1997-11-21

Differential regulation of gastrulation and neuroectodermal gene expression by Snail in the Drosophila embryo

Kirugaval Hemavathy

Et al.

Development 124, 3683-3691 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
DEV5141

Differential regulation of gastrulation and neuroectodermal gene expression by Snail in the *Drosophila* embryo

Kirugaval Hemavathy, Xiangjun Meng and Y. Tony Ip*

Program in Molecular Medicine, Department of Cell Biology, Department of Biochemistry and Molecular Biology, University of Massachusetts Medical Center, Worcester, MA 01605, USA

*Author for correspondence (e-mail: Tony.Ip@ummed.edu)

SUMMARY

The initiation of mesoderm differentiation in the *Drosophila* embryo requires the gene products of *twist* and *snail*. In either mutant, the ventral cell invagination during gastrulation is blocked and no mesoderm-derived tissue is formed. One of the functions of Snail is to repress neuroectodermal genes and restrict their expressions to the lateral regions. The derepression of the neuroectodermal genes into the ventral region in *snail* mutant is a possible cause of defects in gastrulation and in mesoderm differentiation. To investigate such possibility, we analysed a series of *snail* mutant alleles. We found that different neuroectodermal genes respond differently in various *snail* mutant background. Due to the differential response of target genes, one of the mutant alleles, *V2*, that has reduced Snail function showed an intermediate phenotype. In *V2* embryos, neuroectodermal genes, such as *single-minded* and *rhomboid*, are derepressed while ventral invagination proceeds normally. However, the differentiation of these invaginated cells into mesodermal lineage is disrupted. The results suggest that the establishment of mesodermal cell fate requires the proper restriction of neuroectodermal genes, while the ventral cell movement is independent of the expression patterns of these genes. Together with the data showing that the expression of some ventral genes disappear in *snail* mutants, we propose that Snail may repress or activate another set of target genes that are required specifically for gastrulation.

Key words: *Drosophila*, gastrulation, mesoderm, *snail*, repression, neuroectoderm, *single-minded*, *rhomboid*

INTRODUCTION

Gastrulation is the first morphogenetic event during metazoan development. The process transforms a single-cell-layered blastula into a multilayered embryo (McClay, 1991; Costa et al., 1993; Magnuson and Faust, 1993). In *Drosophila*, gastrulation occurs approximately 3 hours after fertilization (Costa et al., 1993; Leptin, 1994). Once cellularization is completed, the ventral cells invaginate first and form the mesoderm. Then the posterior midgut invaginates and, together with the later anterior midgut invagination, brings about the formation of the endoderm. Cells that remain in the periphery become the ectoderm.

The invagination of the presumptive mesoderm is a particularly well-studied process of *Drosophila* gastrulation, mainly because the ventral cell movement occurs first and the ventral furrow is a simple, prominent structure (Costa et al., 1993; Leptin, 1994). The ventral furrow arises from the invagination of a band of cells approximately 60 cells long and 18 cells wide spanning the ventral midline of the blastoderm. Previous studies showed that this ventral band of cells could be divided into two populations (Leptin and Grunewald, 1990; Kam et al., 1991; Sweeton et al., 1991). The first population comprises a central band of about 10 cells wide. Within this band, the ventral most cells first flatten their apical surface, then the whole band of cells constrict apically while their nuclei migrate basally. The cell shape changes lead to the formation of the ventral furrow, which deepens and invaginates into the interior of the embryo. The other population, cells located in immediate adjacent regions, remain unconstricted; these cells follow the ventral cells into the furrow during invagination. Although the initial apical flattening and nuclear migration of ventral cells are stochastic, there is no individual cell invagination during gastrulation. The stochastic cell shape changes somehow are translated into mechanical force that drives the invagination of the ventral epithelium as a sheet.

The establishment of the ventral cell fate depends on the dorsal group genes and *cactus* (Ferguson and Anderson, 1991; Steward and Govind, 1993). These maternal gene products constitute a signal transduction pathway, which regulates the formation of the nuclear Dorsal (Dl) protein gradient in the ventral nuclei of the blastoderm. The nuclear Dl gradient functions as a morphogen which directs precise patterns of zygotic gene expression (Ip and Levine, 1992; Steward and Govind, 1993). High levels of Dl in the ventral regions are required to turn on at least two zygotic genes, *twist* (*twi*) and *snail* (*sna*). Both genes are essential for ventral cell invagination and mesoderm formation (Simpson, 1983; Nusslein-Volhard et al., 1984). While Twi has been implicated as an activator, Sna is known to function as a repressor (Kosman et al., 1991; Leptin, 1991; Rao et al., 1991). In twi^{-} embryos, *sna* expression is attenuated and other early mesodermal markers such as *tinman* (*tin*)

and *nautilus* fail to express (Michelson et al., 1990; Kosman et al., 1991; Bodmer, 1993). In *sna*$^{-}$ embryos, the initial *twi* expression is not affected, while the neuroectodermal genes such as *single-minded* (*sim*), *rhomboid* (*rho*) and *lethal of scute* (*T3*) are derepressed (Kosman et al., 1991; Leptin, 1991). Molecular analyses showed that Sna functions as a DNA-binding protein that interacts directly with the promoters of the neuroectoderm genes to repress their expression in the ventral region (Ip et al., 1992; Kasai et al., 1992).

The presumptive mesoderm invagination is dependent on the combined functions of *twi* and *sna*. However, since the expression of *sna* is attenuated in *twi* mutant embryos, it is possible that the function of Twi at the blastoderm stage is mainly to maintain *sna* expression. Sna then in turn regulates the gene products that directly control gastrulation. This model is supported by the experiments showing that sustained *sna* expression in *twi*$^{-}$ mutant background is capable of promoting ventral cell invagination (Ip et al., 1994). On the contrary, sustained expression of *twi* in the absence of *sna* does not rescue the gastrulation phenotype. These suggest that Sna has a more direct role in the process of invagination. Perhaps Sna represses neuroectodermal genes to prevent them from interfering with other gene products that are required for gastrulation. Alternatively, Sna may repress a separate set of zygotic genes, which when expressed ventrally will block invagination. It is also possible that Sna can activate ventral genes that promote cell movement.

To study the role of Sna in the process of gastrulation, we have analyzed 12 mutant alleles of *sna*. The results suggest that different target genes respond to different functional levels of Sna protein. Due to the differential response, one of the mutant alleles (*sna*^{V2}) that has reduced Sna function shows an intermediate phenotype, with the ventral cells expressing both mesodermal and neuroectodermal markers. These ventral cells, nonetheless, invaginate efficiently. The invaginated cells later on give rise to a reduced mesoderm and an expanded mesectoderm. These results led us to hypothesize that the control of cell movement during gastrulation is independent of mesoderm differentiation. Moreover, recent data and previous results showed that the expression of ventral genes such as *zinc-finger homeobox-1* (*zfh-1*), *dGATAb*, *tin* and *folded gastrulation* (*fog*) (Bodmer et al., 1990; Lai et al., 1991; Abel et al., 1993; Costa et al., 1994) are disrupted in strong *sna* mutants. These indicate that Sna can directly or indirectly activate genes that are expressed in ventral cells. It is possible that, during normal development, Sna acts both to (1) repress neuroectodermal genes to allow the establishment of mesodermal cell fate and (2) regulate a separate set of genes to promote gastrulation.

