Original Article

Microinjected antisera against ductin affect gastrulation in *Drosophila melanogaster*

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ABSTRACT Ductin is a putative connexon-forming protein in gap junctions of arthropods. To analyze the role of gap-junction mediated cell-cell communication during *Drosophila* embryogenesis, we used two different polyclonal anti-ductin sera. One antiserum was directed against ductin isolated from gap junctions of the lobster *Nephrops* whilst the other was raised against a nonapeptide at the N-terminus of ductin from *Drosophila*. Both antisera were found to inhibit, when microinjected into *Drosophila* ovarian follicles, the intercellular exchange of fluorescent tracer molecules between oocyte and follicle epithelium. This result indicates that *Drosophila* ductin plays a decisive role in gap-junctional communication and confirms the cytoplasmic location of the ductin N-terminus in gap junctions. On immunofluorescence preparations and immunoblots, the anti-ductin sera specifically recognized ovarian as well as embryonic antigens. Following microinjections of the antisera into embryos prior to gastrulation, significantly reduced rates of hatching larvae were obtained. Moreover, microinjections into the mid-ventral region of the embryos resulted in specific ventral defects that depended on the concentration of the ductin antibodies. In particular, larvae with ventral holes in their cuticles occurred with high frequency. During gastrulation, antiserum-injected embryos often developed defects in the middle region of their ventral furrow. Here, mesodermal cells failed to invaginate correctly and, thus, no cuticle was formed. We conclude that, during *Drosophila* embryogenesis, gap-junctional communication is required for epithelial integrity and morphogenetic events.

KEY WORDS: *development, cell adhesion, embryogenesis, gap junction, intercellular communication*

Introduction

Gap junctions may promote the transduction of signals within tissues since they provide intercellular routes for electrical communication, via ion fluxes, as well as for biochemical communication, via second messengers such as cAMP, inositol trisphosphate and Ca^{2+} (reviewed in Bennett *et al.*, 1991; Katz, 1995; Paul, 1995). Therefore, gap junctions are supposed to be important for the coordination of developmental processes and, in several organisms, a functional role of these intercellular pathways has been revealed (for reviews, see Caveney, 1985; Green, 1988; Warner, 1988).

In *Drosophila*, only a few developmental mutants have been found so far in which specific defects were correlated with altered gap-junctional communication (e.g., Ryerse and Nagel, 1984; Giorgi and Postlethwait, 1985; Jursnich *et al.*, 1990; Sun and Wyman, 1996). To gain deeper insight into the functional role of gap junctions during *Drosophila* development, other strategies have to be applied. Injecting antisera raised against gap-junction

proteins is a very specific way to block gap-junction mediated cell-cell communication. Using this method, several investigators have found abnormal development in *Hydra*, *Xenopus* embryos, mouse embryos, and chick limb buds (reviewed in Warner, 1992).

During *Drosophila* embryogenesis, gap junctions can first be detected in the early gastrula (Eichenberger-Glinz, 1979; Tepass and Hartenstein, 1994). In order to analyze the role of gap junctions during development, we microinjected different polyclonal

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Abbreviations used in this paper: AD16, affinity-purified anti-*Drosophila* ductin serum; f-AD16, flow-through fraction of the affinity-purification; w-AD16, whole anti-*Drosophila* ductin serum; AN2, anti-*Nephrops* ductin serum; apAN2, affinity-purified anti-*Nephrops* ductin serum; mAB 224-3, monoclonal antibody against the *Manduca* V-ATPase; BSA, bovine serum albumin; LY, Lucifer Yellow CH; NIS, non-immune serum; PBS, phosphate buffered saline; R-14, Robb's tissue-culture medium; RAM, rhodamine-labeled rabbit-anti-mouse serum; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel-electrophoresis; SIT, silicon-intensified target; V-ATPase, vacuolar-type proton pump.
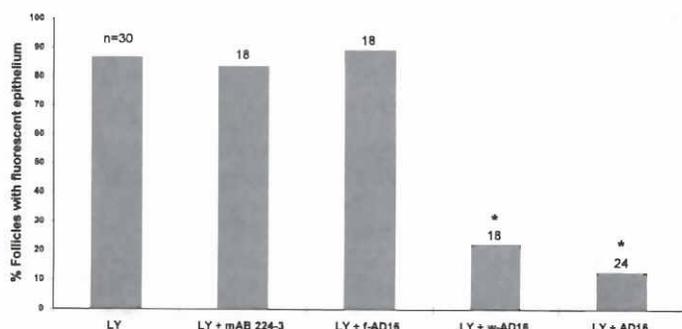
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Fig. 1. Video prints showing two stage-10 follicles about 3 min following microinjections into the oocyte (video-intensified fluorescence microscopy). (A) *Control follicle microinjected with LY alone: All three cell types–the oocyte (ooc), the nurse cells (nc), and the follicle cells (fc)– contain the dye.* **(B)** *Follicle microinjected simultaneously with LY and the affinity-purified anti-ductin serum AD16: No dye-coupling between oocyte and columnar follicle cells can be detected. Bar, 100 μm.*
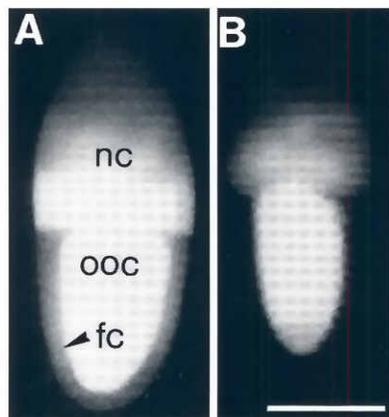
antiserainto embryos of pre-gastrulation stages 2-5. The antiserum AN2 was raised against ductin –the putative connexon-forming protein in gap junctions and the proteolipid component (subunit c or proton channel) in the vacuolar-type H⁺-ATPase– of the lobster *Nephrops norvegicus* (for reviews, see Finbow and Pitts, 1993; Finbow *et al.*, 1995). In *Drosophila* ovarian follicles, AN2 has previously been shown to specifically recognize a 29-kDa protein –a putative dimer of the 16-kDa protein ductin, to bind to cell membranes in a discrete punctate pattern, and to block gap-junctional communication (Bohrmann, 1993; Bohrmann and Haas-Assenbaum, 1993). Using *Drosophila* embryos, we found that AN2 recognizes proteins of 16 and 29 kDa and that it shows a similar staining pattern as in follicles at the cell membranes.

The antiserum AD16, raised against a peptide at the N-terminus of ductin from *Drosophila*, labels cellular antigens in various *Drosophila* tissues (Bonafede and Bohrmann, 1996; Braun and Bohrmann, 1996; Bohrmann and Braun, manuscript submitted). In the present study, we found that AD16 blocks the intercellular exchange of fluorescent tracer molecules via gap junctions, and that it binds to the 16-kDa monomer and to the 29-kDa dimer of ductin in preparations of follicles as well as embryos of *Drosophila*.

Following microinjections of the anti-ductin sera into the embryo's mid-ventral region, we obtained either a disintegration of the epithelium or specific defects during gastrulation and cuticle formation. Since gap-junctional communication is impaired by specific binding of the ductin antibodies, this type of cell signaling seems to be necessary to maintain epithelial integrity and cell adhesion, and to coordinate morphogenetic movements during gastrulation and during subsequent development.

Results

Dye-coupling in follicles

The ovarian follicle of *Drosophila* consists of a cluster of germ-line cells –one oocyte and 15 nurse cells– surrounded by a layer of somatic follicle cells. While the 16 germ-line cells form a syncytium by way of cytoplasmic bridges (King, 1970), cytoplasmic continuity with the follicular epithelium is accomplished via gap junctions (Giorgi and Postlethwait, 1985).

We have previously shown that these gap junctions allow the exchange of the dye Lucifer Yellow CH (LY) between oocyte and follicle cells (Bohrmann and Haas-Assenbaum, 1993). The extent of dye-coupling depends on several parameters: the age of the female, the status of oogenesis, and the developmental stage of the follicle. Moreover, a variety of experimental factors have been found to inhibit or to stimulate this gap-junctional communication.

By analyzing dye-coupling in follicles, specific blockers can be selected that might prove useful in clarifying the role of gap junctions during development. The antisera AN2, raised against isolated *Nephrops* gap junctions, and apAN2, affinity-purified against *Nephrops* ductin (Buultjens *et al.*, 1988; Leitch and Finbow, 1990), have previously been shown to inhibit dye-coupling when microinjected into *Drosophila* follicles (Bohrmann, 1993).

In the present study, the antiserum AD16, prepared and affinity-purified against a N-terminal nonapeptide of *Drosophila* ductin, was tested for its influence on dye-coupling. When LY alone was microinjected into the oocyte, the dye reached the nurse cells

Fig. 2. Dye-coupling between oocyte and follicle cells after microinjections of different solutions. *Dye-coupling was significantly reduced following microinjections of the anti-ductin sera w-AD16 or AD16 into stage-10 follicles. No effect on dye-coupling was observed following control injections of f-AD16 or mAB 224-3. LY +, 2% LY-solution mixed 1:1 with the respective antibody solution; n, number of microinjected follicles; *, value significantly different (P<0.05) from control values (LY, LY + mAB 224-3 or LY + f-AD16).*

TABLE 1

CUTICLE DEFECTS OBSERVED AFTER INJECTIONS FROM DORSAL (HIGHEST ANTIBODY CONCENTRATION ON THE VENTRAL SIDE). (SEE ALSO FIGS. 8-11).

