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Embryonic origin and differentiation of the *Drosophila* heart

Astrid Rugendorff, Amelia Younossi-Hartenstein, Volker Hartenstein

Department of Biology, University of California Los Angeles, Los Angeles, CA 90024, United States of America

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Abstract. We have followed the normal development of the different cell types associated with the *Drosophila* dorsal vessel, i.e. cardioblasts, pericardial cells, alary muscles, lymph gland and ring gland, by using several tissue-specific markers and transmission electron microscopy. Precursors of pericardial cells and cardioblasts split as two longitudinal rows of cells from the lateral mesoderm of segments T2-A7 ("cardiogenic region") during stage 12. The lymph gland and dorsal part of the ring gland (corpus allatum) originate from clusters of lateral mesodermal cells located in T3 and T1/dorsal ridge, respectively. Cardioblast precursors are strictly segmentally organized; each of T2–A6 gives rise to six cardioblasts. While moving dorsally during the stages leading up to dorsal closure, cardioblast precursors become flattened, polarized cells aligned in a regular longitudinal row. At dorsal closure, the leading edges of the cardioblast precursors meet their contralateral counterparts. The lumen of the dorsal vessel is formed when the trailing edges of the cardioblast precursors of either side bend around and contact each other. The amnioserosa invaginates during dorsal closure and is transiently attached to the cardioblasts; however, it does not contribute to the cells associated with the dorsal vessel and degenerates during late embryogenesis. We describe ultrastructural characteristics of cardioblast differentiation and discuss similarities between cardioblast development and capillary differentiation in vertebrates.

Key words: Heart – *Drosophila* – Morphogenesis

Introduction

Recent studies of *Drosophila* embryogenesis have provided important insights into the mechanisms controlling determination and differentiation of the various ectodermally derived cell types, such as epidermis or the nervous system. One general principle that emerges is that ectoderm cells seem to become committed to a particular fate early in development (around or even prior to gastrulation) and that local cell-cell interactions among ectoderm cells play an important role in this process (for a recent review see Ingham and Martinez-Arias 1992). Much less is known about how the mesoderm and the different organs originating from this tissue develop. It appears that mesoderm cells remain uncommitted longer than ectoderm cells. Thus, individual labelled mesoderm cells were transplanted into an unlabelled host at the early gastrula stage, where they produced lineages which comprise more than one cell type (e.g. visceral and somatic muscle) and which cross segment boundaries (i.e. one mesodermal precursor contributes to somatic muscles which may be found in as many as three adjacent segments; Beer et al. 1987).

Genes which play a role in the specification of ectodermal cell types are also expressed and required in the mesoderm. For example, embryos carrying a mutation in one of the neurogenic genes, which control the ratio of epidermal versus neuronal precursors in the ectoderm, also show strong defects in many mesodermal tissues (Corbin et al. 1991; Hartenstein et al. 1992). This finding suggests that the same cellular and molecular mechanisms involved in controlling the early development of the different ectodermal derivatives may also occur in the mesoderm.

One of the structures affected strongly by loss of function of neurogenic genes is the dorsal vessel (Hartenstein et al. 1992). The dorsal vessel is one of the simplest organs found in the *Drosophila* embryo (Poulson 1950; Campos-Ortega and Hartenstein 1985). Associated with it are three types of cell, the cardioblasts, pericardial cells and alary muscles. Cardioblasts form a simple tube which is flanked on either side by a row of pericardial cells. Alary muscles connect the dorsal vessel to the epidermis. In embryos carrying a mutation in any one of the neurogenic genes, the number of dorsal vessel cells is increased; furthermore, all cells of the dorsal vessel express the same, cardioblast-like phenotype. The car-

Correspondence to: V. Hartenstein

dioblast-like cells do not form a tube with a continuous lumen, but are arranged in an irregular, elongated cluster in which multiple small, disconnected clefts appear.

As a basis to further investigate the role of neurogenic and other genes during formation of the dorsal vessel we have undertaken a developmental analysis of this organ. In this paper we present a combined light and electron microscopic study of normal development of the *Drosophila* dorsal vessel. Details regarding the exact origin of the cardioblasts, their dorsal migration and their further differentiation into endothelial cells are described. Of particular interest were the questions: what are the cell contacts formed between cardioblasts, as well as between these cells and adjacent tissues; when do cardioblasts undergo the transition from apolar, undifferentiated mesoderm cells to polarized endothelial cells; how is the lumen of the dorsal vessel formed; and what is the relationship between the amnioserosa (which during dorsal closure is close to the developing heart) and the cardioblasts?

Materials and methods

Markers used in the study of the dorsal vessel. A number of different antibody markers and P*lac*Z insertions were used to label the cells of the dorsal vessel:

1. Polyclonal antibody against muscle myosin (Kiehart and Feghali 1986), which labels somatic muscles, visceral muscles and cardioblasts.
2. Polyclonal antibody against laminin (Fessler et al. 1987), which labels basement membranes.
3. P*lac*Z insertion B2-3-20 (Bier et al. 1989; Hartenstein and Jan 1992), expressed in cardioblasts.
4. P*lac*Z insertion E7-3-63 (Bier et al. 1989; Hartenstein and Jan 1992), expressed in pericardial cells and (at a lower level) cardioblasts.
5. P*lac*Z insertion E2-3-9 (Bier et al. 1989; Hartenstein and Jan 1992), expressed in a subset of cardioblasts.
6. Rhodamine-phalloidine (Molecular Probes) to label microfilaments.

Fly stocks. Flies were cultured on standard yeast-cornmeal-molasses agar medium. Oregon R was used as the wild-type stock.

Antibody labelling. Embryos were collected, dechorionated, and fixed for 30 min in a mixture of 4% formaldehyde in PEMS (0.1 M Pipes, 2 mM $MgSO_4$, 1 mM EGTA, pH 7.0) with heptane. Next they were devitellinized in methanol. After several washes in 0.1 M phosphate buffered saline (PBS) containing 0.1% triton X-100 (PBT), embryos were incubated for 30 min in 0.1 M PBS containing 10% goat serum, 0.1% triton X-100 (PBT + N). Antibodies against muscle myosin (kindly provided by Dr. D. Kiehart and used at a 1:200 dilution), laminin (kindly provided by Dr. L. Fessler; 1:50 dilution), or anti-*b*-galactosidase (Cappel; 1:200 dilution) were then added to the embryos. Incubation was overnight at 4° C. After several washes in PBT, embryos were incubated at room temperature for 2 h in secondary antibody (biotinylated rabbit anti-mouse IgG; Vectastain ABC kit, Vector Labs) diluted at 1:200 in PBT + N. Preparations were washed several times in PBT, then incubated for 2 h at 25° C in a solution of horseradish peroxidase conjugated biotin and avidin (AB solution; Vectastain ABC kit). The embryos were washed twice in PBT and then incubated in a 0.5 mg/ml diaminobenzidine (DAB; Sigma) solution in PBT. After 10 min, 10 ml of 7% hydrogen peroxide was added. The reaction was stopped after 5–10 min by diluting the substrate with 0.1 M phosphate buffer. Preparations were dehydrated in graded ethanol (70%, 90% 95%, 5 min each; 100%, 15 min) and acetone (5 min) and left overnight in a mixture of Epon and acetone (1:1). They were then mounted in a drop of fresh Epon and coverslipped.

Dissections of embryos to produce filet preparations for the rhodamine-phalloidine stains were carried out according to Ashburner (1989). Preparations were incubated in rhodamine-phalloidine (5%, in *Drosophila* Ringer solution), washed several times in PBT, and mounted in 80% glycerol.

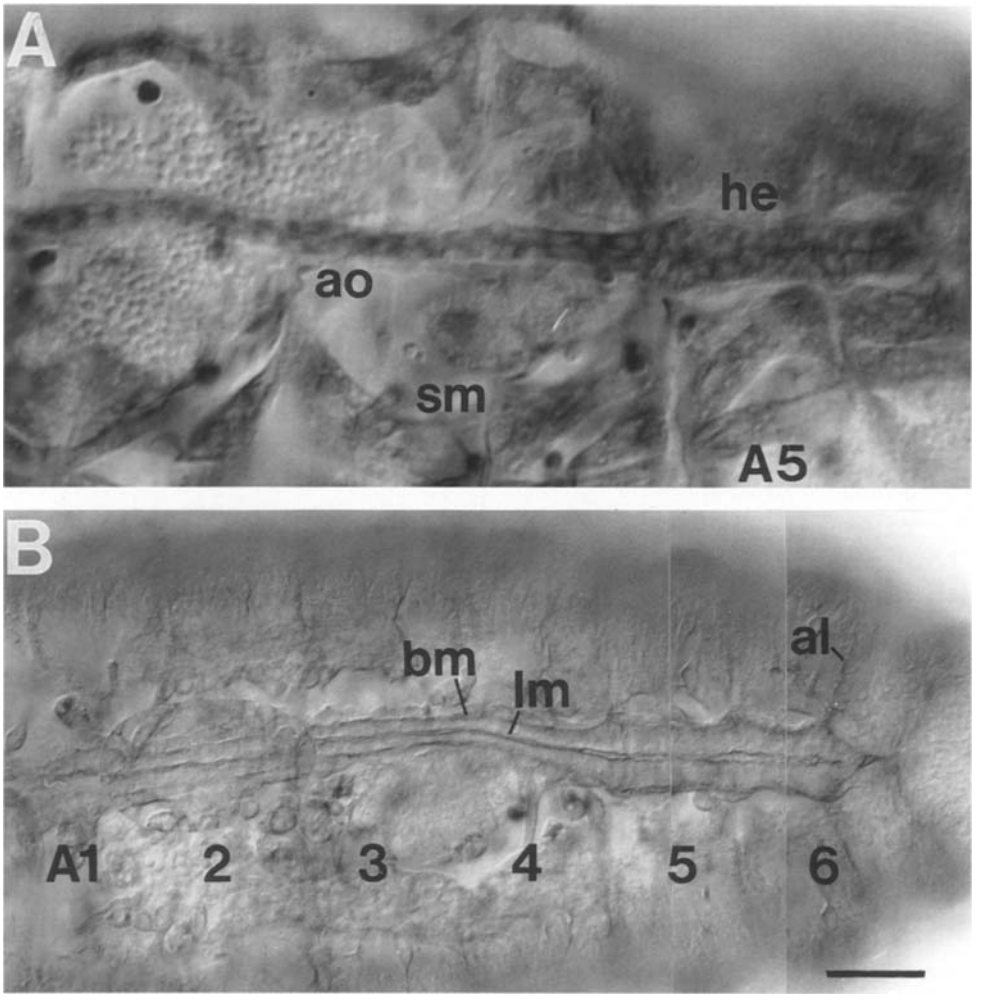
Electron microscopy. Embryos were dechorionated, fixed in 12.5% glutaraldehyde in PBS and heptane for 20 min, then placed on double sided tape and devitellinized by hand. Larvae were dissected (cut into halves) and fixed in 2% glutaraldehyde in PBS for 20 min. Both embryos and larva were postfixed for 30 min in a mixture of 1% osmium tetroxide and 2% glutaraldehyde in 0.15 M cacodylate buffer (on ice). Specimens were washed several times in PBS and dehydrated in graded ethanol and acetone (all steps on ice). Preparations were left overnight in a 1:1 mixture of Epon and acetone and then for 5–10 h in unpolymerized Epon. They were transferred to moulds, oriented, and placed at 60° C for 24 h to permit polymerization of the Epon. Blocks were sectioned (0.1 mm), and sections mounted on net grids (Ted Pella) and treated with uranyl acetate and lead citrate.

