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The *snail* gene required for mesoderm formation in *Drosophila* is expressed dynamically in derivatives of all three germ layers

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Summary

The zygotic effect gene *snail* (*sna*) encodes a zinc-finger protein required for mesoderm formation in *Drosophila* embryos. By *in situ* analysis, *sna* transcripts are first detected at syncytial blastoderm and persist until very late stages of embryogenesis. Expression of *sna* is transient and is observed in tissues derived from all three germ layers. Prior to germband elongation, *sna* RNA accumulation is consistent with its genetically determined role in mesoderm formation. Starting at germband elongation, a second phase of *sna* expression appears to be initiated, characterized by a highly dynamic accumulation of transcripts in the developing central and peripheral nervous systems. Translation of *sna* RNA is apparently delayed as the *sna* protein is not detected before the onset of gastrulation. Its regional distribution generally correlates with that of *sna* transcripts. The complex pattern of *sna* expression strongly suggests that the function of the gene is not restricted to mesoderm formation.

Key words: *Drosophila*, *snail* gene, finger protein, embryogenesis, mesoderm formation, germ layers.

Introduction

The genetic control of *Drosophila* embryogenesis involves the interaction of both maternal and zygotic information. Two independent systems provide the positional information required for correct pattern formation in the embryo. One includes the genes controlling the anterior–posterior pattern and the other the genes controlling the dorsal–ventral pattern (Nüsslein-Volhard, 1979). In each case, maternal effect genes first define the spatial coordinates along the axis. Subsequently, the maternally generated information is interpreted by the zygotic genome, resulting in the spatially restricted expression of genes along the two axes. Whereas the anterior–posterior pattern is a repetition of metameric units, the dorsal–ventral pattern is a nonrepetitive series of cell types (Anderson 1987 for review).

For cells to know if they are to differentiate into epidermis, gut, neurons or muscles they must be provided with information as to their position along the dorsal–ventral axis. Cells surrounding the ventral midline will invaginate at gastrulation to form the mesoderm that will give rise to musculature and other mesodermal derivatives. Ventrolateral cells will give rise to the neuroectoderm that will form the ventral nerve cord and ventral epidermis. Dorsolateral cells will form the peripheral nervous system and dorsal epidermis and extreme dorsal cells will give rise to the amnioserosa. Approximately twenty maternally and zygotically active genes required for the dorsal ventral pattern have been identified (Anderson and Nüsslein-Volhard, 1984; Anderson, 1987). Positional information along the dorsoventral axis is provided by the action of twelve maternal effect genes, eleven of which make up the 'dorsal group'. Null mutations in any one of the genes in this group result in a dorsalising phenotype whereby all embryonic cells follow a dorsal developmental pathway (Anderson and Nüsslein-Volhard, 1984). The current hypothesis is that the product of one of the 'dorsal group' genes, *dorsal* (*dl*), acts as the morphogen that determines dorsal–ventral pattern by selectively activating or repressing the expression of downstream zygotic genes (Rushlow *et al.* 1989; Steward, 1989; Roth *et al.* 1989). The position-dependent formation of the diverse cell types therefore

depends upon the activity of several zygotically active genes, which are selectively expressed in response to the maternal factors. Null mutations in two of these zygotic genes, *sna* and *twi,* result in the lack of normal ventral furrow formation with the subsequent absence of mesodermal structures (Grau *et al.* 1984). Although the phenotypes of the two mutants are similar, they are not identical. Strong *sna* mutant alleles show abnormal development of laterally derived ectodermal structures as well as the ventrally derived mesoderm, whereas in the weaker alleles only the most ventrally derived structures are affected. This variation of phenotypes is not seen in *twi* mutant alleles where only mesodermal derived structures are affected (Simpson, 1983). The *twi* gene is expressed in the midventral region of the embryo, corresponding to the presumptive mesoderm and encodes a protein that contains a putative DNA-binding helix-loop-helix motif (Thisse *et al.* 1988).

The previous characterization of *sna* (Boulay *et al.* 1987) predicted a $43\times10^3 M_r$ protein with five zinc-finger motifs, which suggested that *sna* may be involved in transcriptional regulation. Positive identification of the gene was obtained by inducing *sna* phenocopies in wild-type embryos following the injection of antisense RNA. Subsequent P-element rescue experiments showed 13 kb of flanking sequences to be sufficient to rescue the lethality associated with the sna mutant phenotype, with survival to adulthood (Boulay, 1988). We present the results of studies on *sna* RNA and protein distribution during embryogenesis which show a dynamic expression of the gene in all three germ layers of the developing embryo. The complex pattern of expression suggests that *sna* may be required in a large range of tissues and implies that the function of the gene is not restricted to mesoderm formation. Finally, we present data showing that prior to invagination of the mesoderm, *sna* is expressed in twi^- embryos, and *twi* is expressed in sna^- embryos, suggesting distinct roles for the two genes in the process of mesoderm formation.

Materials and methods

Fly strains

Canton S and Oregon R raised at 25 °C on standard corn meal medium were used as our wild-type reference stocks. The snail allele, sna^{RYI}, and the twist allele, twi^{EY50}, were provided by P. Simpson.

Fusion protein and antisera

Two fragments, one of 717 bp, which codes for amino acids 151–390 (FP-pB1), and the other of 441 bp, encoding amino acids 243–390 (FP-pC1), were excised from plasmids containing DNAaseI-generated deletions of the cDNA clone pcSB (see Boulay *et al.* 1987). A third fragment of 1185 bp, which encodes the complete protein (FP-pD12), was obtained after site directed mutagenesis to introduce a *Hin*dIII site 15 nt upstream of the first ATG. *Hin*dIII fragments of the cDNA were inserted in frame (as well as in the antisense orientation) in the procaryotic vector pUR291 (Rüther and Müller-Hill, 1983) to create *lacZ–sna* fusion genes, which were then introduced into *E. coli* by transformation. Expression was induced by IPTG and *β-gal–sna* fusion proteins were prepared essentially according to Rio *et al.* (1986). For preparations used in immunization, bacteria pellets from induced cultures were lysed in SDS sample buffer (Laemmli, 1970) and loaded onto a preparative polyacrylamide–SDS gel. After electrophoresis, the gel was stained with Coomassie blue, the fusion protein band was excised and pulverized in liquid nitrogen. Alternatively, protein aggregate pellets were suspended in 8 M urea, 50 mM Tris–HCl pH 7.5, 500 mM NaCl, 1 mM EDTA, 50 mM DTT, 10 μM $ZnSO_4$, 1 mM PMSF, anti-protease cocktail (10 μM TPCK, 10 μM TLCK, 0.15 μM pepstatin, 0.1 μM leupeptin), extracted by mild magnetic stirring for 45 min at 0–4 °C and centrifuged for 10 min at 10 000 revs min^{-1}. The extracted proteins were renatured by the stepwise removal of urea after dialysis against solutions of 5 M urea, 50 mM Tris–HCl pH 7.5, 200 mM NaCl, 20 mM DTT, 10 μM $ZnSO_4$, 0.2 mM PMSF, anti-protease cocktail, 10 % glycerol followed by 2 M urea in the same buffer and finally against 50 mM Tris–HCl pH 7.5, 200 mM NaCl, 2 mM DTT, 10 μM $ZnSO_4$, 10 % glycerol. Insoluble material was removed by centrifugation at 10 000 revs min^{-1} for 10 min. These preparations were primarily used for preparing *β-gal–sna* affinity resins (pB1 and pD12) by coupling the proteins to CNBr-activated Sepharose, according to the manufacturer's (Pharmacia) protocol.