Class	Cuticle defects
Normal or slight defects	Cuticle normal; fused denticle belts; mouth hooks or cephalopharyngeal skeleton defective; healed wound visible at dorsal site of injection
Dorsal defects	Small dorsal hole at site of injection (anterior abdominal segments); large dorsal hole (thoracal and anterior abdominal segments)
Ventral defects	Small ventral or ventro-lateral hole (anterior abdominal segments); large ventral or ventro-lateral hole (thoracal and several abdominal segments)
Severe defects	Head and thorax (and anterior abdominal segments) totally absent; cuticle only partially intact
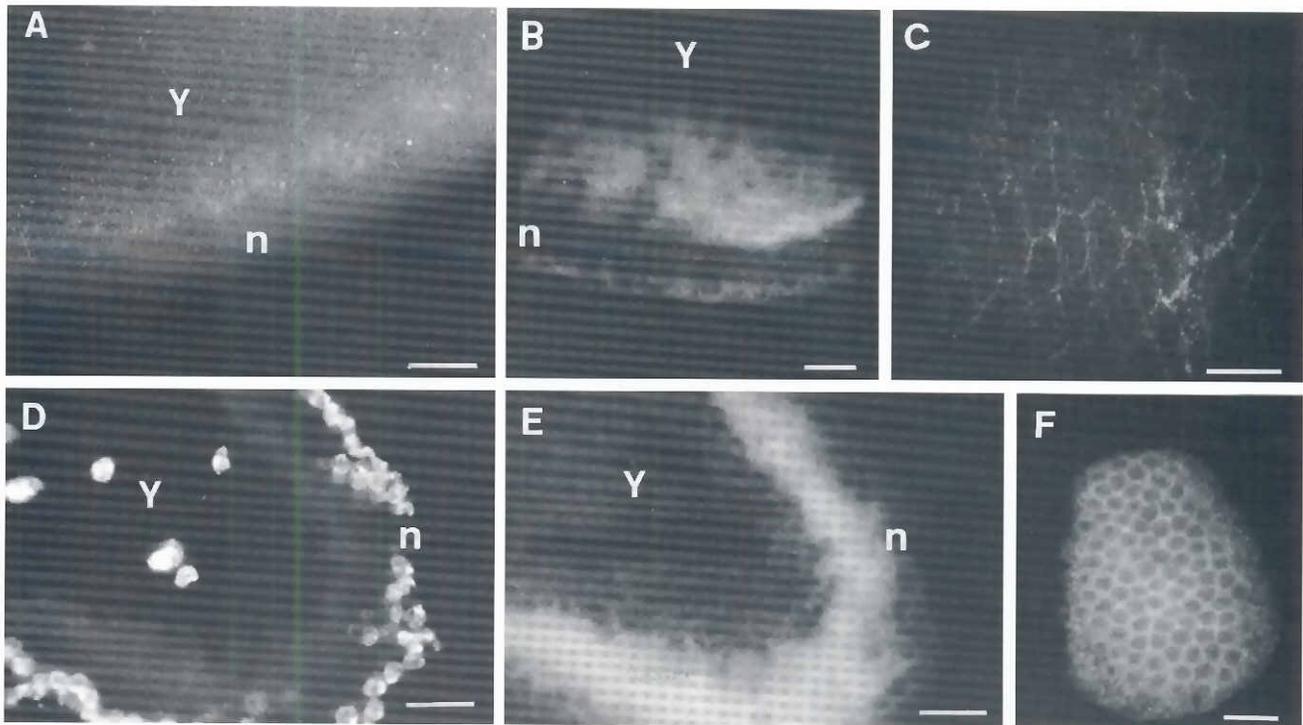
Fig. 3. Immunocytochemical localization of ductin on whole-mounts (A,C) and cryosections (B,E,F) of embryos using the anti-ductin sera apAN2 (A,B), AN2 (C) and AD16 (E,F), respectively. *During stage 4 (A,E), fluorescent antigens were observed at the interface between the cortical layer of nuclei (n) and the central yolk (y) (D: DAPI staining of cryosection shown in E). During later development, granular antigens were detected in the cytoplasm of blastoderm cells (B, stage 5). Thereafter, either a linear pattern (F, stage 6/7, AD16) or a punctate pattern (C, stage 14, AN2) was observed at the cell membranes. Bars in A-C, 10 μm; in D-F, 25 μm.*

within a minute, and about 87% of the follicles showed fluorescence in the columnar follicle cells that cover the oocyte (Figs. 1A,2). When LY was microinjected simultaneously with the affinity-purified antibody fraction AD16, or with the whole anti-ductin serum w-AD16, the percentage of follicles showing dye-coupling was significantly reduced (P<0.05; Figs. 1B,2). On the other hand, following microinjections of LY together with the antibody-deprived flow-through fraction f-AD16, or with the monoclonal antibody mAB 224-3 raised against the V-ATPase of *Manduca sexta*, no reduction of dye-coupling was observed (P>0.05; Fig. 2). Both f-AD16 and mAB 224-3 served as controls since they are not expected to bind to gap junctions.

The mAB 224-3 has previously been used to localize V-ATPase molecules in ovarian follicles of *Manduca* (Janssen *et al.*, 1995) and of *Drosophila* (Braun and Bohrmann, 1996; Bohrmann and Braun, manuscript submitted). It is directed against a 57-kDa protein that co-purifies with the plasma-membrane V-ATPase from *Manduca* midgut, and seems to be associated with the peripheral V_1 sector of the V-ATPase (Jäger *et al.*, 1996).

Indirect immunofluorescence preparations

In *Drosophila* ovarian follicles, AN2 and apAN2 bind to cell membranes in a typical punctate pattern (Bohrmann, 1993), and in *Drosophila* embryos, we observed a similar pattern. Before cell formation (stage 4), fluorescent granules were found in large amounts at the interface between the cortical layer of nuclei and the central yolk (Fig. 3A). The granules are presumed to represent aggregates of maternal precursor proteins since, during oogenesis, they first appear in the nurse cells from where they are delivered to the oocyte

(Bonafede *et al.*, 1995). Later on, the granules become incorporated in the cells of the blastoderm (Fig. 3B) and, during further development, a punctate pattern appears at the membranes (Fig. 3C).

