Fate maps and the morphogenetic movements of gastrulation

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Mechanisms of early *Drosophila* mesoderm formation

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Summary

Several morphogenetic processes occur simultaneously during *Drosophila* gastrulation, including ventral furrow invagination to form the mesoderm, anterior and posterior midgut invagination to create the endoderm, and germ band extension. Mutations changing the behaviour of different parts of the embryo can be used to test the roles of different cell populations in gastrulation. Posterior midgut morphogenesis and germ band extension are partly independent, and neither depends on mesoderm formation, nor mesoderm formation on them. The invagination of the ventral furrow is caused by forces from within the prospective mesoderm (i. e. the invaginating cells) without any necessary contribution from other parts of the embryo. The events that lead to the cell shape changes mediating ventral furrow formation require the transcription of zygotic genes under the control of *twist* and *snail*. Such genes can be isolated by molecular and genetic screens.

Key words: *Drosophila*, mesoderm formation, ventral furrow formation, germ band extension, *twist*, snail.

Introduction

The basic rules for some developmental processes - like pattern formation in the *Drosophila* embryo, or differentiation - are now nearly understood. However, we still know little about the mechanisms and the molecules involved in morphogenesis. How do cells, once they have been assigned their fates and their positions in the developing organism, build ordered structures and organs? Morphogenetic mechanisms include cell proliferation and growth, cell migration, shape changes of individual cells and of groups of cells, for instance epithelia. The most vigorous period of morphogenesis during development is gastrulation, when the spatial relationships of cells within the embryo are continuously changing until the basic body plan is established. All of the morphogenetic mechanisms listed above occur during *Drosophila* gastrulation, although the initial and most dramatic events are mediated only by shape changes of epithelia and by cell intercalation within epithelia. Our own work, and this review, concentrates on an example of epithelial invagination, the formation of the ventral furrow.

Ventral furrow formation is the beginning of mesoderm development. It is the first morphogenetic event of *Drosophila* gastrulation and a particularly clear example of epithelial folding. It has the advantage over the classically investigated cases, such as amphibian gastrulation or neurulation, of being quick and uncomplicated (no cell division or growth, only a single homogeneous cell layer), and of being amenable to genetic analysis. We already know the genes that determine the fates of the cells involved (reviewed in Anderson, 1987 and Stein and Stevens, 1991), and the ventral furrow forms less than an hour after these genes begin to be transcribed. Therefore, the interval between cell fate determination and morphogenetic activity is very short, and hopefully the genetic regulatory cascade, beginning downstream of the known fate determining genes and leading to change in cell behaviour, will be correspondingly simple.

Materials and methods

Staining, in situ hybridisation and sectioning of embryos

Embryos were collected, fixed and processed for in situ hybridisation and antibody staining as described previously (Leptin and Grunewald, 1990).

Stocks

The $twist^{RY50}$ stock was obtained from P. Simpson, Strasbourg. Lethal mutations that had accumulated on the $twist^{RY50}$-carrying chromosome were separated from the $twist^{RY50}$ mutation by recombination. All other stocks were from the Tübingen stock collection (Tearle and Nüsslein-Volhard, 1987). We used the following mutant alleles: sna^{IIG}, Df(2R)twi^{S60} (Simpson 1983), tor^{XR1}, scw^{N5}, fog^{4a6}, $kni^{IID}hb^{7M}$, $Toll^{9QRE}$, $Toll^{r444}$ and $Toll^{10b}$. The *dpp* embryos were transheterozygous for dpp^{Hin37} and Df(2L)DTD2.

cDNA subtraction

RNA was prepared from embryos (blastoderm to early gastrulation) derived from mutant mothers ($Toll^{9QRE}/Toll^{r444}$ and $Toll^{10b}/+$) (details to be published elsewhere), reverse transcribed, digested with *Alu*I and *Rsa*I, linkered, amplified by PCR and enriched through several cycles of sub-

tractive hybridisation as described by Wang and Brown, 1991.

Germ line clones

Germ lines mutant for the gene *flightless1* were generated as described by Wieschaus and Noell, 1986. The *flightless* allele was fli^{WC2} (see Perrimon et al., 1989).

Results and discussion

Drosophila *gastrulation*

Several morphogenetic processes occur in parallel during *Drosophila* gastrulation (Fig. 1). Three invaginations create the germ layers. First, the presumptive mesoderm begins to invaginate as a broad band of cells on the ventral side of the embryo. Then, while mesoderm invagination continues, the endoderm is made from two invaginations, one at the posterior pole (the posterior midgut invagination), and one on the ventral side of the head region (the anterior midgut invagination). During this time, a process called germ band extension moves the posterior end of the embryo onto the dorsal side.

We will describe the cellular events and possible mechanisms that bring about ventral furrow formation and show to what extent mesoderm formation depends on other cell populations and events during gastrulation (an extensive review of these and other aspects of gastrulation has recently been published by Costa et al., 1992). We will conclude with an outlook on how we plan to define the genetic pathway that leads to mesoderm morphogenesis.