Antisera

An equal volume of complete Freund's adjuvant was added to gel-purified fusion protein (pB1 or pD12) suspended in PBS and approximately 200 μg of fusion protein was injected subcutaneously (at several sites) into female rabbits. The animals were boosted after 2 weeks with the same amount of protein in incomplete adjuvant and again after 4 weeks with 100 μg of fusion protein in PBS and were bled 10 days following the second booster. Thereafter the animals were periodically boosted with fusion protein in PBS, bled 10 days later and the titer of the serum was monitored by western blot analysis. Serum was adsorbed on an affinity column prepared from proteins (from IPTG-induced pUR 291 transfected *E. coli*) coupled to CNBr-activated Sepharose. Flow-through material from this column was loaded onto a pB1 (anti-pB1) or pD12 (anti-pD12) fusion protein affinity column and the antibodies eluted with 50 mM glycine–150 mM NaCl, pH 2.5, neutralized immediately and dialyzed against PBS. The specificity of the serum was confirmed by western blot analysis. Antibody–protein complexes were revealed with ^{125}I Protein A in PBS–3 % low-fat milk. All western analyses included appropriate controls, proteins prepared from the vector alone (induced or non-induced), from the non-induced fusion gene plasmid and from plasmids with inserts fused in antisense orientations. The two anti-*sna* sera gave similar qualitative results. However, as the anti-pB1 serum had the higher titer, this preparation was used in immunodetection assays (see below).

Protein extracts

The *sna* cDNA, pcSB, cloned in both orientations into the *Eco*RI site of the eukaryotic expression vector pSG5 (Green *et al.* 1988) was introduced into HeLa cells by calcium phosphate transfection (Ausubel *et al.* 1987). The transfected cells were lysed by three freeze–thaw cycles in TEBG 500 (see Bocquel *et al.* 1989), the proteins separated on 10 % SDS–PAGE and transferred to BA85 nitrocellulose membranes (Schleicher and Schüll). In parallel, proteins were extracted from dechorionated unstaged wild-type embryos essentially as in Soeller *et al.* (1988), separated on 10 % SDS–PAGE and transferred to nitrocellulose membranes.

Immunostaining

Embryos were dechorionated with 50% household bleach, fixed with 4% paraformaldehyde–PBS/heptane (v/v), devitellinized in heptane/methanol–25 mM EGTA (v/v), rehydrated in PBS–0.1% Tween 80 (PBT) and blocked with 5% decomplemented normal goat serum (NGS)–PBT. Embryos were incubated with affinity-purified serum overnight at 4°C and washed with 5% NGS–PBT. Incubation with the second antibody, alkaline-phosphatase-conjugated anti-rabbit IgG (1/1000 dilution), was at room temperature during 2 h and was followed by extensive washings with PBT and with 100 mM Tris–HCl pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$ (coloring buffer). The color was developed by adding 4.5 μl of nitroblue tetrazolium salt (75 mg ml^{-1}, 70% dimethylformamide), 3.5 μl of 5-bromo-4-chloro-3-indolyl phosphate (50 mg ml^{-1}, dimethylformamide) to the last ml of coloring buffer wash and incubating for the appropriate period (5–15 min). The reaction was stopped by the addition of 10 mM Tris–HCl pH 8–1 mM EDTA (TE), embryos were washed with PBS and mounted in 60% glycerol–PBS.

In situ *hybridization*

Antisense *sna* mRNA (for preparation, see Boulay *et al.* 1987) was labeled with ^{35}S-UTP and hybridized to paraffin-sectioned embryos as previously described (Ingham *et al.* 1985). Digoxigenin labeled DNA was synthesized according to the Boehringer-Mannheim protocol and hybridized to whole-mount embryos according to Tautz and Pfeifle (1989).

Results

(A) *Localisation of* sna *transcripts during embryogenesis*

Transient accumulation of sna *RNA in the mesoderm*

In situ hybridization to sectioned and whole-mount embryos was used to determine the spatial distribution of *sna* RNA during embryogenesis. The probes used were ^{35}S-labeled antisense *sna* RNA and a digoxigenin-labeled 547 bp *Hin*dIII fragment of the cDNA, which includes the entire finger coding region. The *sna* transcripts are first detected during late syncytial blastoderm (stage 4) when trace amounts of RNA present at a ventroposterior position are revealed with the ^{35}S probe (not shown). Subsequently, there is an increase in the level of the transcripts and *sna* RNA is readily detected along the ventral surface of the embryo extending into the anterior and posterior poles (Fig. 1A). In the period between late syncytial and early cellular blastoderm, the level of *sna* transcripts in the posterior region begins to decrease (Fig. 1B) and at the completion of cellular blastoderm (stage 5), *sna* RNA is no longer detected in the posterior region of the embryo. Thus, in the period immediately preceding gastrulation (late stage 5 to early stage 6), *sna* RNA is present in the anlagen of the anterior midgut and mesoderm (Fig. 1C).