Using AD16, cytoplasmic staining was more prominent than with AN2 or apAN2 (Fig. 3D,E), and membrane labeling appeared to be continuous rather than punctate (Fig. 3F). This result seems to indicate that not only ductin molecules in gap junctions but also
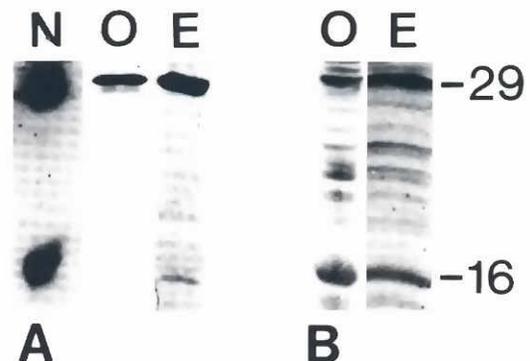
Fig. 4. Immunoblots showing specific binding of the affinity-purified anti-ductin sera apAN2 (A) and AD16 (B) to proteins of *Nephrops* gap-junction preparations (N), *Drosophila* ovary preparations (O) and *Drosophila* embryo preparations (E), respectively. *Molecular masses (M_rx10^{-3}) of the ductin monomer and the dimer are indicated. While apAN2 and AN2 (data not shown) recognize ductin from both Nephrops and Drosophila, AD16 only recognizes Drosophila ductin.*
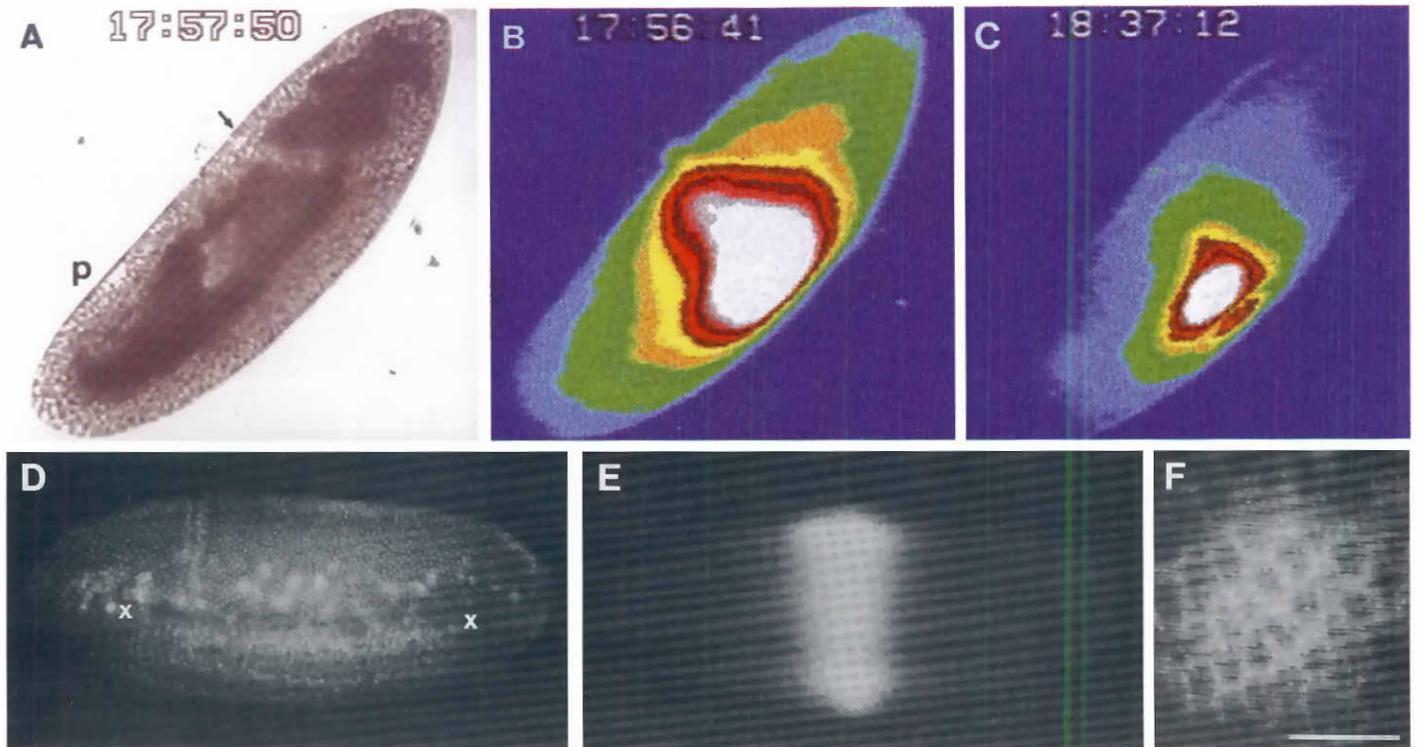
Fig. 5. Distribution of micro-injected solutions. *Video brightfield* **(A)** *and pseudocolor fluorescence image* **(B)** *of a stage-7 embryo. About 30 min earlier (during stage 5), RAM was microinjected from the dorsal side (to the left) at about 50% egg length (anterior at top). The highest RAM concentration is found in the mid-ventral region (to the right) of the embryo, even during later development* **(C;** *stage 8). Pseudocolors indicate different levels of fluorescence intensity (maximum: white; minimum: blue). DAPI* **(D)** *and indirect immunofluorescence staining* **(E)** *of a stage-7 embryo microinjected with apAN2 during stage 5 (ventral view, anterior to the left). The highest antibody concentration is found in the mid-ventral region. Using high magnification and video-intensified fluorescence microscopy* **(F)**, *a punctate staining pattern is seen in the cytoplasm and at the cell membranes in the mid-ventral region of a stage-8 embryo (apAN2-injection during stage 5). The embryos are about 480 μm in length. p, pole cells; arrow, cephalic furrow; x-x, ventral furrow. Bar in F, 25 μm.*

in V-ATPases were labeled. No specific staining was observed in the various control preparations (see Materials and Methods).

Immunoblotting

On immunoblots of *Drosophila* ovary homogenates, AN2 and apAN2 bind specifically to a 29-kDa protein that is immunologically related to the 16-kDa protein ductin (Bohrmann, 1993). The 29-kDa protein is synthesized in the nurse cells and accumulates in the oocyte (Bonafede *et al.,* 1995). In preparations of embryos and first instar larvae, we found a 29-kDa protein as well as a 16-kDa protein (Fig. 4A). Therefore, the 29-kDa protein is presumed to be a maternal cytoplasmic precursor, most likely a dimer, of ductin. Larger amounts of the 16-kDa monomer can be found in membrane-

enriched fractions of ovary cells as well as in other tissues of *Drosophila* (Bonafede and Bohrmann, 1996; Bohrmann and Braun, manuscript submitted). With AN2 and apAN2, also the dimer of *Nephrops* ductin was labeled (Fig. 4A; cf. Finbow *et al.,* 1994).

Using AD16, both the 16-kDa ductin monomer and the 29-kDa dimer were found in ovary homogenates as well as in preparations

Fig. 6. The rate of hatching larvae depends on the treatment of the embryos. *As controls, we used embryos that were either (1) untreated (but covered with oil), (2) dechorionated but not desiccated and not injected (dechor./not inj.), (3) dechorionated and desiccated but not injected (desicc./not inj.), (4) injected with bi-distilled water, (5) injected with NIS diluted 1:5, (6) injected with apAN2 diluted 1:50, or (7) injected with AD16 diluted 1:5. Following injections from dorsal of AN2 1:5, apAN2 1:10 and AD16, respectively, significantly less larvae hatched than in the controls, and the fractions of embryos that developed up to stage 16/17 with an analyzable cuticle (but did not hatch within 48 h) were significantly enlarged (S 16/17; P<0.05). n, number of injected embryos.*
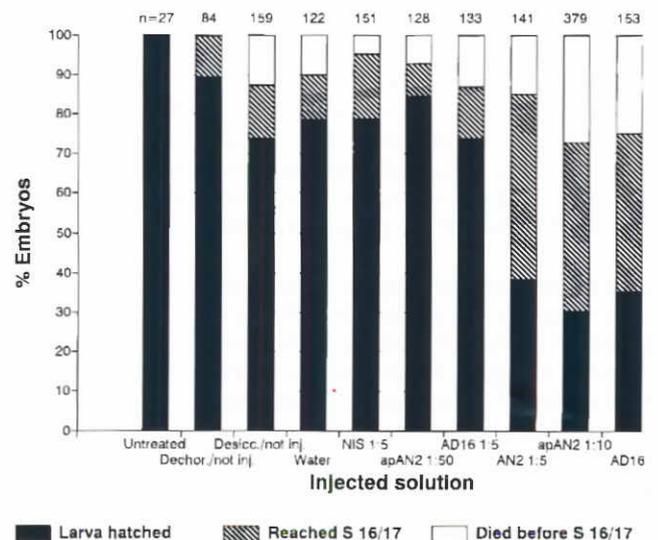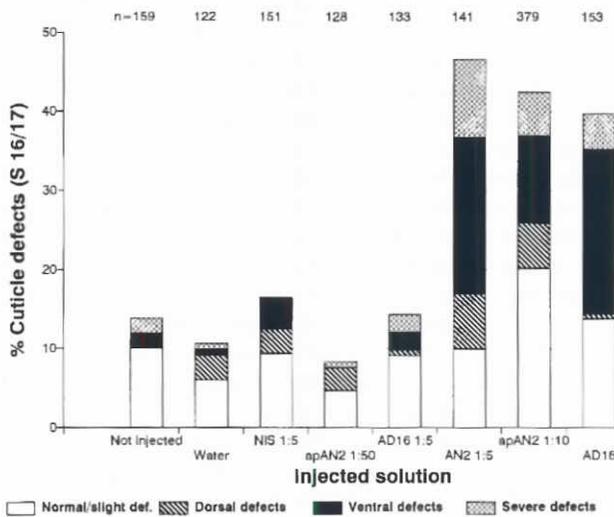
Fig. 7. Relative frequencies of cuticle defects (see Table 1) of non-hatching embryos (S 16/17), expressed as percentages of total embryos that were treated in a specific manner. *Following injections from dorsal of AN2 1:5, apAN2 1:10 and AD16 respectively, the fraction of cuticles showing ventral defects (black columns) was significantly larger than in the controls (P<0.05). Also, the frequency of severe cuticle defects (cross-hatched columns) depended significantly on the concentration of the ductin antibodies (P<0.05). n, number of injected embryos.*

of embryos and first instar larvae (Fig. 4B). AD16 did not recognize *Nephrops* ductin since the N-terminal sequence used as antigen, according to our database searches, is specific for *Drosophila* ductin. In the controls (BSA, NIS, f-AD16), no specific staining was observed (data not shown).

Distribution of microinjected solutions

During cell formation, cytoplasmic continuity still exists between the central yolk and the developing blastoderm of the *Drosophila* embryo. In order to evaluate the distribution of microinjected solutions in the cytoplasm, embryos of stage 5 (cellular blastoderm) were punctured at about 50% egg length on the dorsal side and either of two solutions was applied close to the developing cells on the embryos' ventral side. In one experimental series, we analyzed the distribution of RAM, a rhodamine-labeled rabbit-anti-mouse serum, using video-intensified fluorescence microscopy and a pseudocolor display of fluorescence intensity. This technique clearly revealed that RAM became unequally distributed in the cytoplasm: In most embryos, the highest concentration was found in the mid-ventral region (Fig. 5A-C).

To demonstrate that ductin antibodies became distributed in the same manner, we performed indirect immunofluorescence preparations of embryos that had been microinjected with the affinity-purified anti-ductin serum apAN2. These preparations showed that the antibodies remained concentrated close to the site of their ventral application during several developmental stages (Fig. 5D,E). Moreover, video-intensified fluorescence microscopy revealed a punctate staining pattern in the cytoplasm and at the cell membranes (Fig. 5F).

