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Cell shape changes during gastrulation in *Drosophila*

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Summary

The first morphogenetic movement during *Drosophila* development is the invagination of the mesoderm, an event that folds a one-layered epithelium into a multilayered structure. In this paper, we describe the shape changes and behaviour of the cells participating in this process and show how mutations that change cell fate affect this behaviour.

We divide the formation of the mesodermal germ layer into two phases. During the first phase, the ventral epithelium folds into a tube by a series of concerted cell shape changes (ventral furrow formation). Based on the behaviour of cells in this phase, we conclude that the prospective mesoderm is not a homogeneous cell population, but consists of two subpopulations. Each subpopulation goes through a distinctive sequence of specific cell shape changes which together mediate the invagination of the ventral furrow. In the second phase, the invaginated tube of mesoderm loses its epithelial character, the mesoderm cells disperse, divide and then spread out along the ectoderm to form a single cell layer.

To test how ventral furrow formation depends on cell fates in the mesoderm and in neighbouring cells we alter these fates genetically using maternal and zygotic mutations. These experiments show that some of the aspects of cell behaviour specific for ventral furrow cells are part of an autonomous differentiation programme. The force driving the invagination is generated within the region of the ventral furrow, with the lateral and dorsal cell populations contributing little or none of the force. Two known zygotic genes that are required for the formation of the mesoderm, *twist* and *snail*, are expressed in ventral furrow cells, and the correct execution of cell shape changes in the mesoderm depends on both.

Finally, we show that the region where the ventral furrow forms is determined by the expression of mesoderm-specific genes, and not by mechanical or other epigenetic properties of the egg.

Key words: gastrulation, morphogenesis, *Drosophila*, *snail*, *twist*, *dorsal* group.

Introduction

Morphogenesis is the sum of the processes that create complex three dimensional forms out of simpler structures. One such process is the folding of flat, essentially two-dimensional epithelia into more complicated organs, for example during neurulation, formation of the eye cup, and during gastrulation. Epithelial folding has been studied in many organisms and various models have been proposed to explain the mechanics of cell and epithelial shape changes, but the molecular mechanisms of the processes are not understood. Molecules that are likely to play important roles in shape changes have been identified and analysed biochemically and their roles have been studied *in vitro*, but their functions in intact tissues or the whole organism have hardly been investigated. One way of studying function *in vivo* is by genetic analysis, and for this *Drosophila* is a particularly convenient organism. The first morphogenetic event during *Drosophila* gastrulation, the formation of the ventral furrow, is well suited to study morphogenesis genetically, because it is a relatively simple process and many of the genes that determine the fates of the cells involved are already known.

Before gastrulation begins, the *Drosophila* embryo consists of a single cell layer epithelium of about 5000 morphologically identical cells. On the ventral side of the embryo, a strip of cells invaginates to form a tube, the prospective mesoderm (Fullilove *et al.* 1978; Turner and Mahowald, 1977, see Fig. 1). The mesodermal tube and the overlying ectoderm (the germ band) elongate around the posterior end of the embryo in a process called germ band extension (Fig. 1D–H). At the end of germ band extension, the mesoderm forms a layer on the inside of the ectoderm. The major gastrulation movements and some aspects of cellular behaviour have been described previously (Poulson, 1950; Sonnenblick, 1950; Mahowald, 1963; Rickoll 1976; Turner and Mahowald, 1977; Fullilove *et al.* 1978; Campos-Ortega and Hartenstein, 1985), but to analyze the regulation and mechanics of epithelial folding during gastrulation and to understand the function of the molecules involved, it is necessary to know the details of the events to be investigated. Therefore we began our analysis of gastrulation by describing in detail the behaviour and shape changes of the cells involved in this process.

Because many of the genes that determine the fates of different cell populations in the embryo are known, it

is possible to alter cell fates genetically and thereby analyse the role of the different populations in gastrulation. Fates along the dorsoventral axis of the embryo, including the ventral region that will form the mesoderm, are determined by the *dorsal* group of maternal effect genes (reviewed in Anderson, 1987). One of these, the gene *dorsal*, codes for a nuclear protein, whose uptake into the nucleus is regulated by the other dorsal group genes. The *dorsal* protein becomes localized in the nuclei on the ventral side, but remains in the cytoplasm on the dorsal side of the embryo (Roth *et al.* 1989; Rushlow *et al.* 1989; Steward, 1989). Mutations in *dorsal* group genes can alter the localisation of the *dorsal* protein such that either all or none of the nuclei in the embryo take up *dorsal* protein, or intermediate situations are created. As a result, all cells can take on dorsal or ventral fates, or the regions in which these fates occur can be increased or decreased in size (Roth *et al.* 1989). We use such embryos to test to what extent the behaviour of ventral cells during gastrulation depends on the fate of neighbouring cells and other regions of the embryo.

The localisation of *dorsal* protein determines which zygotic genes are activated or repressed along the dorsoventral axis. Two zygotic genes, *twist* and *snail* (Nüsslein-Volhard *et al.* 1984), known to be required for the development of the mesoderm (Simpson, 1983), are expressed in ventral cells with high nuclear concentrations of *dorsal* protein. Both code for proteins that are probably transcription factors (Thisse *et al.* 1988; Boulay *et al.* 1987) and thus control cell fate *via* the activation of other genes. We show that *twist* and *snail* independently control different aspects of ventral cell behaviour.

Materials and methods

Fly stocks

As wild-type flies we used homozygous *white* flies. The *snail* allele was sna^{IIG}, *twist* was twi^{ID96} or $Df(2R)twi^{S60}$ (Simpson, 1983), the double mutant was sna^{IIG} $Df(2R)twi^{S60}$ and was kindly provided by Kavita Arora. The *string* allele was stg^{7M}. *torso* mutant embryos were derived from tor^{XR1}/tor^{XRi} mothers. The following alleles of maternal dorsalizing genes were used: $dl^{1}/Df(2L)TW119$, pll^{078}/pll^{RM8}, $Tl^{5BRE}/Df(3R)ro^{XB3}$, spz^{RM7}/spz^{197} (completely dorsalized), spz^{67}/spz^{197}(weakly dorsalized at 18°C, Fig. 3), ndl^{046}/ndl^{093}, ea^{1}/ea^{2}, tub as well as the ventralizing *Toll* allele Tl^{10B}. Yash Hiromi provided the P-insertion line P336 in which the mesectoderm cells express β-galactosidase.

Mutant embryos were distinguished from wild-type or heterozygous embryos by the absence of *twist* protein in the case of *twist* mutants and double mutants. In *snail* embryos this criterion could not be used. All embryos expressed the *twist* gene, including the homozygous mutant *snail* embryos, which can be recognized by their phenotype at the extended germ band stage (see Fig. 4). We were initially unable to distinguish mutant embryos at the syncitial and early cellular blastoderm, but after sectioning it became clear that about 25% of the embryos appeared different from wild-type embryos (see Results) and we concluded that these were *snail* mutant embryos. We later confirmed our identification of mutant embryos by using marked balancer chromosomes that allowed us to distinguish mutant from wild-type embryos at early stages.

Antibodies

The antibodies against *twist* and *dorsal* protein were gifts from Siegfried Roth (Roth *et al.* 1989), anti-*zen* from Chris Rushlow and anti-β-galactosidase from Ulrike Gaul. The second antibody was biotinylated goat anti-rabbit from Vector Labs. Embryos were stained according to standard protocols. The antibodies were detected histochemically using the Vectastain ABC Kit from Vector Labs.