MATERIALS AND METHODS

Fly stocks

Canton *S* was used as the wild-type stock. The following *sna* mutant stocks were used for analyzing the expression patterns of downstream genes: *IIG05, 19, 20, V4, HG31, EYI, V2, RY40, V1, EY3, R1* and *EY2*. All these alleles, except *RY40*, are listed in Lindsley and Zimm (1992). The names of the alleles are also listed under different synonyms, such as *sna*^{HG31} is *sna*^{1}. The *RY40* is not listed and therefore should be named as *sna*^{21}. Some of the mutants, especially *V2*, were maintained over the *CyO-ftz-lacZ* marker balancer chromosome. The *lacZ* marker was used to facilitate the identification of homozygous embryos. The *rho*-Gal4 transgenic construct was generated by inserting the 2.2 kb *rho* promoter fragment, in which all the four Sna-binding sites were mutated (Ip et al., 1992), into the pGaTB vector. The UAS-*T3* construct was generated by inserting the *T3* cDNA into the pUAST vector (Brand and Perrimon, 1993). Transgenic flies were generated by P-element-mediated transformation using the yw^{67} stock. All fly stocks were maintained at 25°C on standard yeast-cornmeal-agar media.

RNA in situ hybridization

Embryos from heterozygous parents were collected on apple juice agar plates and aged appropriately. They were dechorionated in bleach for 3 minutes, fixed with vigorous shaking in 4 ml of heptane and 4 ml of 4.6 % formaldehyde in 0.5× PBS-25 mM EGTA for 25 minutes. The embryos in the heptane layer were mixed with 2 volumes of methanol and devitellinized by shaking for 1 minute. The embryos were washed with a few changes of methanol and then ethanol. The ethanol-washed embryos were treated in xylene for 30 minutes, rinsed with ethanol and postfixed in 5% formaldehyde in 1× PBS, 0.1% Tween (PBT). It was followed by a few washes in PBT and digested with proteinase K (4 ug/ml) for 5 minutes. Embryos were washed, postfixed again and prehybridized (in 5× SSC, 50% deionized formamide, 100 μg/ml salmon sperm DNA, 50 μg/ml heparin, 0.1% Tween) at 55°C for 1 hour. Hybridization was carried out in the same buffer, at the same temperature with digoxigenin-labeled antisense RNA probes for 18 hours. The hybridized embryos were washed extensively with hybridization buffer at 55°C, followed by washing with PBT at room temperature. They were then incubated with alkaline-phosphatase-conjugated anti-digoxigenin antibody (Boehringer Mannheim) at 4°C for overnight. The embryos were then washed with PBT and the expression patterns were visualized by incubating with staining solution (100 mM NaCl, 50 mM $MgCl_2$, 100 mM Tris pH 9.5, and 0.1% Tween) containing NBT and X-phosphate as substrates. The stained embryos were mounted in Permount (Fisher) and photographed under Differential Interference Contrast optics using the Zeiss Axiophot microscope.

Antibody staining

An *Nde*I fragment of the *sna* cDNA, which encodes the C-terminal amino acid 103-390, was cloned into the pAR3040 expression vector. The protein was induced in BL21(DE3) *E. coli* strain. Sna antibody was raised in guinea pigs using SDS-PAGE separated, electroeluted Sna protein. The antibody was prepared in Pocono rabbit farm (Canadensis, PA) and RIBI was used as the adjuvant. A 1:500 dilution of the anti-Sna antibody was used in all experiments. Embryos were collected and aged on apple juice agar plates, dechorionated in bleach, fixed in 1:1 mixture of heptane and 1.8 % formaldehyde in a buffer containing 10 mM KPO_4, 15 mM NaCl, 45 mM KCl, and 2 mM $MgCl_2$. After 12 minutes of vigorous shaking, the embryos were devitellinized by shaking the heptane layer with 1:2 mix of methanol. The embryos were washed extensively with methanol. For antibody staining, the embryos were rehydrated in PBT and blocked in 10% BSA/1× PBS for 1 hour at room temperature. The embryos were incubated with the anti-Sna antibody in 1× PBS, 0.1% Tween, 500 mM NaCl, and 1% BSA for overnight at 4°C. The presence of Sna protein was detected histochemically using 1:1000 dilution of biotinylated anti-guinea pig secondary antibody and the Vectastain ABC kit (Vector Labs., USA) using diamino-benzidine as chromogen.

Embryo sectioning

In situ hybridized and stained embryos were rinsed extensively with ethanol and then in propylene oxide. They were infiltrated with 1:1 propylene oxide-Epon (Epon is a mix of 95 g Epox 812, 60 g Nadic methyl anhydrous, 45 g dodecenylsuccinic anhydride, and 2 g DMP-30) (Polysciences Inc., PA) for 2 hours. The embryos were mixed with Epon, transferred to plastic molds and baked at 60°C for 24 hours. 5 μm sections were cut on a Sorvall Porter Blum ultramicrotome, mounted with permount, and photographed under DIC optic.

PCR and sequencing of the mutant alleles

30 homozygous mutant embryos identifiable by their defective cuticle

phenotype were hand picked for DNA isolation. The embryos were incubated in 1× lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 0.01 mg/ml gelatin, 0.045% Nonidet P40, 0.045% Tween) plus 1 mg/ml proteinase K for overnight at 55°C. The embryos were then boiled for 10 minutes and centrifuged at 14,000 ***g*** for 10 minutes, and the supernatant was transferred to a fresh tube. This genomic DNA-containing lysate was used as template for PCR. The genomic regions of the mutant alleles were PCR amplified using different combinations of the primers that cover the whole coding region. Four primers were used. Primer 1 and 4 anneal to the 5' and 3' UTR. Primer 2 and 3 match sequences located midway of the gene. The PCR was performed in two rounds. In the first round, primers 1 and 4 were used to amplify the whole coding region. In the second round, two separate reactions using primers 1 and 2, or 3 and 4 were done employing the first-round product as template. The inner primers were designed in such a way that, when amplified, slightly overlapping sequences would be obtained. The two PCR products were restriction digested and cloned into the bluescript vector. Sequencing reactions were performed using the dye terminator kit FS (Perkin Elmer) and analyzed on an ABI 373 automated sequencer.

Sequences of the primers used for PCR:

(1) 5′-CTA GAA TTC CGG AAA CTA AAA CTT AAT CAC ACA CAC ATC

(2) 5′-CAT GAA TTC GAC GAG GCA CTC ATG GCC GGA GTG TAG

(3) 5′-ATC GAA TTC GCG AAA TGA CAT CCC ACT GCC GGC TC

(4) 5′-ATC GAA TTC TCC TGC TAA GGG ATT CAT ATG CCG AGA

RESULTS

Molecular genetic analysis of a series of *sna* mutant alleles

Previous genetic screens led to the identification of a number of *sna* mutant alleles and some of them have been characterized with respect to their embryonic phenotypes (Ashburner et al., 1983; Grau et al., 1984; Nusslein-Volhard et al., 1984; Lindsley and Zimm, 1992). We were able to collect 12 mutants from various laboratories. To study the detailed phenotype of these alleles, we performed RNA in situ hybridization with molecular probes on embryos collected from heterozygous parents.

The mesectodermal gene *sim* is regulated by Sna in the blastoderm such that, in the absence of Sna function, the two lines of *sim* expression (see Fig. 1A), which coincide with the presumptive mesectoderm, expand into the ventral mesoderm territory (Nambu et al., 1990; Kosman et al., 1991; Leptin, 1991). We used the very simple *sim* pattern to evaluate the strength of the different *sna* alleles. Fig. 1 shows the results of *sim* RNA staining in stage 6-8 embryos, when the mesoderm should have invaginated and *sim* should be expressed only in the ventral midline (Fig. 1A,B). The 12 alleles can be roughly ordered according to the extent of disruption of the *sim* pattern (Table 1). The weak alleles, *EY2* (Fig. 1C), *R1* and *EY3* showed minor derepression of *sim*. *V1* is a stronger *sna* allele since more ventral cells express small patches of the target gene (Fig. 1D). The *RY40* allele clearly showed defects in *sim* expression. About 3 rows of cells juxtapose the mesectoderm-expressed *sim* and there was more derepression into the mesoderm (Fig. 1E). A few *RY40* embryos showed stronger phenotype, in that *sim* was expressed throughout the ventral region (data not shown).