Rates of hatching larvae

Since gap junctions can first be detected in the early gastrula

(stage 6), it is tempting to assume that gap-junctional communication between blastoderm cells is required during gastrulation. We microinjected different sera into embryos prior to gastrulation in the same way as described above, by which the highest concentration was obtained in the cytoplasm close to the prospective ventral furrow.

Embryos that were microinjected during stage 1 rarely developed into hatching larvae. Moreover, embryos microinjected during stages 2-3 were often blocked earlier in development than embryos microinjected during later stages. Therefore, we predominantly used embryos of stages 4-5 (syncytial to cellular blastoderm).

Obviously, the rate of hatching larvae depended on the way that the embryos were treated (Fig. 6). As general controls, we used untreated embryos, dechorionated embryos, dechorionated and desiccated embryos, and embryos that were dechorionated, desiccated and injected with bi-distilled water. As a special control for the anti-ductin serum AN2 (diluted 1:5 with bi-distilled water), we used non-immune serum (NIS; diluted 1:5); as a special control
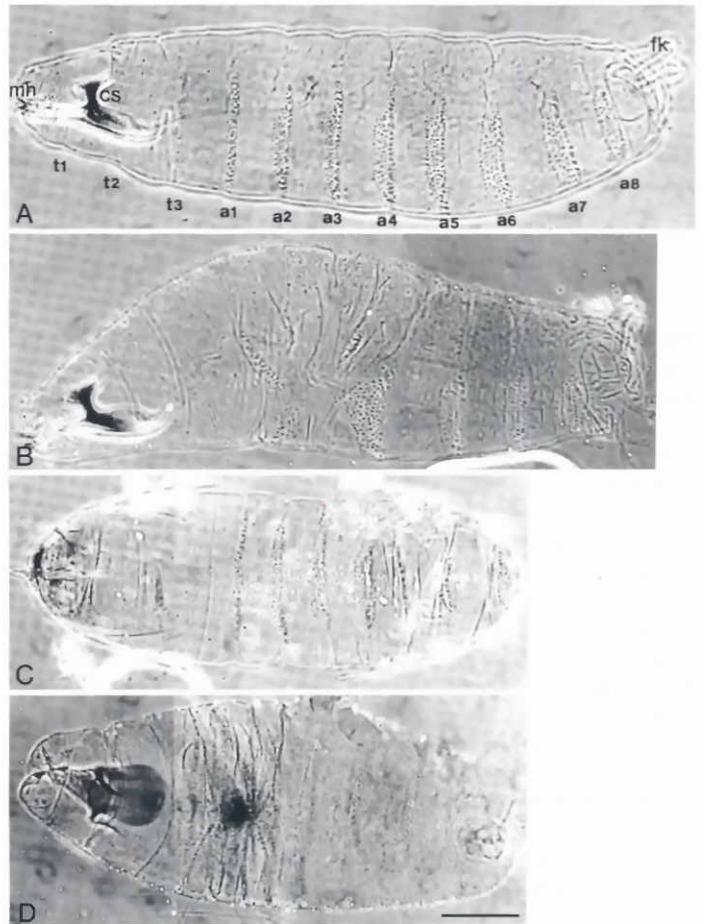
Fig. 8. Examples of normal and slightly defective cuticles (phase-contrast optics, anterior to the left, different solutions injected from dorsal). (A) *Normal cuticle, shown from ventro-lateral (t1-t3, thoracal segments; a1-a8, abdominal segments; cs, cephalopharyngeal skeleton; fk, filzkörper; mh, mouth hooks).* **(B)** *Cuticle with fused ventral denticle belts of abdominal segments a1-a5.* **(C)** *Cuticle with defective mouth hooks and cephalopharyngeal skeleton.* **(D)** *Cuticle, shown from dorsal, with healed wound at site of injection (dark area). Bar, 100 μm.*
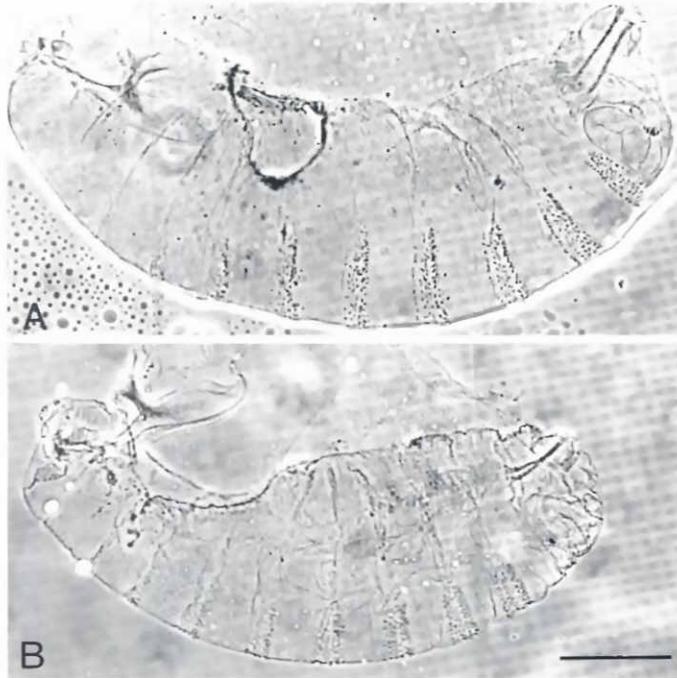
Fig. 9. Examples of cuticles with dorsal defects (phase-contrast optics, lateral view, anterior to the left, different solutions injected from dorsal). (A) *Small dorsal hole comprising abdominal segments a1-a3.* **(B)** *Large dorsal hole comprising the thorax and abdominal segments a1-a2. Bar, 100 μm.*

for the affinity-purified anti-ductin serum apAN2 (diluted 1:10), we used apAN2 diluted 1:50; and as a special control for the affinity-purified anti-ductin serum AD16, we used AD16 diluted 1:5 (Fig. 6).

Following the different control treatments, the rates of hatching larvae did not differ significantly. The same was true for the rates of embryos that died before cuticle formation (stage 16/17; Fig. 6, P>0.05). Compared with untreated control embryos, desiccation (and also dechorionation) was found to affect embryonic survival to a significant degree (P<0.05), whilst the microinjection procedure did not (P>0.05).

For AN2, apAN2 or AD16, the rate of hatching depended significantly on the antibody concentration (P<0.05). However, following injections of AN2 1:5, apAN2 1:10 and AD16, respectively, most of the embryos that did not hatch within 48 h developed up to stage 16/17 showing a differentiated cuticle (Fig. 6).
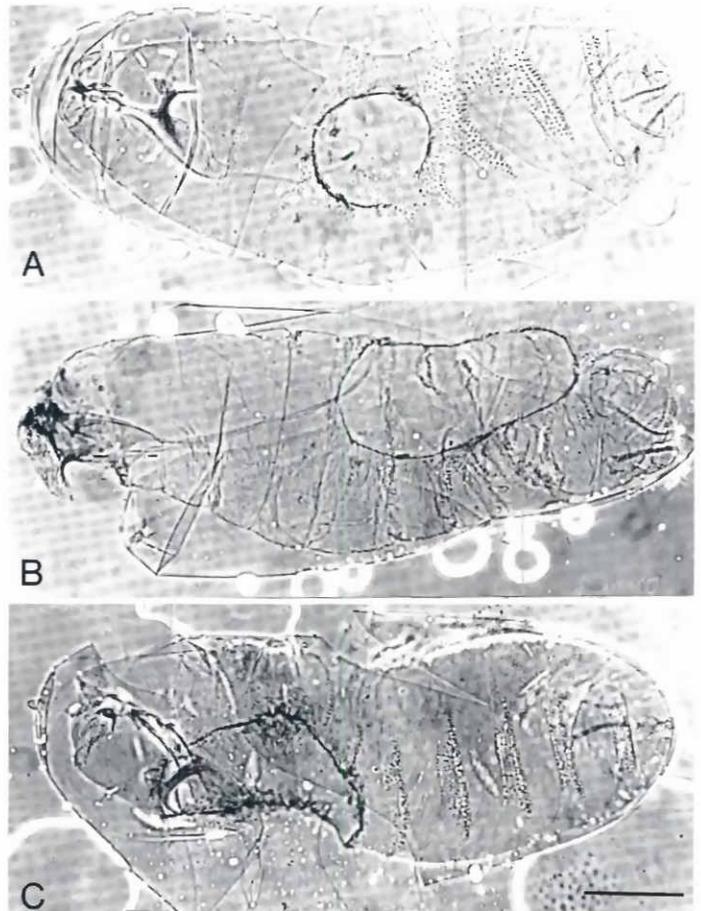
Cuticle defects

Cuticles of non-hatching stage-16/17 embryos that had been either not injected (but dechorionated and desiccated) or injected from dorsal with different solutions were analyzed for defects. The observed cuticle defects were classified according to Table 1. For quantitative analysis, the relative frequencies of cuticles showing specific defects were expressed as fractions of total embryos that had been treated in the same manner (Fig. 7).