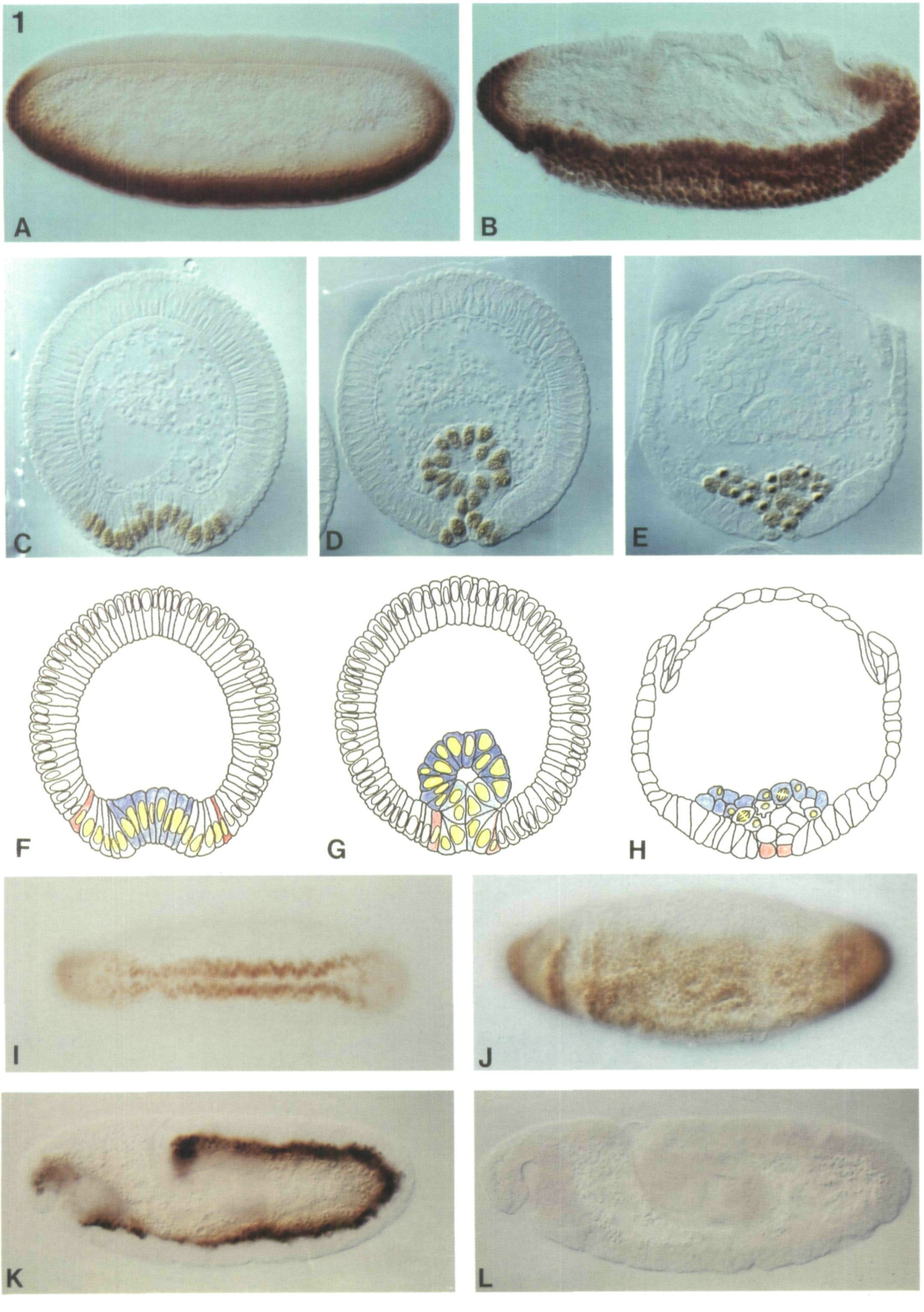
Ventral furrow formation and mesoderm invagination

Before gastrulation begins (Fig. 1A), all 5000 cells in the blastoderm epithelium look morphologically identical (except that cellularisation is not completely finished on the dorsal side). However, cells in different regions are already distinguished by their gene expression patterns. The genes *twist* and *snail* are expressed in ventral cells, including all future mesoderm cells, and some endodermal and ectodermal cells (Thisse et al., 1988; Leptin and Grunewald, 1990). The mesoderm is made from the *snail*-expressing cells between ~10% and ~70% egg length (measured from the posterior end of the embryo). All of these cells also express *twist*, but unlike *snail*, *twist* expression extends slightly beyond the lateral edge of the prospective mesoderm (Kosman et al., 1991; Leptin, 1991).

As soon as the cells on the ventral side of the embryo have formed, they begin to invaginate. The apical sides (facing the outside of the embryo) of the prospective mesodermal cells first flatten and then a subpopulation, a central band of cells approximately 8-10 cells wide, constrict apically and their nuclei migrate towards their basal ends (Fig. 1C. Kam et al., 1991; Leptin and Grunewald, 1990; Sweeton et al., 1991). The cells are thereby turned from a nearly columnar to a wedge shape. The band of cells that undergo these changes will be called the 'central population'. A 4- to 5-cell-wide band of prospective mesoderm cells on each side of the central population, the peripheral population, does not constrict apically. Within about 10 minutes, the changes in the central population result in the appearance of an identation in the ventral surface of the embryo (the ventral furrow), which deepens and invaginates into the interior of the embryo, followed by the peripheral cells on each side which have not constricted apically (Fig. 1B,D). This first phase of mesoderm invagination, which lasts about 15 minutes, is characterized by the absence of individual cell movements (either within the plane of the epithelium, or out of the epithelium), and of cell division or growth. The epithelium remains intact as it invaginates.

It seems likely that the driving force for this phase comes from the cell shape changes in the central population. However, the causal relationships between the events associated with cell shape changes are not clear. Like in other epithelial invaginations (Burnside, 1973), the actin cytoskeleton probably plays a major role in apical constriction. This is supported by the finding that myosin, concentrated at the base of the cell before ventral furrow formation, becomes localized to the apical side as constriction occurs (Young et al., 1991 and Fig. 2). However, apical constriction cannot alone be responsible for the movement of nuclei and shape changes. In *twist* mutant embryos (see below) no apical constriction occurs, but nuclei nevertheless move away from the apical end of the cells and the cells change their shape sufficiently to make a small transient furrow (Leptin and Grunewald, 1990; Sweeton et al., 1991). Surprisingly, apical flattening and nuclear movement occur even in mutants in which the blastoderm is not properly cellularized (Fig. 3; Kristina Straub and M. L., in preparation). All