The *V2* allele exhibited an even more severe phenotype, such that the *sim* staining was intense throughout the mesoderm (Fig. 1F, see also Fig. 2). This derepression was obvious in mid to late blastoderm-stage embryos, and the pattern covered the

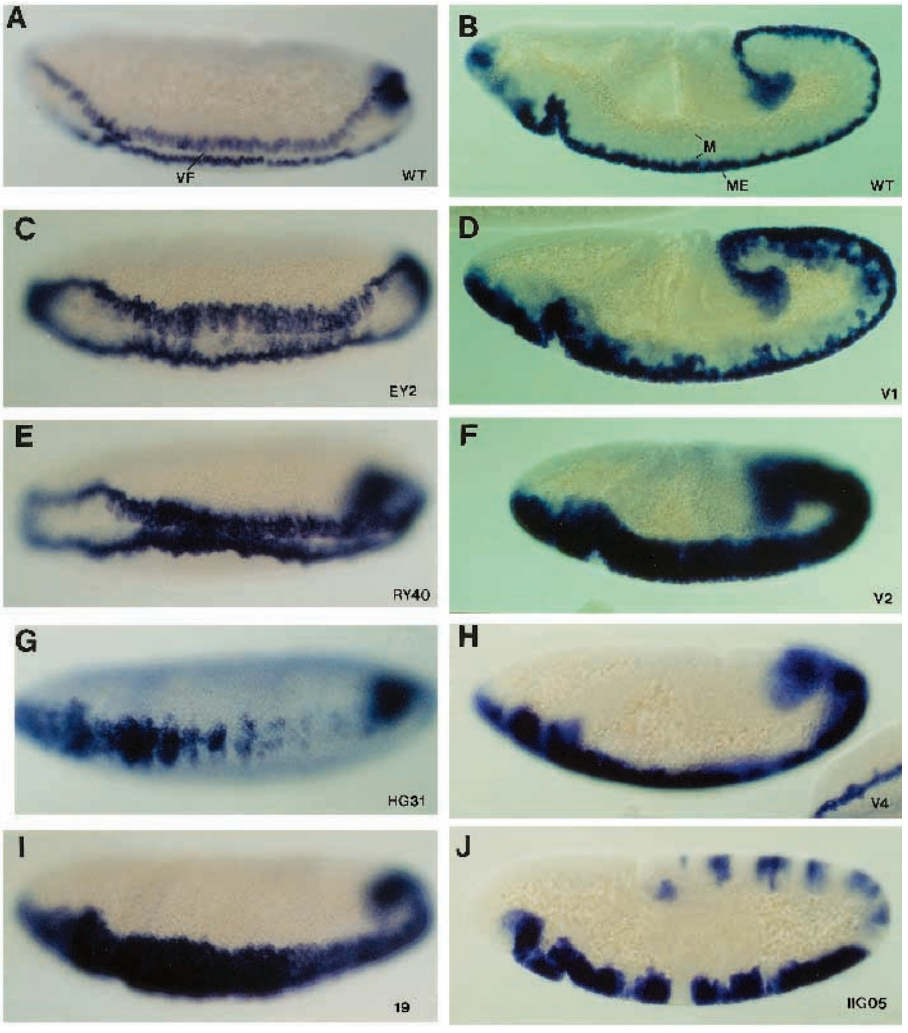

Fig. 1. Expression of *sim* in a series of *sna* mutants. The embryos are at stage 6-8, during germ-band extension. All the embryos were hybridized with a *sim* antisense RNA probe. They are oriented with anterior to the left and dorsal side up. The genotypes are (A,B) wild type, (C) *EY2*, (D) *V1*, (E) *RY40*, (F) *V2*, (G) *HG31*, (H) *V4*, (I) 19, (J) *IIG05*. (A) The VFindicates ventral furrow and (B) M and ME indicate mesoderm and mesectoderm, respectively. The different mutants show different severity in the ability to repress *sim* and to promote invagination. In weak mutants such as *EY2*, there is a slight derepression of *sim* and invagination is not affected. In strong mutants such as *V4* and *IIG05*, there is little *sim* repression or mesoderm invagination. *IIGO5* is rated the strongest allele. In this mutant, *sim* expression is seen consistently in patches.

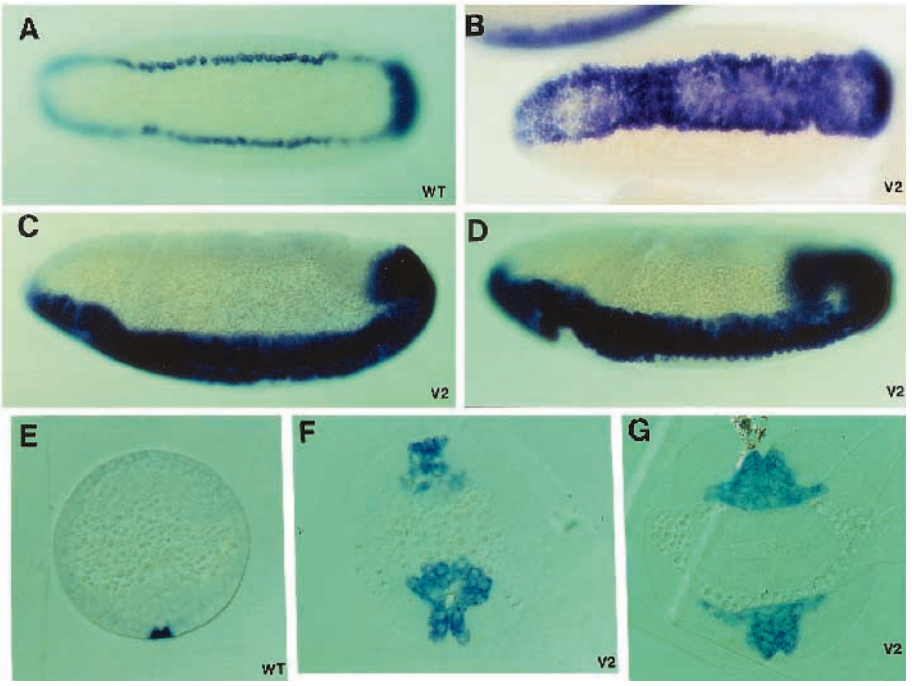

Fig. 2. Invagination is not dependent on the repression of *sim*. All embryos were stained after hybridization with *sim* antisense RNA probe. Whole-mount embryos are oriented with anterior to the left and dorsal side up, and the sections are dorsal side up. Ventral views of (A) wild-type and (B) *V2* blastoderm-stage embryos, showing the derepression of *sim* in the ventral presumptive mesoderm. *sim* is normally expressed as two single cell-wide stripes of mesectoderm and is repressed in the mesoderm by *sna*. (C) Side view of a gastrulating *V2* embryo. There is some invagination at early stage of germ-band extension, but the process is delayed in this mutant embryo compared to the wild type. (D) A similar gastrulating embryo at early to mid extension stage, the invagination has been completed. (E-G) Sections of gastrulating embryos after staining with *sim* antisense RNA probe. (E) Wild type, the two lines of *sim*-expressing cells come together and lie at the ventral midline. The invaginated mesoderm has no staining. (F,G) *V2* embryos at early and late germ-band-extension stages, respectively, show both invagination of the mesodermal cells and misexpression of *sim*.

ventral band of 18 cells (see Fig. 2B), suggesting that the initiation of *sim* expression was not delayed. The other 6 mutant alleles (*EY1*, *HG31*, *V4*, *20*, *19* and *IIG05*) showed a similar strong loss-of-function phenotype (Fig. 1G-J, Table 1). The *sim* patterns were fully derepressed in the ventral regions.