Following all these treatments, a certain fraction of non-hatching embryos showed either normal cuticles or cuticles with slight defects (Table 1; Fig. 7). This fraction was the largest in embryos injected with apAN2 1:10 (with apAN2 1:5, most of the embryos died early), but the other treatments did not differ significantly in this

respect (P>0.05). Thus, by and large, the size of this fraction did not depend on the injected solution. The inability to hatch seemed to result from muscle defects (as indicated by impaired movements and fused segments) as well as from defects of the mouth hooks and the cephalopharyngeal skeleton. Some examples of cuticles with slight defects are shown in Figure 8.

Following microinjections of all solutions, a small fraction of cuticles showed dorsal defects that seemed to be due to deficient wound healing at the site of injection (Table 1; Fig. 7). While such defects appeared more frequently in embryos injected with AN2 1:5 or apAN2 1:10 than in control embryos (P<0.05), this was not the case in AD16-injected embryos. Dorsal defects might, in part, have resulted either from inexact placement of the serum during microinjection or from outflow of excess serum through the injection hole. Examples of dorsal cuticle defects are shown in Figure 9.

Compared to the controls, the fractions of cuticles showing ventral defects were considerably enlarged following injections of AN2 1:5, apAN2 1:10 and AD16, respectively (Table 1; Fig. 7, P<0.05). Thus, embryos injected from dorsal with a critical concentration of ductin antibodies specifically developed cuticles with either small or large ventral holes. Occasionally occurring

Fig. 10. Examples of cuticles with ventral defects (phase-contrast optics, ventral view, anterior to the left, different solutions injected from dorsal). (A) *Small ventral hole in the anterior abdomen.* **(B)** *Large ventral hole comprising most of the abdomen.* **(C)** *Large ventral hole comprising the thorax and abdominal segments a1-a3. Bar, 100 μm.*
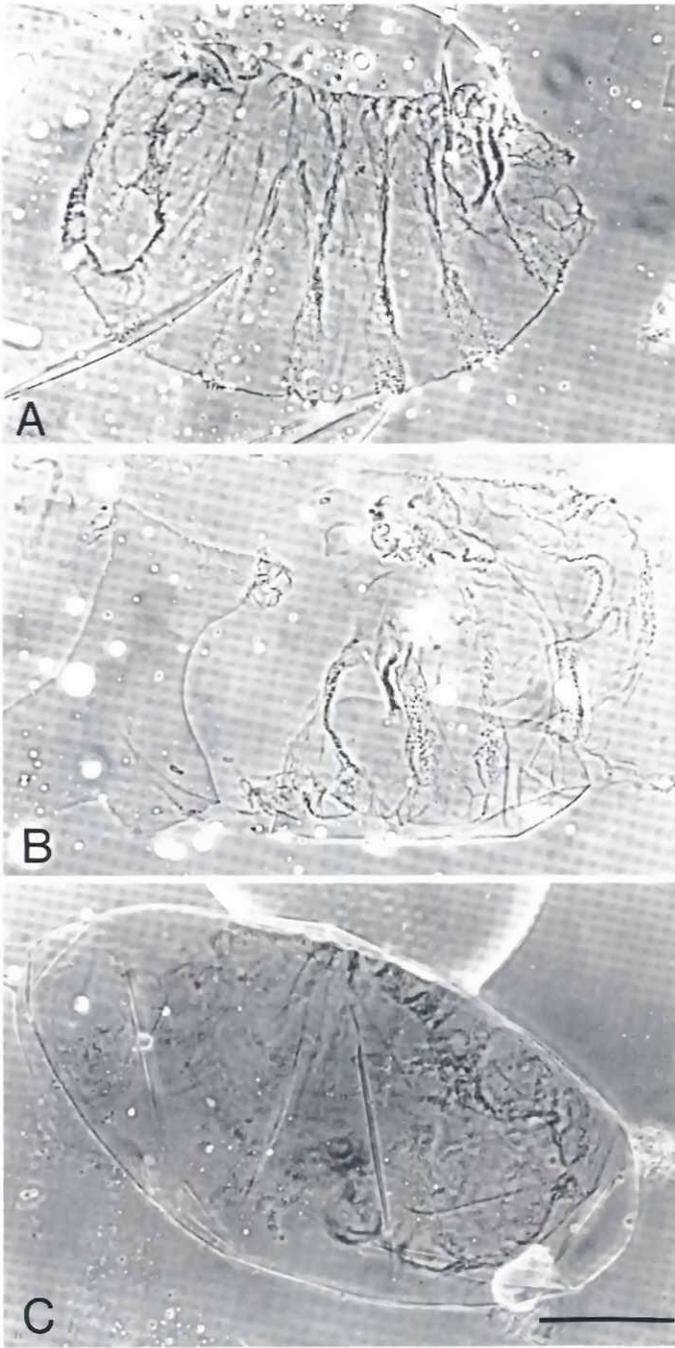
Fig. 11. Examples of cuticles with severe defects (phase-contrast optics, anterior to the left, different solutions injected from dorsal). (A) *Head, thorax and first abdominal segment totally absent.* (B) *Large regions of the cuticle are missing, only a few denticle belts are visible.* (C) *Cuticle only partially intact. Bar, 100 μm.*

ventro-lateral holes seemed to be due to slightly oblique injections. These results indicate that a strong correlation exists between the highest concentration of ductin antibodies and the site of specific cuticle defects. Examples of ventral cuticle defects are shown in Figure 10.

In addition, severe cuticle defects were more frequently observed following injections of AN2 1:5, apAN2 1:10 and AD16, respectively, than after the other treatments (Table 1; Fig. 7, P<0.05). Thus, also

this fraction of defects, where large parts of the cuticle were missing, depended on the concentration of the ductin antibodies in the embryos. Examples of severe cuticle defects are shown in Figure 11.

### Control injections into various regions

To confirm the notion that the predominant class of cuticle defects is always correlated to the region of highest antibody concentration, we further microinjected bi-distilled water or AN2 1:5 into embryos from either ventral (highest concentration in the mid-dorsal region), anterior (highest concentration at the anterior end) or posterior (highest concentration at the posterior end). The results were compared to those following microinjections from dorsal (highest concentration in the mid-ventral region). The regions of highest antibody concentration were inferred from corresponding microinjections of RAM as described above.

Irrespective of the site of injection, compared with water, the rates of hatching larvae were significantly reduced by injections of AN2 (Fig. 12, P<0.05). Of the embryos injected with AN2 from ventral, a fraction larger than that of the embryos injected from dorsal died before cuticle formation (P<0.05). Since gap junctions are present between amnioserosa cells (Tepass and Hartenstein, 1994), we presume that dorsal closure was affected by the antibody treatment. The anterior region of the embryo was more sensitive to the microinjection procedure than the other three tested regions: Even injections of water from anterior had significant adverse effects on development (P<0.05).

The frequencies of specific cuticle defects that were observed in non-hatching stage-16/17 embryos microinjected at different sites with either bi-distilled water or AN2 are summarized in Figure 13. The defects were classified according to Table 1. However, after injections from the anterior or posterior ends, the fractions of
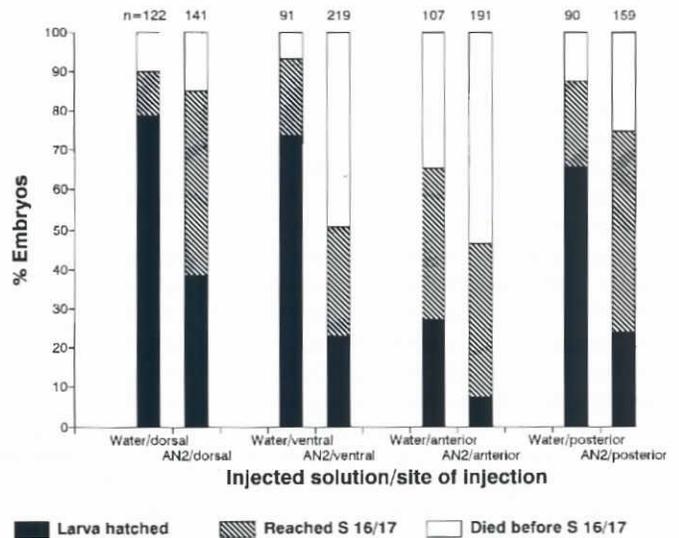
Fig. 12. The rate of hatching larvae depends on the site of injection and on the injected solution. *Compared to water-injected embryos, irrespective of the site of injection, the rates of hatching were significantly reduced by AN2 (P<0.05). Of the embryos injected with AN2 from ventral (highest antibody concentration in the mid-dorsal region), about 50% died before cuticle formation (S 16/17). The anterior region of the embryo appeared to be rather sensitive, even to injections of water. n, number of injected embryos.*
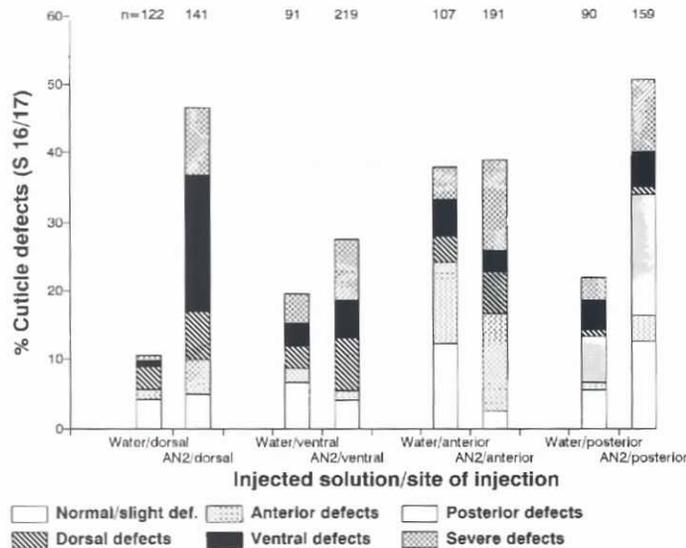
Fig. 13. Relative frequencies of cuticle defects of non-hatching embryos (S 16/17) that had been injected at different sites with either bi-distilled water or AN2 (expressed as percentages of total embryos that were treated in a specific manner). *As additional classes (cf. Table 1, Fig. 3), the fractions of anterior and posterior defects are displayed. In all cases, the frequencies of severe cuticle defects (cross-hatched columns) depended significantly on the presence of ductin antibodies (P<0.05). It is obvious that the fraction of cuticles showing specific ventral defects (black columns) was the largest (P<0.05) following injections of AN2 from dorsal (highest antibody concentration on the ventral side). n, number of injected embryos.*