Results

Structure and position of the dorsal vessel of the mature embryo

The structure and position of the dorsal vessel of a stage 17 embryo is depicted in Fig. 1. The dorsal vessel forms a slender, longitudinally oriented tube located mid-dorsally beneath the epidermis. Anteriorly, it abuts the supraesophageal commissure; posteriorly, it terminates near the A6/A7 boundary. The dorsal vessel is suspended from the body wall by segmentally arranged pairs of filamentous muscle fibres called alary muscles. The first pair of alary muscles inserts at the T3/A1 apodeme; the last (7th) pair inserts at the A6/A7 apodeme. Two caudally directed muscles connect the posterior tip of the dorsal vessel to a middorsal attachment site in A8. The posterior two segments of the dorsal vessel (A5 and A6) are larger in external and internal diameter than the anterior part of the dorsal vessel. Based on the nomenclature used for the larval heart (Rizki 1978), the wide, posterior part of the dorsal vessel is referred to as "heart", whereas the anterior dorsal vessel is called "aorta".

The wall of the dorsal vessel is formed by a double row of contractile myoendothelial cells enclosing a central lumen. These cells during embryonic stages are called cardioblasts; for the mature structure (i.e. larval and adult dorsal vessel), the term "myocardial cell" is commonly used. On either side of the dorsal vessel there is a loose row of pericardial cells. Pericardial cells are large, non-polarized cells involved in ultrafiltration and excretion of haemolymph (for review see Crossley 1985). The anterior segment of the dorsal vessel (rostral of the first pair of alary muscles) does not have pericardial cells. Instead, there is a pair of so called lymph glands (Poulson 1950) and the ring gland, a complex endocrine organ which encircles the anterior tip of the dorsal vessel.



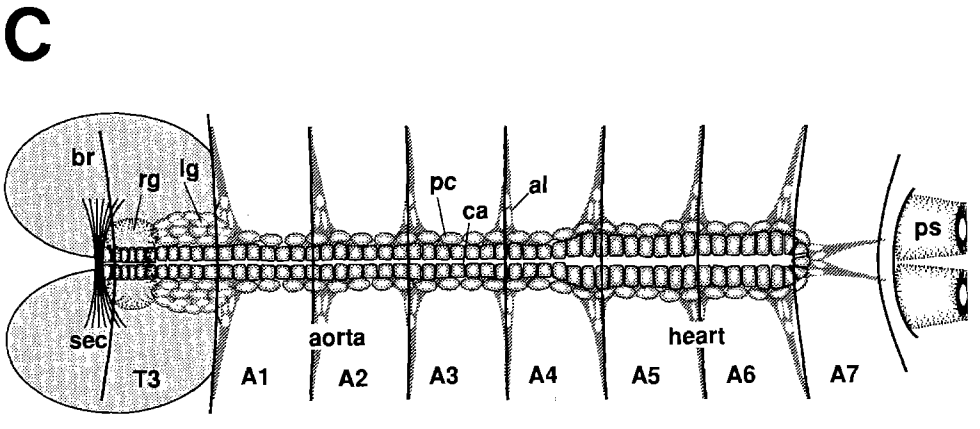


Fig. 1A–C. Structure of the dorsal vessel in the late embryo. **A**, **B** Dorsal views of stage 17 embryos labelled with anti-muscle myosin antibody (**A**) and anti-laminin antibody (**B**). **C** Schematic drawing of the dorsal vessel and associated tissues. In all panels, anterior is to the *left*. The myosin antibody (**A**) diffusely stains the cytoplasm of the cardioblasts. Note the marked difference in diameter of the dorsal vessel comparing its posterior part (the heart; *he*) with its anterior part (aorta; *ao*). Anti-laminin antibody (**B**) stains the basement membrane (*bm*) and the luminal membrane (*lm*) of the dorsal vessel. Also, the basement membrane surrounding the alary muscles (*al*) is labelled. The diagram (**C**) depicts the position of the dorsal vessel and different cell types associated with it. *A1–6*, Abdominal segments 1–6; *br*, brain; *ca*, cardioblasts; *lg*, lymph gland; *pc*, pericardial cells; *ps*, posterior spiracle; *rg*, ring gland; *sec*, supra-esophageal commissure; *T3*, thoracic segment 3

For the larval dorsal vessel, segmentally distributed lateral openings (ostia) have been described through which the haemolymph can enter the lumen (Rizki 1978). These openings are apparently not yet present in the late embryo, as demonstrated by the fact that the extracellular matrix which covers the luminal surface of the cardioblasts ("luminal membrane", see later) forms a closed inner lining of the dorsal vessel.

Mesodermal origin and metameric organization of the heart precursors

Taking advantage of several enhancer trap lines expressed in the precursor cells of the dorsal vessel, their origin and migration has been followed. The results are summarized in Figs. 2 and 3. The precursors of both cardioblasts and pericardial cells (which will be referred to as "heart precursors" in the following) split as two irregular rows of cells from the lateral mesoderm during

