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Mechanisms of dorsal-ventral axis determination in *Drosophila* embryos revealed by cytoplasmic transplantations

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SUMMARY

The establishment of the dorsal-ventral pattern in *Drosophila* embryos depends on a signal transduction process: a putative extracellular ligand released into the perivitelline space surrounding the embryo binds to the *Toll* receptor. *Toll* activation triggers the formation of the nuclear gradient of *dorsal* protein, the morphogen of the dorsal-ventral axis. Here, I analyse the *dorsal* protein distribution and the expression of zygotic dorsal-ventral genes in *Toll*$^{-}$ embryos that have been injected with wild-type cytoplasm under a variety of different injection conditions. Injections into two positions within a single embryo lead to the formation of two dorsal-ventral patterns in one embryo, allowing the analysis of interactions between pattern-forming processes. The results of single and double injections suggest that the spatial information for the embryonic dorsal-ventral axis is largely derived from spatial cues present in the extraembryonic compartment, which restrict the release of the putative *Toll* ligand. They argue against a *Toll*-dependent pattern-formation process employing local self-enhancement and lateral inhibition to enhance a weak initial asymmetry. The putative *Toll* ligand appears to originate from a ventrally restricted zone which extends along the entire anterior-posterior axis. Ligand diffusion or its graded release are required to determine the slope of the nuclear *dorsal* protein gradient. Both the *Toll* receptor and the putative ligand of *Toll* are in excess in wild-type embryos. Since spatial information for the embryonic dorsal-ventral axis is already present in the vitelline membrane or the perivitelline space, it is most likely generated during oogenesis. Oogenic pattern formation is also responsible for the perpendicular orientation the dorsal-ventral axis maintains with respect to the anterior-posterior axis.

Key words: pattern formation, lateral inhibition, origin of spatial information, pattern regulation, *Toll*

INTRODUCTION

The generation of the dorsal-ventral polarity in *Drosophila* embryos requires twelve maternal components encoded by the eleven *dorsal* group genes and *cactus* (for review, see Govind and Steward, 1991). These components constitute a signal transduction pathway. The receptor protein of the pathway is encoded by the *Toll* (*Tl*) gene (Anderson et al., 1985a,b; Hashimoto et al., 1988). *Toll* is a transmembrane protein with homology to the interleukin-1 receptor in its cytoplasmic domain (Schneider et al., 1991). *Toll* protein is evenly distributed in the cell membrane of syncytial blastoderm embryos (Hashimoto et al., 1991). The genes *snake*, *easter* and *spätzle* encode proteins that are secreted into the perivitelline cleft, a fluid-filled space surrounding the embryo. *snake* and *easte*r code for serine proteases and may be involved in processing the putative *Toll* ligand (DeLotto and Spierer, 1986; Chasan and Anderson, 1989; Stein and Nüsslein-Volhard, 1992). Three of the dorsal group genes, *pipe*, *nudel* and *windbeutel*, are required in the somatic tissues of the ovary that produce the egg coverings (Stein et al., 1991, Schüpbach et al., 1991). Therefore, they may provide spatial cues in the vitelline membrane that influence the production of *Toll* ligand (Chasan et al., 1992).

Toll receptor activation leads to the spatially regulated nuclear transport of *dorsal* protein, a NF-κB/rel-like transcription factor (Steward et al., 1988). This results in the formation of a nuclear concentration gradient of *dorsal* protein along the dorsal-ventral axis (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989). *dorsal* protein functions as a concentration-dependent transcriptional activator or repressor of the zygotic genes that specify the dorsal-ventral anlagen (Ip et al., 1991; Jiang et al., 1991; Pan et al., 1991; Thisse et al., 1991).

The embryonic dorsal-ventral axis has an invariable orientation with respect to the egg shell. Since the dorsal-ventral polarity is not derived from a cytoplasmic determinant (Anderson et al., 1985b), a transfer of spatial information from the vitelline membrane to the embryo has to occur (Schüpbach, 1987; Schüpbach et al., 1991). It is, however, not clear how much spatial information relevant for the generation of the nuclear *dorsal* protein gradient is already present in the extraembryonic compartment. If the extraembryonic compartment contained an elaborated prepattern,

the putative *Toll* ligand might be initially produced and distributed in a way that would determine the shape of the nuclear *dorsal* protein gradient. This process would not require additional steps of pattern formation. If, on the other hand, only a weak dorsal-ventral asymmetry were present in the vitelline membrane or perivitelline space, a system with an autonomous pattern-forming capacity would be necessary to generate the spatial information of the nuclear *dorsal* protein gradient. The extraembryonic environment would only determine the orientation of the gradient.

Pattern-formation mechanisms that are able to enhance weak spatial asymmetries have two features in common: local activation and lateral inhibition (Gierer and Meinhardt, 1972). The process of local activation is a self-enhancement or positive feedback process. Lateral inhibition refers to the suppression of new centers of activation in the vicinity of existing activation centers. I will use the term 'autonomous pattern formation' to refer to local activation/lateral inhibition processes with the capacity to generate spatial information.

Previously, two types of tranplantation experiments had been performed to elucidate properties of the dorsal-ventral pattern-formation process: cytoplasmic and perivitelline fluid injections. If wild-type cytoplasm is injected into the dorsal side of wild-type or *Toll*$^-$ embryos, a new dorsal-ventral pattern can be induced only in *Toll*$^-$ embryos while in wild-type embryos there is no effect. The new pattern induced in *Toll*$^-$ embryos has its ventralmost region at the site of injection (Anderson et al., 1985b). The induction of ventral structures is probably due to *Toll* mRNA in the transplanted material (Anderson and Nüsslein-Volhard, 1984a; Hashimoto et al., 1988), which leads to the insertion of *Toll* receptor molecules in a small region of the plasma membrane surrounding the injection site. These experiments suggest that *Toll* receptor molecules present at the dorsal side can only be activated in *Toll*$^-$ embryos, but not in wild-type embryos. If the ligand is produced only at the ventral side, a simple explanation for this inhibition phenomenon is that in wild-type embryos the evenly distributed *Toll* receptor binds all the available ligand ventrally, preventing it from reaching and activating *Toll* receptor molecules on the dorsal side (Stein at al., 1991). However, it is also possible that the ventral activation of *Toll* initiates a process of lateral inhibition which is at least partially responsible for the inactivation of *Toll*-dependent processes in more lateral and dorsal positions.

Transplantations of perivitelline fluid from embryos lacking the *Toll* receptor showed that the perivitelline fluid contains an activity that restores the dorsal-ventral pattern of embryos mutant for the somatic dorsal group genes (Stein et al., 1991). Importantly, the positioning of the injected perivitelline fluid determines the polarity of the new pattern. The polarizing activity probably represents the ligand of the *Toll* receptor because it can only be recovered from *Toll*$^-$ embryos. Presumably, this ligand is only released into the ventral region of the perivitelline space. In wild-type embryos, the ligand is immediately bound and leads to ventral activation of the *Toll* receptor, but in *Toll*$^-$ embryos it diffuses freely. Although these observations demonstrate that the orientation of the dorsal-ventral axis depends on an extracellular signal, they cannot assess the degree of spatial information originally present in the extraembryonic compartment. Thus, we cannot rule out the possibility of an active pattern-formation process required to enhance a weak spatial asymmetry present in vitelline membrane or perivitelline space.

To test whether such an autonomous pattern-formation process exists and whether it involves the *Toll* product, I performed cytoplasmic transplantations into *Toll*$^-$ embryos under a variety of different injection conditions. In particular, I demonstrate that two dorsal-ventral patterns can be generated within a single embryo by the transplantation of wild-type cytoplasm to two different positions. The two patterns induced within one embryo were analysed to detect possible lateral interactions.