At the onset of gastrulation, uninvaginated cells of the presumptive mesoderm are intensely labeled (Figs 1D,2B). As gastrulation proceeds, the strong signal is maintained as the cells invaginate (Fig. 2B), whereas there is a progressive decrease in the levels of *sna* RNA in the invaginated cells (Fig. 2C; compare Fig. 1D and 1E). By the end of gastrulation, only low levels of *sna* RNA are detected in the invaginated mesoderm while high levels are now seen in cells of the mesectoderm (Fig. 1F), an accumulation that lasts until stage 7. The low levels of *sna* transcripts present in the mesoderm persist until stage 8 (Fig. 1G).

Dynamic accumulation of sna *transcripts in the developing nervous system*

The most dynamic phase of *sna* expression occurs during germ band elongation, as shown by a rapidly changing pattern of RNA accumulation. In the early phase of germ band elongation (late stage 6), *sna* RNA is seen in the region of the anterior midgut rudiment as well as in a region on the dorsal surface of the invaginating amnioproctodeum. As germ band extension progresses (stages 7–9), *sna* RNA persists in the anterior midgut until stage 9 at which time transcripts are barely detectable in this tissue (Fig. 1H). Starting at late stage 8, *sna* RNA begins to accumulate in the neuroectoderm (Fig. 1G) particularly in large cells that probably correspond to neuroblast precursors and to segregated neuroblasts (Fig. 1H). In addition, *sna* RNA is present in cells lying between the proctodeum and the posterior midgut which may correspond to the primordium of the Malpighian tubules (Fig. 1H). During the slow phase of germ band extension (stages 9 to 10), *sna* transcripts are evident in segregating neuroblasts of the germ band and in procephalic neuroblasts (Fig. 1H,I). The accumulation in the neuroblasts is not uniform and, within the developing nervous system, *sna* transcripts are detected in some but not in all neuroblasts of an embryo (Fig. 1J,K). However, because of the highly dynamic expression one cannot exclude that *sna* is expressed in all neuroblasts, but not simultaneously. The *sna* transcripts persist in the neuroblasts until stage 11 when they are barely detected. At this time, they are also present in the median cells (Fig. 1M) and persist until stage 14. After germband retraction, staining is again seen in the central nervous system and lasts until stage 16.

sna *RNA persists until late embryogenesis*

Transient accumulation of *sna* transcripts is observed in several groups of cells, some of which we have tentatively identified from their time of appearance, position and morphology. Around stage 10, *sna* RNA appears in laterally positioned individual cells (Fig. 1K) that from the time of their appearance, presumably correspond to precursor cells of the peripheral nervous system (Ghysen and O'Kane, 1989). At the start of germband retraction (late stage 11), the signal in these cells is weaker and *sna* RNA begins to accumulate in a group of cells in each of the first seven abdominal segments (Fig. 1L). On the basis of their number and of their location, these cells probably correspond to progenitor cells of the pentascolopidial chordotonal sensory organs (*lch5*, see Campos-Ortega and Hartenstein, 1985). The accumulation in these organs will continue until stage 14. During stage 13, *sna* RNA