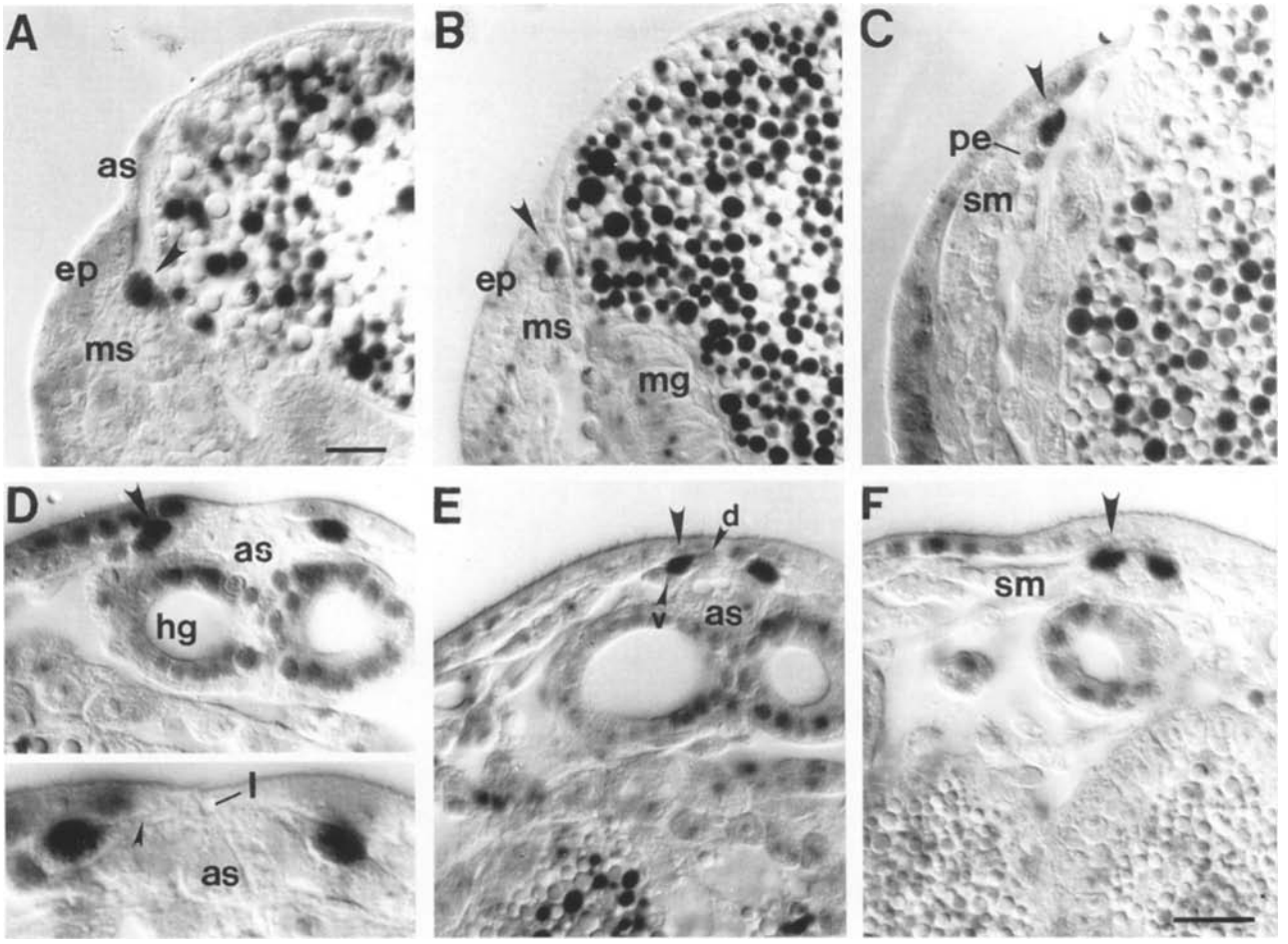


Fig. 2 A–F. Migration of heart precursors during embryonic development, showing portions of transverse sections of embryos at different stages; cardioblasts (*arrowhead*) were labelled with the P*lacZ* insertion B2-3-20. **A** Stage 12 embryo. Cardioblasts form in the lateralmost extension of the mesoderm (*ms*). **B** Stage 13 embryo. Cardioblasts (*arrowhead*) have migrated along with the overlying epidermal primordium. **C** Stage 14 embryo. Cardioblasts and pericardial cells (*pe*; also express *lacZ* at a low level) have separated from the adjacent myoblasts of the dorsal musculature (*sm*). **D** Stage 15 embryo. Shortly before dorsal closure, the amnioserosa (*as*) invaginates and transiently forms a tube with a central lumen (*l*). Cardioblasts have slender leading processes which intercalate inbetween the epidermis and amnioserosa cells (*arrowhead* in lower panel of **D**). **E** Stage 16 embryo. After dorsal closure, the dorsal, leading processes of cardioblasts (*d*) meet in the dorsal midline. The amnioserosa cells (*as*) are still attached to the cardioblasts and will degenerate shortly thereafter. The ventral, trailing processes of the cardioblasts (*v*) then curl around and form the lumen of the dorsal vessel. **F** Stage 17 embryo, after morphogenesis of the dorsal vessel is complete. *Bars*, 10 μm

stage 12. The lateral mesoderm of the stage 12 embryo consists of two layers: an inner layer (splanchnopleura or visceral mesoderm) which gives rise to the visceral musculature, and an outer layer (somatopleura or somatic mesoderm) which will form the lateral and dorsal musculature. The heart precursors originate from within a region located where somatopleura and splanchnopleura merge laterally. We have suggested the term "cardiogenic region" for this lateral part of the mesoderm (Hartenstein et al. 1992).

The expression pattern of the P*lacZ* insertion E2-3-9 indicates that the heart precursors are segmentally organized from stage 12 onward. In mature embryos, the E2-3-9 insertion is expressed strongly in segmentally repeated pairs of cardioblasts which alternate with stretches of four unlabelled cells (Fig. 4); expression at a much lower level is also found in 1–2 pericardial cells adjacent to the labelled cardioblasts. The E2-3-9 expressing cardioblasts differ from the other cardioblasts in two aspects. First, these cells are the first cardioblasts contacted by the alary muscles. Secondly, the E2-3-9 positive cells retain a compact, rounded morphology throughout the larval stages, whereas the other cardioblasts become extremely large and flattened. It is possible that the ostia, segmentally arranged openings in the larval and adult heart wall, develop in spatial relationship with the E2-3-9 positive cardioblasts.

Since E2-3-9 is expressed from stage 12 onward, it is possible to assign metameric complements of cardioblasts ("cardiomeres") to the individual segments. T2 through A6 give rise to 6 cardioblasts each (on each side); only 2–3 cardioblasts originate from A7 and T1. No cardioblasts are produced from A8, A9 and the head segments.

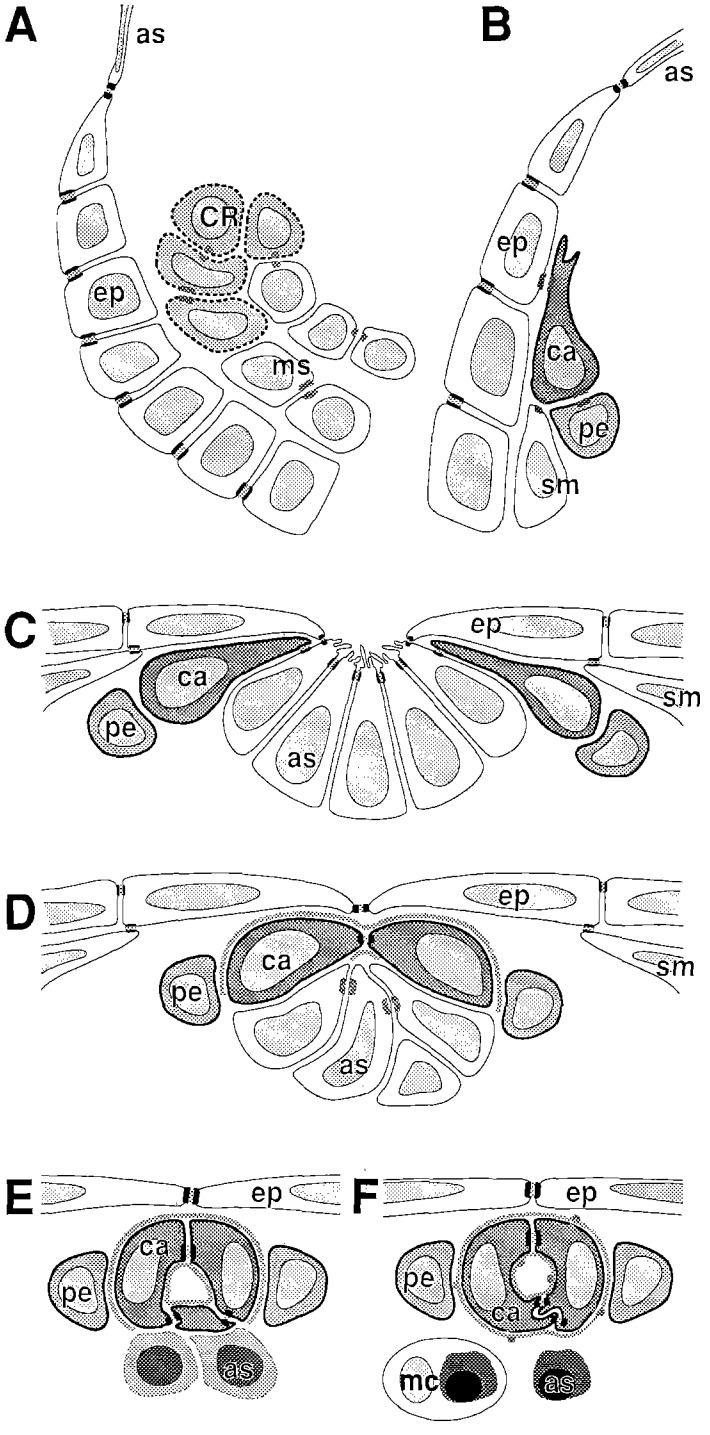


Fig. 3A–F. Schematic representation of development of the dorsal vessel, showing parts of transverse sections of embryos at successive stages. **A** Stage 12 embryo. Precursors of the dorsal vessel appear in the lateral part of the mesoderm (*ms*), for which the term cardiogenic region (*CR*) has been proposed (Hartenstein et al. 1992). **B** Stage 13 embryo. Precursors of cardioblasts (*ca*) and pericardial cells (*pe*) have become distinct from the neighbouring myoblasts (*sm*) and move dorsally. **C** Stage 15 embryo. Immediately before dorsal closure, the amnioserosa (*as*) invaginates. Cardioblasts move in between amnioserosa cells and epidermal cells (*ep*). **D** Early stage 16 embryo. After dorsal closure, the leading edges of the cardioblasts meet along the dorsal midline. Amnioserosa cells are still attached to the cardioblasts. **E** Late stage 16 embryo. Trailing edges of cardioblasts curl around to form a lumen. **F** Stage 17 embryo. Formation of the lumen of the dorsal vessel is complete.

