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A role for the mesoderm in endodermal migration and morphogenesis in *Drosophila*

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SUMMARY

The endodermal midgut arises from two primordia, the anterior midgut (AMG) primordium and the posterior midgut (PMG) primordium, which are separated by almost the entire length of the *Drosophila* embryo. To form the midgut, these two parts have to extend towards each other and to fuse laterally on both sides of the yolk. Shortly before and during that movement, AMG and PMG are arranged as mesenchymal cell masses, but later the midgut cells form an epithelium. We show that these two aspects of midgut development, migration of AMG and PMG and transition to an epithelium, depend on the mesoderm. The extension of the midgut primordia is achieved by cell migration along the visceral mesoderm which forms a continuous layer of cells within the germ band. In mutant embryos lacking the entire mesoderm or failing to differentiate the visceral mesoderm, AMG and PMG are formed but do not migrate properly. In addition, they fail to form an epithelium and instead either remain as compact cell masses anterior and posterior to the yolk (in *twist* and *snail* mutant embryos) or only occasionally wrap around the yolk before embryogenesis is completed (in *tinman*-deficient embryos). We conclude that the visceral mesoderm serves as a substratum for the migrating endodermal cells and that the contact between visceral mesoderm and endoderm is required for the latter to become an epithelium.

Key words: *Drosophila*, visceral mesoderm, endoderm, midgut, morphogenesis, cell migration

INTRODUCTION

Gastrulation in *Drosophila* starts with the formation of the ventral furrow and the amnioproctodeal invagination of hindgut and posterior midgut primordia. While the cells of the posterior midgut invagination and at the anterior tip of the ventral furrow give rise to the endoderm, most of the cells of the ventral furrow form the mesodermal germ layer. The latter differentiates into two major mesodermal tissues: into the somatic mesoderm forming the muscles of the body wall and into the visceral mesoderm forming the musculature surrounding the gut.

Two zygotic genes encoding transcription factors, *snail* (*sna*) and *twist* (*twi*), are required for specification and differentiation of the mesoderm (Simpson, 1983; Nüsslein-Volhard et al., 1984; Boulay et al., 1987; Thisse et al., 1987; Leptin, 1991; Thisse et al., 1991). Both are expressed in the ventralmost region of the embryo, *twi* from the anterior to the posterior pole, *sna* from the anterior pole to about the border of the posterior terminal region of the embryo (Thisse et al., 1988; Alberga et al., 1991; Leptin and Grunewald, 1990). Mutant embryos lacking either of the two genes fail to form a ventral furrow and lack all mesodermal derivatives. Other aspects of gastrulation like amnioproctodeal invagination or germ band extension are not disturbed. A larger set of genes is thought to be involved in mesodermal differentiation. Among these is *tinman* (*tin*, formerly called *msh-2* or *NK-4*), which codes for a transcription factor that appears to be required for positional specification within the mesodermal germ layer and differentiation of the visceral mesoderm (Bodmer et al., 1990; Bodmer, 1993; Azpiazu and Frasch, 1993).

The endoderm has two spatially separated primordia, the anterior midgut (AMG) and the posterior midgut (PMG). In order to form the endodermal part of the digestive tract, the midgut, these two parts have to join and enclose the yolk. At the onset of germ band retraction AMG and PMG extend towards each other and finally meet laterally on both sides of the yolk shortly before retraction of the germ band is completed (Fig. 1). After that, in a second phase of movement, the endoderm wraps ventrally and dorsally around the yolk and finally takes on the shape of a convoluted tube.

Two aspects of midgut development are of particular interest: (i) the mechanism of the movement of the two midgut parts towards each other and (ii) the apparently concomitant transition of the multi-layered, mesenchymal cell masses of AMG and PMG into a highly ordered epithelium. We present a morphological and genetic analysis of both processes and conclude that they require an interaction of the endodermal midgut cells with cells derived from another germ layer, the mesoderm. The arrangement of the midgut cells during the time of extension relative to the visceral mesoderm suggests that AMG and PMG migrate towards

each other guided by the visceral mesoderm. This view is supported by the phenotypes of mutant embryos which fail to develop or differentiate a visceral mesoderm. In these cases, the migration of the midgut parts does not occur. The transition from the mesenchymal cell mass to an epithelium also does not occur and we propose that the visceral mesoderm not only serves as an adhesive substratum during the migration of the midgut parts but is also needed to induce the arrangement of the midgut as an epithelium.