MATERIALS AND METHODS

Fly strains

The wild-type stock was Oregon R. All injections were performed into embryos derived from $Tl^{5BRE}/Df(3R)ro^{XB3}$ females (Anderson et al., 1985a).

Cytoplasmic transplantations

Cytoplasmic transplantations were performed essentially according to Santamaria and Nüsslein-Volhard (1983).

For the dilution experiments, the cytoplasmic content of one wild-type embryo was distributed to 5, 10, 15 or 20 *Toll*$^-$ embryos. The entire cytoplasm of five *Toll*$^-$ embryos, which contained either 1/5, 1/10, 1/15, or 1/20 of the whole cytoplasmic content of a wild-type embryo, was taken up into the injection needle. The mixing was performed by reinjecting the cytoplasm into the empty egg cases and taking it up into the needle again three times. Subsequently, the mixed cytoplasmic content of one egg case was transplanted to 30 recipient *Toll*$^-$ embryos.

For double injections, needles were prepared with flame-polished tips using a microforge.

For non-simultaneous double injections, the first injection was performed before pole cell formation (preblastoderm stage) and the second injection at various time intervals following pole cell formation. The embryos were oriented randomly so that the first transplantation occurred either at the dorsal or at the ventral side. Induction of *twist* expression in response to a second transplantation is possible until the beginning of cycle 14. This is the latest stage of responsiveness to a single injection.

For the formation of stripe-like depositions of cytoplasm, the injection needle was introduced into the embryo from the anterior and moved through the entire embryo to the posterior tip. During retraction of the needle, the cytoplasm was released continuously (Fig. 8A). While the formation of dorsal stripes is relatively easy, the deposition of ventral stripes is hindered by the ventral curvature of the egg. Therefore, in many ventrally injected embryos, the *twist* stripe does not extend along the entire anterior-posterior axis.

Antibodies

The production of antibodies against *twist* and *dorsal* protein is described in Roth et al., 1989. Antibodies against *zen* protein were obtained from C. Rushlow (Rushlow et al., 1987), and anti-*even-skipped* antibodies were supplied by M. Frasch (Frasch et al., 1987). Immunological staining of whole-mount embryos with biotinylated HRP-avidin complexes bound to biotinylated secondary antibody (Vector Laboratories, Avidin/Biotin ABC

system) was carried out as described by MacDonald and Struhl (1986), with the modification that during the washes I added 100 mM NaCl to the solutions. For sectioning, stained embryos were dehydrated (10 minutes 70% ethanol, 2× 10 minutes 100% ethanol, 2× 100% acetone) and mounted in Durcupan-ACM (Fluka). A complete series of transverse sections (10 μm) was prepared to study changes of the staining pattern along the anterior-posterior body axis.

Whole-mount in situs

Whole-mount in situs using a digoxigenin-labeled *sim* probe were done essentially as described by Tautz and Pfeifle, 1989, with minor modifications. Stained embryos were dehydrated (10 minutes 70% ethanol, 2× 10 minutes 100% ethanol, 2× 100% acetone) and mounted in Durcupan-ACM (Fluka).

Cuticle preparations of embryos

For the observation of cuticular structures, differentiated embryos with vitelline membrane or dissected out of the vitelline membrane were mounted in a mixture of Hoyer's medium (Van der Meer, 1977) and lactic acid (1:1).

RESULTS

The dorsal-ventral pattern of *Toll*⁻ embryos with one local transplantation of wild-type cytoplasm

The dorsal-ventral pattern of injected embryos was analysed by studying the *dorsal* (Roth et al., 1989; Rushlow et al., 1989; Steward, 1989), *zerknüllt* (*zen*, Rushlow et al., 1987) and *twist* (*twi*) protein (Thisse et al., 1988) distributions and the distribution of *single minded* (*sim*) transcripts (Thomas et al., 1988). (See Fig. 1 for expression patterns of these markers in wild-type and *Toll*⁻ embryos.) The transplantation of wild-type cytoplasm to a dorsal site in a *Toll*⁻ embryo leads to a local nuclear accumulation of *dl* protein at the site of injection (Fig. 1I). In this region, *zen* protein is absent (Fig. 1L) and *twist* shows a narrow domain of expression surrounding the site of injection (Fig. 1K). If *twist* and *zen* expression are detected simultaneously in injected embryos (Fig. 1M), the three main subdivisions of the induced dorsal-ventral pattern are visible: the region expressing *twist* protein which will give rise to mesoderm, the surrounding region expressing neither *zen* nor *twist* which will contribute to the neuroectoderm, and finally the area of the *zen*-expressing cells which will differentiate as dorsal epidermis and amnioserosa.

To test at the molecular level whether finer subdivisions of the dorsal-ventral pattern are established in injected *Toll*⁻ embryos, the *single minded (sim)* RNA distribution was examined. Correct *sim* expression is only indirectly dependent on the maternal gradient; it is directly regulated by the zygotic genes *twist* and *snail* (Leptin, 1991; Rao et al., 1991; Kosman et al., 1991). In wild-type embryos, *sim* expression is confined to a single line of ventrolateral cells which will form the mesectoderm (Fig. 1D). Injected *Toll*⁻ embryos show a single line of *sim* expression surrounding the site of injection (Fig. 1H). This demonstrates that the pattern induced by cytoplasmic transplantation undergoes processes of refinement similar to the wild-type pattern.

The *dorsal* protein gradient of injected embryos is steeper than that of wild-type embryos

The size of the *twist*-expressing region that forms in a *Toll*⁻ embryo after injection depends on the relative amount of transplanted wild-type cytoplasm. Thus, *twist*-expressing patches with diameters between 10 and 50 cells could be produced. Despite their variability in size, the *twist*-expressing patches are always surrounded by a domain without *twist* and *zen* expression which comprises about 7 cells (Table 1). This domain is considerably smaller than that of wild-type embryos where it comprises approximately 14 cells (Fig. 2B,D). The observed expression patterns were not significantly influenced by the age of the recipient embryos as long as the injections were performed before the beginning of cellularization (early cycle 14, data not shown).

The narrower *zen* repression domain of injected embryos probably results directly from a change in the slope of the nuclear *dorsal* protein gradient. In wild-type embryos, the *dorsal* protein has highest nuclear concentrations in a ventral region which is 12-14 cells wide. Lateral to this region the nuclear concentrations decrease rapidly (Rushlow et al., 1989). Within an approximately 16-cell span, they go from highest to undetectable levels (Roth et al., 1989). In injected embryos, this region of decreasing nuclear concentrations comprises only approximately 8 cells (Fig. 2A,C).

These results demonstrate that domains of *twist* expression and *zen* repression whose sizes differ considerably from those found in wild-type embryos, can still be stable. Furthermore, the size of the domain without *zen* and *twist* expression is independent from the size of the *twist* expression domain. Thus, there is apparently no size regulation at the time of early zygotic dorsal-ventral pattern formation. Rather, the observed patterns directly reflect the shape of the nuclear *dorsal* protein gradient.

