sna*$^{-}$ mutants (Y.T.Ip and D.Kosman, unpublished results). Moreover, the *sna* promoter contains at least one high affinity sna protein binding site (Y.T.Ip and R.Park, unpublished results). Future efforts will address the issue whether this sna site directly mediates transcriptional activation.

sna is expressed at multiple points during *Drosophila* development (Alberga *et al.*, 1991; Kosman *et al.*, 1991; Leptin, 1991), and it is conceivable that the common denominator of *sna* function involves invagination. For example, after being expressed in the invaginating mesoderm, *sna* is activated in neuroblasts as they delaminate from the ventral ectoderm. sna is a member of a family of related zinc finger proteins that includes scratch and escargot (Whiteley *et al.*, 1992; E.Bier, personal communication). These latter genes are not expressed during gastrulation, but are all active during neurogenesis. In this regard it is interesting to note that a recent study of a *sna*-related gene in chick embryos suggests a role in the invagination and migration of primary mesenchyme cells from the primitive streak (Nieto *et al.*, 1994).

Materials and methods

Fly strains

Genetic crosses involved mating *twi sna*/CyO males with females carrying the double balancer, *y w*; Bc Elp/CyO,P{*ftz*−*lacZ*,*ry*$^{+}$};Ki/TM6,*y*$^{+}$. F1 males were collected carrying the genotype *y w*; *twi sna*/Bc Elp;+/TM6,*y*$^{+}$. Separate crosses were performed with males carrying the P-transposon (e.g. Psna$_g$) on the third chromosome. These were mated with females carrying the double balancer described above. F1 females were collected that contain the genotype *y w*/*y w*;+/CyO,P{*ftz*−*lacZ*,*ry*$^{+}$}; P-transposon/TM6,*y*$^{+}$. These F1 females were mated with the F1 males of the preceding cross, and F2 flies were collected with the genotype *y w*; *twi sna*/CyO,P{*ftz*−*lacZ*,*ry*$^{+}$}; P-transposon/TM6,*y*$^{+}$. These were mated with each other to maintain homozygosed stocks. Homozygous *twi*, *sna* embryos were identified by their failure to hybridize with a *lacZ* antisense RNA probe (driven by the *ftz* promoter on the CyO balancer chromosome). Similar results were obtained with two different double mutant stocks, *twi*^{IIH} *sna*^{IIG05} and *twi*^{S60} *sna*^{IIG05}.

Transformation vectors

A *sna* genomic DNA fragment containing ~6 kb of the 5′ flanking region, the entire protein coding sequence, and the 3′ untranslated trailer sequence was digested with *Hin*dIII to yield a 5.6 kb fragment spanning the region from −4.2 kb to +1.4 kb relative to the transcription start site. The purified DNA fragment was cloned into the unique *Hin*dIII site of the pGem7zf(+) vector, and subsequently digested with a mixture of *Eco*RV and *Hin*dIII. This releases a fragment containing the first 1.6 kb of the 5′ flanking region and the *sna* coding sequence. The Psna$_g$ transgene was prepared by inserting this fragment into the 2×PE P-transposon (Jiang and Levine, 1993). 2×PE contains one copy of the *twi* PE sequence placed upstream of a *twi*−*lacZ* fusion gene containing the first 440 bp of the *twi* 5′ flanking region. The *lacZ* sequence was replaced with the *sna* genomic DNA fragment described above. The parental P-transposon corresponds to the pAUG−β-galactosidase vector, which contains the *white* gene as a marker (Thummel *et al.*, 1988).

The Ptwi$_g$ transgene was made by replacing the *lacZ* sequence in the 2×PE P-transformation vector with a 2.3 kb *twi* *Eco*RI fragment (spanning the region from −440 bp to +1.9 kb relative to the transcription start site). The Psna$_c$ transgene was prepared in a similar manner after digesting a full-length *sna* cDNA with *Nde*I. An *Nde*I fragment spanning the region from +160 bp to +1.35 kb was used to replace the *lacZ* sequence in the 2×PE vector.

In situ hybridization and tissue sections

Embryos were harvested, fixed and hybridized with digoxygenin-labeled antisense RNA probes exactly as described previously (Tautz and Pfeifle, 1989; Jiang *et al.*, 1991). Fixed and stained embryos were mounted in Spurr's resin (Sigma) as described by Haines (1992), except that the embryos were not embedded under vacuum. They were allowed to dry at 70°C for 3 days or 65°C for 1 day in rubber molds (purchased from Electron Microscopy Sciences). Hardened blocks were shaved by hand and serial 5 μm sections were prepared with a Sorvall microtome.

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