MATERIALS AND METHODS

Fly stocks

twi^{RY50}: weak *twi* allele (Simpson, 1983); *twi^{ID}* and *twi^{EY53}*: amorphic *twi* alleles (Nüsslein-Volhard et al., 1984; Thisse et al., 1987). *twi^{RY50}* and *twi^{EY53}* chromosomes have been cleaned by meiotic recombination from lethal mutations other than *twi*. Flies homozygous for these chromosomes can be fully rescued by 9 kb of genomic DNA containing the *twi* gene when these are transformed into flies mediated through a P-element vector (Thisse et al., 1991; our unpublished results). *sna^{IIG}* and *sna^{RY1}*: amorphic *sna* alleles (Nüsslein-Volhard et al., 1984; Grau et al., 1984; Boulay et al., 1987). *ush^{IIA}*, *ush^{19}*, *tup^{IIIE}* and *tup^{IIIB}*: homozygous mutant embryos fail to retract their germ band (Nüsslein-Volhard et al., 1984). A490.2M3: enhancer trap line (3rd chromosome) with β-gal expression in the endoderm (Bellen et al., 1989). A183.1F2 and *fasIII^{E25}*: null for fasciclin III protein (Wilson et al., 1989; Elkins, Ferres-Marco and Goodman, unpublished data). *l(1)mys^{xG43}*: null mutation for β1 integrin (PS3, Leptin et al., 1989). *Df(2L)DS6* and *Df(2L)pr-A14*: deficiencies with small overlap which uncovers the locus of the endoderm-specific βν integrin (S. E. Paine-Saunders and R. O. Hynes, pers. commun.; Yee and Hynes, 1993). *Df(1)Dl^{X43}*: small deletion of the *Delta* locus. *Df(1)N^{8}*: small deletion of the Notch locus. *Df(3R) e^{BS2}*, *Df(3R) e^{F1}* and *Df(3R) GC14*: deficiencies for the homoeobox gene *tin* (Bodmer et al., 1990). *Df(3R) hkb^{A321}*: generally used as a *hkb* null allele (Weigelet al., 1990). *tor^{XR1}*: a 9.5 kb deletion of the *tor* locus (Sprenger et al., 1989). *stg^{7B69}* and *stg^{7M53}*: amorphic alleles of *string* (Jürgens et al., 1984; Edgar and O'Farrell, 1989).

Immunohistochemistry and histology

Murine monoclonal anti-β-galactosidase (anti-β-gal) antibodies were purchased from Sigma (St. Louis, USA) and biotinylated goat anti-mouse IgG antibodies or biotinylated goat anti-rabbit IgG antibodies from Jackson (Bar Harbor, USA). Anti-*Drosophila* muscle myosin heavy chain antibody (rabbit) was a gift from Dan Kiehart (Kiehart and Feghali, 1986), murine monoclonal anti-fasciclin III and anti-*Ubx* antibodies from Mike Wilcox (Brower et al., 1980; White and Wilcox, 1985), anti-*caudal* antibody (rabbit) from Uwe Walldorf (Mlodzik and Gehring, 1987) and anti-*twi* antibody (rabbit) from Siegfried Roth (Roth et al., 1989).

Embryos were fixed and stained essentially following standard protocols, and the antibodies were detected histochemically using the Vectastain ABC kit (Vector Labs, USA). Sectioning of the embryos was performed as described (Leptin and Grunewald, 1990). Whole-mount embryos were either mounted individually in Araldite or en masse in methyl salicylate and photographed with a Zeiss Axiophot equipped with Nomarski optics using either Kodak Ektachrome 160T or Agfapan APX 100 film.