Fig. 1. Whole mounts and transverse sections of embryos at various stages of gastrulation, stained with antibodies against the *twist* gene product, a nuclear protein expressed in ventral cells. (A) Cellular blastoderm. The nuclei on the ventral side are beginning to move basally; the posterior midgut primordium with the pole cells is shifting dorsally. (B) Early germ band extension. The mesoderm has invaginated on the ventral side. The posterior midgut has begun to invaginate and move onto the dorsal side, carrying the pole cells with it. (C-E) Transverse sections to show mesoderm invagination. The embryo in C is a few minutes older than that in A, the one in D the same age as that in B. (F-H) Drawings of the sections shown in C-E to illustrate the subpopulations of prospective mesoderm cells with different behaviours. Twist protein (yellow) is expressed in future mesoderm cells (blue), but initially a gradient of twist protein extends through the mesectodermal cells (red) into the extoderm (Kosman et al., 1991; Leptin 1991). All cells between the mesectodermal cells will invaginate to form the mesoderm, but in wild-type embryos only the central cells (dark blue) undergo the typical shape changes that we believe to bring about mesoderm invagination, while the peripheral cells (light blue) appear to follow into the furrow passively. These two populations are no longer distinguishable at the stage shown in E and H. The colour differences in this panel are therefore based entirely on conjecture. (I-L) Comparison of mesoderm formation in wild-type (I,K) and mutant (J,L) embryos. (I) Ventral view of a wild-type embryo at the same age as that in B. (J) Ventral view of a *snail* mutant embryo at the same age. The mesodermal region has not invaginated, but has formed irregular small folds. (K) Wild-type embryo at the extended germ band stage. The mesoderm, still expressing *twist*, has spread out as a single cell layer on the inside of the ectoderm. (L) *twist* mutant embryo at the extended germ band stage. No mesodermal cell layer has formed.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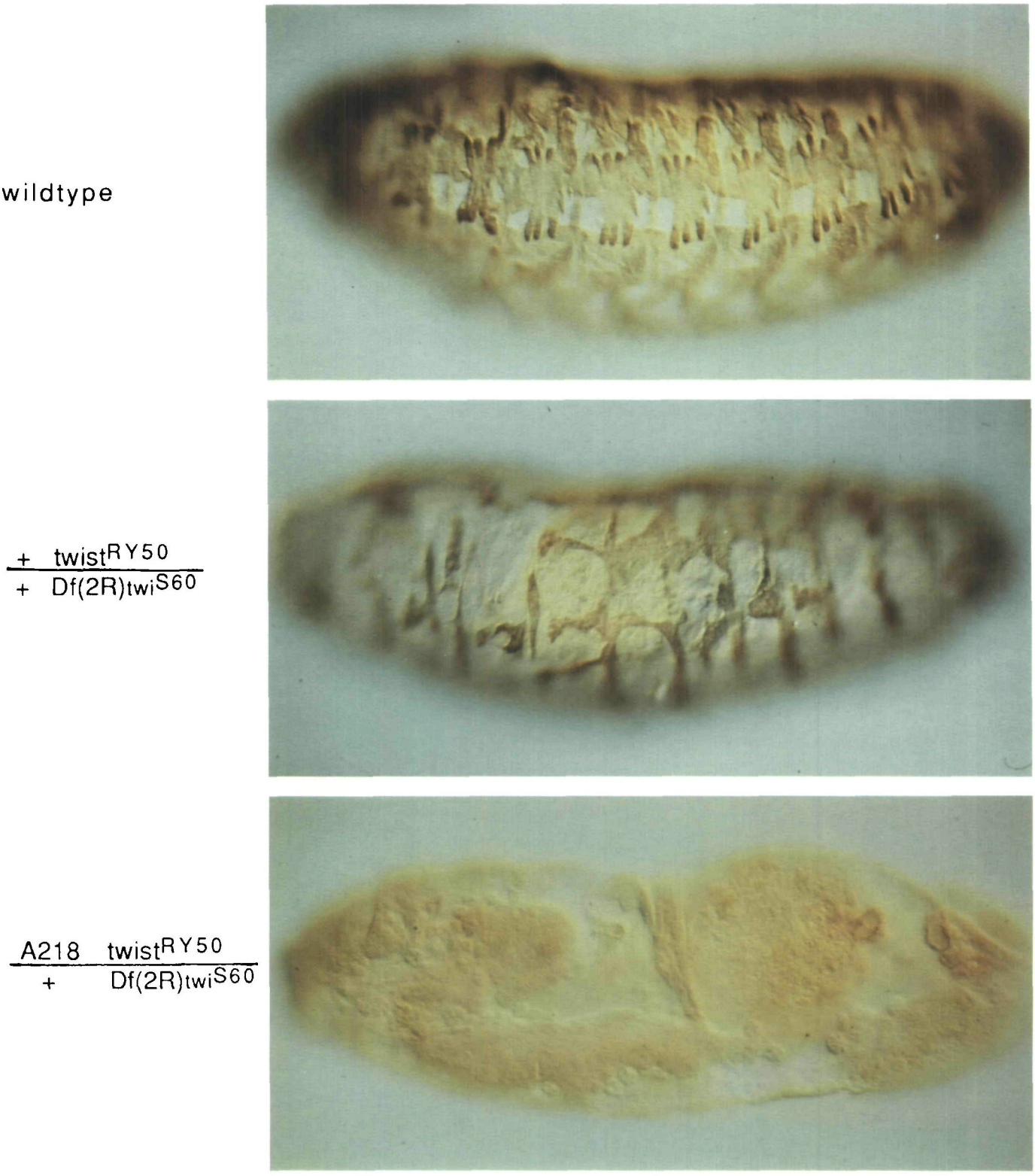

Fig. 7. Muscle development in mutant embryos. Late embryos were stained with antibodies against muscle myosin to visualize muscles. The typical wild-type muscle pattern (top) is disrupted in embryos carrying a weak *twist* allele over a *twist* deficiency (middle). If the embryos are also heterozygous for a mutation in another gene (*twist*-enhancer A218), muscles fail to develop altogether.