anterior defects (anterior holes; defective mouth hooks or cephalopharyngeal skeleton) or posterior defects (posterior holes) were significantly larger than after the other treatments (P<0.05). Hence, compared with Table 1 and Figure 7, these cuticle defects are displayed in Figure 13 as separate classes.

Obviously, ventral cuticle defects occurred much more frequently following injections of AN2 from dorsal than following the other treatments (Fig. 13, P<0.05). Compared with dorsal injections, injections of AN2 from ventral did not significantly enlarge the fraction of dorsal defects (P>0.05) since many of these embryos died prior to stage 16/17.

Irrespective of the site of injection, with AN2 the fractions of severe cuticle defects were always significantly larger than with water (P<0.05). Especially, in embryos injected from anterior, the anterior half of the cuticle was often totally missing, whilst the corresponding result was found after injections from posterior. Less severe anterior defects (see above) occurred in almost equal amounts (P>0.05), following anterior injections of either water or AN2. However, for the less severe posterior defects, this was not true: Compared with water, this fraction was significantly enlarged following injections of AN2 from posterior (P<0.05).

Taken together, this comparison revealed two facts: (1) Concerning the microinjection procedure proper, the anterior region of the embryo is more sensitive than the other three analyzed regions. (2) The predominant class of cuticle defects is strongly correlated to the region of the highest antibody concentration. In particular, a high frequency of ventral defects clearly depends on a high concentration of ductin antibodies on the ventral side of the embryo.

Early developmental defects

Concerning the presumed involvement of gap-junction mediated cell-cell communication during gastrulation, we were predominantly interested in the ventral cuticle defects. To gain deeper insight in the origin of these defects, we analyzed living embryos using bright-field optics. Following injections from dorsal of AN2 1:5, apAN2 1:10 and AD16, respectively, a considerable number of embryos showed specific defects during gastrulation. Examples of such defects are shown in Figure 14. In most cases, the embryo's ventral region was affected, but sometimes also the dorsal region was impaired. In several cases, we observed rather severe defects where the anterior half of the embryo was degenerated or delayed by several developmental stages compared to the posterior half.

Using DAPI staining of embryos fixed during or after gastrulation (stages 6-7), we found that, following injections from dorsal with either of the anti-ductin sera, a considerable number of embryos showed defects in the middle region of the ventral furrow (Lämmel and Bohrmann, 1996). Obviously, in the region of highest antibody concentration, cell-cell communication was affected to such a
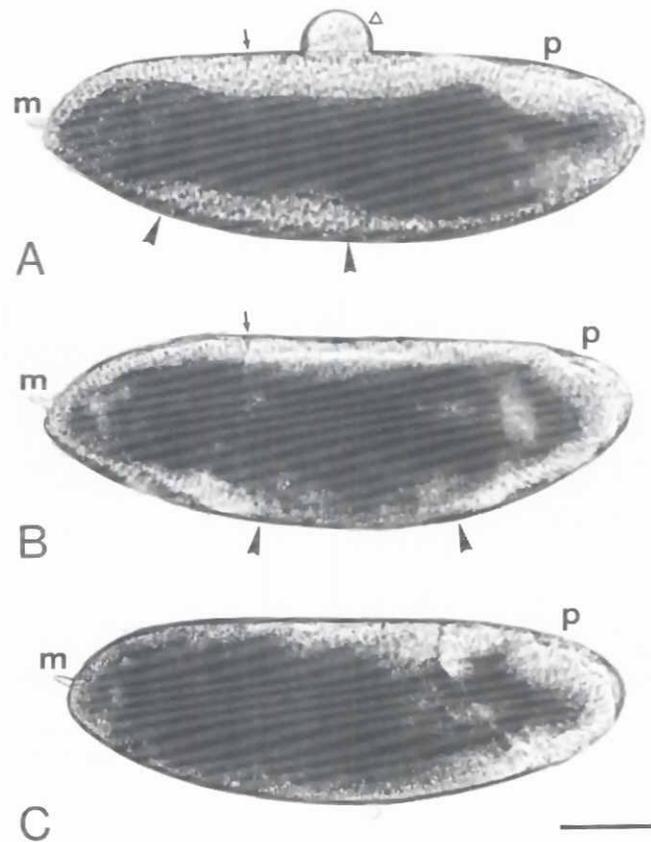
Fig. 14. Examples of early defects as observed in living embryos (stage 6/7, lateral view) injected with anti-ductin sera during stage 4/5 from dorsal (at top; anterior to the left; bright-field optics). (A) *Different phases of ventral furrow formation in the anterior half (between arrowheads) and posterior half.* (B) *Abnormal epithelium and disturbed gastrulation (between arrowheads).* (C) *Anterior half of the embryo is degenerated. m, Micropylar cone; p, pole cells; arrow, cephalic furrow; triangle, outflow of excess serum and cytoplasm through the injection hole. Bar, 100 μm.*
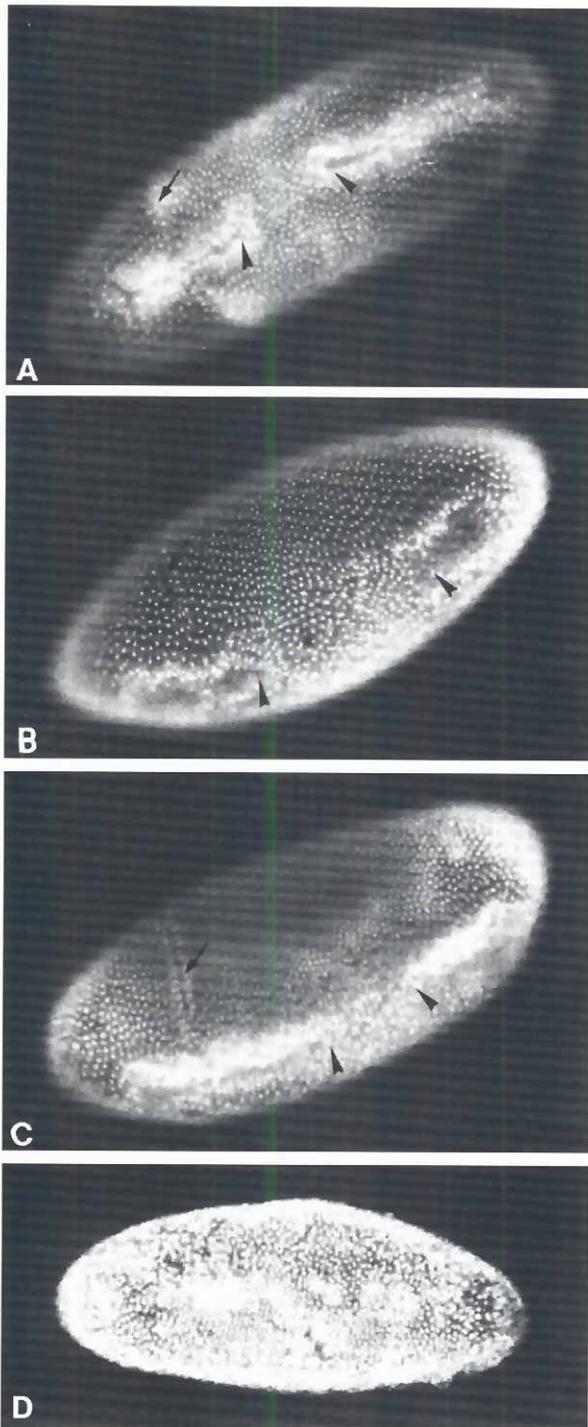
Fig. 15. Examples of defects in ventral furrow formation as observed using DAPI staining (stage-6/7 embryos; ventral or ventro-lateral views, anterior to the left; fluorescence optics). *The embryos were injected with anti-ductin sera from dorsal during stage 4/5 (highest antibody concentration in the mid-ventral region). As a consequence, variable amounts of prospective mesodermal cells failed to invaginate during gastrulation* (**A-C**, *between arrowheads). Such cells are not able to secrete a cuticle, which results in the observed ventral cuticle holes. Other embryos developed no ventral furrow at all and showed small holes between neighboring cells, an indication of impaired cell adhesion* (**D**). *The embryos are about 480 μm in length. Arrow, cephalic furrow.*

degree that invagination of prospective mesodermal cells could not take place correctly (Fig. 15A-C). This phenomenon seemed to result in a continuous superficial exposure of part of the mesoderm and, consequently, in a ventral area where no cuticle developed.