RESULTS

Midgut development

We followed the development of the midgut with the enhancer trap A490.2M3 which, beginning from stage 8, displays homogeneous gene activity throughout the cells that contribute to the midgut (Fig. 1, Bellen et al., 1989). Two hours after the onset of gastrulation, the midgut primordia are two separate groups of cells: the AMG primordium and the PMG primordium. At stage 10 the AMG primordium is contiguous with the invaginating stomodeum. The PMG is positioned at the end of the germ band and is contiguous with the prospective hindgut (Fig. 1A). While the AMG is already mesenchymal at this stage, the PMG will undergo a visible transition from an epithelium (Fig. 1A) to a mesenchyme at the end of stage 10 (Fig. 1B, see also Foe, 1989; Hartenstein et al., 1992). The cells are small and round and form a large mass which is several cells deep. At this time, the AMG begins to split into two arms which extend towards the posterior. Slightly later, during stage 11, this extension becomes more pronounced (Fig. 1C), and the PMG has also extended along the germ band in a similar fashion. The midgut primordia now span almost three times as much of the germ band (which is still almost fully extended) as they did a stage earlier. Moreover, both the AMG and the PMG are arranged in symmetrical bi-lobate structures with lobes right and left of the yolk (Fig. 1D). Shortly before the completion of germ band retraction the leading tips of AMG and PMG meet on both sides of the yolk, and by the time the germ band has retracted AMG and PMG will have fused (Fig. 1E,G). In a second phase of movement, the midgut cells then spread in transversal directions, both ventrally and dorsally, and completely enclose the yolk at the end of stage 14 (not shown). Two aspects of the midgut development are the topic of our paper: the transition of the mesenchymal cell mass of AMG and PMG into an ordered epithelium and the spatially accurate movement of the midgut parts towards each other.

Morphological analysis of the endodermal migration

How are AMG and PMG directed towards each other during their migration? The leading tips of the two midgut parts are precisely positioned below the narrow band of visceral mesoderm that forms one continuous layer of cells along the inner surface of the germ band on either side of the embryo (Fig. 1F,F'). The visceral mesoderm becomes distinguishable in such a form during the course of stage 10 (see below Fig. 4F). It therefore might fulfill the spatial and temporal requirement for a structure that could guide the midgut parts. If the visceral mesoderm had such a guiding function one would expect it to be in close contact with the midgut. In serial cross-sections (Fig. 2A-C) through the bilobate AMG of a stage 11 embryo, we observe a tight attachment of the endoderm along its entire length to the underlying mesoderm. The visceral mesoderm expresses fasciclin III, a cell surface molecule (Snow et al., 1989, see also Fig. 4F) before it becomes morphologically distinct from the somatic mesoderm. A cross-section through the AMG of a stage 11 embryo immunostained for the fasciclin III protein (Fig. 2G) reveals that indeed the entire visceral mesoderm makes contact with the endoderm, and that it is only the visceral mesoderm to which the endoderm attaches. This also holds true for the PMG (Fig. 2H). In the particular embryo shown, the PMG is attached to a more posterior (upper) stretch of