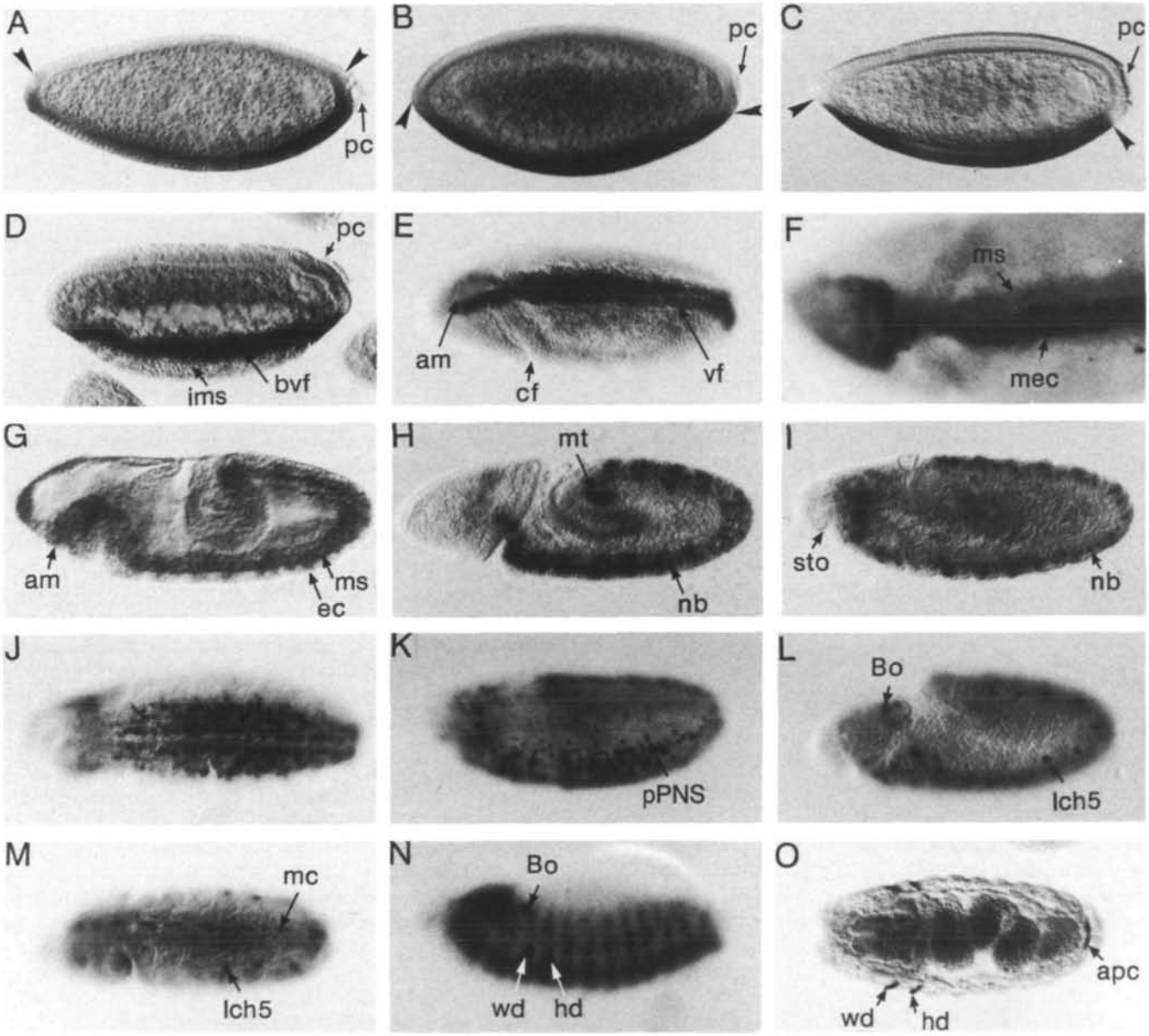

Fig. 1. Pattern of *sna* expression in wild-type embryos. Whole-mount embryos at various stages of development were hybridized with a digoxigenin-labeled 547 bp *Hin*dIII fragment from the cDNA. For all embryos, anterior is to the left and dorsal is up (except E, F, J, M, O). Staging is according to Campos-Ortega and Hartenstein (1985). Embryos were photographed using Nomarski optics. Arrows indicate labeled structures or landmarks. (A) Syncytial blastoderm embryo (stage 4). Arrowheads indicate the anterior and posterior limits of the region of expression, note reduction in early stage 5 embryo (B) and in late stage 5 embryo (C); pole cells (pc). (D) Early gastrulating embryo (stage 6), invaginating mesoderm (ims), border ventral furrow (bvf). (E) Ventral view of late gastrulating embryo (early stage 7), anterior midgut (am), cephalic furrow (cf); cells on the border of the ventral furrow (vf) are about to invaginate. (F) Detail of the ventral furrow region at the end of gastrulation (stage 7, embryo slightly older than in E). Note stronger signal in the mesectoderm (mec) than in the mesoderm (ms). (G) Stage 8 embryo (germband extension) transcripts start to accumulate in the ectoderm (ec). (H) Stage 9 embryo, neuroblasts (nb), Malpighian tubules (mt). (I–K) Stage 10 embryos (extended germband) strong signal in the neurogenic region (J, ventral view); stomodeum (sto), presumed precursor cells of the peripheral nervous system (pPNS). (L,M) Lateral and ventral views, respectively, of a stage 11 embryo, Pentascolopidial chordotonal (*lch5*) and Bolwig organ precursors (Bo) and median cells (mc) are indicated. (N) Germband retracted embryo (stage 13), presumed precursors of wing disc (wd) and haltere disc (hd). (O) Embryo at the end of embryogenesis (stage 16–17), cells in the region of the anal plate (apc).

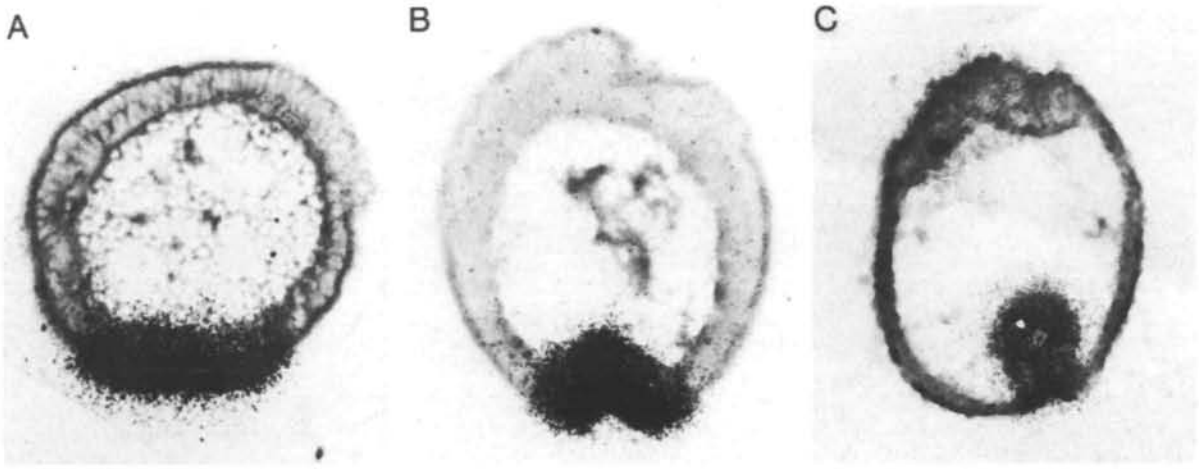

Fig. 2. Distribution of *sna* transcripts during mesoderm formation. A ^{35}S labeled antisense RNA probe was hybridized to 8 μm cross sections through gastrulating embryos. (A) End of stage 5, strong signal in the anlage of the mesoderm. (B) Stage 6, start of mesoderm invagination. (C) later stage 6, note the reduced signal in invaginated mesoderm.

accumulates in two clusters of cells of the mesothoracic and metathoracic segments, which probably correspond to precursors of the wing and haltere imaginal discs (Fig. 1N). Transcripts are also detected in cells that from their position and morphology, presumably correspond to Bolwig photoreceptor organs (Bolwig, 1946). At the end of embryogenesis (stages 16–17), some *sna* RNA is still present in the presumed wing and haltere disc precursors as well as in a group of cells located in the vicinity of the anal plate (Fig. 1O).

(B) *Distribution of* sna *protein*

We analyzed the distribution of *sna* protein during embryonic development by staining whole-mount embryos with antibodies raised against *lacZ-sna* hybrid proteins. All immunodetection analyses were performed with monospecific antibodies prepared by affinity chromatography (see Materials and Methods). These antibodies recognized a single protein band with an apparent relative molecular mass of approximately 48×10^3 in wild-type embryonic extracts as well as in extracts of HeLa cells transiently expressing the *sna* protein from the eukaryotic expression vector pSG5 (Green *et al.* 1988) but did not recognize any protein produced when the coding region was inserted in pSG5 in the antisense orientation (Fig. 3). The signal obtained with the antiserum was either abolished or significantly lowered if the serum was preincubated with fusion protein (2 h at 25 °C) before incubation with embryos or with nitrocellulose transfers of protein extracts. Finally, no specific staining was detected with the antiserum in homozygous *sna* embryos (not shown).