Neuroectodermal genes show variable response to Sna

A number of neuroectodermal genes, including *sim*, *rho*, *T3* and an *Enhancer-of-split* gene, *m7*, are expressed in the lateral regions and are repressed by Sna in the ventral regions (Kosman et al., 1991; Leptin, 1991; Rao et al., 1991). The study was extended to examine the expression of other target genes using embryo in situ hybridization.

The mutants, when ordered according to the severity of the phenotype, showed a gradual decrease in their ability to repress *sim*, *rho* and *T3* (Table 1). Among the three genes, *rho* seemed to be the most sensitive one. In the weaker *EY2* and *RI* alleles, the expression of the three genes was largely normal although some sporadic derepression was observed. We therefore assigned ++ for these repression compared to the +++ in wild type. In the *EY3* and *V1* alleles, *rho* was derepressed but only

Table 1. Molecular genetic analysis of a series of *sna* mutant alleles

snail allele	Origin	snail RNA	Snail Protein	Invagination	Neuroectodermal gene repression			Gene activation	
					sim	rho	T3	zfh1	dGATAb
Wild type		+	+	+++	+++	+++	+++	+	+
EY2 (3)	EMS	+	+	++	++	++	+++	+	+
R1 (5)	X-ray	+	+	++	++	++	+++	+	+
EY3 (4)	EMS	+	+	++	++	+/–	+++	+	+
V1 (9)	EMS	+	+	++	+	+/–	+++	+	+
RY40 (21)	X-ray	+	+	++/–	+/–	+/–	+/–	+	+
V2 (10)	EMS	+	+	++	–	–	++	+	+
EY1 (2)	EMS	+	+	–	–	–	–	–	–
HG31 (1)	EMS	+	+	–	–	–	–	–	–
V4 (12)	EMS	+	+/–	–	–	–	–	–	–
20 (20)	X-ray	+	+	–	–	–	–	–	–
19 (19)	X-ray	+	+	–	–	–	–	–	–
IIG05 (18)	EMS	+	+	–	–	–	–	–	–

The synonyms of the alleles are listed in the brackets in the left-most column. The expression of *sna* RNA and proteins in the various *sna* mutants were analyzed using in situ hybridization and antibody stainings. All the alleles showed detectable RNA and protein, except the *V4* allele, which makes a truncated protein and had very weak protein staining. We did not attempt to grade the expression levels of the RNA and protein, and a (+) was assigned as long as there was detectable staining. The embryos were also analyzed for the expression of various downstream genes including *sim*, *rho*, *T3*, *zfh-1* and *dGATAb*. The influence of the mutations on invagination was observed under microscope and at least a few hundred embryos were examined. The mutants are tabulated according to increasing severity of the mutant phenotypes. The *RY40* embryos showed mostly intermediate and occasionally severe phenotypes. It was therefore rated (+/–) in gene repression. The activation of *zfh-1* and *dGATAb* is defined genetically, since the molecular mechanism is not known. Also we did not attempt to rate the activation into different classes other than (+) or (–) because the wild-type expression is somewhat variable.

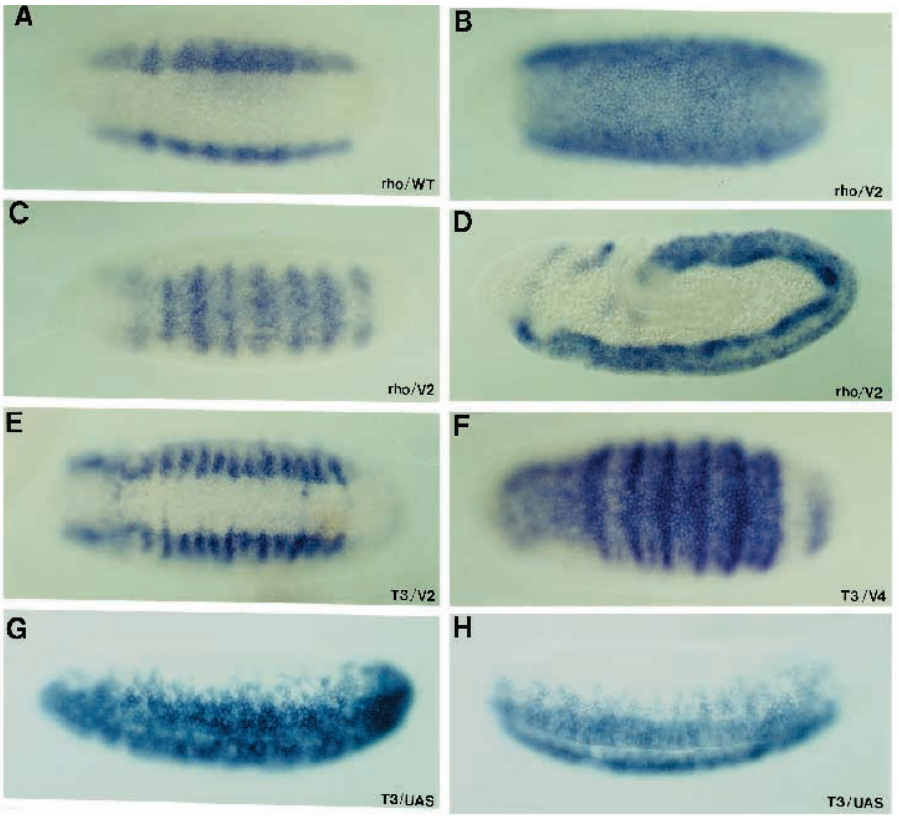

Fig. 3. The derepression of neuroectodermal genes does not affect gastrulation. Embryos stained after hybridizing with *rho* (A-D) or *T3* (E-H) antisense RNA probe. The embryos are (A,B,C,E,F) ventral views, and (D,G,H) side views. (A) Wild-type embryo showing the expression of *rho* as two broad bands in the neuroectoderm and is excluded from the ventral cells. (B-D) *V2* mutants stained with *rho*, showing complete derepression of this gene in blastoderm stage, gastrulating, and germ-band-extended embryos. (E) A *V2* embryo showing normal *T3* staining as compared to its derepressed pattern seen in a strong mutant *V4* in F. (G,H) The embryos contain both *rho* promoter-*Gal4* and UAS-*T3* transgenes. This *rho* promoter contains mutations in 4 Sna-binding sites so that it drives the expression of Gal4 in both lateral and ventral regions. (G) An early gastrulating embryo and (H) an embryo at mid germ-band extension. The staining showed that *T3* expression was detected throughout the ventral half of the embryos.

at early blastoderm stage; the *rho* repression was quite normal at late blastoderm stage and therefore +/– was assigned. This result may reflect the timing of expression, since it may need more time for the mutant *EY3* and *V1* Sna proteins to accumulate to sufficient level at late blastoderm stage in order to repress *rho* efficiently. While the derepression of *sim* and *rho* in these weaker alleles was increasingly obvious, the *T3* expression was not affected. This is particularly clear in the intermediate *V2* allele, which showed a dramatic loss of repression of the other two genes, but *T3* is rather normal. In the strong alleles, all the target genes were fully derepressed.