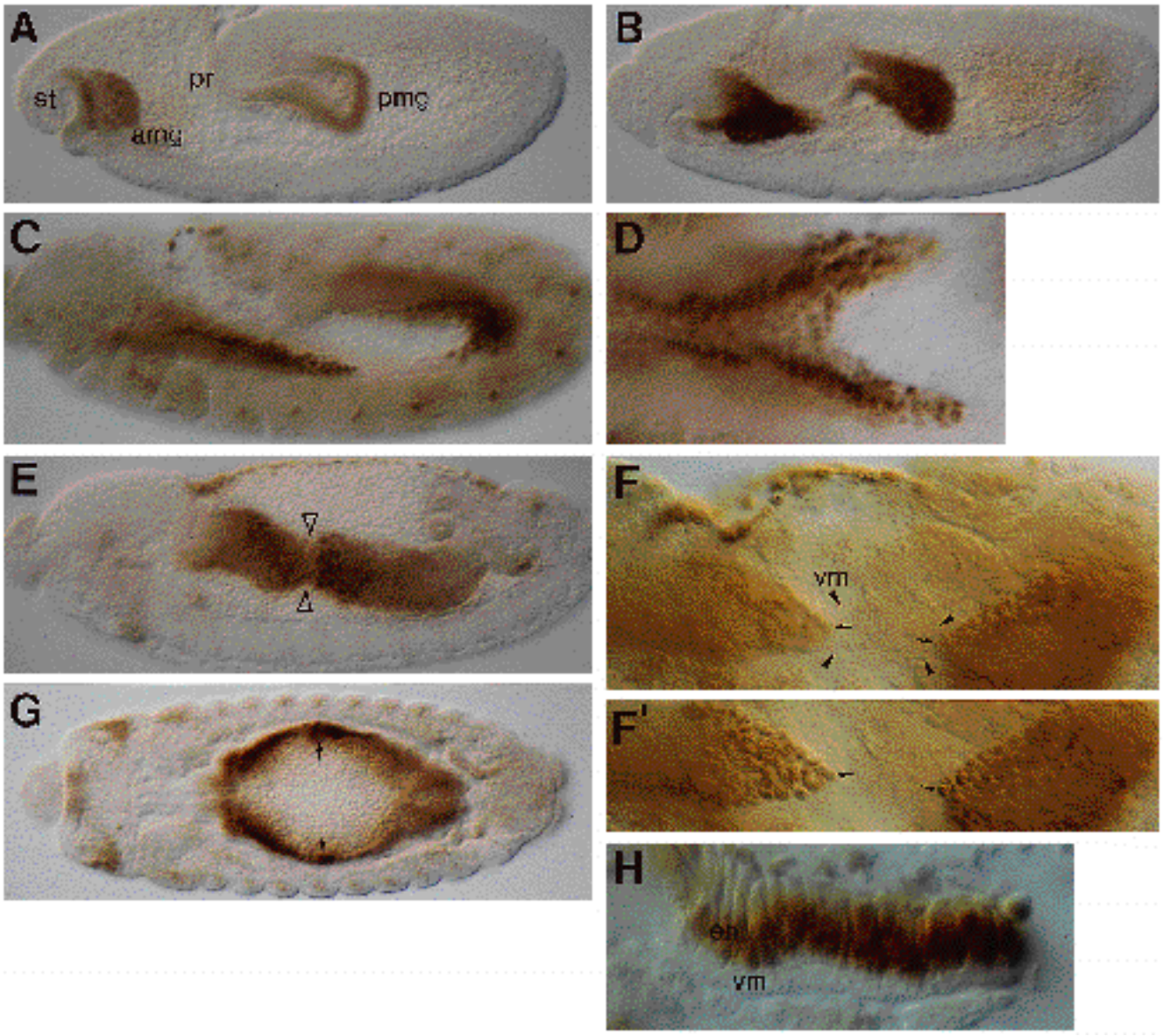

Fig. 1. Midgut morphogenesis. (A) At early stage 10 the posterior midgut (pmg) is a simple epithelium and contiguous with the proctodeum (pr). The anterior midgut (amg) is in contact with the stomodeum (st). (B) The PMG has formed a multilayered cell mass (late stage 10). (C) During stage 11, AMG and PMG extend significantly. (D) Horizontal view of the two arms of the AMG of an embryo slightly younger than that shown in panel C. (E,G) AMG and PMG have joined right and left of the yolk and acquired the properties of a simple epithelium. The open arrowheads point to the junction of AMG and PMG. Posterior to this position, i.e. at the anterior tip of the PMG, the visceral mesoderm expresses *Ubx* protein over the length of one parasegment (not shown, but see Fig. 7C) while internally a cluster of large endodermal cells is located on each side (arrows). (E, optical parasagittal section; G, optical horizontal section; early and late stage 13). (F) Higher magnification of the visceral mesoderm (vm, between arrowheads) and (F′), in a slightly lower focal plane, of the leading tips (arrows) of AMG and PMG (stage 12). (H) Higher magnification of a piece of midgut of a stage 13 embryo showing the endodermal epithelium (en) attached to the visceral mesoderm. All embryos carry the enhancer trap A490.2M3 and were immunostained for β-gal protein.

visceral mesoderm but not yet to the more anterior (lower) region. Thus AMG and PMG are apparently moving towards each other along the cell layer of the visceral mesoderm.