Sectioning of embryos

Stained embryos were dehydrated through a methanol series followed by two 10 min incubations in 100% ethanol. They were then transferred to dry acetone and finally to a 1:1 mixture of dry acetone and Araldite. Araldite was Durcupan ACM from Fluka and was prepared by mixing 100 ml of reagent A with 100 ml of reagent B, then adding 3.5 ml of reagent D and finally 2 ml of reagent C. This mixture was stored frozen until use. Embryos were left in the acetone:Araldite mixture in an open dish overnight until the acetone had evaporated. They were then oriented for sectioning, polymerized at 60°C for 2 days and sectioned on a Reichert–Jung Supercut 2050 microtome. The sections were mounted in Araldite or permount and photographed on a Zeiss Axiophot with Nomarski optics.

In situ *hybridisation*

The insert from the cloned DNA containing the *snail* gene was purified from agarose gels and labelled with Digoxigenin using the kit from Boehringer Mannheim, following the directions provided. The labelled probe was hybridized to embryos and detected as described by Tautz and Pfeifle (1989). Labelled embryos were treated for sectioning and photography in the same way as the antibody-stained embryos described above.

Results

Wild-type development

Fig. 1 shows whole mounts of embryos at successive stages of gastrulation. The embryos are stained with antibodies against the *twist* protein, a nuclear protein expressed in prospective mesoderm and endoderm cells. The mesoderm cells initially occupy the ventral surface of the embryo (Fig. 1B) and invaginate during gastrulation to form the mesodermal cell layer underlying the ectoderm (Fig. 1H). The formation of the tube of mesoderm from the ventral epithelium takes about 15 min (Fig. 1B–1D), the conversion of the invaginated tube into the final mesodermal cell layer (Fig. 1D–1G) just over an hour. To analyse the behaviour of the invaginating mesoderm cells in detail, we made transverse sections of gastrulating embryos.

Fig. 2A shows an embryo at the beginning of cellularization. The nuclei are lined up at the periphery of the egg in a thick layer of cortical cytoplasm which takes up about one-third of the diameter of the egg. The centre of the egg is filled with yolk. About 25% of the periphery is occupied by nuclei that contain the *twist* protein and will later make the mesoderm. At the edge

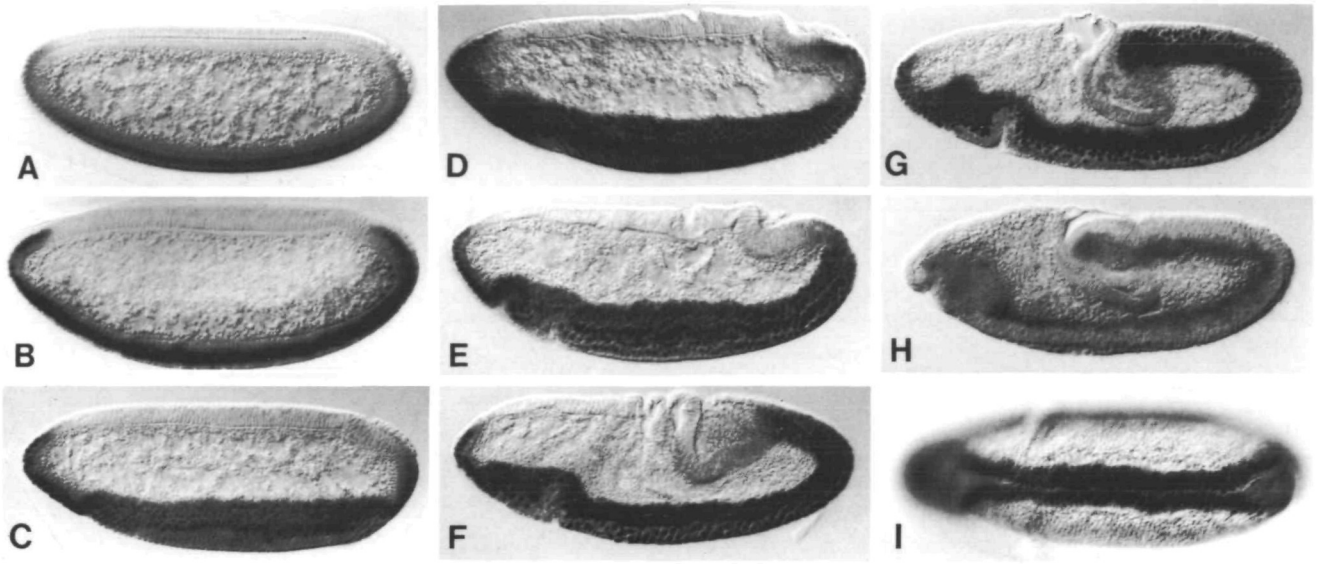

Fig. 1. Whole mounts of embryos stained with *twist* antibodies to demonstrate to which stages the sections in Fig. 2 correspond. (A) Syncytial blastoderm (Fig. 2A; stage 5a according to Campos-Ortega and Hartenstein, 1985). (B) Cellular blastoderm. The ventral nuclei have begun to move inwards in some places. (Fig. 2D; stage 5a/b). (C) Ventral furrow formation (Fig. 2F; stage 6). (D) Beginning germ band extension (Fig. 2G,H; stage 7). (E) Germ band extension (Fig. 2I; stage 7a). (F) Germ band extension. The midgut has invaginated on the dorsal side (Fig. 2J; stage 8). (G) Germ band extension (Fig. 2K, stage 8d). (H) Fully extended germ band. The mesoderm now forms a single cell layer underneath the ectoderm (Fig. 2L; stage 10). (I) Ventral view of the ventral furrow, approximately same stage as Fig. 1E.

of the region of strongly stained nuclei there are one or two nuclei that are stained much more weakly. These are the future mesectoderm cells, which become joined at the ventral midline when the mesoderm has invaginated (see also Fig. 5S), and later form part of the nervous system (Campos-Ortega and Hartenstein, 1985). In Fig. 2A membranes have started to invaginate between the nuclei, this process being more advanced on the ventral side. This is the first morphologically visible difference between the dorsal and ventral sides of the embryo (apart from the overall egg shape) (Mahowald, 1963). This asymmetry is maintained until the completion of cellularization (Fig. 2B), so that the cells on the dorsal side do not take up all of the cytoplasmic layer and are shorter than the ventral cells. As soon as the cellular blastoderm has formed, the ventral cells become flat on their apical surfaces, while the surfaces of the other cells remain rounded (Fig. 2B,C). This flattening may be the beginning of the contraction of the ventral surface, which becomes clearly visible in Fig. 2D. Simultaneously, the nuclei of the central 8–10 cells begin to move inwards (Fig. 2C,D).

At this stage two subpopulations can be distinguished within the prospective mesoderm, a central and a peripheral population. The central population is about 8–10 cells wide. These cells contract apically and their nuclei move away from the periphery of the egg. We will refer to this population of cells as 'central cells'. The other cells (which we will call 'peripheral cells'), lying on either side of the central population in strips about four cells wide, show the opposite behaviour. Their nuclei remain close to the peripheral surface of the egg, their apical membranes expand (or are stretched), while their basal surface becomes very narrow. Both populations express the *twist* protein. The processes of apical narrowing and nuclear movement continue and the ventral epithelium forms an indentation (Fig. 2E,F). The central cells transiently become taller and narrower (Fig. 2F). In some strains bulbous projections from the apical surfaces of the central cells appear at this stage (see Fig. 6 and Rickoll, 1976). The dorsal cells become tall and thin and their nuclei lose their regular alignment as the dorsal epithelium begins to form transverse folds (Fig. 2G,H and Fig. 1D–F). The ventral indentation increases and folds into the embryo as a tube (Fig. 2G–I). Figs 2G,H show embryos at approximately the same stage of development. The wide opening of the ventral side of the embryo in Fig. 2G is artificial. The live embryo develops inside a membrane (the vitelline membrane) which has to be removed for antibody staining and which holds the developing ventral fold together. In some cases (Fig. 2H) but not always (Fig. 2G) this state is maintained after fixation and removal of the vitelline membrane. After much longer and stronger fixations and in live embryos the open state of the ventral furrow is not seen. The fact that the ventral furrow can open up in this way shows that at this stage no intercellular bonds have been formed between the lips of the ventral furrow. In the tube that is formed by the invagination of the ventral furrow, the two subpopulations of the mesoderm can still be recognized (Fig. 2H). The central cells form the tube proper, while the two strips of