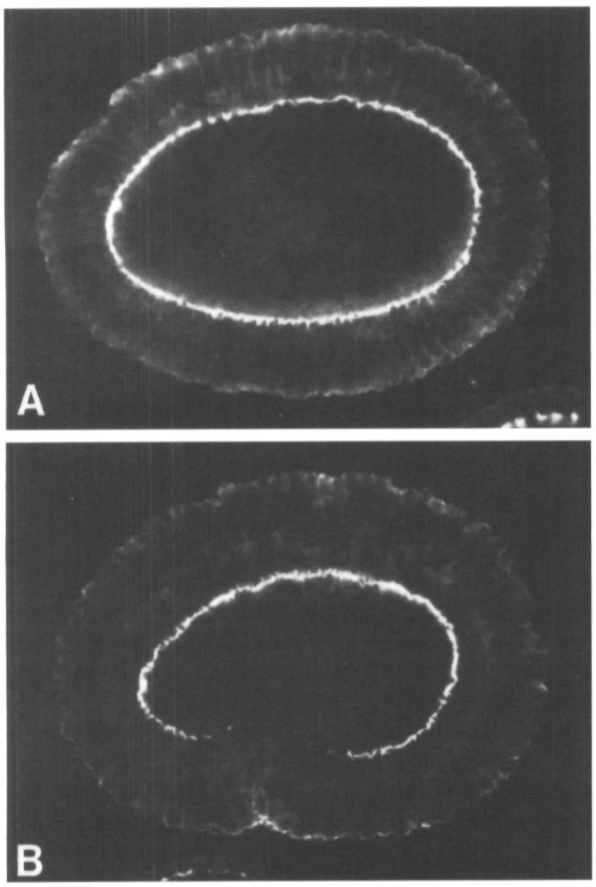

Fig. 2. Transverse sections of embryos before gastrulation begins (A), and when the ventral furrow is forming (B), stained with antibodies against cytoplasmic myosin. Myosin is initially concentrated at the basal end of each cell, but relocates to the apical sides of the ventral cells as they constrict.

of these findings are consistent with the notion that the movement of nuclei is passive, i. e. that nuclei are merely being released from the apical end of the cell. This would mean that the role of nuclear movement is to permit the cell shape changes rather than to cause them.

The actual mesodermal germ layer is formed in the next phase of mesoderm invagination. *snail* expression is now lost from the mesoderm, but *twist* continues to be expressed (Thisse et al., 1988; Alberga et al., 1991; Leptin 1991; Kosman et al., 1991). The tube of prospective mesoderm created by the invagination of the ventral furrow loses its epithelial structure and disperses into single cells (Fig. 1F). These divide, migrate out on the ectoderm to form a single cell layer (Fig. 1H) and then divide again. The cell divisions during this phase have no morphogenetic role, as shown by the finding that the mesoderm disperses and spreads out normally in mutant embryos in which these divisions fail to occur (Leptin and Grunewald, 1990).

Relationship between ventral furrow formation and other morphogenetic events in the embryo

Mutations that affect different parts of the embryo can be used to assess the roles of different cell populations in gastrulation. Mutations in both maternal-effect and zygotic

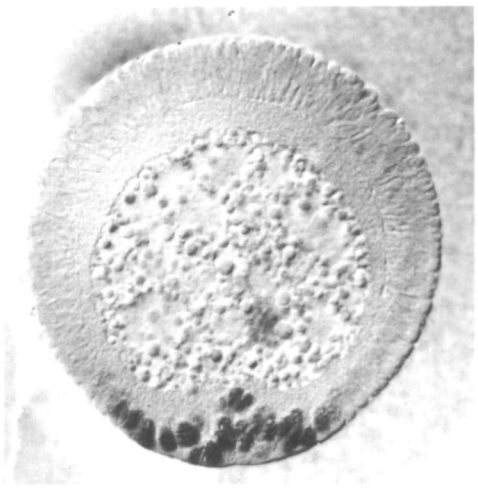

Fig. 3. Transverse section of an embryo derived from a germ line mutant for the gene *flightlessI*. Although no proper cell membranes are formed in ventral cells, apical flattening and nuclear migration still take place.

genes have been useful for this purpose. Most of these are mutations that delete or change certain cell fates in the developing embryo either along the anterior-posterior axis, or along the dorsoventral axis.

Mutations in maternal-effect genes that set up pattern along the dorsoventral axis affect the fates of most cells along the dorsoventral axis (Roth et al., 1989; Ray et al., 1991). Ventralizing mutations lead to the expression of ventral-specific genes in all cells of the embryo, while dorsalizing mutations produce embryos in which no ventral genes are expressed. In contrast, mutations in zygotic genes have more restricted effects. Usually only the fates of the cells that normally express the gene are changed, while other cells in the embryo develop according to their normal programme. Mutations in the genes *twist* and *snail* abolish or change the fates of the cells that normally give rise to the mesoderm, and mutant embryos fail to make a mesoderm (Fig. 1J,L; Simpson, 1983). Another group of genes is responsible for the development of dorsal fates (Ferguson and Anderson, 1992; Arora and Nüsslein-Volhard, 1992), and embryos mutant for these genes fail to differentiate the amnioserosa and varying proportions of the dorsal ectoderm.

Observations in mutants with changed ventral fates:regional and cellular autonomy of ventral furrow formation

Maternally dorsalized embryos do not form a ventral furrow, nor do any of their cells undergo the changes usually seen in ventral cells. Their apical surfaces remain rounded and the nuclei stay in the apical parts of the cells (Leptin and Grunewald, 1990). In contrast, in completely ventralized embryos, the apical surfaces of all cells flatten, and nuclei at all positions along the dorsoventral axis migrate basally, behaviour characteristic of the most ventral cells in wild-type embryos. This indicates that this behaviour is part of the autonomous morphogenetic program of cells expressing ventral genes, and not simply a

mechanical response to activities from neighbouring non-ventral cells.

A similar argument can be made for the formation of the ventral furrow. Some ventralized embryos still have residual dorsoventral polarity such that they still form a ventral invagination although *all* cells express ventral genes. Since these embryos contain no cells with lateral or dorsal fates, one can conclude that the activities of dorsal and lateral cells are not required for ventral furrow formation. That their activities are also not sufficient can be seen in *twist* and *snail* mutant embryos. These mutants make no ventral furrow, although dorsal and lateral cells develop normally. These findings suggest that all information for furrow formation resides within the cells that make the furrow, and that the necessary forces are generated within this region (Leptin and Grunewald, 1990).