Whereas *sna* transcripts first appear during syncytial blastoderm (stage 4), the *sna* protein is first detected at the onset of gastrulation (stage 6); thus there is a significant lag in the appearance of the translation product. At the start of gastrulation, weak staining of *sna* protein is observed in individual cells along the ventral surface (Fig. 4A) and shortly afterwards in the primordium of the anterior midgut and in the mesoderm (Fig. 4B). The protein is detected only in a subset of the invaginated cells and is apparently located in the nucleus (Fig. 4B,C). During the early phases of ventral furrow formation, cells within the furrow are more intensely stained than those bordering the furrow (Fig. 4C,D), which is in contrast to the RNA accumu-

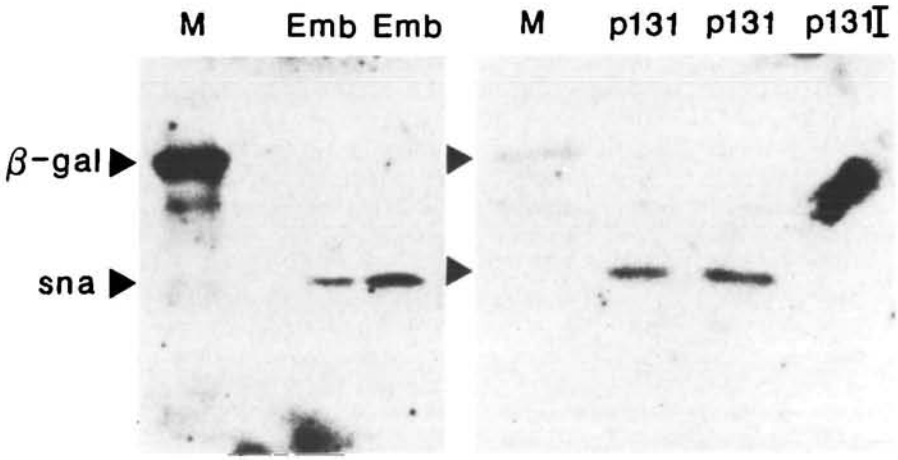

Fig. 3. Monospecific *sna* antiserum recognizes recombinant and endogenous *sna* protein. Western blots of extracts of wild-type embryos (Emb) and of HeLa cells transiently transfected with pSG5 based *sna* expression vectors with *sna* cDNA inserted in the sense orientation (p131) and in the antisense orientation, (p131I). Two separate preparations of Emb and p131 were analyzed. See Material and methods for preparation of protein extracts and immunodetection procedure.