Toll product is present in excess in wild-type embryos

The injection of a small amount of wild-type cytoplasm (approximately 1% of total egg contents) is sufficient to form a complete set of dorsal-ventral pattern elements in a region spanning about 50% of the anterior-posterior axis (Anderson et al., 1985b). Therefore, either the activated *Toll* product induces a process of self-enhancement or it is present in vast excess in wild-type embryos. To address this question, injection experiments were performed with diluted wild-type cytoplasm. (see Materials and Methods). The cuticle phenotype of the injected embryos was scored for the presence of ventral denticles and filzkörper which serve as markers for ventrolateral and dorsolateral fates, respectively (Fig. 3). The formation of ventral denticles in more than 50% of the injected embryos can be achieved using cytoplasm diluted up to15-fold. With 20-fold diluted cytoplasm no ventral denticles were observed, but dorsolateral stuctures (filzkörper) still formed. In the case of 10-fold diluted cytoplasm, injected embryos were also stained for *twist* expression. 26 out of 42 injected embryos showed a patch of *twist*-expressing cells, demonstrating that 10-fold diluted cytoplasm still leads to the formation of the ventralmost structures in a majority of injected embryos.

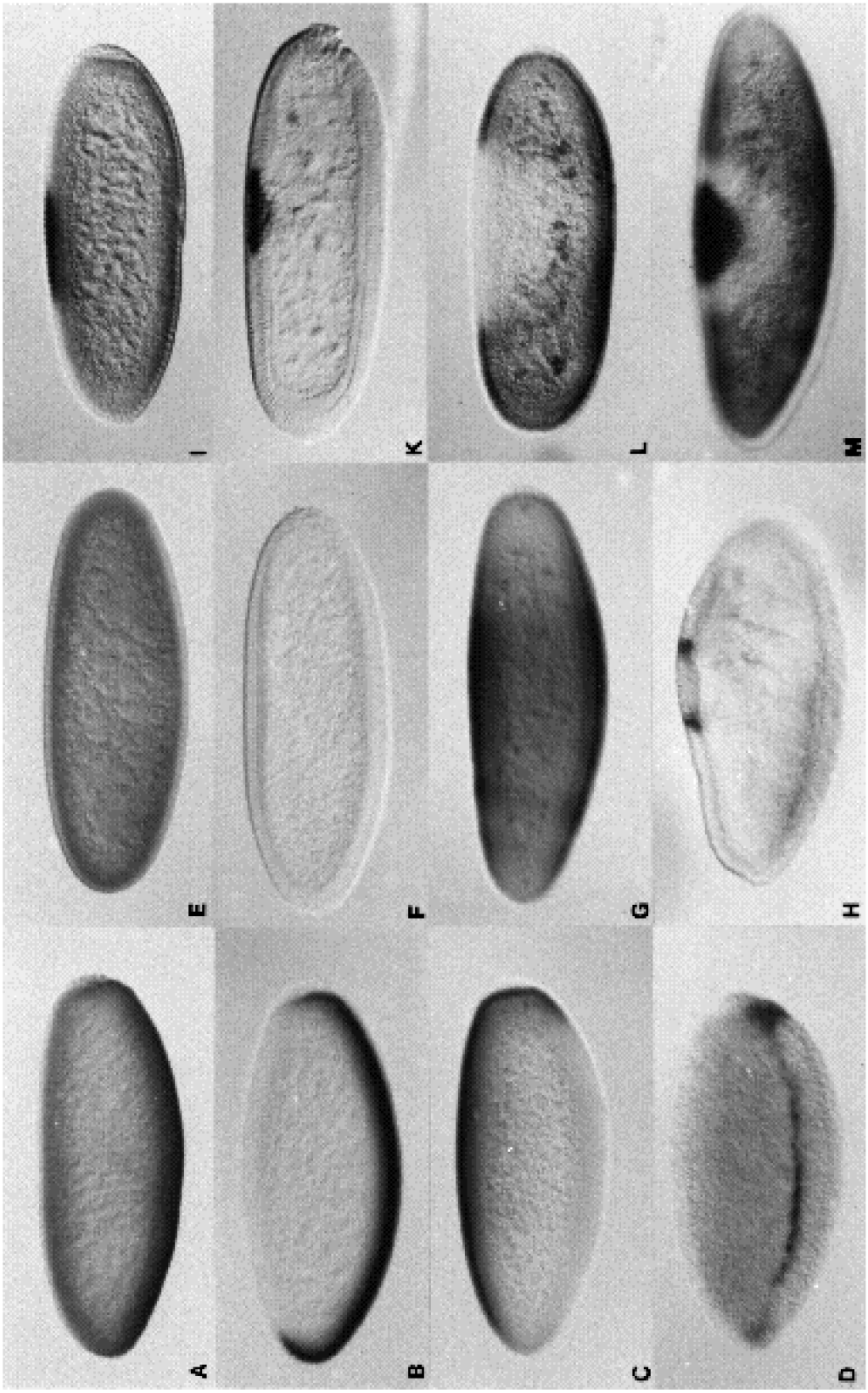


Fig. 1. The dorsal-ventral pattern of singly injected *Toll*$^{-}$ embryos. All panels show optical midsections of cellular blastoderm embryos. (A-D) Wild-type embryos; (E-G) embryos derived from $Tp^{5BRE}/Df(3R)ro^{XB3}$ females; (H-M) embryos derived from $Tp^{5BRE}/Df(3R)ro^{XB3}$ females that were injected with wild-type cytoplasm at the dorsal side. (A, E, I) *dorsal* protein distribution; (B, F, K) *twist* protein distribution; (C, G, L) *zen* protein distribution. (D, H) *sim* mRNA distribution; (M) Simultaneous visualization of *zen* and *twist* protein distributions. In wild-type embryos, *dorsal* (*dl*) protein forms a nuclear concentration gradient, which peaks ventrally; *twist* and *zen* proteins are respectively expressed in a ventral and a dorsal stripe of cells spanning the entire anterior-posterior axis. In *Toll* mutant embryos *dorsal* protein remains cytoplasmic, no *twist* protein can be detected and *zen* protein is uniformly expressed. See text for further explanations.

Table 1. The size of the domain without *zen* and *twist* expression is independent of the number of *twist*-expressing cells

Number of *twi*-expressing cells	Number of embryos	Number of cells showing neither *zen* nor *twi* expression
10-20	7	6-8 (average 7.0)
20-30	12	5-9 (average 7.0)
30-40	7	6-9 (average 7.6)

Preblastoderm embryos derived from $Tl^{5BRE}/Df(3R)ro^{XB3}$ females were injected with varying amounts of wild-type cytoplasm, simultaneously stained with *zen* and *twist* antibodies and sectioned. The number of *twist*-expressing cells and the number of cells expressing neither *twist* nor *zen* (*zen* repression domain) was counted along the embryonic circumference.

The varying degrees of rescue observed after injection of different amounts of *Toll* product show that a graded relation exists between *Toll* concentration and the number of dorsal-ventral pattern elements formed in injected embryos. This result argues against a strong self-enhancement process initiated by the *Toll* product since such a process should cause an all-or-none response rather than a graded response upon injection. It is therefore more likely that the rescue response reflects a vast excess of *Toll* product present in wild-type embryos. Assuming that *Toll* RNA in the injected cytoplasm is as efficiently translated as in wild type, the local concentration of *Toll* protein in wild-type embryos is in approximately 10-fold excess.