These conclusions are further supported by several findings that suggest a high degree of cellular autonomy of the processes that cause the ventral furrow to form. First, in certain maternally dorsalized embryos, ventral cell fates can be induced by injection of wild-type cytoplasm from wild-type embryos (Anderson et al., 1985), and a furrow forms at the site of injection (Siegfried Roth and M. L., in preparation). The shape, direction and dimension of the furrow depends on the shape of induced ventral gene expression. This indicates that the shape and site of the furrow do not depend on the geometry of the egg, but are determined only by the patch of cells expressing ventral genes. Second, the cells of the central population in normal embryos begin their shape changes nearly, but not completely simultaneously. There is no particular order in which they constrict (Kam et al., 1991; Sweeton et al., 1991), suggesting that each cell begins its shape change independent of its neighbours. Certainly cells at all positions within the prospective mesoderm have the capacity to constrict, even when they are surrounded by cells that are genetically unable to constrict (M. L. and Siegfried Roth). Therefore, no large scale coordination by cell interactions appears to be necessary.

These conclusions apply to the *initiation* of ventral furrow formation and the mechanisms of cell shape changes. The speed and efficiency of the process may be coordinated by communication between cells. This view is based on the mutant phenotypes of two genes, *concertina* and *folded gastrulation*, (Sweeton et al., 1991) and the predicted structure of one of their products (Parks and Wieschaus, 1991). Ventral cells in mutant embryos have the capacity to constrict apically and begin these activities at the appropriate time, but the furrow then forms too slowly and with an irregular appearance. Since *concertina* codes for a G-protein homolog, these findings suggest the involvement of signal transduction mechanisms, and therefore possibly cell communication in the process of furrow invagination (Parks and Wieschaus, 1991; Costa et al., 1992).

Timing of ventral furrow formation

If all cells that make the ventral furrow initiate their cells shape changes independently of each other, how is the starting point for these changes determined? Since the changes begin as soon as cellularisation of the blastoderm is completed ventrally, cellularisation itself might provide the signal. However, this cannot be the case, since apical flattening and nuclear movement occur even in the absence of proper cellularisation. It seems much more likely that the accumulation of one or more crucial zygotic gene products above a critical level initiates ventral furrow formation. This notion is based on the observation that embryos that have only one functional copy of the zygotically active gene *twist* (and therefore probably only half the amount of this transcription factor) begin ventral furrow formation several minutes later than embryos with two copies (unpublished observation). Thus, the product of one or more genes transcribed under the control of *twist* must be limiting for furrow formation.

Several aspects of ventral furrow formation are easily explained by this interpretation. The cells begin to change their shapes approximately at the same time, because they all transcribe their genes at approximately the same rate. The apparently random initiation of cell shape changes is due to slight differences in the time when the critical level of zygotic gene products is reached in individual cells. The cells nearest the midline have a higher chance of constricting early compared to more lateral cells because the earliest *twist* (and *snail*) expression is restricted to a narrow ventral band corresponding in width to the central population of prospective mesoderm cells (Leptin, 1991) and, within this band, *twist* levels are highest near the midline. Finally, the early expression of *twist* and *snail* in this region might be the only genetic difference between the central and the peripheral population of prospective mesoderm cells. The peripheral cells would then in principle also have the capacity to constrict, but only accumulate enough of the critical gene product by the time that most of the central cells have invaginated and begun to pull the peripheral cells into the deepening furrow. This view is supported by mosaics in which patches of wild-type cells in the region of the peripheral population do constrict if they are in an environment of mutant, non-constricting central cells (M. L. and Siegfried Roth, in preparation).

Observations on mutations affecting other fates: relationships between germ band extension, endoderm formation and mesoderm formation

Endoderm formation

The posterior midgut invaginates by the same cell shape changes as the mesoderm (Sweeton et al., 1991). The cell surfaces flatten and then constrict apically while cell nuclei move basally. Shortly afterwards, the posterior midgut forms an indentation which invaginates, drawing neighbouring non-constricting cells along. It appears that posterior midgut formation is more sensitive to subtle interference with these shape changes since the severity of the phenotypes of *concertina* and *folded gastrulation* differs in ventral furrow and posterior midgut although the progression through cell shape changes is affected in both (Sweeton et al., 1991).

The anterior midgut is formed by different mechanisms (for review see Costa et al., 1992) and will not concern us further here.

Germ band extension

Germ band extension begins as soon as the ventrolateral

furrow has begun to invaginate and the posterior midgut cells are beginning to change their shapes. The germ band consists of the invaginated mesoderm and the overlying ectoderm, situated between the head fold and the hindgut primordium. This region begins to lengthen, but since the embryo is enclosed in membranes and cannot change its overall dimensions, this results in the posterior midgut primordium being displaced dorsally. The dorsal epithelium of the embryo, the future amnioserosa, does not lengthen but becomes thin and folds up as the advancing posterior midgut invagination is pushed dorsally. Germ band extension is later reversed by germ band retraction so that the original anterior-posterior order is re-established. The function of germ band extension and retraction are not understood. They may have more to do with anterior-posterior pattern formation within segments than with morphogenesis (Wieschaus et al., 1991).

Mutations interfere with germ band extension in two ways. They can abolish the mechanisms that provide the driving force (the active mechanisms) or they can interfere with processes that allow the gastrulating embryo to respond to these forces by proper morphogenetic movements.

The driving force for germ band extension appears to be cell intercalation in the ventral ectoderm (Wieschaus et al., 1991). This process is disrupted by mutations that abolish positional values along the anterior-posterior axis (mutations in maternal axis determining genes and segmentation genes; Fig. 4). In the most extreme cases, no germ band extension movements occur at all (Wieschaus et al., 1991). However, the mesoderm and the anterior and posterior midgut invaginate normally in these embroyos.

The second aspect of germ band extension affected by mutations is the movement of the posterior midgut primordium. For the germ band to extend properly, the posterior midgut has to move dorsally. In mutants that do not form a posterior midgut (e.g. *folded gastrulation*, or *torso*), the posterior pole of the embryo cannot be pushed onto the dorsal side. Instead, the germ band stops extending or buckles into folds on the ventral side of the embryo.

The movement of the posterior midgut towards the head is made possible by the dorsal epithelium (the amnioserosa) becoming very thin and folding up between head and posterior midgut. In mutants whose amnioserosa does not form, the posterior midgut begins to be pushed dorsally, but then stalls and eventually sinks into the inside of the embryo, underneath the mutant amnioserosa, which has not folded up (Fig. 4).