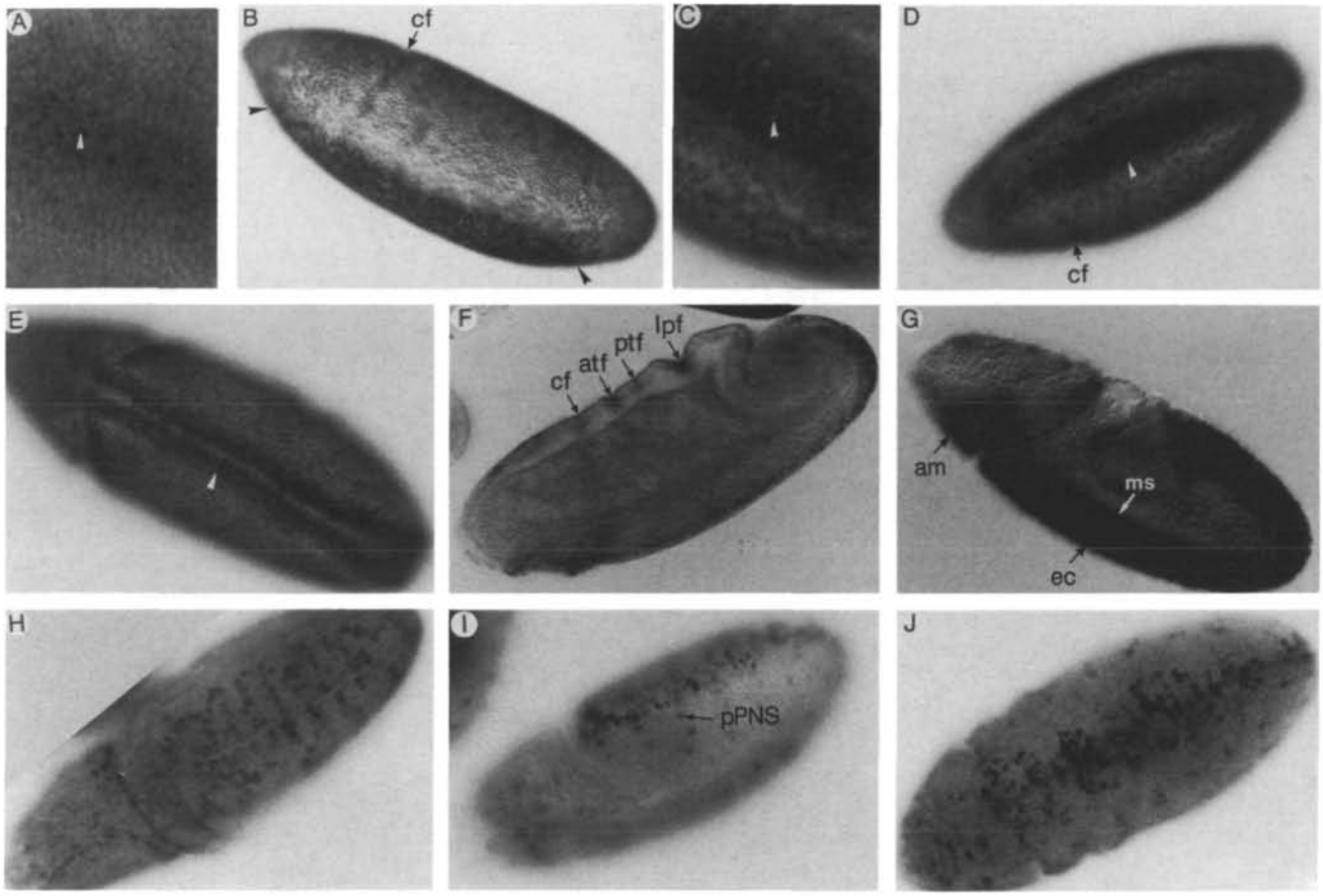

Fig. 4. Localisation of *sna* protein in wild-type embryos. All embryos were incubated with affinity-purified anti-*β-gal–sna* protein antiserum (pB1). Anterior is to the left and dorsal is up (except D,E,H,J). Staging is according to Campos-Ortega and Hartenstein (1985) and photographs were taken with Nomarski optics. (A) Detail of the ventral furrow at the onset of gastrulation (stage 6). Arrowhead indicates a cell expressing the protein. (B) lateral view of an early gastrulating embryo (stage 6). Arrowheads indicate the limits of the region of expression, cephalic furrow (cf). (C) Detail of the ventral furrow of a mid gastrulating embryo (stage 6). Arrowhead indicates a non-expressing cell. (D) Ventral view of a mid gastrulating embryo (stage 6), arrowhead indicates less intensely stained cells on the border of the furrow. (E) Ventral view of a late gastrulating embryo (slightly older than in D), cells on the border of the furrow are more intensely stained (arrowhead) than cells inside the furrow. (F) Lateral view of embryo during early germband elongation (stage 7, focus is on the dorsal folds), cephalic furrow (cf), anterior transversal fold (atf), posterior transversal fold (ptf), lateral proctodeal fold (lpf). (G) Stage 8 embryo (germband elongation). Low protein levels are detected in the mesoderm (ms), anterior midgut (am), ectoderm (ec). (Note that staining was extended deliberately.) (H) Dorsal view of a stage 9 embryo, neuroectodermal cells are stained. (I) Lateral view of embryo at about stage 10–11, showing staining in presumed precursor cells of the peripheral nervous system (pPNS). (J) Ventral view of a stage 11 embryo, neuroectodermal cells (as in H) are stained for the protein.

lation (see above). However, at the end of gastrulation, cells located on the border of the ventral furrow become more strongly stained for protein than cells of the invaginated mesoderm. Thus, at this stage of embryogenesis, the distribution of the protein coincides with the accumulation pattern of *sna* transcripts (compare Figs 2E and 4E). The regional distribution of the protein generally correlates with that of transcripts; however, with the start of germband elongation, some differences are observed. During the early phase of germband elongation (stage 7), weak staining of the protein is seen in the regions of the cephalic furrow and transversal folds (Fig. 4F). In later phases of germband elongation, the intensity of the signal increases and, in the neurogenic region, the protein is detected only in a subset of the ectodermal cells (Fig. 4H–J). However, as the distribution pattern of the protein does not correspond to that seen during the wave of neuroblast segregation (Hartenstein and Campos-Ortega, 1984), it is not known if all cells that express *sna* protein will become neuroblasts or if all neuroblasts express the protein. Surprisingly, in the neurogenic region, staining is apparently restricted to the cytoplasm in the majority of cells (Fig. 4H–J). During the later stages of embryogenesis (until stages 16–17), only low levels of the protein are detected (data not shown). This late expression includes the presumptive wing and haltere disc precursors.

(C) sna *expression in* twi$^-$ *embryos and* twi *expression in* sna$^-$ *embryos*

The two zygotic genes, *sna* and *twi*, occupy similar positions in the regulatory hierarchy required for the development of the mesoderm (reviewed by Anderson, 1987) and may act sequentially with one gene controlling the other. To investigate this possibility, we looked at the expression of *twi* protein in *sna* embryos by immunodetection using antibodies directed against the *twi* protein (Thisse *et al.* 1988). At blastoderm, there is no detectable difference in the distribution of *twi* protein between heterozygous ('wild-type') and homozygous *sna* embryos. The first visible difference between the two types of embryos is seen at the end of gastrulation (stage 6–7). In the heterozygous embryo, *twi*-positive cells invaginate (Fig. 5A,C) whereas in a homozygous *sna* embryo, these cells do not invaginate and remain at the surface (Fig. 5B,D). During germband elongation (late stage 8), *twi* expression decreases and only a few *twi*-positive cells remain in the small ventral fold which is formed in *sna*$^-$ embryos (compare Fig. 5E,F). Thus it would appear that, prior to gastrulation, *sna* is not essential for *twi* expression.

As the *sna* protein is not detected before the start of gastrulation, the expression of *sna* in early *twi* mutant embryos was analyzed by the distribution of *sna* transcripts. The results show that, at blastoderm, *sna* transcripts are present in *twi*$^-$ embryos. The expression of *sna* in *twi*$^-$ embryos and of *twi* in *sna*$^-$ embryos, prior to gastrulation, excludes a simple hierarchical relationship between the two zygotic genes for formation of the mesoderm. The spatial expression of *sna* is more restricted in *twi*$^-$ embryos and the transcripts accumulate in a narrower region than in wild-type embryos (compare Fig. 6A and B). However, this may be a general effect of a reduction in the size of the mesoderm anlage in *twi* embryos (see below).