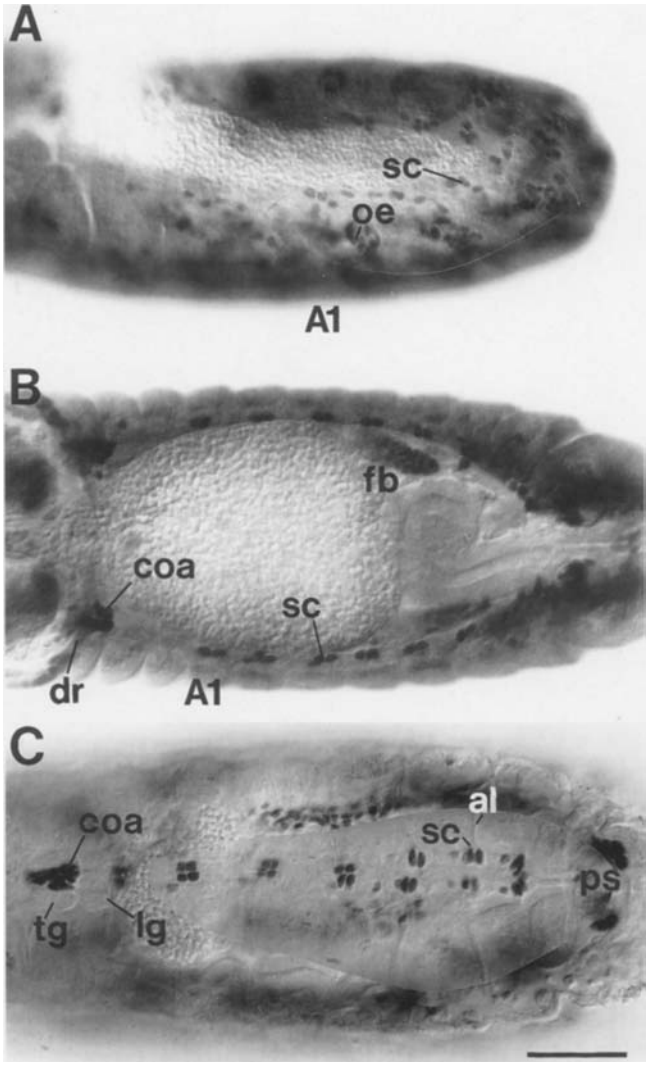


Fig. 4A–C. Segmental origin of the dorsal vessel, showing whole-mounts of embryos carrying the P*lacZ* insertion E2-3-9 which is expressed in segmentally repeated subsets of cardioblasts and some other cell types. **A** A lateral view; **B** ,**C** dorsal views. In all panels, anterior is to the *left*. **A** Stage 12 embryo. Irregular clusters of segmentally repeated heart precursors (*sc*) located in segments A1-A7 are labelled. Expression also appears in the oenocytes (*oe*) and the fat body precursors (out of the focal plane). **B** Stage 14 embryo. Segmentally repeated pairs of cardioblasts in A1–A7 are labelled. Further posteriorly and medially, labelling is seen in the fat body (*fb*). Anteriorly, the cells which will form the corpus allatum (*coa*) appear in the mesoderm of T1 and the dorsal ridge (*dr*). **C** Stage 17 embryo. Segmentally repeated cardioblasts (*sc*) of the fully formed dorsal vessel are located at the levels where alary muscles (*al*) insert. The corpus allatum (*coa*) forms an unpaired structure above the dorsal vessel; it is flanked by the (unlabelled) thoracic glands (*tg*). Together, corpus allatum and thoracic glands form the ring gland. *lg*, Lymph gland; *ps*, posterior spiracle; *Bar*, 50 μm

Amnioserosa cells have degenerated and are taken up by macrophages (*mc*). In all panels, adherens junctions are indicated by *fat double lines*; *thick grey lines* around cardioblasts (**D–F**) indicate extracellular matrix deposited around these cells

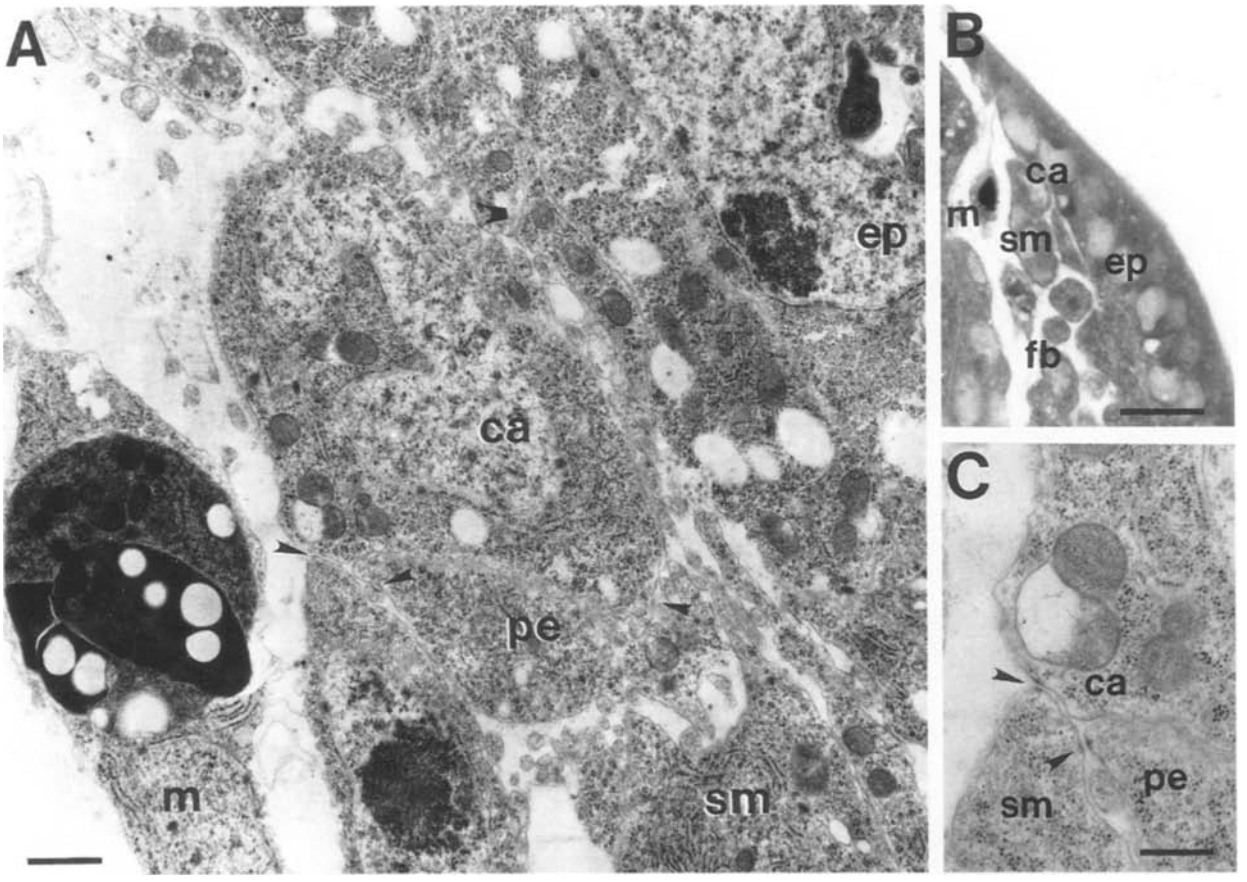


Fig. 5A–C. Morphology of the heart precursors during their dorsal migration. **A** Electron micrograph of a part of a transverse section of a stage 14 embryo. The precursors of cardioblasts (*ca*) and pericardial cells (*pe*) are closely attached to each other and to the overlying epidermis (*ep*). Small focal adherens junctions (*arrowheads*) exist between the heart precursors and the neighbouring myoblasts (*sm*). Contacts of this type (*large arrowhead*) are also present between the cardioblast and the thin process to the *top right* of this cell, which most probably belongs to an epidermal cell. **B** Light micrograph of part of a transverse section of a stage 14 embryo. Spatial relationship between heart precursors (*ca*, cardioblasts) and neighbouring tissues (*sm*, dorsal myoblasts; *fb*, fat body; *m*, macrophage; *ep* epidermis) is shown. **C** Electron micrograph of a part of a transverse section of a stage 14 embryo. Focal adherens junctions (*arrowheads*) between precursors of cardioblasts (*ca*), pericardial cells (*pe*), and somatic muscles (*sm*) are shown at higher magnification. *Bars*, 1 μm (**A**); 10 μm (**B**); 0.5 μm (**C**)

Dorsal migration of the heart precursors

During embryonic stages preceeding dorsal closure (stages 12 to 14), the heart precursors are displaced dorsally along with the rest of the body wall (Figs. 2, 3). Relative to the overlying epidermal primordium, the heart precursors move only little. Thus, throughout development, they are two to three cell diameters away from the dorsal edge of the epidermal primordium. Medially and ventrally, the heart precursors contact the myoblasts of the dorsal musculature. Whereas the pericardial precursors retain their rounded, apolar morphology, the cardioblast precursors become polarized; these cells flatten and develop small dorsally directed leading processes (Fig. 5).

Formation of the dorsal vessel during dorsal closure

At the end of stage 15 the dorsal edges of the epidermal primordia of both sides meet along the dorsal midline. The amnioserosa, which during earlier stages had covered the dorsal aspect of the embryo, invaginates. During this process, the formerly squamous amnioserosa cells adopt a high columnar shape. The cardioblast precursors squeeze in between the amnioserosa and epidermis (Fig. 6). Subsequently, the epidermal primordia fuse along the dorsal midline; at the same time, the amnioserosa cells detach from the epidermis.

Once amnioserosa and epidermis have separated, the leading processes of the cardioblast precursors meet their contralateral counterparts in the midline (Fig. 7). During stage 16 and into the beginning of stage 17, the trailing edges of the cardioblast precursors of either side bend around medially and contact each other. Thereby the cardioblasts adopt their definitive morphology as endothelial cells enclosing a central lumen (Fig. 8). At this stage cardioblasts have five different surface compartments: an apical (luminal) compartment facing the internal lumen, a basal (abluminal) compartment facing outward, an anterior/posterior compartment in between adjacent cardioblasts of the same side, a dorsal medial