Independence of gastrulation movements

From the phenotypes of the mutants described so far, it is already clear that proper germ band extension depends on proper posterior midgut development, while the posterior midgut develops independently of germ band extension. Neither germ band extension nor posterior midgut formation depend on mesoderm development, since they occur normally in *twist* and *snail* mutants, which do not make any mesoderm (Simpson, 1983; Leptin and Grunewald, 1990; Sweeton et al., 1991).

How is mesoderm development affected by disruptions of germ band extension or posterior midgut formation? The ventral furrow forms and invaginates normally even if the posterior midgut is absent, for example in *torso* embryos. This is also the case if the germ band does not extend, either because the driving force for extension is abolished by mutations in anterior-posterior patterning genes, or if posterior midgut movement is inhibited by the failure of the amnioserosa to form due to mutations in dorsoventral patterning genes (Fig. 4). However, the spreading of the invaginated mesoderm on the ectoderm appears abnormal in these mutant embryos. Especially in *dpp* mutant embryos, which lack all dorsal fates, the mesoderm migrates all the way to the dorsal midline (Fig. 4). This is seen to a lesser extent in embryos mutant for the other genes of this group. This behaviour has two causes. One is the reduction of germ band extension. In the absence of germ band extension, the germ band does not fold over the back of the embryo, and the path is free for the mesoderm to migrate much further dorsally than normal. However, in mutants that fail to extend their germ band due to anterior-posterior patterning defects, the mesoderm migrates less far dorsally than in *dpp* mutants (B. G. and M. L., in preparation). Therefore the fate of the underlying ectoderm probably also plays a role in determining the extent of mesoderm migration.

Genetic regulation of ventral cell behaviour

We know the genes responsible for determining the cells that invaginate to form the mesoderm (the maternal dorsal group genes) and we know what the activities of ventral cells are that bring about the first steps of the invagination. We do not know how these two are linked. What is the cascade of gene activities that leads from fate determination to morphogenetic activity?

The first manifestation of ventral identity under the control of the maternal dorsoventral morphogen gradient is the expression of *twist* and *snail*. These genes code for transcription factors that are found in the nuclei of prospective mesoderm (and a few other) cells (Alberga et al., 1991; Boulay et al., 1987; Kosman et al., 1991; Leptin, 1991; Leptin and Grunewald, 1990; Thisse et al., 1987). They are the only known genes that are essential for all aspects of mesoderm differentiation and morphogenesis (Fig. 1). In *twist* and *snail* mutants, no mesoderm develops and the failure is visible already at the time when the ventral cells should begin their characteristic shape changes. Ventral cells in *twist* mutants flatten but do not constrict their apical sides (Leptin and Grunewald, 1990; Costa et al., 1991). They become tall and thin and then often form a small, transient furrow. Ventral cells in *snail* mutants do not flatten. They become short and wide, so that the ventral epithelium turns into a thin sheet which buckles into irregular folds (Fig. 1). In embryos mutant for both genes, ventral cells undergo none of these changes. Thus, *twist* and *snail* regulate separate aspects of early mesoderm formation.

Since *twist* and *snail* code for transcription factors, their roles can be shown more directly by assaying the expression of their potential target genes in mutant embryos (Leptin, 1991). Several genes are known that are expressed early in the mesoderm, or excluded from the mesoderm, or expressed only at the boundary between mesoderm and ectoderm. The function of *twist* is to activate genes in the