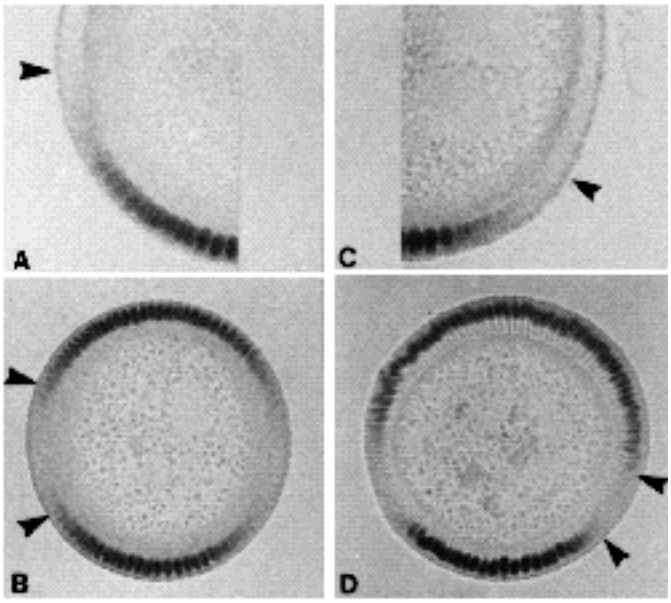


Fig. 2. *dorsal* protein gradient and *zen* repression domain of injected embryos. (A, B) Transverse sections of wild-type embryos. (C, D) Transverse sections of embryos derived from $Tl^{5BRE}/Df(3R)ro^{XB3}$ females and injected with wild-type cytoplasm at the dorsal side. For comparison with the wild-type pattern, the injected embryos are oriented with the ventralmost region of the induced pattern downward. (A, C) Embryos stained using *dorsal* antibodies. Only the lower left (A) or the lower right sector (C) of the transverse sections is shown to facilitate the comparison of the relevant parts of the *dorsal* protein gradients. The arrows indicate the extension of the *dorsal* protein gradients. (B, D) Embryos stained simultaneously with *zen* and *twist* antibodies. (C, D) Embryos injected at preblastoderm stage. The arrows indicate the extension of the *zen* repression domain.

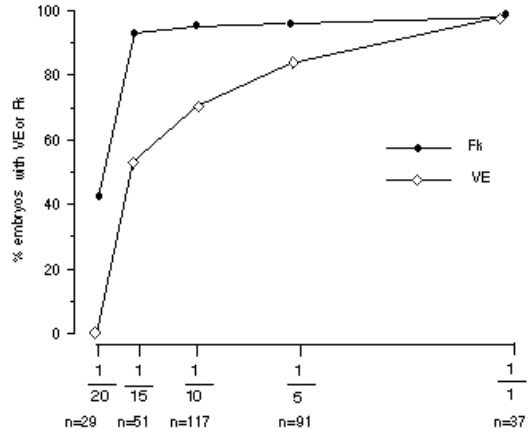


Fig. 3. Injections with diluted wild-type cytoplasm. Preblastoderm embryos derived from $Tl^{5BRE}/Df(3R)ro^{XB3}$ females were injected with nondiluted or diluted wild-type cytoplasm (see Materials and Methods). The different dilutions are plotted on the horizontal axis using a logarithmic scale. The numbers of injected embryos scored for the differentiation of ventral epidermis (VE) and filzkörper (Fk) are indicated.

No interaction between dorsal-ventral and anterior-posterior pattern-forming systems

The shapes of the patterns induced by injection often show radial symmetry (Figs 1M, 5B,C). In Fig. 4A, this property is demonstrated using *sim* RNA as a marker. The radial symmetry implies that these embryos possess dorsal-ventral gradients not only perpendicular, but at all angles to the anterior-posterior axis. The blastoderm-stage $Toll^{-}$ embryo seems to have no axial bias with respect to the orientation and spreading of the induced dorsal-ventral pattern. This can be demonstrated more dramatically by injecting wild-type cytoplasm into the anterior or posterior tip of $Toll^{-}$ embryos. Embryos injected posteriorly form a posterior cap of *twist* expression (Fig. 4D) and a *sim* stripe encircling the embryo (Fig. 4C). Therefore, in these embryos the dorsal-ventral axis is parallel to the anterior-posterior axis. Despite this fact, the early anterior-posterior pattern formation of dorsally or posteriorly injected embryos is not significantly disturbed, since they form seven *even-skipped* expression stripes (Frasch et al., 1987) with normal orientation (Fig. 4B,D).

In summary, the spatial and temporal properties of the single injection experiments demonstrate that, in $Toll^{-}$ embryos, all cells of preblastoderm and syncytial blastoderm stages respond to small amounts of transplanted wild-type cytoplasm in a similar way. The developing patterns show no preference in orientation with respect to the anterior-posterior or dorsal-ventral egg axes. These characteristics, together with the ability to manipulate the size of the induced patterns, make it feasible to study the interaction

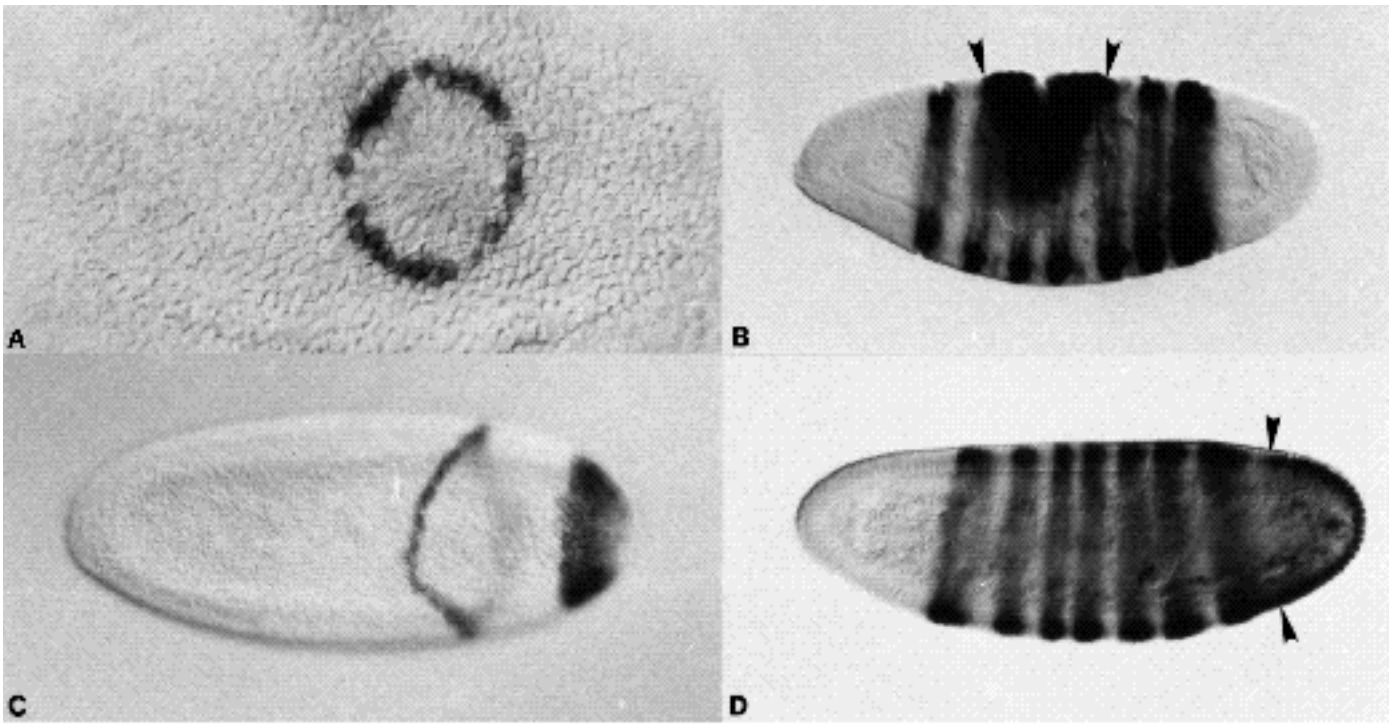


Fig. 4. The independence of anterior-posterior and dorsal-ventral pattern formation in blastoderm embryos. Preblastoderm embryos derived from $Tl^{5BRE}/Df(3R)ro^{XB3}$ females were injected with wild-type cytoplasm at the dorsal side (A, B) or at the posterior pole (C, D). (A) Dorsal surface view; (B-D) optical midsections. (A, C) *sim* mRNA distribution. The embryo in C shows also *sim* expression at the posterior pole. This is due to the induction of *twist* expression in a region were *snail* is repressed by the terminal system (Ray et al., 1991). It reflects the regulatory interactions, which normally govern *sim* expression in the ventral region (Leptin, 1991; Rao et al., 1991; Kosman et al., 1991). (B, D) Distribution of both *even-skipped* protein to show the segmentation pattern and of *twist* protein to show the site of injection. The arrows mark the extent of the *twist*-expression domain.

of two dorsal-ventral patterns induced simultaneously or sequentially in the same embryo.