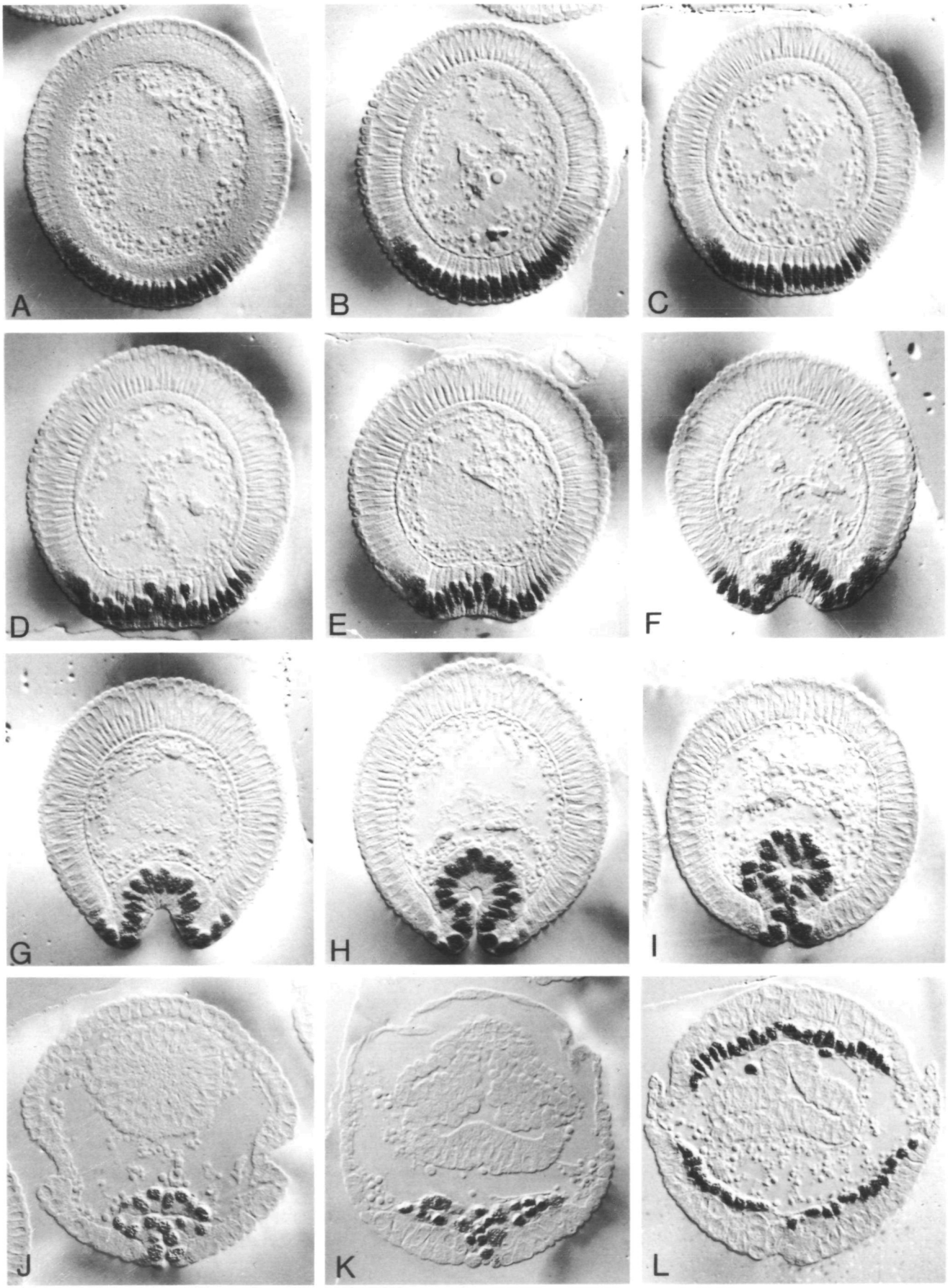
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Fig. 2. 5 μm sections through embryos at successive stages of embryogenesis. Embryos are stained with anti-*twist* antibodies. Sections are taken at approximately 50 % egg length. Dorsal is up, ventral down. The apical side of the blastoderm cells is on the outside of the embryo. (A) Syncytial blastoderm. (B) Cellular blastoderm. (C) Beginning nuclear migration and ventral contraction. (D) Central contraction. A central and peripheral population of mesoderm cells is distinguishable at this stage. (E) Beginning of invagination. Note that the apex of the cell just to the left of the ventral midline is very wide, while the neighbouring cells on either side are already contracted. (F) Further invagination. (G,H) Similar stages of invagination, but in G the embryo shows an artificially wide opening of the ventral furrow. This section is included to show that no junctions have formed between the lips of the closing ventral furrow at this stage. (I) Nearly complete invagination. Two or three mesoderm cells still lie on the surface of the embryo. (J) Flattening of the tube of invaginated mesoderm. In this and the following sections the posterior midgut is visible, and has invaginated on the dorsal side of the embryos (cf. Fig. 1F). At this point, only the mesectoderm cells are left at the surface of the embryo. (K) Mitosis and dispersal of the mesoderm. Note that the distribution of *twist* protein differs in the individual cells, as they are in different parts of the cell cycle. (L) Completion of mesoderm invagination. The mesoderm is cut twice in this section, because the germ band has now extended fully and stretches around the dorsal side of the embryos (cf. Fig. 1H). The mesectoderm cells now lie at the ventral midline and have lost the *twist* protein from their nuclei. Neuroblasts have begun to delaminate from the ectoderm.

peripheral cells form the stem. The most peripheral cells of the prospective mesoderm still lie on the surface of the embryo.

The tube now loses its typical epithelial appearance, flattens out (Fig. 2J) and disperses into single cells that undergo mitosis (Fig. 2K). The dispersal does not depend on mitosis, since it also occurs in *string* mutant embryos (see Fig. 7A), in which this and later cell divisions do not take place (Edgar and O'Farrell, 1989). The mesoderm cells begin to spread out laterally until they form a single cell layer on the inside of the ectoderm (Fig. 2L). The mesectoderm cells come to lie next to each other on the ventral midline, slightly invaginated. At this stage, the *twist* protein has disappeared from their nuclei.

In summary, the invagination of the mesoderm can be divided into two phases. During the first phase, the cells within the ventral epithelium change their shapes in such a way that the central part of the epithelium bends and is displaced towards the inside of the embryo. The more peripheral cells follow. During this phase all cells remain attached to each other within the epithelium. In the second phase, the epithelial character of the mesoderm is lost as the cells enter mitosis and spread out to form the second germ layer. It is probably in this second phase that cell–cell and cell–matrix interactions become important. For example, the *Drosophila* integrins are first expressed on the cell surface at this stage (see Fig. 7), supporting genetic evidence that at least this class of cell interaction molecules plays no role in the first phase of gastrulation (Leptin *et al.* 1989).