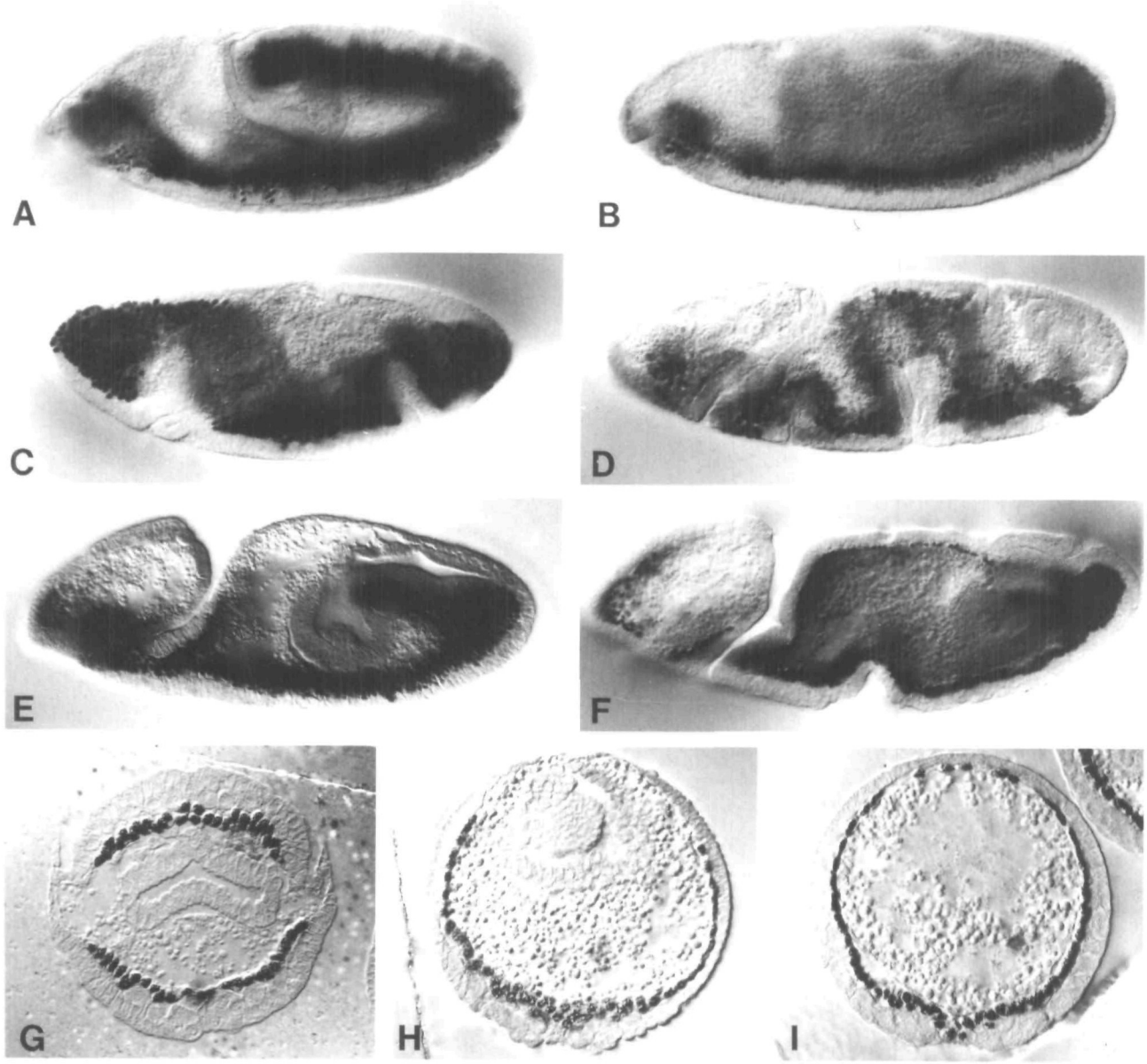

Fig. 4. Germ band extension and mesoderm spreading in wild-type and mutant embryos. All embryos are stained with *twist* antibodies and are at the stage corresponding to the extended germ band stage of the wild-type embryo shown in A. (A) Wild-type extended germ band. (B) Embryo that has failed to undergo proper germ band extension due to pattern formation defects along the anterior-posterior axis (*hunchback knirps* double mutant embryo). (C,D) Mutant embryos without posterior midgut invagination: *torso* embryo (C; derived from homozyogus *torso* mutant mother), and *folded gastrulation* embryo (D). (E,F) Embryos with defects in dorsal fate determination in which the amnioserosa fails to form. *screw* embryo (E) and *dpp* embryo (F). (G-I) Transverse sections through embryos similar to those in A, E and F. G, wild-type; H, *screw*; I, *dpp*.

region of the prospective mesoderm, but it has no effect on genes transcribed in the ectoderm. In contrast, *snail* is not required directly for the activation of mesodermal genes (at least those examined so far), but in the mesodermal region it represses genes destined to be active only outside the mesoderm. Of course *snail* can thereby be indirectly required for the activation of mesodermal genes if one of its targets normally represses mesodermal genes. *twist* and *snail* together define the mesoderm/ectoderm border and the expression of genes expressed only at this border (see also paper by Levine in this issue).

We have shown that the ventral cellular activities that cause the ventral furrow to form do not occur properly in *twist* and *snail* mutants, and that *twist* and *snail* act by regulating the expression of other genes in the ventral region of the embryo. To understand the pathway from fate determination to morphogenesis, one has to identify these genes. They are unlikely to be easily recognisable by their mutant phenotypes. Neither saturation screens for embryonic lethal mutations, nor a specific screen of the whole genome for early acting zygotic genes have identified any genes whose mutant phenotypes resemble that of *twist* or *snail*. There-