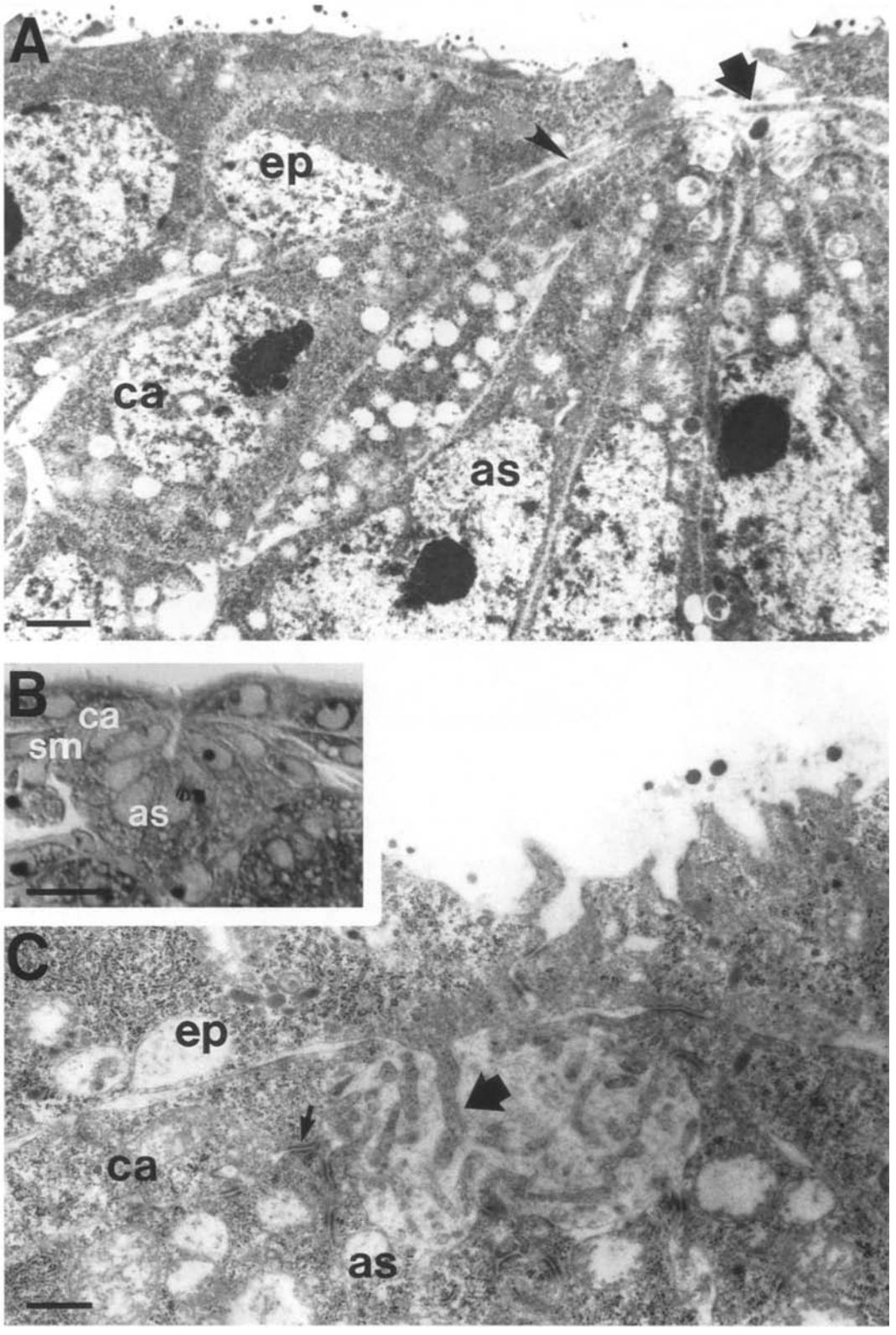


Fig. 6A–C. Invagination of the amnioserosa during dorsal closure. **A** Electron micrograph of a dorsal part of a transverse section of a late stage 15 embryo. Amnioserosa cells (*as*) have elongated in the apical-basal axis and constricted apically. Their apical membrane is folded into numerous microvillus-like processes (*wide arrow*; see also *arrow* in **C**). Cardioblasts (*ca*) have long leading processes (*arrowhead*) which reach in between the epidermis (*ep*) and amnioserosa cells. **B** Light micrograph of a dorsal part of a transverse section of a late stage 15 embryo. Spatial relationship between cardioblasts (*ca*) and neighbouring tissues (*sm*, dorsal myoblasts; *as*, amnioserosa) is shown. **C** Electron micrograph showing adherens junctions (*small arrow*) between invaginating amnioserosa cells and between the cardioblast and an amnioserosa cell. *Bars*, 1 μm (**A**); 10 μm (**B**); 0.5 μm (**C**)

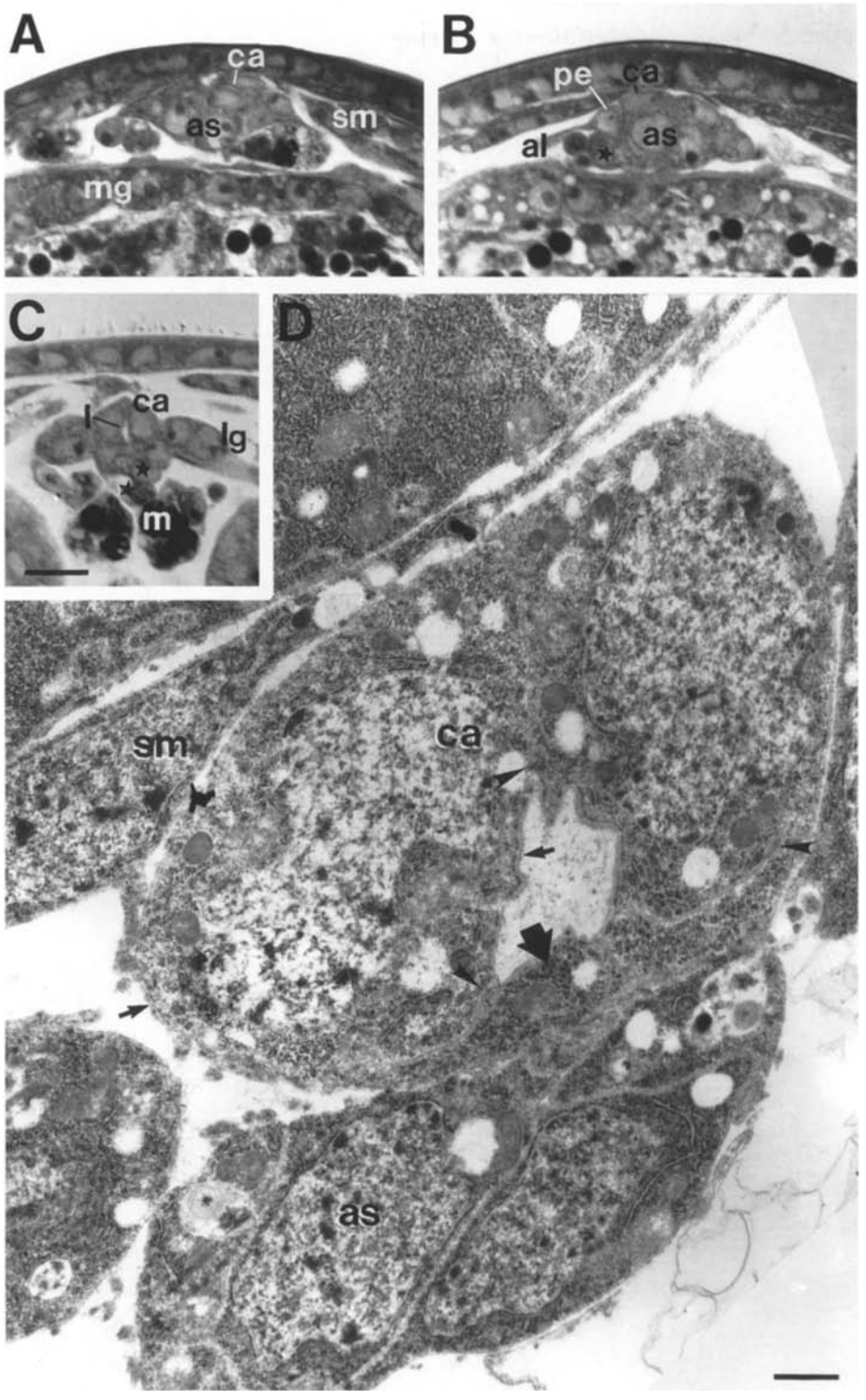


Fig. 7 A–D. Closure of the dorsal vessel and degeneration of the amnioserosa. **A–C** Light micrographs of dorsal parts of transverse sections of early stage 16 (**A**), late stage 16 (**B**) and early stage 17 (**C**) embryos. **A**, **B** The leading edges of cardioblasts (*ca*) have met in the dorsal midline; trailing edges still point laterally and no lumen has formed. Amnioserosa cells (*as*) are attached to the ventral surface of the cardioblasts; *asterisk* (**B**) marks an amnioserosa cell which has started to degenerate (basophilic cytoplasm and nucleus). **C** Trailing edges of the cardioblasts have come around, thereby forming the lumen (*l*) of the dorsal vessel. Amnioserosa cells have largely degenerated (*asterisks*) and are taken up by macrophages (*m*). The photograph shows a more anterior section containing the lymph glands (*lg*). **D** Electron micrograph of an early stage 17 embryo. Cardioblasts have formed a lumen. Morphogenesis of the ventral wall of this lumen is still in progress. The ventral wall is still thin; *large arrow* points at a process of a neighbouring cardioblast which has squeezed between the two cardioblasts lying in the plane of section. *Small arrows* indicate extracellular matrix material which form a luminal membrane (lining the lumen of the dorsal vessel) and a basal membrane. *Arrowheads* demarcate focal adherens junctions in between cardioblasts. *lg*, Lymph gland; *mg*, midgut; *pe*, pericardial cell; *sm*, somatic musculature; *Bars*, 10 μm (**A–C**); 1 μm (**D**)

compartment in between the former leading processes of cardioblasts of opposite sides, and a ventral medial compartment in between the former trailing edges of cardioblasts of opposite sides.

Before their trailing edges come around and meet, the luminal surface of the cardioblasts is in contact with the former amnioserosa cells (Fig. 7). The emerging lumen of the heart is transiently filled by these cells. Later during stage 17 the amnioserosa cells separate from the heart and undergo apoptotic cell death (Fig. 7). At this stage, numerous macrophages can be seen in close association with the dorsal vessel. When the trailing edges of cardioblasts of opposite sides first meet, their zone of contact (ventral medial surface compartment) is small and irregular. Subsequently, the ventral medial surface expands. Prominent interdigitations of the opposing cardioblasts appear (Fig. 8).