Mutants that change cell fate

The following paragraphs describe the behaviour of cells in mutant eggs in which the determination and the differentiation of the mesoderm are affected. The maternal effect genes of the *dorsal* group are required for determining the different fates of cells along the dorsoventral axis (Anderson, 1987). Situations can be created genetically in which all cells have identical fates, either dorsal (dorsalized embryos) or ventral (ventralized embryos). We use four molecular markers (visualized with antibodies) for specific cell populations along the dorsoventral axis. First, the subcellular location of the *dorsal* gene product is a marker for fate along the dorsoventral axis: *dorsal* protein in the cytoplasm corresponds to dorsal fates, while *dorsal* protein in the nucleus determines ventral fates (Roth *et al.* 1989; Rushlow *et al.* 1989; Steward, 1989). Second, we use the *twist* gene product as a marker for ventral cells (Thisse *et al.* 1988); third, the product of the gene *zen* is a marker for dorsal cells (Rushlow *et al.* 1987). Finally, the expression of β-galactosidase under the control of an enhancer that directs expression in mesectoderm cells is used to mark these cells.

In Fig. 3 we show that mutant embryos in which all cells have either dorsal or ventral fates, all cells behave identically during cellularization. The cell membranes invaginate equally at all positions around the egg and at the cellular blastoderm stage all cells look alike. After the completion of cellularization, dorsalized and ventralized embryos begin to develop differently. In dorsalized embryos the nuclei of all cells remain at the periphery of the egg (Fig. 3A), as they do in dorsal cells in wild-type embryos (Fig. 1H). In contrast, in ventralized embryos (in $Toll^{10B}$ mutant embryos), nuclei at many positions move towards the centre of the egg and the apical surfaces of most cells flatten (Fig. 3B). These are characteristics of the central population of ventral cells in wild-type embryos. Thus, the above aspects of the behaviour of individual dorsal and ventral cells do not require interactions with neighbouring cells with different fates but are part of an autonomous process of differentiation.

The phenotype of $Toll^{10B}$ mutant embryos is variable, and often incompletely ventralized embryos show interesting abnormalities in ventral furrow formation. For example, although all cells around the periphery appear equally ventralized in the embryo shown in Fig. 3C, as judged by *twist* expression, there is still some dorsoventral asymmetry, and a furrow is formed ventrally. Thus, there are two types of *twist*-expressing cells in this embryo, and only those on the ventral side behave like the central population in wild-type embryos. This region is larger than in the wild type, more cells than usual are recruited into the furrow and, probably as a consequence, their shapes appear more elongated. These cells invaginate and generate a fairly normal looking tube of mesoderm, except that both the tube and the stem contain more cells than normal (Fig. 3D).

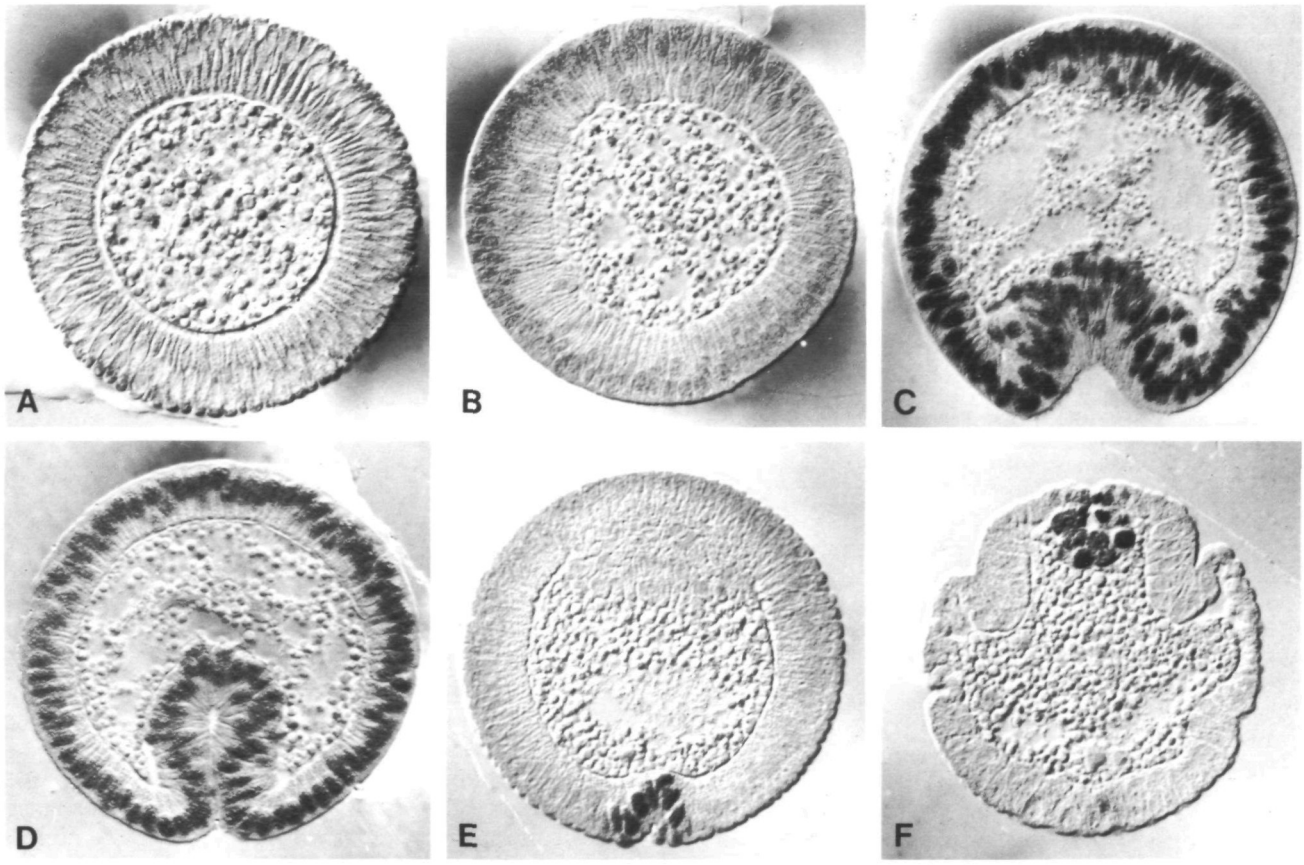


Fig. 3. Maternally dorsalized and ventralized embryos stained with anti-*dorsal* (A,B) or anti-*twist* (C–F) antibodies. In embryos in which all cells have a dorsal fate (A) the *dorsal* protein is found in the cytoplasm, in embryos with only ventralized cells (B), it is localized in the nucleus. (A) Late cellular blastoderm of a dorsalized embryo (*pelle*). All cells have rounded apical surfaces and the nuclei are at the apical ends of the cells. (B) Late cellular blastoderm of a ventralized embryo ($Toll^{10B}$). All cells have flattened apical surfaces, and nuclei have moved inwards at many positions around the periphery of the egg. These embryos never form furrows. (C,D) Gastrulating incompletely ventralized ($Toll^{10B}$) embryos. Although all cells express the *twist* protein, only ventral cells invaginate (indicating a residual difference in cell fate between ventral and dorsal cells in these embryos). (E,F) Gastrulating embryos with reduced mesoderm (weak *spätzle*). (F) A section through an extended germ band, in which the ventral furrow is only visible on the dorsally extended part of the germ band. The more anterior (here ventrally located) part of the embryo is dorsalized in these mutants (S. Roth, personal communication) and does not express *twist* or invaginate.

In incompletely dorsalized embryos, the mesoderm and the ventral furrow are reduced in size (Roth *et al.* 1989). Nevertheless, this narrow band of *twist*-expressing cells is able to invaginate completely (Fig. 3E,F; since the mesoderm develops only in the posterior part of these embryos, the ventral furrow is only seen in the part of the extended germ band that has folded around the dorsal side of the embryo, and not on the ventral, more anterior, part). These mutant phenotypes demonstrate that the overall concerted behaviour of the invaginating cell population does not depend on the size of the population.