Doubly injected embryos

If wild-type cytoplasm is delivered to two different positions of a *Toll*$^-$ embryo, a dorsal-ventral pattern is derived from each site of injection. Fig. 5 shows embryos that are injected in a dorsal and in a ventral position. The embryos form nuclear *dorsal* gradients at opposing dorsal and ventral regions (Fig. 5A). The detection of zygotic gene expression reveals radially symmetric zones of *twist* expression surrounded by a ring of *zen* repression at both sites of injection (Fig. 5B,C). The two patterns can be separated by *zen*-expressing cells, indicating that both have the major subdivisions of the wild-type dorsal-ventral pattern (Fig. 5D,E). The doubly injected embryos form two mesoderm invaginations and differentiate cuticular patterns which often consist of complete mirror-image duplications (data not shown). Thus, two locally induced dorsal-ventral patterns can form in one embryo without detectable interference or competition. This behaviour differs strikingly from that of wild-type embryos where the normal dorsal-ventral pattern-formation process completely inhibits the induction of a second pattern by cytoplasmic injection (Anderson et al., 1985b). In the following, I describe a series of experiments that are designed to detect the inhibition phenomenon seen in wild-type embryos and to determine whether it results merely from the sequestering of a ventrally released ligand or from a process of autonomous pattern formation. In the latter case, activation zones should be surrounded by regions of lateral inhibition regardless of their locations with respect to the egg shell.

No *Toll*-dependent lateral inhibition process

The suppression of one pattern by another pattern might be detected only if they are induced close together. In doubly injected *Toll*$^-$ embryos, greater amounts of wild-type cytoplasm result in larger regions of *twist* expression (Fig. 5F). The zones of *zen* repression are closer together and sometimes they are not separated by *zen*-expressing cells (Fig. 5G). However, no inhibition of one pattern by the other is observed. If the injection sites are closer together, either along the embryonic circumference or at the dorsal side of the embryo, the induced patterns still exert no inhibition on each other (Fig. 6A-C). These observations suggest that the two dorsal-ventral patterns induced in one embryo develop independently if far enough apart and overlap if generated close together.

In the transplantations described thus far, both injections were carried out almost simultaneously during preblastoderm stages. If a pattern-forming center requires time to generate a zone of inhibition, this inhibition might only be observed in non-simultaneous double injections. Therefore, double injections were performed with a time delay

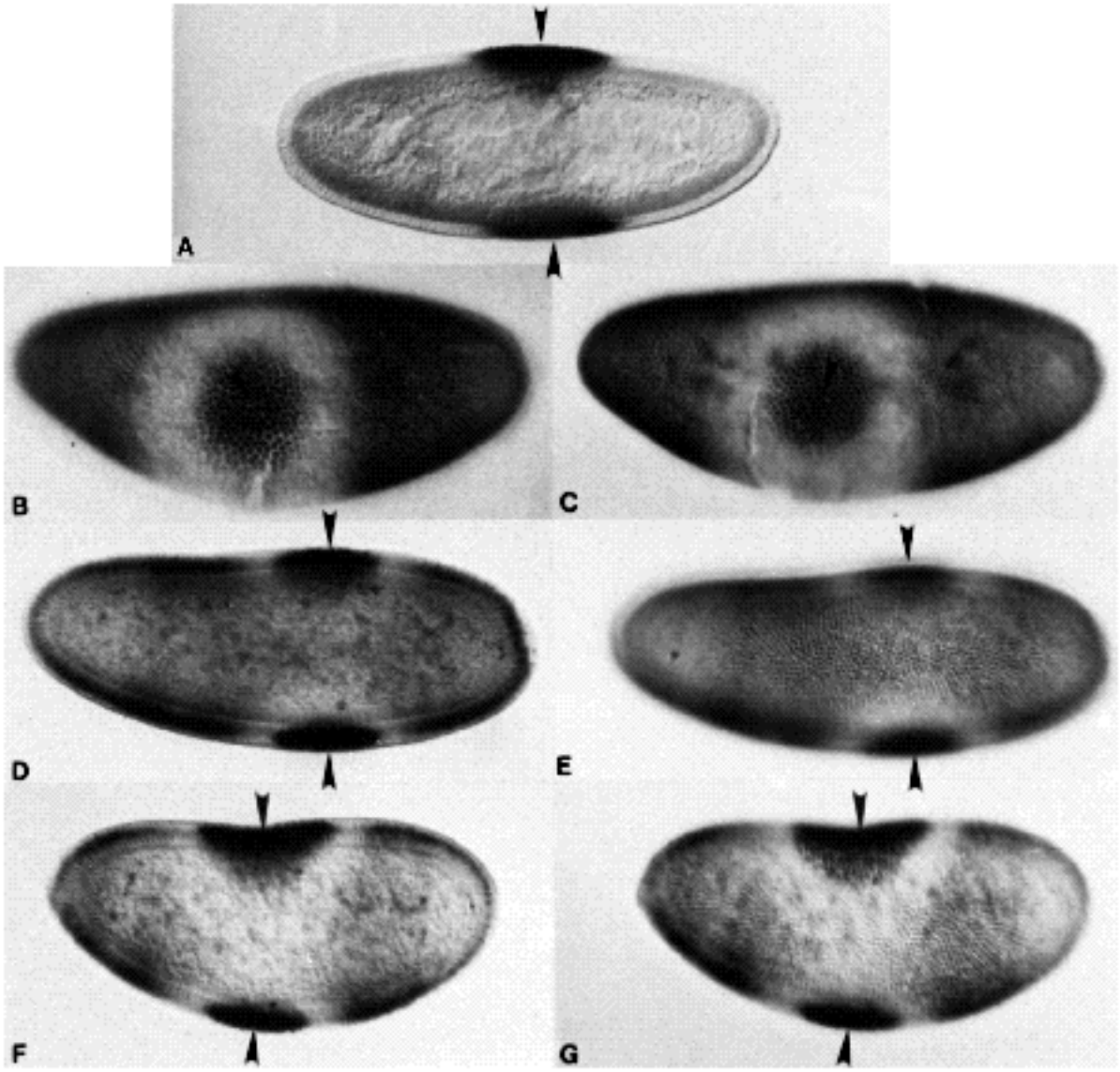


Fig. 5. Embryos with two dorsal-ventral patterns. Preblastoderm embryos derived from $Tl^{5BRE}/Df(3R)ro^{XB3}$ females were injected simultaneously at the dorsal and ventral side with wild-type cytoplasm. (A) *dorsal* protein distribution; (B-G) *zen* and *twist* protein distribution. (A) Optical midsection; (B) dorsal surface view; (C) ventral surface view of the embryo shown in B; (D, F) optical midsections; (E, G) lateral surface views of the respective embryos shown in D and F.

betweentransplantations (see Materials and Methods). If the second injection was performed before cycle 14, the induced pattern was not consistently smaller than that derived from the first injection, regardless of the time delay between both injections (Fig. 7). Furthermore, the results were not influenced by the site (ventral or dorsal) of the first injection. Thus, non-simultaneous double injections also reveal no sign of lateral inhibition.