The results shown in Table 1 reveal that there is differential repression of neuroectodermal genes in *sna* mutants with increasing severity. It seems to indicate that the regulation of these genes involves different functional levels of Sna. A possible explanation is that the promoters of these genes contain different number of Sna-binding sites, or binding sites with different affinity. For instance, the *T3* gene may contain more binding sites and therefore was less affected in the weak mutants.

Ventral invagination is not inhibited by derepressed neuroectodermal genes

Although ventral cell invagination and mesoderm differentiation are tightly linked, it is not clear whether the ventral cells have to assume mesodermal cell fate before they are allowed to invaginate. In addition to analyzing the target gene expression, we therefore also examined ventral cell invagination in the *sna* mutants. The invagination of ventral cells in the weak alleles, including *EY2*, *R1*, *EY3* and *V1*, did not seem to be different from that of wild type except a little delayed (Fig. 1C,D). By the mid germ-band-extension stage, the invagination looked almost the same as in wild type. Although *sim* expression was quite abnormal in *RY40* embryos, gastrulation proceeded rather normally during germ-band extension (Fig. 1E). The strong alleles (*EY1*, *HG31*, *V4*, *20*, *19* and *IIG05*) all showed very little ventral cell invagination by mid to late germ-band extension (Fig. 1G-J).

The most interesting phenotype was observed in the *V2* embryos. Although the mutant embryos exhibited a complete derepression of *sim*, they did not show much defect in gastrulation (Fig. 1F). A detailed analysis of this mutant is shown in Fig. 2. In blastoderm-stage *V2* embryos, *sim* showed a dramatic derepression into the presumptive mesoderm (Fig. 2B). Although the invagination was a bit delayed in the mutant embryo (Fig. 2C), ventral furrow formation was quite coordinated. Most mutant embryos had deep furrows already present at early germ-band-extension stage (Fig. 2D). Sectioning of the embryos showed that most, if not all, of the mesodermal anlage had invaginated by early germ-band extension (Fig. 2F). On many occasions, the invaginated cells also spread out laterally to form a mesodermal layer similar to those in the wild-type embryos (Fig. 2G). The lateral cell movement, however, was not as extensive as in the wild type.

Not only was *sim* repression affected in the *V2* allele, *rho* was also fully derepressed in this mutant embryo. Although *V2* was classified as an intermediate mutant, the *rho* expression was very similar to that in the strong alleles. In early blastoderm stage, both the ventral cells and the lateral cells expressed *rho* RNA (Fig. 3B), suggesting a total derepression. At the onset of gastrulation, the stripy pattern of *rho* was seen throughout the ventral regions (Fig. 3C). The expression was also detected in the invaginated mesoderm in germ-band fully extended embryos (Fig. 3D).

In contrast to *sim* and *rho*, the *T3* expression in the *V2* mutant was very similar to that of wild type at gastrulation (Fig. 3E); while in stronger mutants the *T3* should express in both the ventral and lateral cells (Fig. 3F). To test whether *T3* could disrupt invagination when present in the ventral cells, we ectopically expressed the *T3* gene using the UAS-Gal4 system (Brand and Perrimon, 1993). In such an experiment, the Gal4 gene was placed under the control of a *rho* promoter in which the Sna-binding sites were mutated so that the reporter gene was driven to express in both lateral and ventral cells (Ip et al., 1992). The misexpression of *T3* alone in the ventral cells, again, had no effect on the invagination of the presumptive mesoderm (Fig. 3G,H).

Ventral cells that invaginate do not necessarily possess mesodermal fate

Since the invaginated cells in *V2* embryos express both mesodermal and neuroectodermal determinants (Table 1), we investigated which tissue type these cells eventually become. The mutant embryos were stained with mesodermal markers including *tin*, *S59* and *Pox meso*, as well as *slit*, a mesectodermal marker and a downstream target of *sim*. The spatial pattern of *tin* at stage 12 was rather normal since it was expressed in the visceral mesoderm. The number of cells expressing the gene, however, was much reduced (Fig. 4A,B). A similar observation was obtained with the *S59* probe. The pattern of this gene marks the position of a subset of somatic muscle precursor cells (Fig. 4C) (Dohrmann et al., 1990). There was an obvious reduction of cells that expressed *S59* in the *V2* embryo (Fig. 4D), while the spatial pattern still resembled that of the wild type. The result obtained with *Pox meso*, which also marks some of the somatic mesoderm (Bopp et al., 1989), showed a decrease of the mesodermal derivatives as well (Fig. 4E,F).

In contrast, the *slit* probe revealed an increase in the mesectodermal cell number. The normal pattern of *slit* in a stage 12 embryo resides in the central nervous system midline (Fig. 4G) (Rothberg et al., 1988; Nambu et al., 1991). In *V2* mutants, the staining became several cells wide (Fig. 4H). This result is consistent with the notion that there is an expansion of the mesectoderm at the expense of the mesoderm. However, the expanded *slit* staining was organized around the ventralmost region and did not scatter around the mesoderm. Therefore, the reduced mesodermal staining may indicate that some invaginated cells do not express any of the markers used and do not differentiate into either tissue type. These cells may transform into other cell types or die during embryogenesis.

The reduced expression of mesodermal markers in *V2* embryos is likely due to the interference by the misexpression of neuroectodermal determinants. In addition, the slight reduction of cell invagination (see Fig. 2G) may also contribute to the reduced mesodermal gene expression, as previosuly reported (Maggert et al., 1995).

Molecular analysis of the mutant alleles

To study the molecular defects of the Sna protein in the different alleles, we analysed the *sna* RNA and protein expression pattern in the mutant embryos. In situ hybridization revealed that all the mutants, including the two X-ray-induced strong alleles, still expressed *sna* RNA (Table 1). Protein staining on whole-mount embryos using anti-Sna antibodies showed that all the alleles also produced detectable proteins (Table 1; Fig. 5). Only *V4* allele showed significantly less but detectable protein staining in early embryos and in the neuroblasts, and therefore was assigned +/– (Table 1). The results indicate that all the mutations probably affect the coding but not the promoter regions of the *sna* loci.

Since the *V2* allele showed an intermediate phenotype, we examined the exact molecular defect of this mutant protein.

