Uncoupling gastrulation and mesoderm differentiation in the Drosophila embryo

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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−1 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.

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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-kB (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 spitzel 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; Thissel 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; Thissel 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 since 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 Psnag 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 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 Ptwig 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 Psnag 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 Psnag 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; Thiss 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 PsnaA 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 PsnaB 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− embryos (Jiang et al., 1991). This observation suggests that twi might exert a weak auto-regulatory effect on the PE. Nonetheless, the staining pattern shown in Figure 2B clearly demonstrates that the PsnaA fusion gene is efficiently expressed in the absence of endogenous twi+ and sna+ products.

Fig. 2. PsnaA 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 digoxigenin-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 snaB iso null allele, which encodes a stable RNA that fails to produce detectable protein. The loss of this gene 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 PsnaB 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 PsnaB 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 PwiG 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 PsnaG transgene. A ventral furrow is restored that includes invagination of the ventral-most 8-10 cells. The sim lines bracket the furrow.

(Leptin and Grunewald, 1990; Figure 3B). Both sim expression and a ventral furrow are restored in twi− sna− double mutants that contain the PsnaG 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 zfhh (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 PsnaG 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 PsnaG fusion gene. Foremost among these is the observation that the dorso-ventral limits of PsnaG 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 PsnaG 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 Psnag 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 Psnag 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 Psnag 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; Figure 1B) was expressed in twi' sna' double mutants. The Ptwi 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 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 Grunwald, 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, see Figure 1B). The Psna 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; 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 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 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 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 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 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 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).

**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...
ventral furrow is a two-step process, which is initiated by the stochastic invagination of the ventral-most cells (Leptin and Gruenwald, 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 mesodermal 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 euchromatin (AS-C) and Enhancer of split [E(spl)] complexes (e.g. Campuzano and Model, 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.

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**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
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 involving mating twi snaCyO males with females carrying the double balancer, y w; Be ElpCyO.P (fla−/−lacZ+/−) Kp/TM6,y. F1 males were collected carrying the genotype y w; twi sna/Be Elp++;TM6,y. Separate crosses were performed with males carrying the P-element (e.g. Pnna+) 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/+; CyO.P (fla−/−lacZ+); P-element/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 snaCyO.P (fla−/−lacZ+); P-element/TM6,y. These were mated with each other to maintain homozygous stocks. Homozygous twi sna embryos were identified by their failure to hybridize with a lacZ antisense RNA probe (driven by the fla promoter on the CyO balancer chromosome). Similar results were obtained with two different double mutant stocks, twi114 sna1806 and twi114 sna1606.

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 HindIII 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 HindIII site of the pGem7zf (+) vector, and subsequently digested with a mixture of EcoRV and HindIII. This releases a fragment containing the first 1.6 kb of the 5′ flanking region and the sna coding sequence. The Pnna+ 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 Pw7 transgene was made by replacing the lacZ sequence in the 2×PE P-transformation vector with a 2.3 kb twi EcoRI fragment (spanning the region from −440 bp to +1.9 kb relative to the transcription start site). The Pnna+ transgene was prepared in a similar manner after digesting a full-length sna cDNA with NdeI. An NdeI 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 digoxigenin-labeled antisense RNA probes exactly as described previously (Tautz and Pfeifle, 1989; Jiang et al., 1991). Fixed and stained embryos were mounted in Synapane (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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