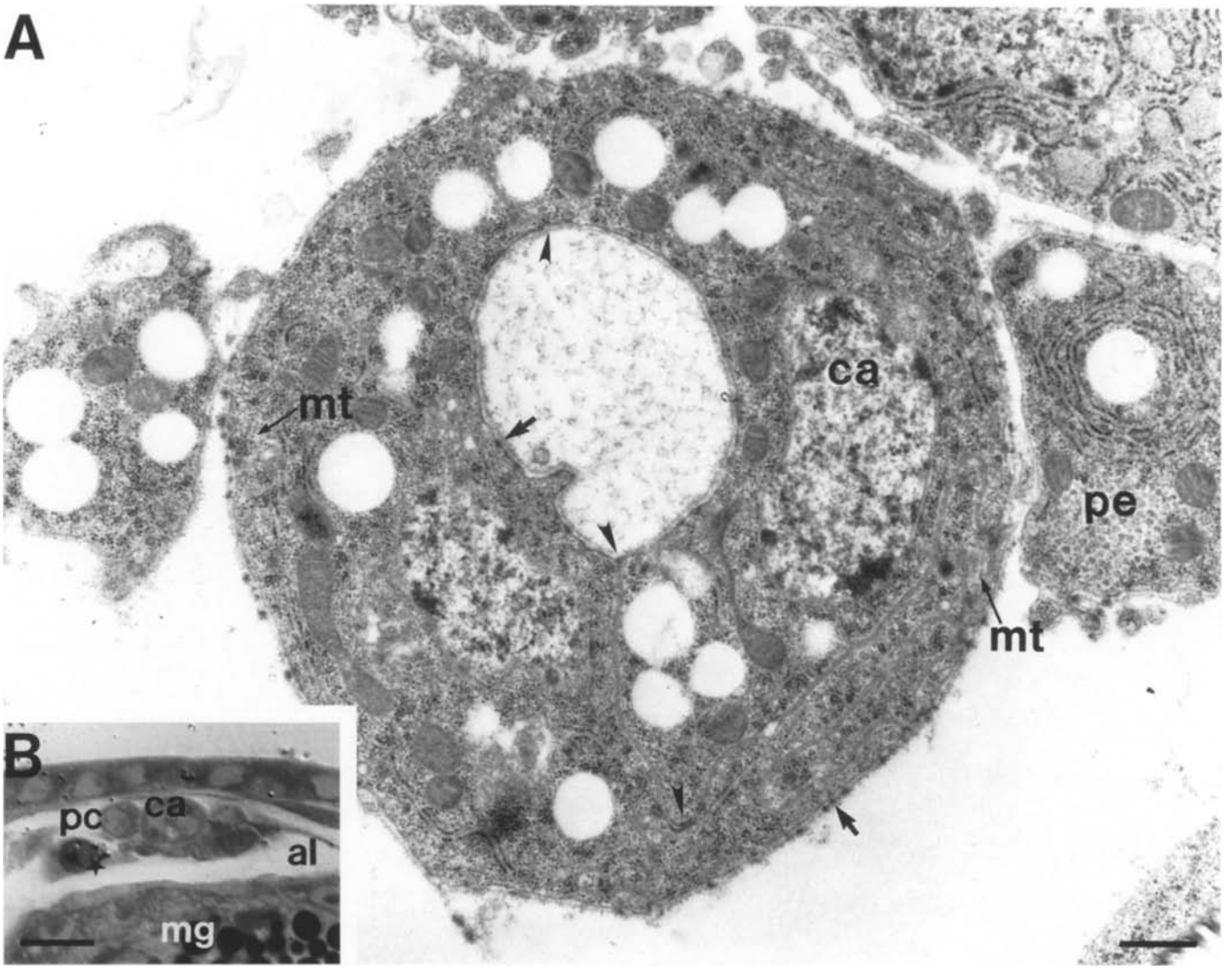


Fig. 8A, B. Structure of the late embryonic dorsal vessel. **A** Electron micrograph showing transverse section of the dorsal vessel of a late stage 17 embryo. *Small arrows* indicate hemi-adherens junctions inbetween the cell membrane (both luminal and abluminal) of cardioblasts (*ca*) and the extracellular matrix. *Arrowheads* show focal adherens junctions inbetween cardioblasts. Microtubules (*mt*) and microfilaments start to assemble into discrete longitudinal bundles. *Circular spaces* in the cytoplasm of cells (in this and all other electron micrographs) correspond to lipid droplets which occur at a high frequency in embryonic cells. **B** Light micrograph of transverse section of late stage 17 embryo, depicting the spatial relationship between cardioblasts (*ca*) and neighbouring tissues (*mg*, midgut; *pe*, pericardial cell; *al*, alary muscle; *asterisk*, degenerated amnioserosa cell). *Bars*, 1 μm (**A**); 10 μm (**B**)

Ultrastructural differentiation of the dorsal vessel

Three types of cell-cell contacts, spot adherens junctions (spot AJs), hemi-adherens junctions (hemi AJs; between membranes and extracellular matrix), and gap junctions (GJs) are formed by the cells of the heart and their precursors. Early mesodermal cells form small numbers of irregularly scattered spot AJs amongst each other. The same type of contacts can be observed between the heart precursors during stages 13–15. Small spot AJs are also formed between heart precursors and epidermal cells on the one hand, and heart precursors and myoblasts on the other (Fig. 5). During invagination of the amnioserosa, prominent AJs connect the trailing process of the cardioblast precursors to the amnioserosa (Fig. 6). These junctions are short-lived and disappear when the amnioserosa cells lose contact with the heart.

The cardioblast during stages 16 and 17 show multiple spot AJs (Fig. 8). They are preferentially localized close to the luminal surface, although they can occur also at more basal levels. A type of junction similar to this in structure and distribution has been described for vertebrate capillary cells and was called endothelial junction (for review, see Franke et al. 1988). The authors of this study could show that, biochemically, the endothelial junctions of capillaries represent adherens junctions.

During late stage 17, longitudinally oriented bundles of microtubules and microfilaments start to appear in the cardioblasts. These fibres precede the definitive myo-

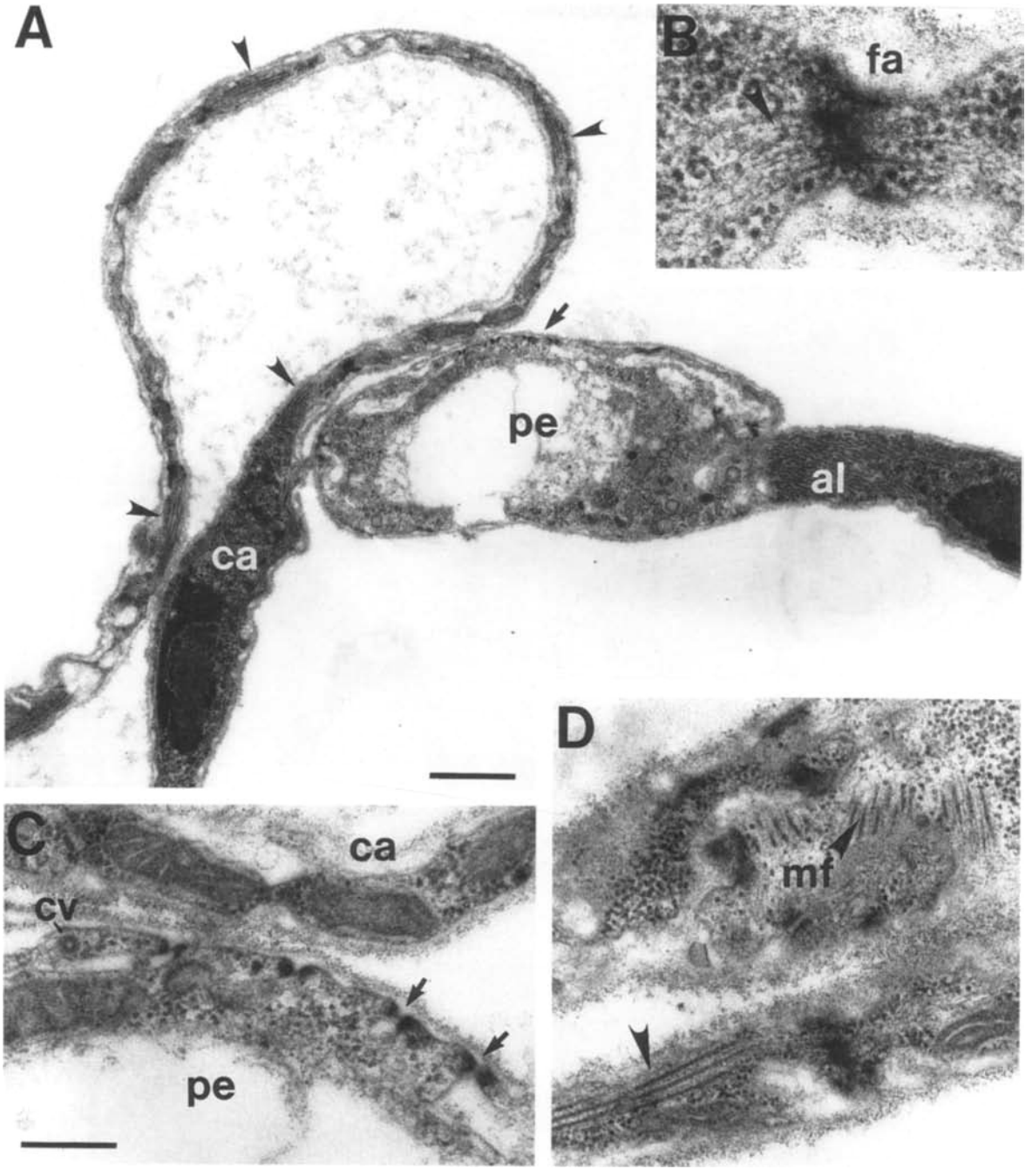


Fig. 9A–D. Structure of the larval dorsal vessel. **A–D** Electron micrographs of transverse sections of a first instar larva. Cardioblasts (*ca*) have dramatically elongated and become very thin and electron dense. Myofilaments (*arrowheads*; *mf* in **D** which shows them at a higher magnification) form several discrete longitudinal bundles. At the boundary between neighbouring cardioblasts (shown in **B**), myofilaments insert head on in prominent adherens type junctions ("intercalated disc", *fa*). Pericardial cells (*pe* in **A** and **C**) still form rounded cells laterally attached to the cardioblasts; they show membrane specializations which were described for nephrocytes (*arrows* in **C**); parts of the cell membrane invaginate, and adherens junctions are formed around the neck of these invaginations. The *large hole* in the centre of the pericardial cell is an artefact. In **A**, one of the alary muscles (*al*), containing massive bundles of microfilaments, is visible. *Bars*, 2 μm (**A**); 0.5 μm (**B–C**)

fibrils of the mature heart. Microfilaments (or, at later stages, the actin fibres of myofilaments) insert head on in adherens junctions present between neighbouring cardioblasts of the same side. In the larva, these junctions have developed into large contacts with prominent cytoplasmic undercoats ("intercalated discs"; Fig. 9). Larval cardioblasts ("myocardial cells") are extremely flat, electron dense cells. The pericardial cells have developed characteristic membrane specializations called nephrocyte junctions. In these specializations, which seem to be restricted to that surface of the pericardial cell facing the cardioblasts, small parts of the cell membrane inva-