Discussion

sna *expression during mesoderm formation*

The first evidence of *sna* expression is obtained during

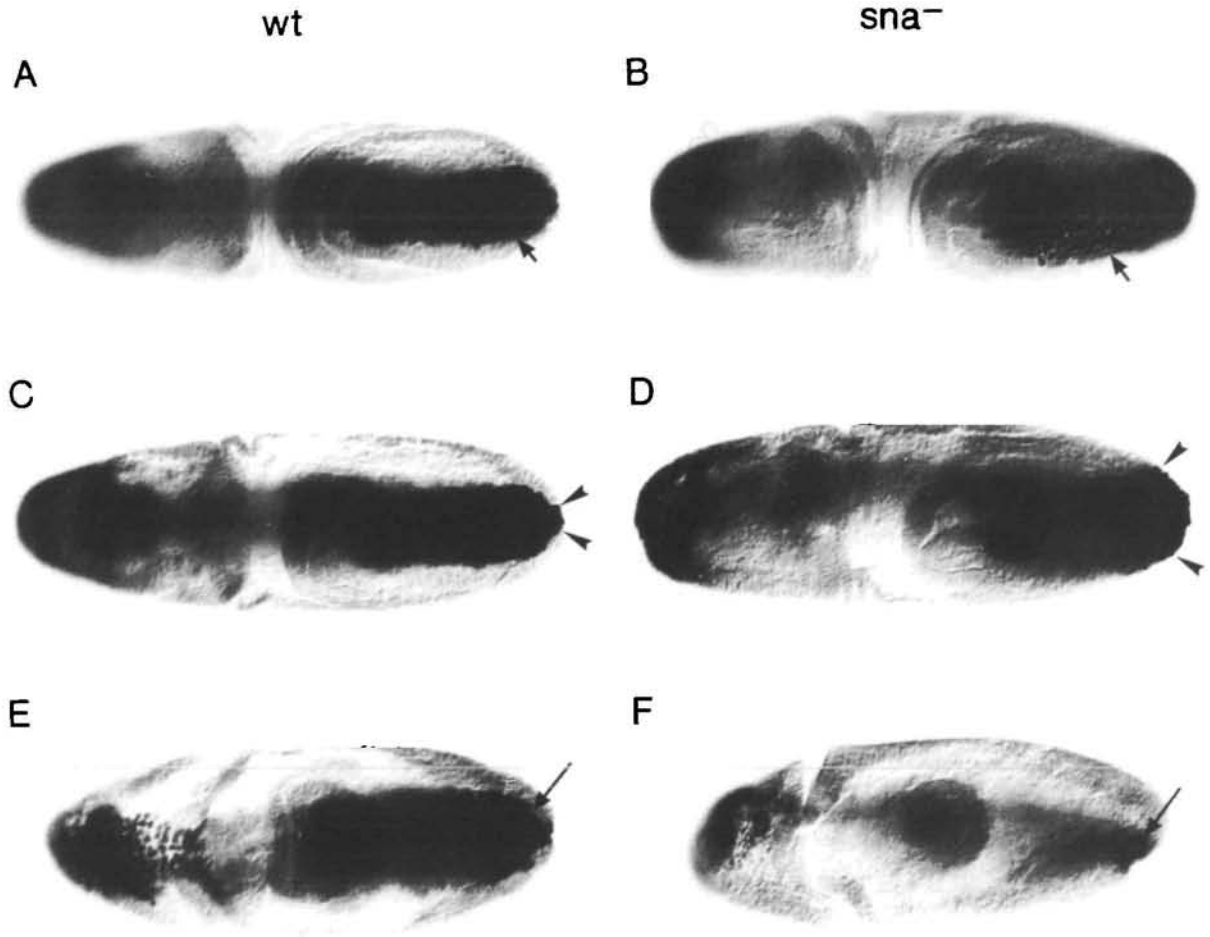

Fig. 5. Expression of *twi* in wild-type and *sna*^{RY1} embryos. All embryos were incubated with anti-*twi* protein antiserum (Thisse *et al.* 1988). (A,C) early stage 8 wild-type embryo (same embryo, different focal plane); (E), late stage 8 wild-type embryo; (B,D), early stage 8 *sna*^{RY1} embryo (different planes of focus of the same embryo); (F) late stage 8 *sna*^{RY1} embryo. Arrows indicate invaginated *twi*-positive cells in A and E and non-invaginated *twi*-positive cells in B and F. Arrowheads in C and D give limits of *twi*-positive cells which remain at the surface.

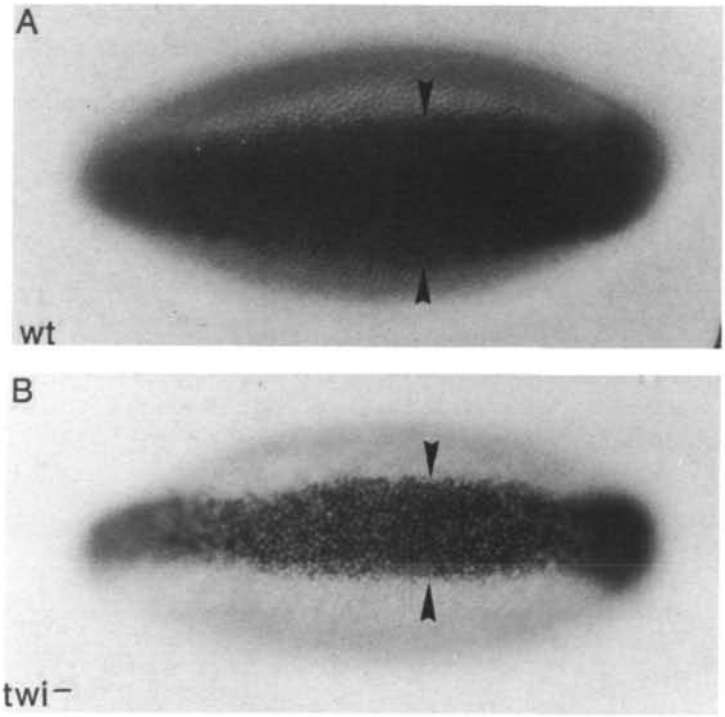

Fig. 6. Expression of *sna* transcripts in wild-type and twi^{EY50} embryos. Whole-mount embryos were hybridized with digoxigenin-labeled *sna* probe. Ventral views of stage 5 wild-type embryo (A) and stage 5 twi^{EY50} embryo (B). Arrowheads indicate limits that define the anlage of the mesoderm.

syncytial blastoderm when low levels of transcripts are detected in the region of the proctodeum anlage. These then accumulate progressively along the ventral surface of the embryo and extend into the posterior and anterior poles. At this developmental stage, the spatial distribution of *sna* transcripts is similar to that of the *dorsal* (Steward *et al.* 1988; Roth *et al.* 1989) and *twist* proteins (Thisse *et al.* 1988). This is not surprising as the expression of all three genes is required for mesoderm formation. It has been shown that the *dorsal* gene product is the morphogen that controls dorsal–ventral pattern by activation or repression of zygotic regulatory genes (Rushlow *et al.* 1989; Steward, 1989; Roth *et al.* 1989). In addition, Simpson (1983) reported synergistic interactions between *dl* and *twi* and *dl* and *sna*, suggesting possible interactions between the products of these three genes. At blastoderm, in embryos issued from homozygous *dl* females, *sna* transcripts are not detected in the mid-ventral region of the embryo (region of the presumptive mesoderm in wild-type embryos) though low levels of the RNA are detected in other regions of the embryo (A. Alberga, unpublished results). The fact that *sna* is expressed in twi^{-} embryos and that *twi* is expressed in sna^{-} embryos excludes a simple relationship in which the product of one gene is essential for the expression of the other. Nevertheless, the spatial expression of *sna* is reduced in the absence of the *twi* gene product (see Fig. 6B). A comparable reduction in the size of the mesoderm anlage was also observed at blastoderm, in twi^{-} embryos hybridized with probes corresponding to the transcripts of $E(spl)m^{8}$ and *Delta* (Kempe, Haenlin and Campos-Ortega, unpublished results). Therefore, the modification of *sna* expression may reflect a general effect of a reduction in the size of the mesoderm anlage in twi^{-} embryos. In contrast, the absence of the *sna* gene product has no effect on *twi* expression, prior to gastrulation (see Fig. 5). Together, these results suggest that, if a hierarchial relationship exists between these two genes, then *twi* is epistatic to *sna* in the process of mesoderm formation.