Zygotic genes

The maternal genes determine fate along the dorsoventral axis by regulating (directly or indirectly) the transcription of zygotic genes. Two zygotic genes, *twist* and *snail* (Nüsslein-Volhard *et al.* 1984), are known to be required for the development of the mesoderm (Simpson, 1983). Both genes have been cloned and their sequences suggest that they may be DNA-binding proteins (Boulay *et al.* 1987; Thisse *et al.* 1988). Their products are localized in the prospective mesoderm (Thisse *et al.* 1988; this work).

Fig. 4 shows embryos mutant for *twist*, for *snail* and mutant for both genes (double mutants). All embryos were stained with *twist* antibodies. At the beginning of germ band extension, when the ventral furrow has formed in wild-type embryos (Fig. 1D), no invagination is visible in the three types of mutant embryos. Later, at the fully extended germ band stage, there are two cell layers in wild-type embryos (Fig. 1H; the mesoderm and the ectoderm), but only one in mutant embryos (Fig. 4, second row). However, in ventral views of mutant embryos small furrows can be distinguished (Fig. 4, bottom row and Simpson, 1983). In *snail* embryos these folds are irregular and are neither centrally located nor parallel to the anterior–posterior

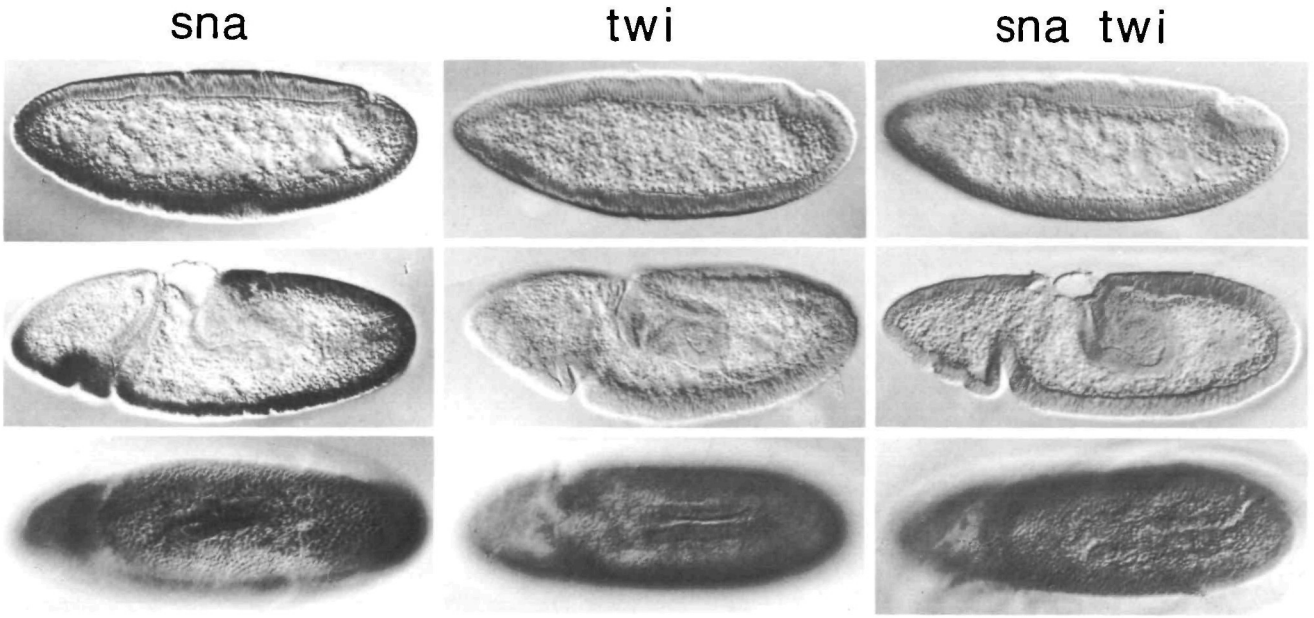


Fig. 4. Whole mounts of *snail*, *twist* and double-mutant embryos stained with anti-*twist* antibody. The top row shows embryos at the beginning of germ band extension, when the ventral furrow has already formed in wild-type embryos (cf. Fig. 1D) The next row is the fully extended germ band. No internal mesodermal cell layer is present (compare to Fig. 1H). The bottom row shows ventral views of ventral furrows at approximately the same stage as the top row (cf. Fig. 1K for wildtype).

axis. In *twist* mutants, the furrows are often quite deep and long and always run along the ventral midline. In double mutants, folds are rare and never very long.

In the following we describe how the absence of *twist* and *snail* function affects the behaviour of ventral cells as seen in sections. In all three classes of mutants, the first signs of shape changes in ventral cells occur much later than in wild-type embryos, beginning around the same time as germ band extension, when the ventral furrow is already invaginating in wild-type embryos (Fig. 5, A–D).

In *twist* embryos, the ventral furrow is formed by a strip 8–10 cells wide. These cells become cylindrical and invaginate. During this process, they produce membrane protrusions on their apical surfaces, like wild-type cells, but their apical surfaces do not flatten (Fig. 6). Nevertheless, a furrow forms, showing that the apical contraction is not absolutely required for the formation of a furrow. No specific changes are seen in the neighbouring more lateral cells, and their apical sides do not become stretched (Fig. 6). Although the ventral furrow in *twist* mutant embryos is quite deep, it is not stable. By the end of germ band extension it flattens out again and forms a continuous epithelium with the ectoderm. The region of uninvaginated mutant mesoderm is bordered by the two rows of mesectodermal cells, which would normally have become joined at the midline.

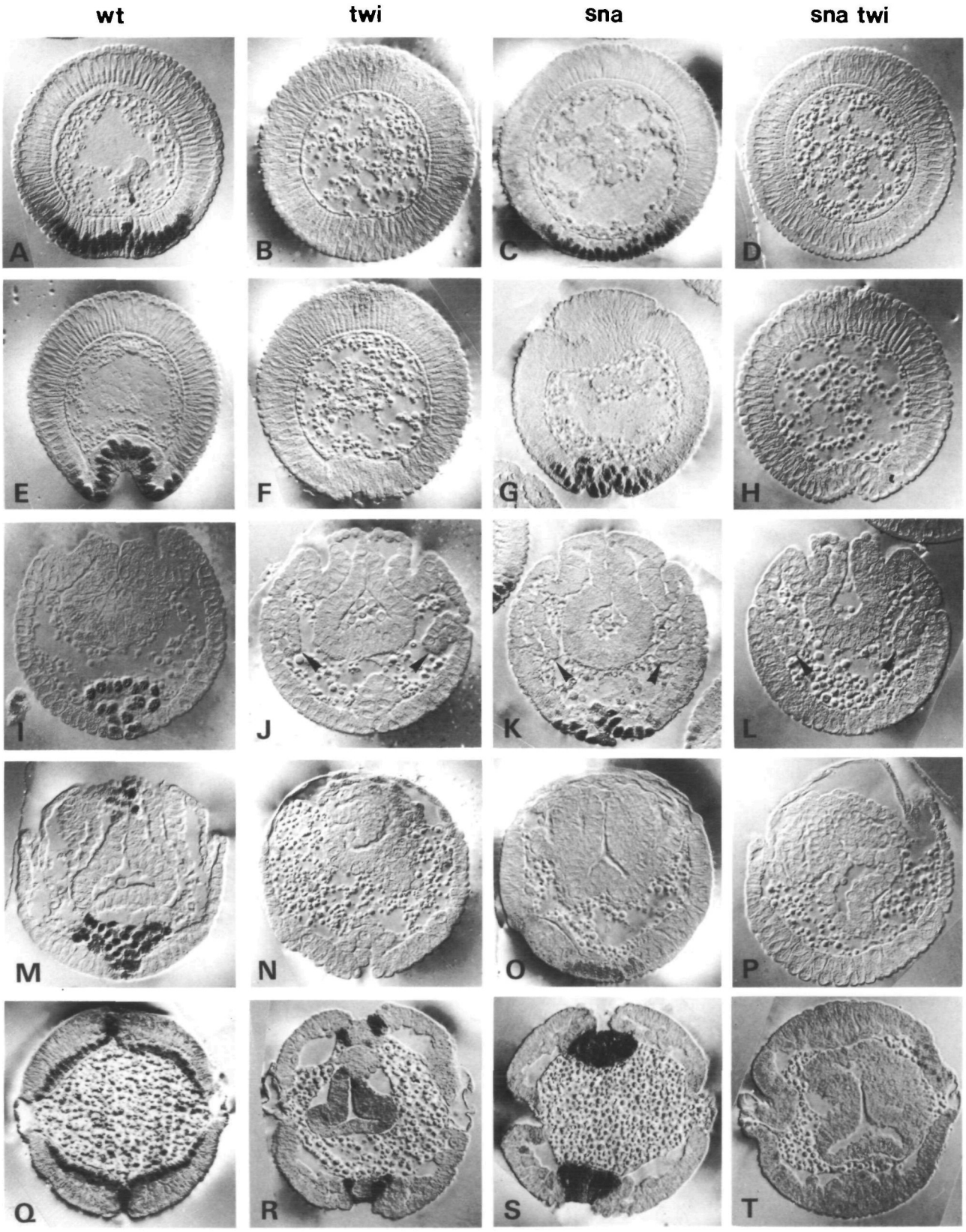
The first sign of a difference between ventral and other cells in *snail* mutant embryos is a thinning of the whole ventral epithelium (Fig. 5C). The ventral cells become shorter and more cuboidal and their nuclei remain very close to the apical side of the ventral cells. As the flattening becomes more extreme, the epithelium begins to buckle (Fig. 5G). The irregular folds do not necessarily appear along the midline of the epithelium, but can form anywhere. As the germ band elongates, the epithelium straightens out again and the folds disappear.

