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Kirugaval Hemavathy

Xiangjun Meng

University of Massachusetts Medical School

Y. Tony Ip

University of Massachusetts Medical School

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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; Sweeten 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 (snaV2) 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 mese- ectoderm. 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 zic-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: IGG50, I9, 20, V4, HGC31, EyE1, 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 snaHGC31 is sna1. The Ry40 is not listed and therefore should be named as sna21. Some of the mutants, especially V2, were maintained over the Cy0-flc-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/1 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 0.5x PBS-25 mM EGTA for 25 minutes. The embryos in the heptane layer were mixed with 2 volumes of methanol and devitelinized 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 1x 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 pre-hybridized (in 5x 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 MgCl2, 100 mM Tris pH 9.5, and 0.1% Tween) containing NBT and X-phosphate as substrates. The stained embryos were mounted in Permoun (Fisher) and photographed under Differential Interference Contrast optics using the Zeiss Axiopt microscope.

**Antibody staining**

An Ndel 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, electrophoet 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 KPO4, 15 mM NaCl, 45 mM KCl, and 2 mM MgCl2. After 12 minutes of vigorous shaking, the embryos were devitelinized 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 diaminobenzidine 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 Epoxy 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 Sorval Porter Blum ultramicrotome, mounted with permount, and photographed under DIC optic.

**PCR and sequencing of the mutant alleles**

30 homozygous mutant embryos identified 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 GGC 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), RI and EY3 showed minor derepression of sim. VI 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](image_url)  
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 VFinicates 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. IIG05 is rated the strongest allele. In this mutant, sim expression is seen consistently in patches.
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 Snail**

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 Snail 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 R1 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

<p>| Table 1. Molecular genetic analysis of a series of sna mutant alleles |
|-----------------------|-------------------|------------------|-------------------|------------------|</p>
<table>
<thead>
<tr>
<th>allele</th>
<th>Origin</th>
<th>snail RNA</th>
<th>snail Protein</th>
<th>Neuroectodermal gene repression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>EMS</td>
<td>+</td>
<td>+</td>
<td>+++</td>
</tr>
<tr>
<td>EY2 (3)</td>
<td>EMS</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>R1 (5)</td>
<td>X-ray</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>EY3 (4)</td>
<td>EMS</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>V1 (9)</td>
<td>EMS</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>V2 (10)</td>
<td>EMS</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>EY1 (2)</td>
<td>EMS</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>HG31 (1)</td>
<td>EMS</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>V4 (12)</td>
<td>EMS</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>20 (20)</td>
<td>X-ray</td>
<td>+</td>
<td>+</td>
<td>−</td>
</tr>
<tr>
<td>19 (19)</td>
<td>R1</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IIG05 (18)</td>
<td>EMS</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

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.
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 II605) 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 stripe 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 previously 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.

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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.
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 invariant 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. 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 snaV2 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).
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 transheterozygous 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. 7.** Expression of mesodermal genes dGATAb and zfh-1 are affected in sna mutants. (A,C) Wild-type and (B,D) sna19 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 sna19 mutant embryos, no mesodermal staining is detected.

**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 Snail 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 Snail protein may contain one transcriptional regulatory domain that is essential for both activation and repression. It could be that by interacting with different factors Snail can function as both an activator and a repressor, as in the case of DI (Jiang et al., 1993; Kirov et al., 1993; Lehming et al., 1994; Ip, 1995). Alternatively, Snail can indirectly activate mesodermal genes by repressing a yet to be identified repressor. Regardless of the mechanism, the analysis of Snail 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, Shigee Kayasho and Umea Stock Center. We thank John Nambu, Zhichun Lai, Roth Bodmer, Manfred Frisch, Markus Noll, Ted Abel and Rolf Reuter for providing one transcriptional regulatory domain that is essential for both more, none of the strong mutants showed a defect in repressing neuroectodermal genes while being normal in activating mesodermal genes. This suggests that the Snail protein may contain one transcriptional regulatory domain that is essential for both activation and repression. It could be that by interacting with different factors Snail can function as both an activator and a repressor, as in the case of DI (Jiang et al., 1993; Kirov et al., 1993; Lehming et al., 1994; Ip, 1995). Alternatively, Snail can indirectly activate mesodermal genes by repressing a yet to be identified repressor. Regardless of the mechanism, the analysis of Snail target genes required for gastrulation will further our understanding of this important developmental process.

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