In the interval between syncytial blastoderm and gastrulation, the spatial expression of *sna* is consistent with a role in mesoderm formation. During cellular blastoderm, *sna* expression is apparently turned off in cells of the posterior pole and its transcripts extend anteriorly to include the anlagen of the anterior midgut and mesoderm. Thus immediately preceding the onset of gastrulation, the spatial expression of *sna* singularizes since during the same period, both *dl* and *twi* protein expression continue in the posterior pole (Steward, 1989; Roth *et al.* 1989 and Thisse *et al.* 1988, respectively). In the gastrulating embryo, high levels of *sna* transcripts are present in the presumptive mesoderm before and during invagination whereas only low levels are found in the invaginated tissue. Thus the expression of *sna* in mesodermal cells is apparently limited to a relatively brief period during the early stages of embryogenesis. In comparison, *twi* expression is maintained in the mesoderm until much later in embryogenesis (Thisse *et al.* 1988). The difference in their temporal patterns of expression contributes to the increasing evidence for separate roles of these two genes in the development of the mesoderm (see Leptin and Grunewald, 1990).

Although *sna* transcripts are present at syncytial blastoderm (stage 4), *sna* protein is not detected before the onset of gastrulation (stage 6). Similar delays between the appearance of RNA and gene products have been reported for other *Drosophila* developmental genes, e.g. *zerknüllt* (Rushlow *et al.* 1987), *slit* (Rothberg *et al.* 1988) and *single-minded* (Crews *et al.* 1988). While the ventral furrow is being formed, the *sna* protein is detected in nuclei of cells within the furrow and these are more intensely stained than cells surrounding the furrow. This suggests that during gastrulation the protein is essentially nuclear (see below) and accumulates primarily in cells that are in the process of invagination (see Fig. 4A–D). In comparison, during this period, cells that are about to invaginate have higher levels of *sna* RNA than cells that are in the process of invagination (compare Figs 1D and 4D). In the presumptive mesoderm, *sna* RNA and *twi* protein are expressed in the same cells; however, not all *twi*-positive cells invaginate during gastrulation (Leptin and Grunewald, 1990). The localization of the *sna* gene product in invaginating cells suggests that *sna* may be required for the invagination process of the future mesodermal cells.

sna *expression is not restricted to the mesoderm*

If the early expression of *sna* is consistent with the phenotypes of the mutants, its later expression is somewhat unexpected. Throughout embryogenesis *sna* transcripts accumulate transiently in a variety of tissues in which there is no known function for the gene. These include the endodermal derived midgut and ectodermal derived structures, in particular, that of the developing nervous system. Around stage 8, *sna* RNA is first detected in cells from which neuroblasts will segregate and afterwards in the segregating neuroblasts (stage 9). In addition, the transcripts are present during the formation of the peripheral nervous system. The accumulation of *sna* RNA in the nervous system is very dynamic, which makes it difficult to describe fully its spatial pattern of expression. At present, we have no explanation for the accumulation of *sna* RNA in the precursors of the nervous system. Preliminary observations on *sna* mutants (El Messal, 1987) showed that, in *sna* mutants, the segregation of neuroblasts occurs; however, it is not known if their number and/or their identity are correct. The neural cord formed is often abnormal with ganglion-like structures, its left and right sides often fail to fuse and later to condense into a normal CNS. These defects could be due either to the lack of the *sna* gene product or to a secondary mechanical defect, resulting from the absence of mesodermal structures and the twisted nature of the mutant embryo. It remains to be seen if *sna* is involved in neurogenesis and in the formation of the endoderm. A temperature-sensitive allele would be useful for this analysis. In very late stages of embryogenesis (16–17), *sna* transcripts are found in the presumed precursors of the wing and haltere discs. This result is of particular interest and it is likely that failure of *sna* activities in these discs leads to the missing halteres and hemithorax phenotypes seen in both genetic (Grau *et al.* 1984) and transgenic studies (Boulay, 1988).

During germband elongation in the neurogenic region, the protein is accumulated only in a subset of the cells that accumulate RNA. A differential accumulation of transcript and protein was reported for the *Drosophila* segmentation gene *Krüppel* (Gaul *et al.* 1987) and for *lethal of scute*, one of the genes in the *achaete–scute* complex (Cabrera, 1990). In both cases, post-transcriptional regulation was invoked as the basis for this difference. As there is a significant delay between the appearance of *sna* RNA and *sna* protein, it is possible that post-transcriptional regulation is also involved in the case of the *sna* protein.

Subcellular localisation of the sna *protein*

Although in some cells the protein appears to be in the nucleus, the majority of the cells in the neurogenic region clearly show cytoplasmic localisation of the protein (see Fig. 4H–J). Note that in HeLa cells the transiently expressed protein is nuclear (A. Alberga and T. Ylikomi, unpublished results), which confirms that the protein contains a functional signal for nuclear accumulation. What could be the significance of the cytoplasmic localisation? If the *sna* protein acts only as a nuclear transcription factor then it will be active during mesoderm formation when the protein is apparently located in the nucleus (see Fig. 4A–C) and to a much lesser extent in the developing nervous system. This would be similar to the case of *dorsal* where it has been shown that the *dl* protein is located in the nucleus in regions where the gene is known to have a function and is cytoplasmic where there are no known genetic requirements (Rushlow *et al.* 1989: Steward, 1989; Roth *et al.* 1989). It is however, possible that the cytoplasmic *sna* protein has a distinct function. By virtue of its zinc fingers, it shows structural homology to *Xenopus* TFIIIA transcription factor and we note that TFIIIA binds to the internal control region of 5S RNA genes (Engelke *et al.* 1980) as well as to the gene product, 5S RNA (Picard and Wegnez, 1979). Klug and Rhodes (1987) suggested that the ability to bind to both DNA and RNA could be a general property of TFIIIA-like finger proteins (i.e. bearing the Cys-Cys . . . . His-His motif), thus providing the possibility for an additional mechanism for regulation. It is therefore not excluded that the *sna* protein could be binding to RNA and it remains to be seen if the cytoplasmic localisation is indeed indicative of a novel regulatory role of the *sna* protein.

In spite of this difference in the localisation of the protein in early and late embryogenesis, is there a unifying feature of *sna* expression? It is noteworthy that in both the early and late phase, *sna* is expressed in regions that undergo a morphogenetic movement and that the expression apparently ceases once the movement is completed. This suggests a possible role of *sna* in the process of cell movement.

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