In embryos mutant for both *twist* and *snail* very few morphogenetic changes occur in the ventral epithelium. Occasionally, small transient furrows are formed. The example shown in Fig. 5H is one of the strongest cases we observed.

Twist, *snail* and double mutants have one feature in common: towards the end of germ band extension, the mutant mesoderm is still part of the outer cell layer of the embryo. Thus, the ventrolateral ectoderm (the neurectoderm) in these mutants cannot move ventrally as far as it does in wild-type embryos, and the more dorsal epithelium (which flattens and expands during the normal course of gastrulation) can also not spread as far ventrally. As a result, it forms two deep folds that invaginate where the dorsal ectoderm and the neurectoderm meet (Fig. 5J,K,L) This shows that the expansion of the dorsal epithelium does not exert enough pressure on the lateral epithelium to push it ventrally, and therefore the invagination of the ventral furrow in wild-type embryos cannot merely be a passive response to pressure from the lateral epithelium.

Genetic regulation of cell fates in the prospective mesoderm and spatial regulation of ventral furrow formation

The shapes of cells in the ventral furrow of wild-type embryos suggest that the ventral epithelium consists of



two subpopulations, both expressing the *twist* protein. This distinction could be a mechanical consequence of the way the furrow forms, but the appearance of the furrows in *twist* and in incompletely ventralized *Toll*^{10B} embryos makes this unlikely. Alternatively, the difference between central and peripheral cells could be determined by a gene specific to either of these populations. The *twist* gene cannot be this gene, since it is

Fig. 5. Sections through wild type, *twist*, *snail* and double mutant embryos. All embryos were stained with anti-*twist* antibodies. The last row (extended germ band) was stained both for *twist* and for the β-galactosidase marker in mesectoderm cells. (A–D) Around the time when the ventral epithelium in wildtype embryos begins to invaginate (A), the nuclei of the 10 ventralmost cells in *twist* mutant embryos have moved away from the periphery of the embryo and the cells have become irregular in shape (B). The dorsalmost nuclei in this section, as well as in F and N are faintly stained with anti-*zen* antibodies. In *snail* embryos, all mesodermal cells become shorter and more cuboidal (C). There are no visible differences between ventral and other cells in the double mutant (D). (E–H) Beginning of germ band extension. In *twist* mutants, the central 8–10 cells lose their conical shape and become cylindrical and elongate slightly (F). In *snail* embryos, the ventral epithelium buckles in an irregular way. The resulting folds are not always in the middle of the epithelium and sometimes one sees two parallel folds (G). In the double mutant slight indentations in the centre of the ventral surface often appear at this stage, but not always as strongly as the one shown here (H). (I–L) As germ band extension progresses, the dorsal epithelium and lateral epithelia flatten and expand. In the mutants, less of the surface area of the embryos is available for these epithelia (because the uninvaginated ventral epithelium occupies part of the surface), and the dorsal epithelium folds inwards (arrowheads). (M–P) Later stages of germ band extension. In *twist* embryos, the epithelium that had invaginated begins to move outwards again (N). In *snail*, the *twist* protein begins to disappear (O), as it does in mesectodermal cells in wild-type embryos (Fig. 2K,L). (Q–T) The final result of gastrulation at the fully extended germ band stage. In these embryos the mesectoderm cells are marked with β-galactosidase. In the wild-type embryo the mesoderm has invaginated completely and forms a single cell layer. The mesectoderm cells lie close together at the ventral midline. In the mutants the mesodermal cells remain at the surface of the embryo. In *twist* mutants, they lie between the two rows of mesectoderm cells. In *snail* mutants, they inappropriately express the mesectoderm marker and also lie at the surface of the embryo between the adjoining neurectoderm regions. In the double mutant, the expression of the mesectoderm marker is lost from both mesoderm and mesectoderm, and the mesodermal cells cannot be distinguished morphologically from the neighbouring neurectoderm. Note that the mesectoderm cells in both wild-type and mutants have become wedge-shaped. In the mutants, this shape change appears sufficient to lead to a slight invagination of the mutant mesoderm cells.

expressed in both populations (unless differences in *twist* protein level undetectable by our methods distinguish the two populations). Since the only other gene known to affect the mesoderm early is *snail*, we tested whether the *snail* gene was specifically expressed in either subpopulation. We find that the *snail* RNA is expressed in a strip of the same width as the *twist* protein (Fig. 7), that is in the whole prospective mesoderm, and is therefore probably not responsible for the subdivision of the mesoderm into central and peripheral cells.

In wild-type embryos the ventral furrow always forms along the ventral midline, over about 80% of the anterior–posterior length of the egg. Thus, the region of invagination is not co-extensive with *twist* expression along the anterior–posterior axis, since *twist* staining extends all the way to the anterior and posterior poles of the egg. It seemed possible that the region of invagination is dependent partly on the mechanical properties of the egg, which might not allow furrow formation close to the narrower poles of the egg. However, the following results indicate that the length of the invagination along the anterior–posterior axis is also genetically determined.

In *torso* mutant embryos the posterior and anterior poles take on more central fates (reviewed in Nüsslein-Volhard *et al.* 1987; Klingler *et al.* 1988). In such embryos, the ventral furrow extends over the whole length of the embryo (Fig. 7). Thus, there are no mechanical constraints that prevent mesoderm invagination near the poles. Therefore, the cells near the poles that do not invaginate in wild-type embryos are probably distinguished genetically from the more central cells that do. This is confirmed by *in situ* hybridisations with a probe for the *snail* gene (Fig. 7). Only the region that invaginates is labelled with the *snail* probe, while the termini of the embryo do not express *snail*. In *torso* mutant embryos, which form a furrow along their whole length, the expression domain of *snail* extends to the poles (Fig. 7).