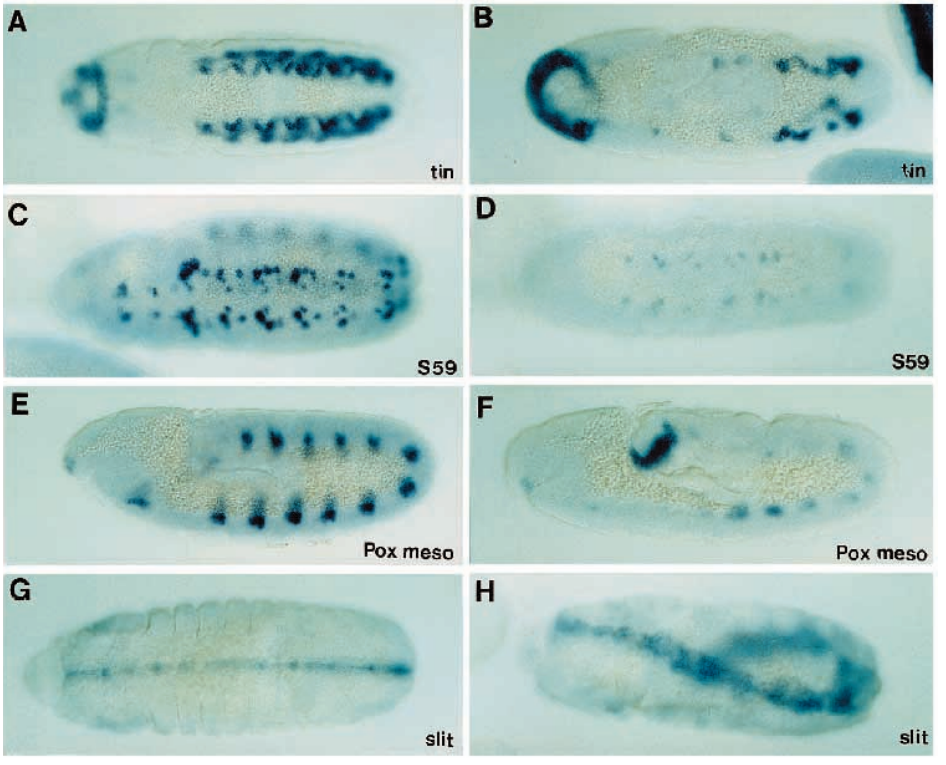

Fig. 4. The expression of mesodermal and mesectodermal markers in *V2* mutant embryos. (A,C,E,G) Wild-type embryos and (B,D,F,H) *V2* embryos. The embryos in E and F are sagittal views, and all other embryos are either dorsal or ventral views. The expression of the mesodermal gene *tin*, *S59* and *Pox meso* are reduced, but their patterns resemble the wild type. It suggests that the number of both somatic and visceral mesodermal cells is reduced. The mesectodermal gene *slit*, on the contrary, has expanded expression. The expansion remains around the midline.

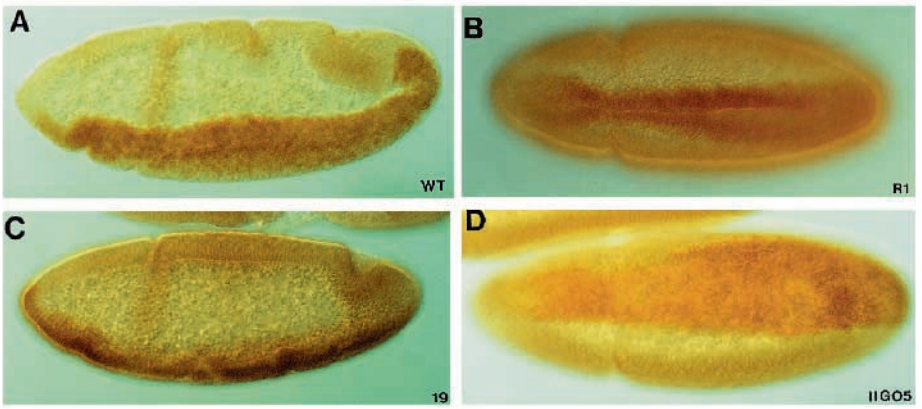

Fig. 5. Embryos of some of the *sna* mutants immunostained for Sna protein. Sna is detected in the ventral region comprising the mesodermal anlage at early stages of gastrulation. Embryos from all the mutant alleles make detectable Sna proteins. (A) Wild type, (B) *R1*, (C) *19*, (D) *IIG05*.

Restriction mapping and sequence analysis of the *sna* locus revealed that there is no intron present in the genomic DNA (data not shown). We therefore used PCR to amplify directly the *sna* genomic DNA isolated from the homozygous mutant embryos and analyzed the clones by sequencing. The results, as presented in Fig. 6, showed that *V2* contains a missense mutation that changes the amino acid (a.a.) 330 from a glycine to a glutamic acid (GGA to GAA). The glycine residue is located between the zinc fingers no. 3 and no. 4, a region highly conserved among the Sna homologs isolated from different species (Fig. 6B). Particularly, the glycine residue is invariable in all the homologs. The *V2* mutant protein may have reduced DNA-binding affinity or different specificity.

Similar analyses revealed that two strong alleles, *V4* and *HG31*, contain nonsense mutations. The a.a. 141 was changed from a tryptophan to a stop codon (TGG to TGA) in *V4*. This result is consistent with the antibody staining (see Table 1). The *HG31* protein also contains a stop codon (TGT to TGA) at a.a.

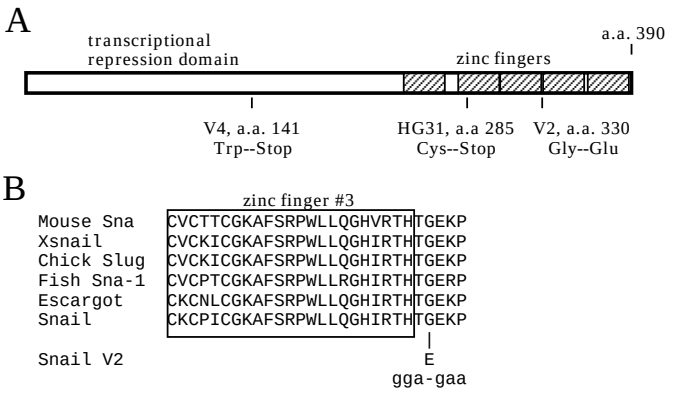

Fig. 6. Molecular defects of *sna* mutant alleles. (A) A schematic diagram of the Sna protein, which is predicted to contain 390 amino acids. The five DNA-binding fingers are located at the C terminus. The transcriptional repression domain is located at the N terminus, as suggested by Gray and Levine (1996). Three alleles, *V2*, *HG31* and *V4*, were sequenced. The *V4* has a mutation that changes the tryptophan at amino acid 141 to a stop codon; *HG31* also has a stop codon, at amino acid 285. The *V2* has a missense mutation, changing a neutral glycine residue at amino acid 330 to a negatively charged glutamic acid. (B) The alignment of the region around zinc finger no. 3 of all the Sna-related proteins. Xsnail is from *Xenopus*, and Escargot is from *Drosophila*. The glycine residue mutated in the *V2* allele is invariant.

285, in the second zinc finger. Due to the strong phenotypes exhibited by this mutation, we suggest that all the in vivo functions of Sna depend on the DNA-binding fingers.

Expression of mesodermal markers that require Sna for activation

The results presented so far indicate that the repression of neuroectodermal gene is required for establishing mesodermal cell fate but not required for gastrulation. It may be that Sna regulates other target genes that are present in the ventral region and are responsible for invagination. Previous results showed that the ventral expression patterns of *tin* and *fog* are affected in *sna* mutants (Bodmer, 1993; Costa et al., 1994). To gain further support for the model, different genes that are expressed in the ventral regions were examined in *sna* mutant embryos. The results revealed that Sna function is also required for the activation of genes such as *dGATAb* and *zfh-1*. *dGATAb* is first expressed after cellularization in the primordia of anterior midgut, posterior midgut, cephalic mesoderm, the vitellophages and the amnioserosa (Fig. 7A) (Abel et al., 1993). *zfh-1* is first expressed in the presumptive procephalic mesoderm and, at gastrulation, spreads throughout the entire mesoderm (Fig. 7C) (Lai et al., 1991).

Whole-mount in situ hybridization showed that, in strong *sna* mutant embryos, *dGATAb* expression in the cephalic mesoderm and the anterior midgut was lost (Fig. 7B). *zfh-1* staining in the invaginated mesoderm also disappeared in the mutants (Fig. 7D). In weaker alleles, the expression of both genes appeared normal (Table 1). The regulation of mesodermal genes can be achieved by Sna binding to their promoters and directly activating transcription. Alternatively, Sna can repress a repressor, and indirectly allow the expression of *zfh-1* and *dGATAb*.