In several embryos, either the furrow showed minor irregularities or larger parts of the furrow did not form. In some cases, the ventral furrow was totally absent, and the embryos showed small holes between neighboring cells, most likely due to impaired cell adhesion (Fig. 15D). Presumably, such embryos would have developed severe cuticle defects, or they would have died before cuticle formation.

Discussion

Between gap junctions of arthropods and those of vertebrates, many structural and functional differences have been found (for a review, see Berdan, 1987). In general, different members of the large connexin family are supposed to form the connexon channels in gap junctions of different species and different tissues (e.g., Goodenough and Musil, 1993; Wolburg and Rohlmann, 1995). However, the search for connexin homologs in arthropods has been unsuccessful so far, and the results of several studies suggest that there may be limited, if any, homology between the genes of arthropod and vertebrate gap-junction proteins (reviewed in Ryerse, 1995a). On the other hand, the channel-forming 16-kDa protein ductin has been found in gap junctions of both arthropods and vertebrates. At least in arthropods, where it appears to be the predominant gap-junction protein, ductin is the best candidate for the connexon-forming component (John *et al.*, 1997; for reviews, see Finbow and Pitts, 1993; Finbow *et al.*, 1995).

Recently, Pietrantonio and Gill (1995,1997) have described an anti-peptide serum directed against the putative loop 2 of ductin from *Heliothis virescens*. Immunohistochemically, this antiserum binds in the midgut to regions of high V-ATPase activity as well as to areas of cell-cell contact, supporting the gap-junction involvement of ductin in this species. On immunoblots, besides a 17-kDa protein, this antiserum recognizes a 28-kDa protein and further proteins, presumed oligomers of ductin. Comparable aggregation patterns of ductin have been observed in previous studies (Finbow *et al.*, 1992,1994). According to our investigations, it is very likely that the dimer of 29 kDa is the maternal cytoplasmic precursor of ductin in the oocyte of *Drosophila* (Bohrmann, 1993; Bonafede *et al.*, 1995; Bohrmann and Braun, manuscript submitted).

Possibly, ductin is not the only connexon-forming protein in invertebrates: proteins of the OPUS family, that show no homologies with connexins or with ductin, also seem to be components of gap-junction structures (Phelan *et al.*, 1998; for reviews, see Barnes, 1994; Bryant, 1997). Moreover, Ryerse (1995b,c) has described antisera directed against 40-kDa candidate gap-junction proteins from *Drosophila* and *Heliothis virescens*. These antisera label gap junctions in cell fractions or in intact testis, but the proteins seem to have no homologies with connexins or with ductin. The antisera specifically recognize various proteins that seem to be either proteolytic fragments or oligomers of gap-junction proteins.

In order to elucidate the role that gap-junction mediated cell-cell communication plays during *Drosophila* embryogenesis, we tempted to specifically obstruct the embryonic connexon channels by microinjecting different anti-ductin sera. Injections into *Drosophila* ovarian follicles revealed that these antisera inhibit dye-coupling

between oocyte and follicle cells, which means that they are useful tools to block gap-junctional communication (Bohrmann, 1993; the present study). Moreover, *Drosophila* follicles microinjected with anti-ductin sera failed to complete oogenesis normally when cultivated either *in vivo* (in sterile females) or *in vitro* (in R-14 medium; our unpublished observations).

The structure and dual membrane orientation of ductin in gap junctions and V-ATPases has been described and discussed in several publications (Finbow *et al.*, 1992; Finbow and Pitts, 1993; Dunlop *et al.*, 1995). In V-ATPases, the C- and N-termini as well as the putative loop 2 are located either vacuolar or extracellular, whereas they are located on the cytoplasmic side of the membrane in gap junctions. The antisera AN2 and apAN2 are directed against ductin epitopes on the cytoplasmic side of gap junctions, and they have been found to bind to gap junctions from a variety of species (reviewed in Finbow and Pitts, 1993). Since the ductin N-terminus is largely species-specific (Dow *et al.*, 1992), in *Drosophila*, AN2 and apAN2 are likely to cross-react with epitopes located in the C-terminus or in loop 2.

The antiserum AD16 is directed against a nonapeptide at the N-terminus of *Drosophila* ductin. In *Drosophila*, ductin in gap junctions and V-ATPases appears to be the same polypeptide (Finbow *et al.*, 1994). Therefore, AD16 is presumed to bind to both gap junctions and V-ATPases. However, when microinjected into living cells, antibodies specific for epitopes in the C- or N-terminus or in loop 2 can only bind to ductin in its gap-junction orientation but not in its V-ATPase orientation.

According to our database searches, the epitope recognized by AD16 is specific for *Drosophila* ductin. Correspondingly, with tissue preparations from several other species, no specific reaction was obtained using AD16 (unpublished observations). Thus, our dye-coupling experiments confirm the cytoplasmic location of the N-terminus in *Drosophila* gap junctions, and they show that ductin plays a decisive role in gap-junctional communication in this species.

Taken together, there is strong evidence that the embryonic defects obtained in the present study using microinjections of AN2, apAN2 and AD16, respectively, were exclusively due to the blockade of gap-junctional communication, whilst the function of V-ATPases was not affected. Dow *et al.* (1997) have reported that homozygous defects of ductin are lethal for the third larval instar of *Drosophila*. In this case, it will be difficult to decide, whether the effect is due to defective gap junctions or V-ATPases.

Becker *et al.* (1995) have injected site-directed antisera raised against different connexins into mouse embryos and have found site-specific inhibition of dye-coupling as well as specific developmental defects. Similarly, Finbow *et al.* (1993) have injected antisera raised against an N-terminal peptide of mouse ductin into cells in culture and obtained inhibition of dye-coupling, which confirmed the cytoplasmic location of the ductin N-terminus in mammalian gap junctions. Although these anti-peptide sera were also found to recognize ductin in V-ATPases, they did not bind to intact bovine chromaffin granules (N-terminus of ductin vacuolar) and did not inhibit V-ATPase activity (Finbow *et al.*, 1993). In mice, there is evidence that ductin and connexins are components of similar or the same gap-junctional complexes (Finbow and Meagher, 1992).

The importance of gap junctions for specific cell adhesion and thus, for morphogenesis, has been demonstrated in various systems

(reviewed in De Haan, 1994). For example, in the mouse embryo, the early stages of compaction do not require gap-junctional communication but gap junctions are important for the maintenance of the compacted state and for subsequent development (Lee *et al.*, 1987). Similarly, delamination and extrusion of cells during gastrulation after expression of a dominant-negative connexin mutation indicates that gap-junctional communication is necessary for the maintenance of embryonic cell adhesion in *Xenopus* (Paul *et al.*, 1995). Although the results were somewhat different from those obtained with antibody blockade (Warner *et al.*, 1984), a loss of specific embryonal structures was observed in both studies.

In insects, there is evidence that cell-cell communication via gap junctions plays a role during pattern formation, for example in the epidermis (reviewed in Warner, 1992) and in the midgut (Baldwin *et al.*, 1993). It seems not very likely that the deficiencies observed in the present study result from the inability to establish or change morphogenetic gradients, since such gradients appear to be based upon molecules too large to pass through gap junctions. However, our results indicate that gap-junctional communication is involved in the coordination of morphogenetic movements.

The first gap-junction proteins in the embryo are obviously maternal products. During *Drosophila* oogenesis, ductin is synthesized in the nurse cells and then becomes stored in the egg, predominantly as a dimer (Bohrmann, 1993; Bonafede *et al.*, 1995). In the *Xenopus* egg, a comparable maternal connexin precursor was observed (Warner *et al.*, 1984). Recently, it has been found that cytoplasmic oligomers of ductin are assembled and stored in the ER (Dunlop *et al.*, 1995).

Our immunocytochemical analysis suggests that gap-junctional precursors appear in the cell membranes of the early gastrula in a diffuse distribution at first. This corresponds with electronmicroscopic results (Eichenberger-Glinz, 1979): in early embryonic stages, incipient gap junctions are small and individual gap-junction particles are distributed throughout the membranes. On the other hand, in our preparations, besides gap-junction particles also V-ATPases might have become labeled.

The described microinjection experiments strongly indicate that gap junctions are important during gastrulation. In the region of the highest antibody concentration (i.e., in the middle of the embryo's ventral side), prospective mesodermal cells failed to invaginate and the exposed cells did not secret a cuticle. Moreover, this impaired gastrulation is likely to result in various defects in mesodermally derived tissues. We conclude that, during *Drosophila* embryogenesis, gap-junctional communication is required to maintain epithelial integrity and cell adhesion which is a precondition for gastrulation and other morphogenetic events.