Discussion

Mechanics of epithelial folding during ventral furrow formation

The description of wild type ventral furrow formation presented here was compiled as a basis for the analysis of the molecular mechanics of this process. A number of models and mechanisms have been proposed to explain epithelial folding (reviewed in Ettensohn, 1985). Some aspects of these models are inconsistent with the data presented here. Of the forces that have been considered to account for epithelial invagination – cell growth and division, cell adhesion, and cytoskeleton-mediated changes in cell shape – the first (cell growth and division) cannot be active in *Drosophila* ventral furrow formation, as changes in cell size are not apparent and cell division does not occur in the mesoderm until after it has invaginated.

It has been suggested (Gustafson and Wolpert, 1967) that an increase in cell adhesiveness could cause neighbouring cells in an epithelium to increase their area of contact. If this occurred only at the apical end of the cells, this 'zippering-up' would cause an invagination. However, as the ventral furrow forms, the areas of membrane contact between neighbouring ventral cells do not increase noticeably. Instead, spare plasma membrane is protruded in bulbous villi from the apical surfaces of cells that change shape. These phenomema are inconsistent with shape change through increased membrane adhesion.

Cytoskeleton mediated cell shape change is the

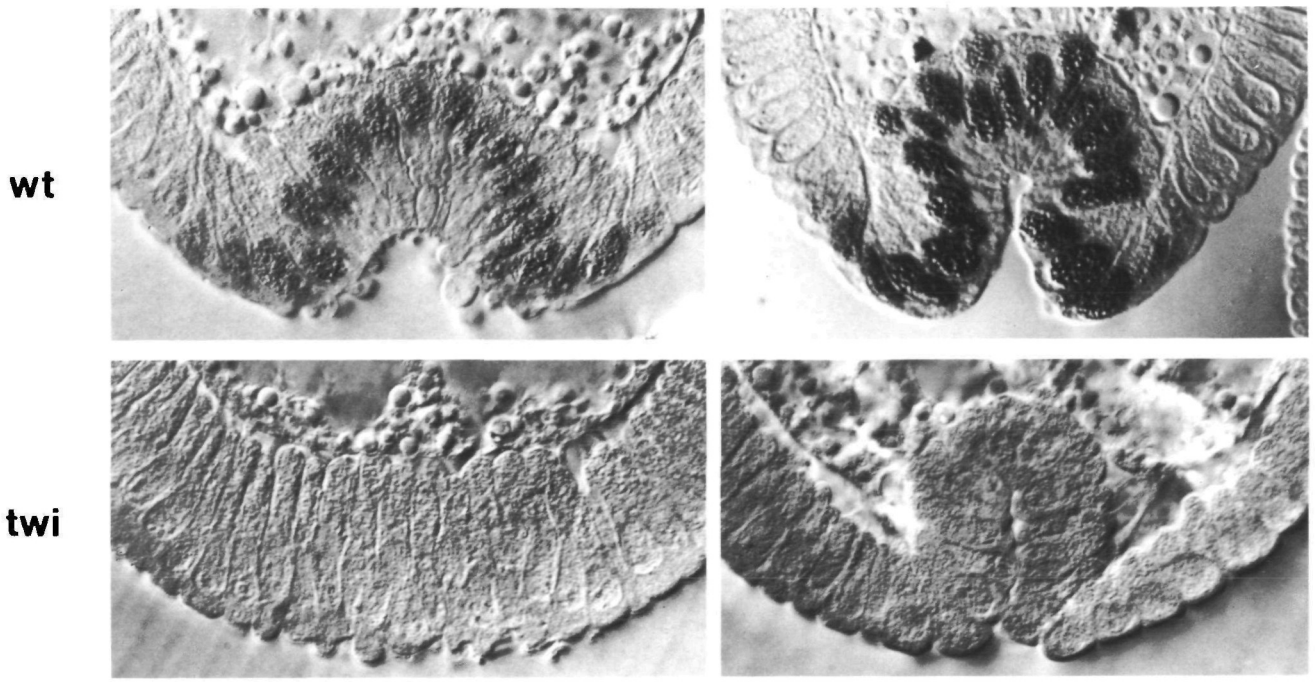


Fig. 6. Higher magnifications of ventral furrows in a wild-type embryo and *twist* mutants at two stages of furrow formation.

mechanism best supported by histological and experimental evidence (reviewed in Ettensohn, 1985). Apical contraction of the actin filament system has been suggested as a driving force in neural tube formation (Burnside, 1973), and computer modelling (Odellet *al.* 1981) shows that a wave of apical contractions of cells in an epithelium can cause an invagination in the epithelium. Here, however, such a wave of contractions does not seem to occur. Rather, the 8–10 ventralmost cells go through all their morphogenetic changes within a short period in an apparently random order. One often sees sections in which lateral cells have narrow apical ends, while closer to the midline they still have their original large apical surface area. (Such a stochastic sequence of cell shape changes in an invaginating epithelium is also observed in gastrulating amphibian embryos; Hardin and Keller, 1988.) The only cells of the prospective mesoderm that are ever seen to have expanded apical surfaces are the outer three or four on each side. We cannot exclude the possibility that a wave of contractions passes through the epithelium very quickly (between the stages that we show in Fig. 2B,C) and that later events are a consequence of this. However, in over 200 embryos we sectioned we never found one that would represent an intermediate in this process.

The cell shape changes in the central population of the prospective mesoderm are probably driven by more than one mechanism. The two main events occurring in these cells are the movement of the nuclei away from the apical surface of the cells and the contraction of the apical surface. Neither the sections we show here nor observations of live embryos indicate whether these events are causally related. However, the way the ventral furrow forms in *twist* mutant embryos suggests that they are not. In *twist* embryos, the nuclei of the ventral cells move away from the apical surface and the cells redistribute their contents such that they become more cylindrical, but the apical surface does not flatten or contract. The cells invaginate later and in a less orderly way than in wild-type embryos, but they do form a deep furrow. Thus, apical contraction and the displacement of nuclei are largely independent, and apical contraction is not an essential component of furrow formation. The combination of several mechanisms producing shape change might be a way of ensuring the reproducible formation of a stable furrow and of protecting the process from random external disturbances.

From the observations in $Toll^{10B}$, *twist* and *snail* mutant embryos, we conclude that the formation of the ventral furrow is an autonomous action of the ventral epithelium and does not depend on the behaviour of lateral or dorsal cells. As in wild-type embryos, the dorsal and lateral epithelia in *twist* and *snail* mutants flatten and expand during gastrulation. This expansion, however, does not exert enough force to push the ventral epithelium in *snail* or *snail twist* double mutant embryos into a furrow. The small transient folds in *snail* mutants might be a response to some pressure from the lateral epithelium (and also show that the epithelium is capable of folding), but they are much smaller than wild-type furrows. In incompletely ventralized $Toll^{10B}$ embryos, the dorsal epithelium does not flatten or expand at all, because it has been transformed towards a more ventral fate. Nevertheless, a ventral furrow forms and invaginates completely. Therefore, the expansion of the dorsal and lateral ectoderm is neither