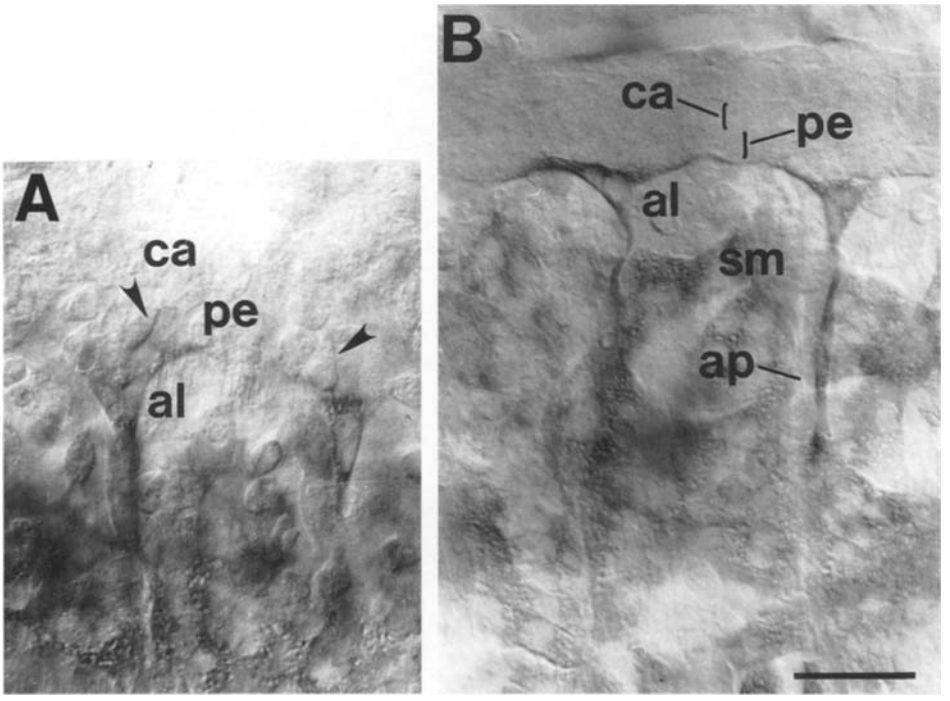


Fig. 10A, B. Development of the alary muscles. **A**, **B** Parts of whole-mounts of embryos (dorsolateral view) stained with an antibody against muscle myosin. During stage 14 (**A**), alary muscles (*al*) form stout, segmentally repeated structures which send thin processes (*arrowhead*) through the row of pericardial cells (*pe*) which flank the cardioblasts (*ca*). At stage 17 (**B**), alary muscles have adopted a tripolar shape. One process has elongated and inserts at a dorso-lateral level at the intersegmental apodeme (*ap*; actual point of insertion is out of the focal plane). The other two processes elongate anteriorly and posteriorly alongside the dorsal vessel and insert via thin filaments (not clearly visible in whole-mount preparations) at the cardioblasts. Longitudinal processes of neighbouring alary muscles meet in the middle of a segment. *Bar*, 20 μm

ginate, and adherens junctions form around the neck of these invaginations (Fig. 9). Similar membrane specializations have been previously noted in nephrocytes (of which the pericardial cells represent a subpopulation) of other species (for review see Crossley 1985).

Beginning around stage 15, a structurally distinct basement membrane is laid down at the basal surface of the cardioblasts. A similar structure appears at the same time at the luminal surface of cardioblasts ("luminal membrane"). A large number of small hemi-adherens junctions (for nomenclature, see Lane and Flores 1990) appear on both luminal and abluminal surface between the cardioblast membrane and the basement membrane or luminal membrane, respectively. In mature embryos, similar contacts occur in several different tissues, such as the epidermis and the trachea. A peculiarity of the dorsal vessel are densities which appear in the luminal membrane and basement membrane and which are not accompanied by densities of the apposed cardioblast membrane (Fig. 8).

Development of the alary muscles

The alary muscles are among the first muscles of the *Drosophila* embryo to differentiate. In the late stage 13 embryo, when they can be first visualized using an anti-muscle myosin antibody, the alary muscles appear as segmentally repeated triangular syncytia with 2–3 nuclei each (Fig. 10). Dorsally, the alary muscles are in broad contact with the dorsal vessel precursors. During dorsal closure, the alary muscles lengthen considerably. Their dorsal surface forms thin processes which penetrate through the row of pericardial cells to contact the cardioblasts to which they become firmly attached. The ventral processes form long and thin ligaments which cross the inner surface of the main tracheal trunks and reach a dorso-lateral level at which they insert at the intersegmental apodeme.

Relationship of the anterior dorsal vessel to head structures

The mesoderm of the embryonic head in *Drosophila* does not contribute to the formation of the dorsal vessel. However, since the head in Dipteran larvae is withdrawn posteriorly into the interior of the body, it comes into close contact with the dorsal vessel. In the stage 17 embryo, the anterior tip of the dorsal vessel (aorta) is located between the two brain hemispheres, right behind the prominent supra-oesophageal commissure (Fig. 11). Slightly more posteriorly, the aorta is flanked by the ring gland, which is followed by the lymph gland. The ring gland completely encircles the aorta. The dorsomedian part of the ring gland, called the corpus allatum, contacts the posteriormost extension of the dorsal pouch.

One of the enhancer trap lines used to study heart development was also expressed in the lymph glands (E7-3-63), another one in the corpus allatum (E2-3-9). The thoracic gland, represented by the lateral segments of the ring gland (King et al. 1966) expresses *engrailed* (unpublished observation). Using these markers, we could follow how the ring gland and lymph glands become associated with the aorta (Fig. 11). In the stage 13 embryo, the primordium of the lymph glands appears as a local thickening in the row of heart precursors of segment T3. It is not clear at this moment whether heart precursors in T3 are mitotically more active, or whether