If the strength of inhibition depends on the size of the induced pattern, a larger pattern might suppress the development of a smaller pattern. Furthermore, since inhibition was observed only in wild-type embryos, patterns approaching the size of the wild-type pattern might be required. In Fig. 8, an injection technique is described that allows the formation of stripe-like regions of *twist* expression in injected Tl^{-} embryos (see also Materials and Methods). If stripes are produced that extend along the entire anterior-posterior axis, wild-type-like embryos can be generated. In the case of the delivery of cytoplasm in a stripe along the dorsal midline, the embryos develop in the egg case with reversed orientation. 50% of the ‘upside down’ embryos show a complete or almost complete restoration of the dorsal-ventral cuticle pattern (Fig. 8D).

If embryos with a dorsal stripe of injected cytoplasm also receive a ventral spot-like injection, both the dorsal stripe and the ventral spot of cytoplasm give rise to *twist*-expressing regions (Fig. 8E,F; Table 2). The larger dorsal pattern was never observed to repress the smaller ventral pattern, indicating that there is no detectable repressive activity dependent on the size of the pattern. This result holds also

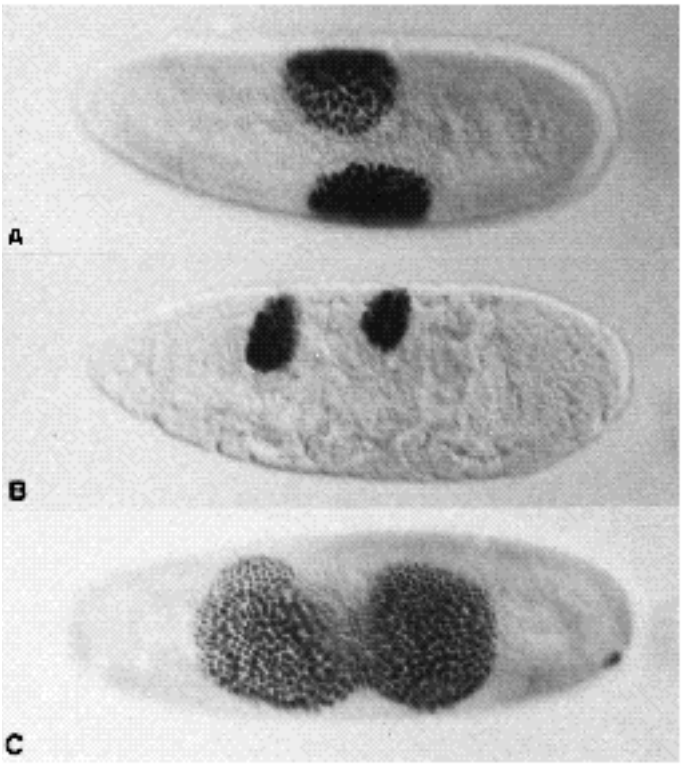
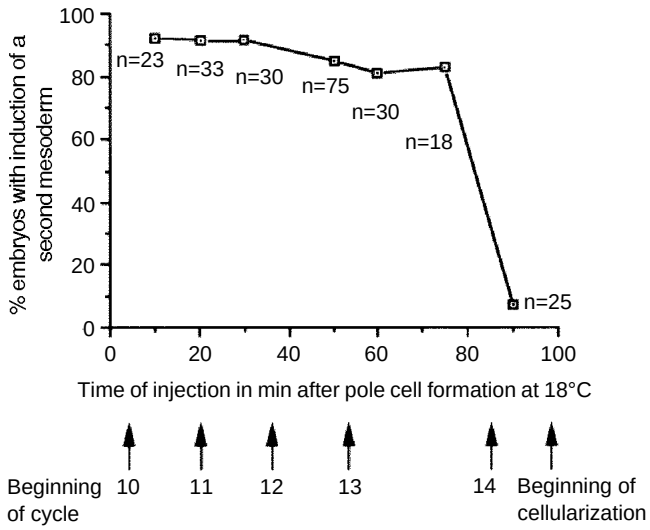


Fig. 6. Variation of the distance between sites of injection. *Toll*⁻ embryos were injected at two different sites with wild-type cytoplasm and later stained with *twist* antibodies. (A) Embryo with a dorsal and a lateral injection. (B) Embryo with two dorsal injections placed closely together. (C) The injections were performed as in B, but larger amounts of cytoplasm were transplanted. The *twist* expression zones derived from the two sites of injections have fused.



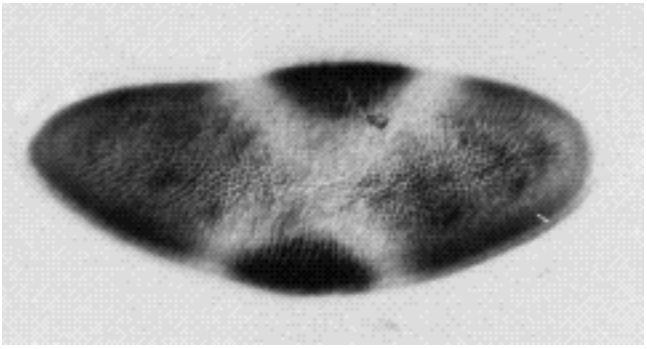


Fig. 7. Non-simultaneous double injections. *Toll*⁻ embryos were injected with wild-type cytoplasm at a dorsal and a ventral position with a delay between injections. The first injection was performed before pole cell formation, the second at different time intervals after pole cell formation. The doubly injected embryos were stained using *twist* and *zen* antibodies. For each time interval the number of embryos scored for a second patch of *twist*-expressing cells is indicated. Below the graph: Lateral surface view of a doubly injected embryo with two dorsal-ventral patterns of similar size. The ventral injection was performed 60 minutes after the dorsal injection.

when the ventral injection was performed substantially later than dorsal injection (data not shown). Thus, although these embryos can form a wild-type-like pattern, they do not behave like wild-type embryos with respect to a second cytoplasmic injection.

An inhibition dependent on the orientation with respect to the egg shell

The embryos injected with a dorsal stripe of cytoplasm differ in one major respect from wild-type embryos: their dorsal-ventral pattern forms inside the egg shell with opposite orientation as compared to wild type. To reconstruct the wild-type situation fully, I also produced *Toll*⁻ embryos with a ventral stripe of wild-type cytoplasm (Fig. 8G). Despite some experimental constraints (see Materials and Methods), double injections with a ventral stripe and dorsal spot of cytoplasm lead to results significantly different from the experiments with the reverse orientation. While a dorsal stripe never inhibited *twist* induction on the opposite side, in 38% of the embryos with a ventral stripe, a second dorsal injection failed to induce a zone of *twist* expression (Table 2). Interestingly, a correlation could be seen between the anterior-posterior extension of the ventral *twist* stripe and the ability to induce a dorsal pattern. The inhibition was usually only observed if the *twist* stripe extended along the entire anterior-posterior axis (compare Fig. 8G and H). Therefore, the inhibition of a second pattern seems to depend on both the size and orientation of the other pattern with respect to the egg shell. In summary, the observed inhibition phenomenon is not an intrinsic property of the pattern-formation process inside the embryo, but rather reflects a strong spatial asymmetry present in the vitelline membrane or the perivitelline space.