DISCUSSION

We have presented evidence showing that the derepression of some neuroectodermal genes, while affecting mesoderm development, does not necessarily block gastrulation. In the intermediate sna^{V2} mutant background, we observed that one process (repression of neuroectodermal genes and mesoderm differentiation) is affected while the other process (ventral cell invagination) is normal. Previous results showed that, in strong *sna* mutants, there is no ventral invagination and no mesoderm derivative is formed (Simpson, 1983; Nusslein-Volhard et al., 1984).

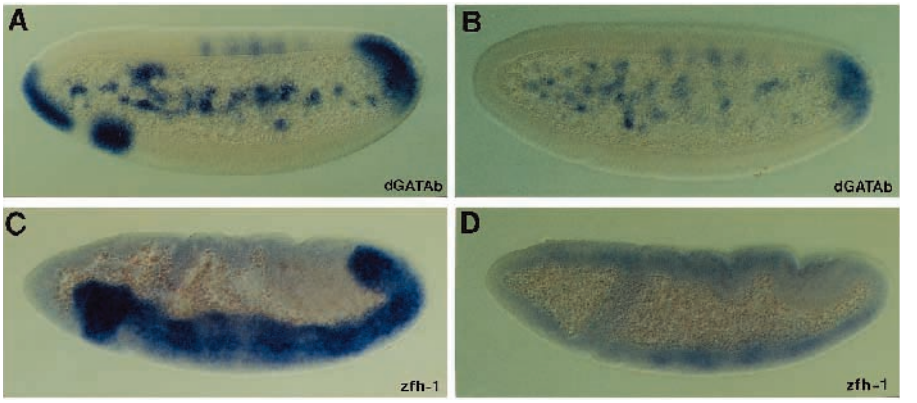

Fig. 7. Expression of mesodermal genes *dGATAb* and *zfh-1* are affected in *sna* mutants. (A,C) Wild-type and (B,D) sna^{19} embryo stainings are shown after hybridization with *dGATAb* (A,B) or *zfh-1* (C,D) antisense RNA probes. The embryos are (A,B) at blastoderm stage and (C,D) at early germ-band-extension stage. Embryos are oriented with anterior to the left and dorsal side up. The cephalic mesodermal and anterior midgut stainings of *dGATAb* are not observed in the *sna* mutant embryos, suggesting that only these patterns are under the control of *sna*. In *zfh-1*-stained sna^{19} mutant embryos, no mesodermal staining is detected.

The two processes, therefore, are tightly linked. Our results, however, reveal that the cells that invaginate are not necessarily destined to be mesodermal cells unless the correct determinants are also expressed. Furthermore, the activation of some ventrally expressed genes requires the input of Sna. These lead us to postulate that, during normal development, separate sets of Sna target genes are responsible for regulating ventral cell movement versus mesodermal cell fate determination (Fig. 8).

Several lines of evidence support the notion that different sets of Sna target genes may be responsible for the two processes. First, Sna not only represses neuroectodermal gene expression, but also participates in ventral gene activation (Fig. 7) (Bodmer et al., 1990; Costa et al., 1994). Whether the activation of these genes requires the direct binding of Sna to the promoter sequence is not known. The mutants of *zfh-1* and *dGATAb* showed defects in the formation of mesodermal derivatives, but not the invagination of mesodermal cells (Lai et al., 1993; Rehorn et al., 1996). It suggests that they may not be the ones that regulate gastrulation. However, it is possible that the combination of these two genes and other Sna target genes present in the ventral cells is responsible for gastrulation. These genes may be functionally similar to *fog*. *fog* encodes a putative diffusible molecule and participates in the coordination of ventral invagination (Costa et al., 1994). In *sna* mutants, *fog* expression is also affected. The mutant phenotype of *fog*, however, is milder than that of *sna*. Hence there should be additional gastrulation genes downstream of *sna*. The search for such gastrulation genes will help to further understand this developmental process.

Second, the derepression of three of the known Sna targets is not responsible for the gastrulation phenotype. It was logical to postulate that the repression of neuroectodermal genes by Sna is essential for the normal invagination and cell differentiation processes since these are the observed phenotypes in strong *sna* mutants. However, there has been no direct evidence showing that misexpression of these genes in the ventral region can lead to the change of cell fate or the block of cell movement. Indeed, the results reported here suggest that misexpression of *sim*, *rho* and *T3* are not sufficient to block invagination. Two possible scenarios can be envisaged to explain the results. One is that a massive derepression of many neuroectodermal genes at the same time causes all the observed sna^{-} phenotypes. Another possibility is that the repression of neuroectodermal genes is required only to establish mesodermal cell fate. In fact, in the *V2* mutant many of the muscle precursor markers show reduced expression. Taken together, the cell movement may require the proper function of another set of Sna target genes that are active in the ventral cells.

Sequence analysis revealed that the *V2* allele contains a point mutation in the zinc-finger DNA-binding domain. Previous result showed that the transcriptional repression domain of Sna is likely present in the N-terminal and is separable from the zing-finger region (Gray and Levine, 1996). Therefore, the mutant protein may be partially defective in promoter recognition. We favor the possibility that the *V2* protein has a reduced binding affinity to all promoters and represents a hypomorph rather than a neomorph. First, there is good evidence for the differential response of target genes in progressively stronger mutants, and *V2* fits into the trend (Table 1). Second, we have crossed the *V2* allele to all the other *sna* mutants and the result showed that *V2* behaves as an hypomorphic allele, such that there are about 3% survivors when in *trans*heterozygous with some weak alleles but there is no survivor in combination with strong alleles (data not shown). Thus, it may be that the target promoters contain binding sites with different affinity such that *sim* and *rho* are very sensitive to the change in Sna activity, while the regulation *T3* and other targets can be circumvented with lower activity. Therefore, the partial loss of binding affinity is translated to a partial loss of target gene regulation and to an intermediate phenotype, which allows us to detect the differential regulation of gastrulation and mesoderm differentiation.

Although the repression of *sim* and *rho* requires a higher functional level of Sna compared to the activation of mesodermal genes, we cannot yet assign a distinct threshold concentra-

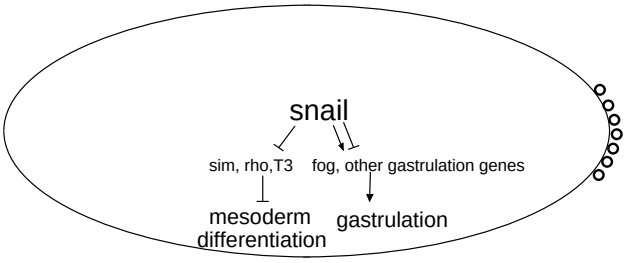

Fig. 8. A working model of how Sna may regulate the process of gastrulation versus the process of mesoderm differentiation. Sna binds to promoters of neuroectodermal genes and excludes their expression in the ventral cells; the derepression of these genes will interfere with the proper establishment of mesodermal cell fate. Sna also regulates a separate set of target genes, including *fog*, which controls the cell shape changes and cell movement during gastrulation.

tion for Sna to function as an activator versus a repressor. This is because in *V2* embryos *T3* is also fully repressed. Furthermore, none of the strong mutants showed a defect in repressing neuroectodermal genes while being normal in activating mesodermal genes. This suggests that the Sna protein may contain one transcriptional regulatory domain that is essential for both activation and repression. It could be that by interacting with different factors Sna can function as both an activator and a repressor, as in the case of Dl (Jiang et al., 1993; Kirov et al., 1993; Lehming et al., 1994; Ip, 1995). Alternatively, Sna can indirectly activate mesodermal genes by repressing a yet to be identified repressor. Regardless of the mechanism, the analysis of Sna target genes required for gastrulation will further our understanding of this important developmental process.