Materials and Methods

Preparation of follicles and microinjection procedure

Drosophila melanogaster Oregon R females were reared and dissected when 2-3 days old as described previously (Bohrmann, 1993; Bohrmann and Haas-Assenbaum, 1993). Stage-10 follicles, in which the oocyte occupies about 1/2 of the follicle's volume (for stages, see King, 1970), were carefully isolated and immediately transferred to the microinjection chamber. The follicles were incubated in R-14 medium (Robb, 1969; Bohrmann, 1991) from about 5 min prior to about 5 min after microinjection.

The microinjection procedure has been described elsewhere (Bohrmann, 1997; Bohrmann and Schill, 1997). In short, micropipettes were pulled from 1-mm glass capillaries containing a filament, and microinjections were

carried out on an inverted epifluorescence microscope (Zeiss Axiovert). The injection pipette (tip diameter 1-2 μm) was mounted on a motorized micromanipulator equipped with a piezo translator (Märzhäuser PM 20), and coupled to a microinjector (Eppendorf 5242). The recipient follicle was held in place with a suction pipette (tip diameter 30-50 μm) mounted on a second micromanipulator, and coupled to a screw-adjustable syringe. The volume injected into each oocyte (using a pressure of about 300 hPa) was in the order of 100 pl, which is equivalent to about 5% of the oocyte volume (Bohrmann and Sander, 1987).

Solutions microinjected into follicles

The following solutions were microinjected: (1) the fluorescent tracer Lucifer Yellow CH [LY; 1% solution (w/v) in bi-distilled water], (2) a whole antiserum raised against the 16-kDa membrane-channel protein ductin from *Drosophila melanogaster* (w-AD16; mixed 1:1 with 2% LY-solution), (3) the affinity-purified anti-ductin serum (AD16; antibody concentration 150 μg/ml; mixed 1:1 with 2% LY-solution), (4) the flow-through fraction of the affinity purification (f-AD16; mixed 1:1 with 2% LY-solution), and (5) a monoclonal antibody raised against the *Manduca sexta* V-ATPase (mAB 224-3; antibody concentration 1.1 mg/ml; mixed 1:1 with 2% LY-solution).

The mAB 224-3 (Klein *et al.*, 1991; Jäger *et al.*, 1996) was kindly provided by Ulla Klein (München, Germany); w-AD16, AD16 and f-AD16 were obtained from nanoTools (Denzlingen, Germany). The w-AD16 was raised in the chick against the nonapeptide Met-Ser-Ser-Glu-Val-Ser-Ser-Asp-Asn (-Cys) from the N-terminus of *Drosophila* ductin. For the sequence of the ductin gene *Vha16*, see Meagher *et al.* (1990) and Finbow *et al.* (1994). Affinity-purification of the whole serum (w-AD16) on a peptide-affinity column resulted in the purified antibody-fraction (AD16) and in the antibody-deprived flow-through fraction (f-AD16).

Video-intensified fluorescence microscopy

Our low-light-level video system consisted of a SIT camera (Hamamatsu C-2400), a digital image processor (Hamamatsu Argus-10), a time-lapse recorder, a monochrome monitor, and a color monitor (for details, see Bohrmann, 1997; Bohrmann and Schill, 1997). With this system, it was possible to minimize photobleaching, and to detect rather weak levels of fluorescence intensity. Video prints were produced on a video-copy processor (Mitsubishi P66E). Statistical significance of differences between relative frequencies of dye-coupling was established at the $\alpha=0.05$ level using the χ^2 test (Sachs, 1978).

Indirect immunofluorescence preparations of embryos

For the immunostaining of cryosections, embryos of various stages (see Campos-Ortega and Hartenstein, 1985) were dechorinated with diluted bleach, fixed in formaldehyde/PBS/heptane, embedded in a cryosectioning medium according to Hartmann (1984) and frozen in dry ice/acetone. Sections of 10 μm were cut on a cryotome, collected on glycerol/gelatine-coated slides, incubated for 5 min each in 0.01% Tween-20/PBS and 50 mM NH₄Cl/PBS, and blocked for 30 min at 37°C with 5% skimmed milk powder/PBS. The sections were then incubated overnight at 4°C in one of the following solutions: (1) 0.5% BSA/PBS (control), (2) a rabbit non-immune serum (NIS; diluted 1:200 with 0.5% BSA/PBS; control), (3) a rabbit antiserum raised against gap-junctional preparations from the lobster *Nephrops norvegicus* (AN2; diluted 1:200), (4) AN2 affinity-purified against the 16-kDa membrane-channel protein ductin from *Nephrops* (apAN2; diluted 1:200), and (5) AD16 (diluted 1:20), respectively. With these dilutions, the best results were obtained. NIS, AN2 and apAN2 (Buultjens *et al.*, 1988; Finbow *et al.*, 1988; Leitch and Finbow, 1990) were kindly provided by Malcolm Finbow (Glasgow, UK). Following incubations in the appropriate biotinylated goat anti-rabbit or rabbit anti-chicken antibodies (Dianova, Hamburg, Germany), streptavidin-Texas Red and DAPI were applied as described previously (Bohrmann, 1993). The preparations were mounted in 0.5% propylgallate/0.5 M Tris pH 9 to reduce photobleaching and photographed on 400 ISO film (Kodak T-Max) using a Zeiss epifluorescence microscope.

For the immunostaining of whole-mounts, embryos of various stages were dechorionated, fixed in formaldehyde/PBS/heptane and the vitelline envelopes removed using methanol (e.g. Miller, 1995). Thereafter, the embryos were immunostained, mounted and photographed as described above.

Immunoblotting

Immunoblots were performed as described previously (Bohrmann, 1993). In short, proteins from homogenates of *Drosophila* ovaries, embryos and first instar larvae as well as from *Nephrops* gap-junction preparations (kindly provided by M. Finbow) were separated using 15% SDS-PAGE and transferred to nitrocellulose membranes. Nonspecific binding sites were blocked and the blots incubated in 1% BSA/PBS (control), in NIS (diluted 1:200; control), in f-AD16 (diluted 1:100; control), in AN2 (diluted 1:200), in apAN2 (diluted 1:200), and in AD16 (diluted 1:50), respectively. With these dilutions, the best results were obtained. Subsequently, the appropriate biotinylated secondary antibodies (see above), streptavidin-peroxidase and H₂O₂/chloronaphthol were applied, and photographs were taken on 12 ISO film (Agfaortho).

Preparation of embryos and microinjection procedure

Embryos were collected, washed, dechorionated with diluted bleach and arranged in rows on sticky coverslips using standard methods (e.g., Santamaria, 1986; Spradling, 1986). After slight desiccation for 20 min using a stream of air, the embryos were covered with Voltalef 10S oil and transferred to a microinjection set-up, consisting of a Leitz Laborlux microscope, a micromanipulator and an Eppendorf microinjector.

The micropipettes (tip diameter 3-6 μm) were filled with one of the following solutions: (1) bi-distilled water, (2) NIS (diluted 1:5 with bi-distilled water), (3) AN2 (diluted 1:5), (4) apAN2 (diluted 1:50 or 1:10), and (5) AD16 (diluted 1:5 or undiluted). For each solution, several concentrations had been tested before.

Embryos of stages 2 to 5 (early cleavage to cellular blastoderm) were punctured at about 50% egg length on the dorsal side, and the solutions were applied close to the embryo's ventral side. The volume injected into each embryo was in the order of 250 pl, i.e. 3-4% of the egg volume. Further embryos were microinjected either from ventral, from anterior or from posterior, using AN2 1:5 and water, respectively.

In order to evaluate the distribution in the cytoplasm, embryos were microinjected with a rhodamine-labeled rabbit-anti-mouse serum (RAM, Dianova) and observed using video-intensified fluorescence microscopy (see above). Pseudocolor prints of fluorescence intensity were produced using the digital image processor, a personal computer equipped with a video-frame grabber (Fast Screen Machine) and a color printer (HP DeskJet 850). We also analyzed the cytoplasmic distribution of ductin antibodies by fixing and immunostaining embryos at various stages after microinjections of apAN2 1:10.

Analysis of defects following microinjections

The embryos were allowed to develop for at least 48 h and cuticles of non-hatching embryos of stages 16/17 (end of embryogenesis) were prepared using Hoyers medium with lactic acid (e.g., Wieschaus and Nüsslein-Volhard, 1986). The cuticle preparations were analyzed using a Zeiss microscope equipped with phase-contrast optics; photographs were taken on 25 ISO film (Agfapan APX). Following the different treatments, statistical significances of differences between (1) rates of hatching larvae, and (2) relative frequencies of specific cuticle defects, were established at the $\alpha=0.05$ level using the χ^2 test.

Using bright-field optics, the development of microinjected embryos was observed, and photographs were taken on 25 ISO film. After having reached stages 6-7 (i.e., after gastrulation), the embryos were fixed in formaldehyde/PBS/heptane, individually removed from their vitelline envelopes and the nuclei stained with DAPI. Photographs were taken on 400 ISO film using an epifluorescence microscope.

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