Not only the *Toll* protein, but also the *Toll* ligand is in excess

Interestingly, the doubly injected embryos often have more *twist*-expressing cells than wild-type embryos. 15 embryos injected with a dorsal stripe and a ventral spot of cytoplasm were subsequently sectioned to estimate the size of the induced mesodermal regions. In 13 of the sectioned embryos, the dorsal *twist* stripe was 25 to 30 cells wide and

thus broader than the 20- to 22-cell-wide *twist* expression zone of wild-type embryos. Fig. 8F shows an example in which the dorsal *twist*-expressing zone constitutes as much as 60% of the embryonic circumference (55 cells). If the zone of *twist* expression formed by the ventral spot-like cytoplasmic injections is also included, the total area of *twist* expression is often considerably larger than in wild-type embryos. The ability to induce more ventral structures

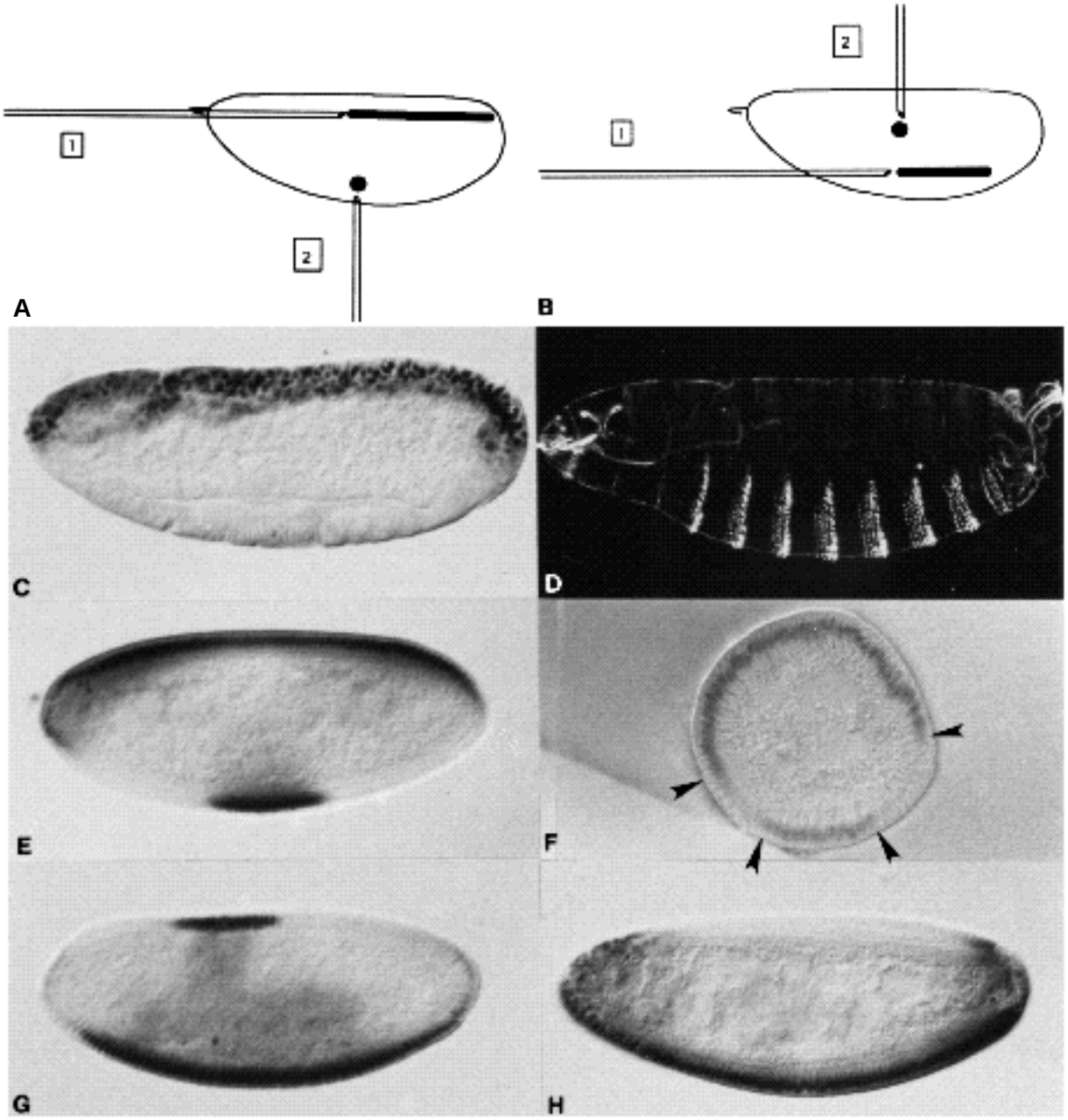


Fig. 8. Double injections with stripe-like deposition of cytoplasm. (A, B) The injection technique. (A) The injection of a dorsal stripe of cytoplasm is followed by a ventral spot-like injection. (B) The injection of a ventral stripe of cytoplasm is followed by a dorsal spot-like injection. (C, E, F, G, H) Injected *Toll*$^-$ embryos stained using *twist* antibodies. (C, D) *Toll*$^-$ embryo injected with a dorsal stripe of wild-type cytoplasm. (C) The mesoderm formation occurs dorsally and the germband extends towards the ventral side. (D) Dark-field photograph of the cuticle produced by an injected embryo. The cuticle pattern resembles that of a wild-type larva. The larva is oriented with the ventral side down, although it developed inside the egg shell in the opposite direction. (E, F) *Toll*$^-$ embryos injected with a dorsal stripe of wild-type cytoplasm followed by a ventral spot-like injection as depicted in (A). (E) Optical midsection. (F) Transverse section. Arrows indicate the extension of the *twist*-expressing regions. In this specific case the dorsal *twist*-expressing region comprises about 60% of the embryonic circumference. (G, H) *Toll*$^-$ embryos injected with a ventral stripe of cytoplasm followed by a dorsal spot-like injection as depicted in B. (G) In this embryo, both injections cause regions of *twist* expression. (H) In this embryo, the dorsal injection does not lead to a region of *twist* expression.

Table 2. Double injections with stripe-like depositions of cytoplasm

	Number of embryos with a spot-like second injection resulting in		
	Spot-like twist expression	No twist expression	No assignable pattern
Dorsal stripe ventral spot	40 (72%)	0 (0%)	16 (28%)
Ventral stripe dorsal spot	46 (47%)	37 (38%)	14 (14%)

For explanation of injection technique see Fig. 8. 'no assignable pattern' includes embryos with an irregular *twist*-expression domain that might have formed by a fusion of the dorsally and ventrally induced patterns.

than present in a wild-type embryo suggests that, like the *Toll* receptor, the ligand of *Toll* is present in excess (for further explanation see discussion).

DISCUSSION

The pattern-forming capacity of the dorsal-ventral system in *Drosophila* embryos

The existence of an autonomous pattern-formation process that controls the dorsal-ventral axis formation in lower insects has been suggested on the basis of classical embryological studies (Sander, 1976). For example, in experiments with the cicada *Euscelis*, longitudinal ligations separating left and right or dorsal and ventral egg halves caused pattern duplications producing two complete embryos from the two separated fragments (Sander, 1971). These self-regulatory properties not only rule out the existence of prelocalized cytoplasmic determinants, but also are incompatible with a strict dependence of the embryonic dorsal-ventral axis on a prepattern present in the extraembryonic compartment. However, they can be explained by a mechanism of lateral activation of mutually exclusive states (Meinhardt and Gierer, 1980) or by a local activation/lateral inhibition mechanism (Meinhardt, 1989).