We would like to thank Xiaodi Hu for excellent technical assistance. The *snail* mutant stocks were kindly provided by Michael Levine, Pat Simpson, Maria Leptin, Michael Ashburner, Shigeo Hayashi and Umea Stock Center. We thank John Nambu, Zhichun Lai, Roth Bodmer, Manfred Frasch, Markus Noll, Ted Abel and Rolf Reuter for providing plasmid DNA. We also thank Shirwin Pockwines for help with tissue sectioning. The critical reading of the manuscript by John Nambu, Li Zeng and Steve Small is greatly appreciated. The work in Y. T. I. lab is supported by grants from Lucille P. Markey Charitable Trust and NIH. Y. T. I. is a Scholar of the Leukemia Society of America.

REFERENCES

Abel, T., Michelson, A. M. and Maniatis, T. (1993). A *Drosophila* GATA family member that binds to *Adh* regulatory sequences is expressed in the developing fat body. *Development* **119**, 623-633.

Ashburner, M., Detwiler, C., Tsubota, S. and Woodruff, R. C. (1983). The genetics of a small autosomal region of *Drosophila melanogaster* containing the structural gene for alcohol dehydrogenase. VI. Induced revertants of Scutoid. *Genetics* **104**, 405-431.

Bodmer, R. (1993). The gene *tinman* is required for specification of the heart and visceral muscles in *Drosophila*. *Development* **118**, 719-729.

Bodmer, R., Jan, L. Y. and Jan, Y. N. (1990). A new homeobox-containing gene, *msh-2*, is transiently expressed early during mesoderm formation of *Drosophila*. *Development* **110**, 661-669.

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

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotype. *Development* **118**, 401-415.

Costa, M., Sweeton, D. and Wieschaus, E. (1993). Gastrulation in *Drosophila*: Cellular mechanisms of morphogenetic movements. In *The Development of Drosophila melanogaster*, pp. 425-465. (ed. M. Bate and A. Martinez Arias). Cold Spring Harbor Laboratory Press.

Costa, M., Wilson, E. T. and Wieschaus, E. (1994). A putative cell signal encoded by the *folded gastrulation* gene coordinates cell shape changes during *Drosophila* gastrulation. *Cell* **76**, 1075-1089.

Dohrmann, C., Azpiazu, N. and Frasch, M. (1990). A new *Drosophila* homeo box gene is expressed in mesodermal precursor cells of distinct muscles during embryogenesis. *Genes Dev.* **4**, 2098-2111.

Ferguson, E. L. and Anderson, K. V. (1991). Dorsal-ventral pattern formation in the *Drosophila* embryo: the role of zygotically active genes. *Curr. Topics Dev. Biol.*, **25**, 17-43.

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

Gray, S. and Levine, M. (1996). Short-range transcriptional repressors mediate both quenching and direct repression within compelx loci in *Drosophila*. *Genes Dev.* **10**, 700-710.

Ip, Y. T. (1995). Converting an activator into a repressor. *Curr. Biol.* **5**, 1-3.

Ip, Y. T. and Levine, M. (1992). The role of the *dorsal* morphogen gradient in *Drosophila* embryogenesis. *Sem. Dev. Biol.* **3**, 15-23.

Ip, Y. T., Maggert, K. and Levine, M. (1994). Uncoupling gastrulation and mesoderm differentiation in the *Drosophila* embryo. *EMBO J.* **13**, 5826-5834.

Ip, Y. T., Park, R. E., Kosman, D., Bier, E. and Levine, M. (1992). The *dorsal* gradient morphogen regulates stripes of *rhomboid* expression in the presumptive neuroectoderm of the *Drosophila* embryo. *Genes Dev.* **6**, 1728-1739.

Jiang, J., Cai, H., Zhou, Q. and Levine, M. (1993). Conversion of a *dorsal* dependent silencer into an enhancer: evidence for *dorsal* corepressors. *EMBO J.* **12**, 3201-3209.

Kam, Z., Minden, J. S., Agard, D. A., Sedat, J. W. and Leptin, M. (1991). *Drosophila* gastrulation: analysis of cell shape changes in living embryos by three-dimensional fluorescence microscopy. *Development* **112**, 365-370.

Kasai, Y., Nambu, J. R., Lieberman, P. M. and Crews, S. t. (1992). Dorsal-ventral patterning in *Drosophila*: DNA binding of Snail protein to the *single-minded* gene. *Proc. Natl. Acad. Sci. USA* **89**, 3414-3418.

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

Kosman, D., Ip, Y. T., Levine, M. and Arora, K. (1991). Establishment of the mesoderm-neuroectoderm boundary in the *Drosophila* embryo. *Science* **254**, 118-122.

Lai, Z.-C., Fortini, M. and Rubin, G. (1991). The embryonic expression patterns of *zfh-1* and *zfh-2*, two *Drosophila* genes encoding zinc-finger homeodomain proteins. *Mech. Dev.* **34**, 123-134.

Lai, Z.-C., Rushton, E., Bate, M. and Rubin, G. M. (1993). Loss of function of the *Drosophila zfh-1* gene results in abnormal development of mesodermally derived tissues. *Proc. Natl. Acad. Sci. USA* **90**, 4122-4126.

Lehming, N., Thanos, D., Brickman, J. M., Ma, J., Maniatis, T. and Ptashne, M. (1994). An HMG-like protein that can switch a transcriptional activator to a repressor. *Nature* **371**, 175-179.

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

Leptin, M. (1994). Control of epithelial cell shape changes. *Curr. Biol.* **4**, 709-712.

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

Lindsley, D. L. and Zimm, G. G. (1992). The Genome of Drosophila melanogaster. San Diego, CA: Academic Press, Inc.

Maggert, K., Levine, M. and Frasch, M. (1995). The somatic-visceral subdivision of the embryonic mesoderm is initiated by dorsal gradient thresholds in *Drosophila*. *Development* **121**, 2107-2116.

Magnuson, T. and Faust, C. (1993). Genetic control of gastrulation in the mouse. *Curr. Opi. Genet. Dev.* **3**, 491-498.

McClay, D. R. (1991). Gastrulation. *Curr. Opi. Genet. Dev.* **1**, 191-195.

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

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

Nambu, J. R., Lewis, J. O., Wharton, K. A. and Crews, S. T. (1991). The *Drosophila single-minded* gene encodes a helix-loop-helix protein that acts as a master regulator of CNS midline development. *Cell* **67**, 1157-1167.

Nusslein-Volhard, C., Wieschaus, E. and Kluding, H. (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.

Rao, Y., Vaessin, H., Jan, L. Y. and Jan, Y.-N. (1991). Neuroectoderm in *Drosophila* embryos is dependent on the mesoderm for positioning but not for formation. *Genes Dev.* **5**, 1577-1588.

Rehorn, K.-P., Thelen, H., Michelson, A. M. and Reuter, R. (1996). A molecular aspect of hematopoiesis and endoderm development common to vertebrates and *Drosophila*. *Development* **122**, 4023-4031.

Rothberg, J. M., Hartley, D. A., Walther, Z. and Artavanis-Tsakonas, S. (1988). *slit*: an EGF-homologous locus of D. melangoaster involved in the development of the embryonic central nervous system. *Cell* **55**, 1047-1059.

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

Steward, R. and Govind, S. (1993). Dorsal-ventral polarity in the *Drosophila* embryo. *Curr. Opi. Genet. Dev.* **3**, 556-561.

Sweeton, D., Parks, S., Costa, M. and Wieschaus, E. (1991). Gastrulation in *Drosophila*: the formation of the ventral furrow and posterior midgut invaginations. *Development* **112**, 775-789.

(Accepted 21 July 1997)