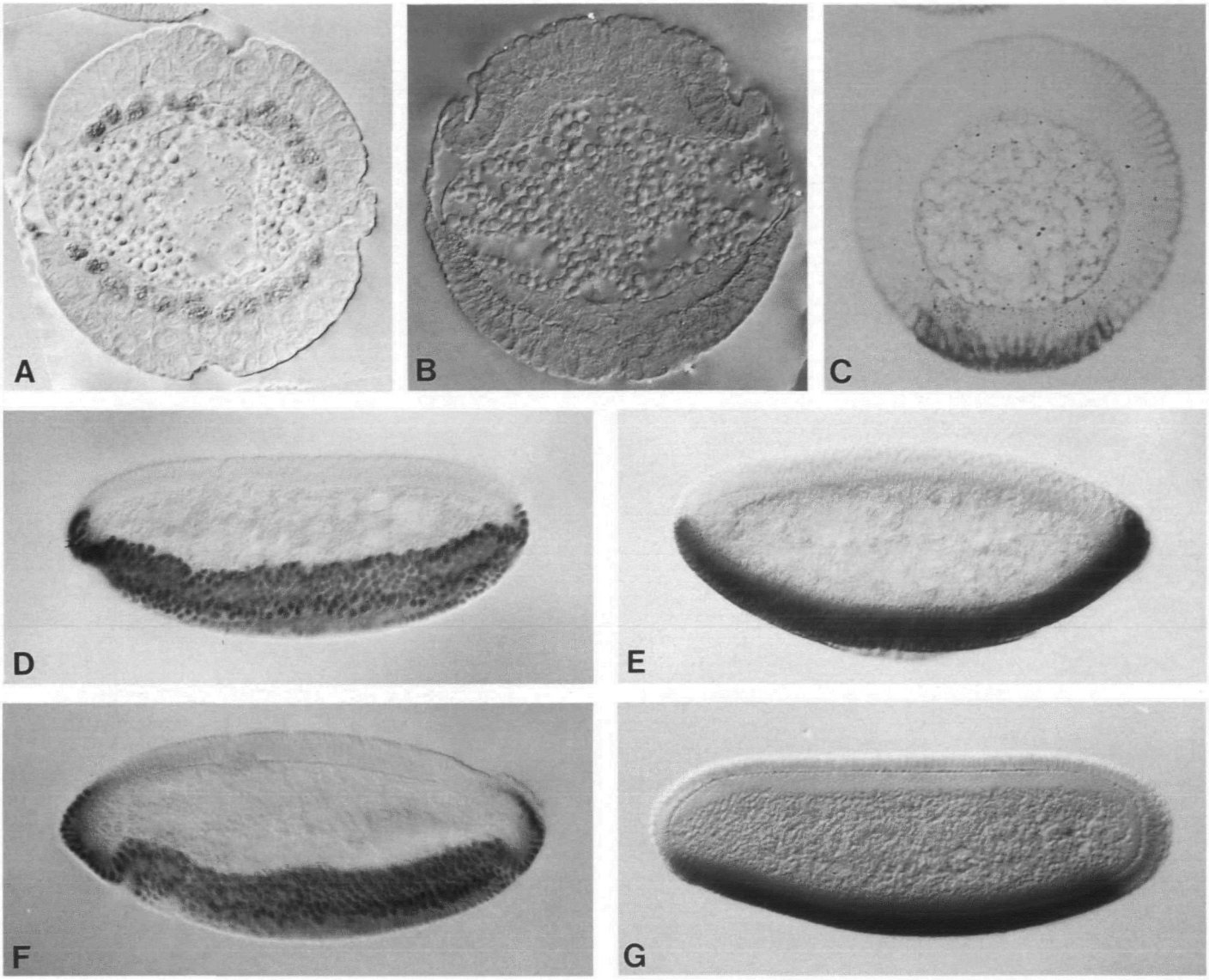


Fig. 7. (A) *string* mutant embryo. The mesoderm, stained with anti-*twist* antibodies, has not undergone any cell divisions and contains only one-quarter as many cells as in wild-type embryos. Nevertheless, it has dispersed and spread out normally. (B) Extended germ band embryos stained with antibodies against the *Drosophila* integrin PS1. (C) Section through a wild-type embryo hybridized with a probe for *snail* RNA. (D) Gastrulating *torso* mutant embryo stained with anti-*twist* antibodies. (E) *snail* expression pattern in *torso* mutant embryos. (F) Gastrulating wild-type embryo. (G) *snail* expression in wild-type embryo.

sufficient nor required for ventral furrow formation and the only force driving the invagination must be the cell shape changes in the prospective mesoderm.

Dependence of cell behaviour on cell fate

In amphibians, it has been shown by explanting parts of gastrulating embryos into tissue culture that many cell movements and shape changes can be carried out autonomously even by cells separated from their normal environment (reviewed in Gerhart and Keller, 1986). A similar situation is created genetically in *Drosophila* embryos in which all cells have identical fates. Ventralized embryos show that the apical flattening of ventral cells and the movement of their nuclei towards the interior does not depend on interactions with the lateral or dorsal epithelia. Thus, the nuclei are not squeezed inwards as a passive response to the action of forces from neighbouring cells (no inward movement is seen in dorsalized embryos), but are probably moved by forces from within the cells. At the very least, there must be a specific and ventral cell autonomous event that releases the nuclei to move inwards, or a specific dorsal mechanism that holds them at the periphery.

In mutant eggs in which the region that expresses ventral-specific genes is changed in size or shape, the ventral furrow is changed correspondingly. Our results suggest that the region of invagination along the anterior–posterior axis correlates more closely with the region of expression of the *snail* gene than that of the *twist* gene. Unlike the ventral furrow, the *twist* domain extends to the anterior and posterior poles of the embryo, whereas the *snail* expression domain is confined to the region that forms the ventral furrow and later the mesoderm. In *torso* mutant embryos, the *snail*-expressing region extends to the poles of the egg and so does the ventral furrow.

Also along the dorsoventral axis (i.e. the right-left axis in the mesoderm), the *twist*-expressing region consists of populations of cells showing different types of behaviour. In wild-type embryos, two subpopulations can be distinguished in the ventral furrow, a central and a peripheral population. Judging only from the description of the wild type, one might contribute this merely to mechanical reasons. It is possible that all cells expressing *twist* have a propensity to change their shape in the way the central cells do, but that cells at the edge of the field are subject to mechanical influences from cells outside the *twist*-expressing region which prevent this shape change. However, this seems very unlikely in view of the ventral furrows in incompletely ventralized $Toll^{10B}$ embryos. In these embryos, all cells express the *twist* protein, but only a band of cells on the ventral side (and only ever on the ventral side) invaginate. Therefore, not all *twist*-expressing cells are determined to go through the cell shape changes characteristic of the central cells. The invaginating region in $Toll^{10B}$ is wider than the ventral furrow in wild-type embryos, showing that there are no mechanical reasons why the whole *twist*-expressing region in wild-type embryos should not invaginate. It seems likely that the region that invaginates in $Toll^{10B}$ corresponds to the wild-type central population, while the remaining cells correspond to the peripheral population, and that these regions are genetically distinct.

We do not know which gene might be responsible for the subdivision of the *twist*-expressing region, since both *twist* and *snail* are expressed in all cells. Differences in levels of *twist* protein might play a role, but even in very weakly stained embryos, cells on either side of the border between the central and peripheral cells are stained with equal intensity. The visible gradient of *twist* protein expression begins only in the most lateral part of the peripheral region. The region of peripheral cells could be determined by nonautonomous influences from genes expressed in more lateral cells, or the ventral region could be subdivided by an as yet unknown gene.

Twist and *snail* both affect the same process, ventral furrow formation and mesoderm development. One might have expected that they would act at successive steps in a hierarchy of gene activity, and that one gene is under the control of the other. However, this cannot be the case, since the phenotype of the double mutant is more severe than that of either single mutant. Their phenotypes in terms of the behaviour of the mutant ventral cells differ, which means that they themselves control different aspects of cellular behaviour. It is important to point out that we cannot infer from the cell behaviour in *twist* and *snail* mutant embryos what aspects of cell shape change these genes control in wild-type embryos. The *twist* and *snail* gene products probably act by regulating the transcription of other genes, and do not affect cell shape directly. Therefore, the shape changes in ventral cells must require the transcription of at least two zygotic genes (one controlled by *twist*, the other by *snail*) in the ventral region, whose products interact with the pre-existing cellular machinery that mediates cell shape changes. Understanding how the ventral furrow is formed depends not only on identifying these downstream genes, and the postulated gene that distinguishes the central from the peripheral region of the ventral furrow, but also on determining how these genes affect the cytoskeleton to induce cells to change their shape.

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