As shown by perivitelline fluid injections, the extracellular signal that is required to establish the dorsal-ventral axis of *Drosophila* embryos has a polarizing influence and thus confers some spatial information (Stein et al., 1991). From these experiments, however, it is not clear whether the extracellular spatial cues fully contain the spatial information present in the final pattern. The situation could be similar to the cell fate determination of the vulval precursor cells in *C. elegans*, which requires both a graded inductive signal from the anchor cell and a lateral inhibition between the cells receiving the signal (for review see Horvitz and Sternberg, 1991). Therefore, a *Toll*-dependent local activation/lateral inhibition process might exist. The spatial cues present in the vitelline membrane or perivitelline space may trigger a process, which then would lead autonomously to the elaboration of the final pattern.

However, the described experiments presented here fail to demonstrate either a *Toll*-dependent local self-enhancement process or a *Toll*-dependent lateral inhibition mechanism. Only *Toll*$^-$ embryos that had received a ventral stripe-like injection of wild-type cytoplasm show an inhibition phenomenon. If the process under investigation has an autonomous pattern-forming capacity, its behaviour should not depend strongly on orientation with respect to the egg shell. My results argue against an autonomous pattern-formation mechanism occurring at the level of *Toll* activation or downstream of *Toll*. Therefore, it is unlikely that *Drosophila* embryos possess the regulatory capacity found for the dorsal-ventral pattern-formation process in more primitive insects (Sander, 1976).

The transfer of spatial information from the extraembryonic compartment to the embryo

The failure to detect autonomous pattern formation, strongly supports the previously proposed model (Stein et al., 1991; Schüpbach et al., 1991) that the *Toll* ligand is released from a restricted ventral region of the perivitelline space and that its amount is limited with respect to the number of *Toll*-binding sites. Under these conditions, binding and sequestering of the ligand prevents its further spreading to more lateral regions thereby limiting the size of the activation zone. This mechanism attributes two functions to the *Toll* receptor: *Toll* is not only required to transmit the extracellular signal to the cytoplasm, but *Toll*, uniformly distributed in the plasma membrane, is also necessary to localize the extracellular signal to the ventral side.

A similar model has been proposed for the terminal system of the anterior-posterior axis. Here, the *torso* protein, a receptor tyrosine kinase, is uniformly distributed in the plasma membrane (Sprenger et al., 1989; Casanova and Stuhl, 1989). The *torso* receptor binds a putative extracellular ligand, which is released from the terminal region of the perivitelline space or the vitelline membrane (Stevens et al., 1990; Stevens and Nüsslein-Volhard, 1991). The injection of *torso* RNA into central regions of *torso*$^-$ embryos leads to the formation of terminal structures at the site of injection (Sprenger and Nüsslein-Volhard, 1992). Similar results have been obtained by a non-uniform expression of *torso* protein in *torso*$^-$ embryos (Casanova and Struhl, 1993). Thus, as in the dorsal-ventral system, the ligand for the terminal system can diffuse and its localization to the terminal regions depends on a uniform distribution of the receptor protein.

If this model applies, receptor density and amount of ligand are important parameters. The *Toll* gene shows no detectable dosage sensitivity (Anderson et al., 1985a) implying that *Toll* product is present at least in two-fold excess. The dilution experiments with wild-type cytoplasm indicate that *Toll* is in approximately 10-fold excess. Suprisingly, it seems that not only the *Toll* receptor, but also the ligand of *Toll* is produced in excess. By injection of dorsal stripes of cytoplasm, embryos can be produced that have significantly more *twist*-expressing cells than wild-type embryos (Fig. 8F). Since *twist* expression requires the ligand-dependent activation of *Toll*, the increased amounts of mesodermal cells indicate that more ligand is present than is necessary to form a mesoderm of normal size. Under injection conditions, the density of *Toll* protein in the plasma membrane may be lower than in wild type, so that

the same amount of ligand may activate a larger area of receptor molecules. This implies that at the ventral side of a wild-type embryo more ligand is bound than is necessary to achieve the highest nuclear concentrations of *dorsal* protein. Thus, it is not the absolute amount of the ligand, but the relative concentrations of ligand and receptor molecules that determine the size of the activation zone.

The final shape of the nuclear *dorsal* protein gradient, and especially its slope, might depend on events occurring at several different levels. Gradient formation by diffusion could occur downstream of *Toll* in the cytoplasm or upstream of *Toll* in the perivitelline space. It is even possible that the slope of *dorsal* gradient is already determined by spatial cues in the vitelline membrane. If gradient formation occurred entirely downstream of *Toll* in the cytoplasm, the nuclear gradient of injected embryos should have either the same slope as in wild-type embryos or a reduced slope due to the lateral diffusion of *Toll* RNA in the cytoplasm or *Toll* receptor molecules in the plasma membrane. The fact that the *dorsal* gradient of injected embryos is steeper than in wild type strongly suggests that extracellular events contribute to the gradient formation in wild-type embryos.

The role of oogenesis and the perpendicular orientation of the body axes

Since no active *Toll*-dependent mechanisms of pattern-sharpening seem to govern the formation of the nuclear *dorsal* protein gradient, the question arises how the spatial information contained in the extracellular signal for *Toll* is generated. Given the apparent precision of this extracellular prepattern, it is likely that processes upstream of *Toll* exist that have properties of autonomous pattern-formation systems. These processes may involve activities of dorsal group genes, which are necessary to produce the ligand. They may occur in the perivitelline space after egg deposition or in the follicular epithelium during oogenesis. In the latter case, they would be responsible for defining the ventral region of the vitelline membrane where the production of the ligand can be initiated. In fact, pattern duplications in embryos that are derived from ventralized *torpedo*, *gurken* or *cornichon* eggs suggest the existence of an autonomous pattern-formation system operating upstream of *Toll* (Roth et al., unpublished data).

A feature of the dorsal-ventral patterns induced in *Toll*$^-$ embryos is the complete lack of a bias that restricts their orientation with respect to the anterior-posterior axis. It seems that the blastoderm embryo is isotropic with respect to the orientation of dorsal-ventral gradients. Thus, no general mechanism operates in blastoderm embryos that accounts for or stabilizes the perpendicular orientation of the body axes and the interpretation of dorsal-ventral and anterior-posterior positional information occurs largely without interference. This idea has been proposed previously based on the observation that the majority of maternal-effect and zygotic mutations disrupt either the anterior-posterior or the dorsal-ventral pattern (Anderson and Nüsslein-Volhard, 1984b; Nüsslein-Volhard et al., 1987).

An interesting exception is the interaction between terminal and dorsal-ventral system, which influences the expression pattern of dorsal-ventral zygotic genes in terminal regions of the embryo (see for example the terminal *sim* expression in Fig. 4C; Ray et al., 1991; Casanova, 1991). However, this exception does not affect the general conclusion: the Cartesian coordinate system reflected in the expression pattern of early zygotic genes depends strongly, if not entirely, on the correct localization within the egg of the RNAs that determine the anterior and posterior pattern (for review see St Johnston and Nüsslein-Volhard, 1992) and on the correct positioning in the egg shell of the extracellular signals that determine the terminal and dorsal-ventral pattern. The process of axis orientation relies, therefore, on a not yet understood pattern-formation system, which operates during oogenesis and determines with high precision the 90° angle between the dorsal-ventral and anterior-posterior axes by defining the relative positions of cytoplasmic determinants and extracellular signals in the growing egg.

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