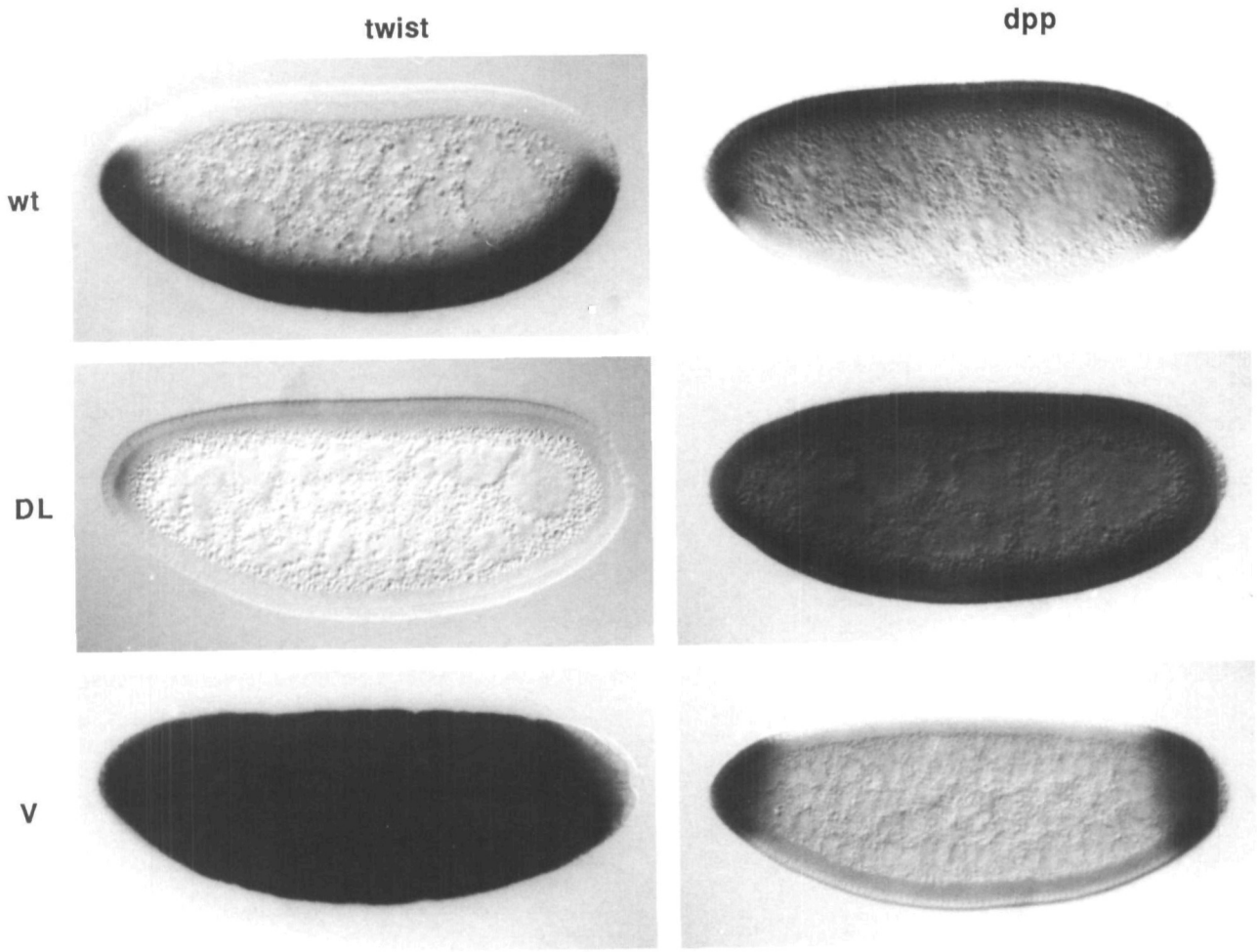

Fig. 5. Dorsal and ventral gene expression in maternally ventralized and lateralized embryos. The left column shows the expression pattern of *twist*, the right column the expression of *dpp* in wild-type (wt), 'dorsolateral' (DL) and 'ventralized' embryos. The DL embryos were derived from mothers transheterozygous for the *Toll* alleles $Toll^{9QRE}/Toll^{r444}$, the V embryos from mothers heterozygous for the dominant ventralizing Toll allele $Toll^{10b}$ (Anderson et al., 1985).

fore, mutations in *twist* and *snail* target genes involved in furrow formation may give very subtle phenotypes. Indeed, one gene that was identified only because its mutation causes defects in posterior midgut formation turned out upon further analysis to show defects in ventral furrow formation as well (Sweeton et al., 1991). If the desired genes cannot be found in a conventional screen for visible phenotypes, other methods have to be designed to identify them.

Search for new genes active in mesoderm morphogenesis

We have made use of two aspects of ventral furrow formation to conduct molecular and genetic screens. A genetic screen is based on the dosage sensitivity of the ventral-fate-determining system, and a molecular screen on the knowledge that at least some of the genes that we are searching for have to be expressed ventrally.

The molecular screen

Genes that are expressed ventrally in wild-type embryos are not expressed in maternally dorsalized embryos, but are expressed in all cells of maternally ventralized embryos (Roth et al., 1990 and Fig. 5). Therefore ventral genes can be identified by using subtractive hybridisation to isolate all those genes expressed only in ventralized and not in dorsalized embryos. Theoretically one could also find ventrally expressed genes by isolating all genes expressed in wild-type embryos but not in *twist* mutant embryos. However, this is more difficult, because *twist* mutant embryos constitute only one quarter of the progeny of a cross and would have to be hand-selected at the appropriate time of development. In contrast, **all** embryos from a mother carrying a maternal effect mutation express the phenotype, and large numbers of appropriately staged embryos can be collected. Fig. 6 shows cDNA from ventralized and dorsalized embryos before and after several cycles of subtractive hybridisation. cDNA from the dorsally expressed gene *dpp* becomes enriched in dorsalized cDNA, while *twist* becomes enriched in ventralized cDNA. At the same time, RNA from ubiquitously expressed genes like tubulin disappear from both populations. A library constructed from the enriched ventral cDNA should be a good source for new ventrally expressed genes, as has indeed been confirmed by the analysis of the first 72 clones isolated.

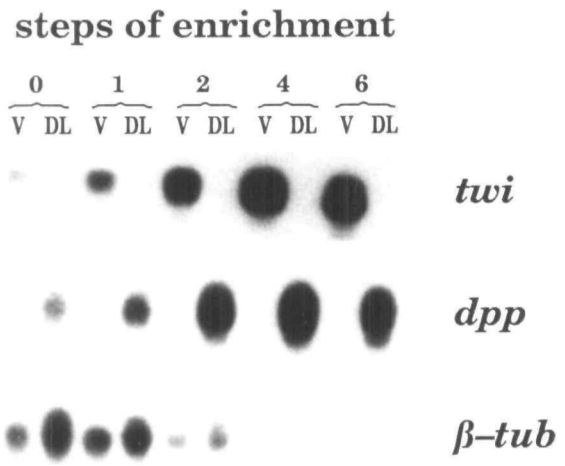

Fig. 6. Subtraction of 'ventral' (V) versus 'dorsolateral' (DL) cDNA. Amplified cDNA populations isolated from mutant embryos as those shown in Fig. 5 were subtracted against each other in successive cycles as described by Wang and Brown, 1991. Southern blots of the cDNAs were probed for a ventrally expressed gene (*twist*), a dorsally expressed gene (*dpp*) and a ubiquitously expressed gene (β-tubulin).

The genetic screen

As described above, embryos heterozygous for *twist* mutations gene begin ventral furrow formation several minutes later than embryos carrying two intact copies of *twist*. If the embryo also has only half the normal amount of maternal *dorsal* product (a condition that normally has no effect on development), it cannot survive at 27°C (Simpson, 1983). Similarly, embryos carrying a particular combination of *twist* alleles survive at 22°C, but die at raised temperatures (Thisse et al., 1987), or, more importantly, when the dose of *snail* is reduced by half. In both cases, the product of a gene (or genes) acting in parallel or downstream of *twist* is probably reduced below a critical level required for viability. We based a screen on the assumption that halving the level of such a gene product by inducing a mutation in it should also cause lethality in embryos carrying the above combination of *twist* alleles. The effect of one of the mutations found in this screen is shown in Fig. 7. Embryos carrying the *twist* alleles mentioned above show the characterstic pattern of larval muscles when stained with antibodies against muscle myosin. The introduction of an additional mutation in a new, unrelated gene that was isolated because it causes lethality leads to the loss of these muscles (Fig. 7). Thus, rather than causing a non-specific enhancement of lethality, the new mutation does indeed interfere with mesoderm formation and therefore probably represents a gene whose product normally interacts with *twist* or is controlled by *twist*.

The genetic and molecular approach together should identify genes acting in the pathway from fate determination to the expression of ventral fate by ventral-specific cell behaviour. It will also be important to investigate directly the cellular mechanisms involved in ventral cell shape changes. Determining the role of the actin and tubulin cytoskeleton and finding the cytoskeleton-associated proteins that mediate the rearrangement of cellular components during shape changes will be a main task towards this goal. By working down from the genes directly controlled by the fate-determining genes, and up from the genes whose products control the state of the cytoskeleton in ventral cells, we hope to be able to fill in the pathway from cell fate determination to morphogenesis.

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