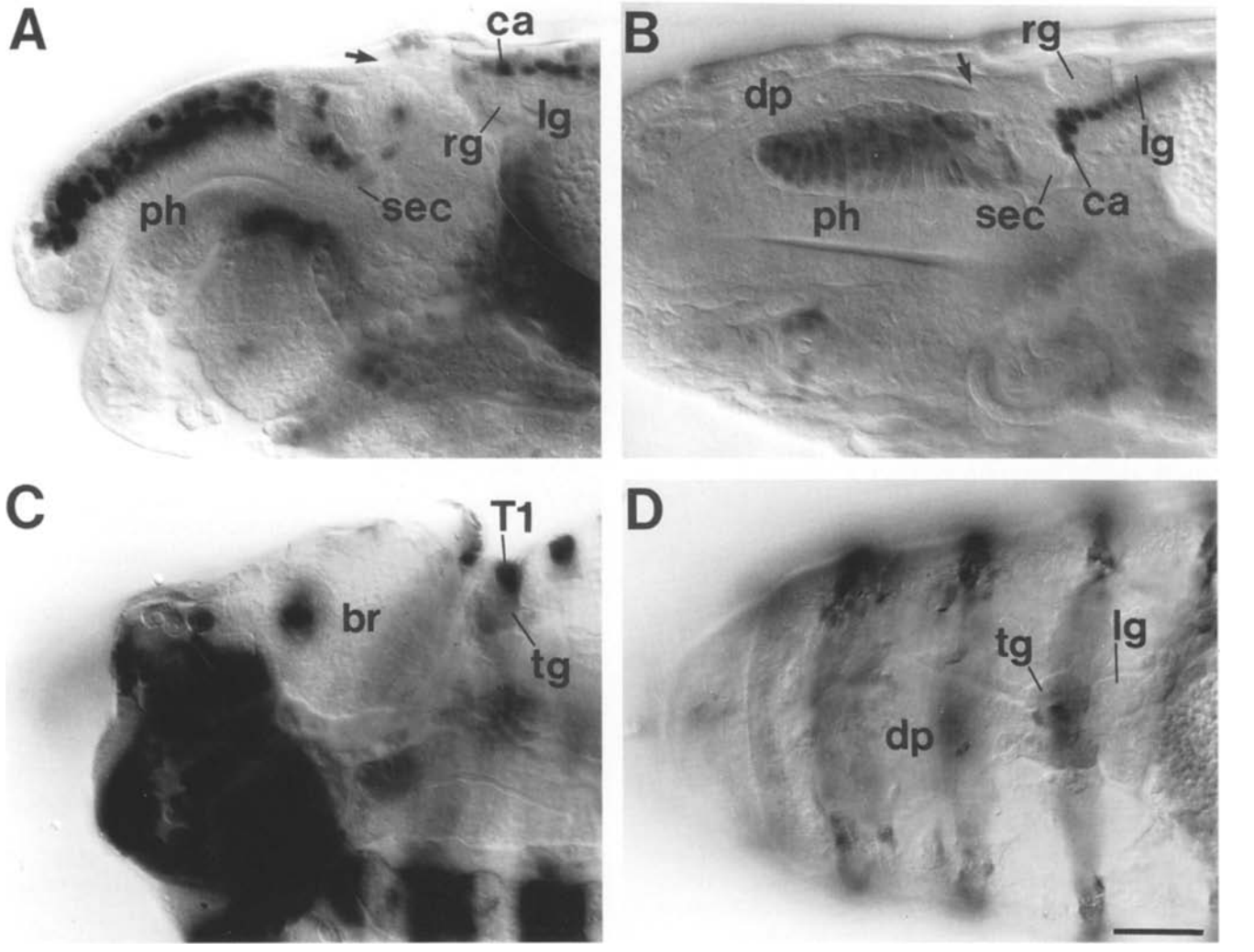


Fig. 11 A–D. Development of the anterior tip of the dorsal vessel and the ring gland. **A** Wholemount of a stage 15 embryo (lateral view, anterior to the *left*) in which the cardioblasts are labelled with the P*lac*Z insertion B2-3-20. Cardioblasts form a loose row which ends at the fundus of the dorsal fold (*arrow*). Two other structures which flank the anterior end of the dorsal vessel, the ring gland (*rg*) and the lymph gland (*lg*; both seen as unstained clusters of cells in Nomarski optics), are already in place alongside the row of cardioblasts. **B** Wholemount of a stage 17 embryo (lateral view, anterior to the *left*) in which the cardioblasts are labelled with the P*lac*Z insertion B-2-3-20. After completion of head involution, the supraesophageal commissure (*sec*) has come in contact with the anterior tip of the dorsal vessel. **C, D** Wholemount embryos (**C**, lateral view; **D**, dorsal view; anterior to the *left*) carrying the *lac*Z insertion rhy25 which is expressed in the *engrailed* expression domains (Hama et al. 1990). Note group of labelled cells (*tg*) in **C** (stage 14 embryo) which have segregated from the dorsal part of the T1 *en* domain. In **D**, which shows a stage 17 embryo, these cells form the lateral parts of the ring gland, which are called thoracic glands. *br*, Brain; *dp*, dorsal pouch; *ph*, pharynx; *Bar*, 30 μm

they accumulate by directed cell migration at this position. At around the same stage at which the lymph gland primordium becomes apparent, a group of cells enclosed within the dorsal mesoderm of the gnathal segments expresses the P*lac*Z insertion E2-3-9. These cells during dorsal closure move dorsally and finally meet and fuse above the level of the cardioblasts to form the corpus allatum. For other insects, it was reported that the corpus allatum is a derivative of the ectoderm of the gnathal segments (Snodgrass 1935). Since expression of lacZ in the corpus allatum precursors of the *Drosophila* E2-3-9 line commences as late as stage 13, it is possible that these precursors had originally segregated from the ectoderm, instead of originating from the mesoderm.

The thoracic glands originate from the dorsal ectoderm of T1. They segregate from the ectoderm during stage 15 and form a cluster of cells on either side of the heart precursors (Fig. 11). Later, they fuse with the corpus allatum. The origin of the ventral part of the ring gland (corpora cardiaca) could not be analysed, since no specific marker is known for this structure at the present time.

Discussion

The development of the dorsal vessel has been described for several insect species. Its origin in the lateral meso-

derm was first described in the mole cricket by Korotnier (1883) and has subsequently been confirmed by investigations in a variety of other insects (for review of the older literature, see Schroeder 1925). Most of the modern studies of the insect dorsal vessel focus on later developmental events, i.e. the differentiation of myofibrils, which mainly takes place during postembryonic development (Sedlak and Whitten 1976; Tadkowski and McCann 1980a, b). No ultrastructural information existed regarding the morphogenesis of the dorsal vessel. Snodgrass (1935) describes the cardioblasts as crescent-shaped cells which migrate dorsally during dorsal closure and, after meeting with their contralateral counterparts in the dorsal midline, form a lumen. This description suggests that the mechanism by which the lumen of the dorsal vessel is formed in *Drosophila*, namely the bending around of the trailing edges of the cardioblasts, may be a general phenomenon in insect development.

During the larval and adult stage, myocardial cells show very similar ultrastructural characteristics in all species investigated (Edwards and Challice 1960; Myklebust 1975; Jensen 1977; for review, see Jones 1977). They form a contractile endothelium whose cells do not fuse into a syncytium. As described in this paper for the *Drosophila* first instar larva, myofilaments mainly form longitudinal bundles which insert at the boundary between adjacent cardioblasts. Specialized adherens junctions ("fasciae adherentes") are formed at these sites. In analogy to the vertebrate heart, the complex junction between adjacent cardioblasts which anchors the myofilaments is called "intercalated disc".

The pericardial cells form a subpopulation of cells called nephrocytes. These cells were found to take up haemolymph substances (Kowalevsky 1892) and, together with the Malpighian tubules, form the excretory system of insects (for review see Crossley 1985). Beside the pericardial cells, cells of the lymph gland and suboesophageal body (so called garland cells) also belong to the class of nephrocytes. The ultrastructural "hallmarks" of nephrocytes described in previous studies (e.g. Kessel 1960; Crossley 1985) abundant membrane invaginations which are sealed at their opening by specialized adherens junctions, are also evident in the *Drosophila* larva. Three-dimensional reconstructions of nephrocytes showed that these membrane invaginations form a complex network of tubules or lacunae junctions ("nephrocyte junctions"). It has been suggested that this system of submembraneous tubules found in nephrocytes represents a device for ultrafiltration (for review see Crossley 1985), similar to the podocyte membrane in the vertebrate kidney.

Regarding its structure and development, the insect dorsal vessel closely resembles a vertebrate capillary. The capillary wall is formed by flattened endothelial cells. The junctional complex of these cells ("endothelial junction"; Franke et al. 1988) ultrastructurally closely resembles the junctions described here for the cardioblasts. Capillary endothelial cells are derived from mesodermal (mesenchymal) precursors which can be studied in an in vitro system. Thus, capillary formation and its dependence on matrix components has been studied extensively utilizing cultures of human umbilical vein endothelial cells (HUVECs) and human dermal microvascular endothelial cells (HDMECs; Folkman and Haudenschild 1980; Maciag et al. 1982; Kubota et al. 1988; Grant et al. 1989a, b). In order to identify the components of the basement membrane that are necessary for the formation of capillary structures, antibody blocking studies and substrate variation studies were conducted and identified laminin and collagen IV as such components (Kleinman et al. 1981; Furcht 1986; Dejana et al. 1988; Kubota et al. 1988; Grant et al. 1989a, b).

Intracellular morphological changes during capillary formation and their associated cytoskeletal and protein synthesis requirements have been investigated (Grant et al. 1991). It has been shown that endothelial cells, after attaching to the matrigel substrate, align and form cylindrical structures that contain a lumen and polarized organelles. During the process of endothelium formation the cytoskeleton becomes reorganized into longitudinal bundles of microfilaments, similar to what happens in cardioblasts during the formation of myofilaments. Drugs interfering with either microtubule or microfilament function do not interfere with the attachment of capillary precursors to the substrate, but inhibit subsequent tube formation (Grant et al. 1991). It has also been suggested that the resting tension of the cytoskeleton itself could be a means by which changes in the extracellular matrix triggers morphogenetic movements of capillary precursors. Tensile forces are generated by contractile microfilaments and resisted by the attachment of these filaments (via membrane molecules) to the extracellular matrix. Variation in the balance of forces results in alterations of cell and tissue form (Ingber and Folkman 1989).

The morphogenetic movements of cultured capillary precursors, i.e. the linear alignment and subsequent curling around, are very similar to those described for the development of the dorsal vessel in *Drosophila*. The dorsal vessel may therefore represent a model system to study endothelium formation by using a genetic-molecular approach. The genes coding for homologues of the vertebrate extracellular matrix proteins known to be involved in capillary development (e.g. laminin; collagen) are known (Fessler et al. 1987; Montell and Goodman 1989; Lunstrum et al. 1988). These proteins are expressed abundantly in the extracellular matrix surrounding the developing dorsal vessel. The homologues of the integrins are expressed in the early mesoderm (Bogaert et al. 1987; Leptin et al. 1989); their later expression in the dorsal vessel has not been reported. Future studies of the phenotypic consequences of mutations in the corresponding genes may shed some light on if and how the extracellular matrix controls dorsal vessel development. Other extracellular proteins have been identified which are expressed in the dorsal vessel and may be required for its differentiation. An example is *slit*, an excreted protein with EGF homology and abundant expression in the luminal membrane of the dorsal vessel (Rothberg et al. 1990). Our own preliminary observations indicate that the morphogenesis of the dorsal vessel in *slit* mutant embryos is normal; however, the lumen

of the dorsal vessel shows irregularities and cardioblasts dissociate in late embryos. Finally, in order to test the requirement for various embryonic tissues (e.g. amnioserosa, epidermis) for the development of the dorsal vessel, mutations in which these tissues are deleted can be used. In regard to this possibility, we are currently looking at dorsal vessel development in TI^{D} mutant embryos which lack all ectodermal derivatives (Ray et al. 1991). Preliminary results show that the differentiation of mesoderm into different cell types (i.e. somatic and visceral muscle, macrophages, dorsal vessel) takes place. Using electron microscopy and various cell type specific markers, we are carrying out a detailed analysis of the differentiation of cardioblasts and pericardial cells in this mutant.

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