Morphological analysis of the transition from mesenchyme to epithelium

During their extension, AMG and PMG each consist of a mass of cells with mesenchymal character and show little sign of specialization apart from their organization into two arms (Figs 1C.D, 2A-D). At stage 13, when the germ band is retracted, the midgut has acquired its typical arrangement of an epithelial layer of columnar cells between yolk and visceral mesoderm (Fig. 1G,H). While the columnar shape is only transient and disappears when the cells flatten to enclose the yolk in stage 14 (Fig. 1G), the epithelial character is maintained. When and how does the transition from mesenchyme to epithelium occur? The extending arms

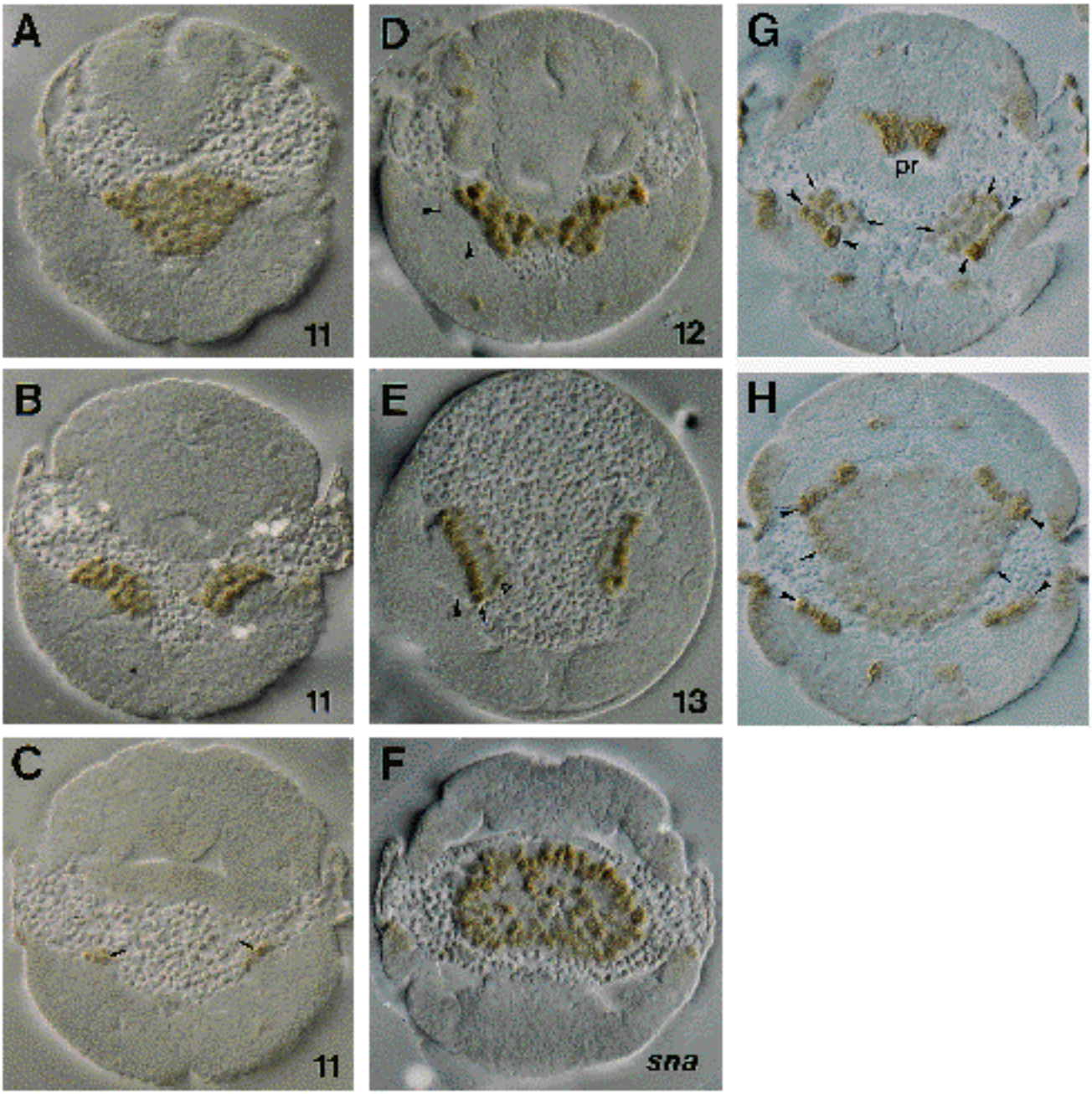

Fig. 2. Midgut endoderm and visceral mesoderm are in close contact at the time of midgut extension. (A-C) Serial 10 μm cross-sections through the AMG (every other section shown). Arrows in C point to the cells of the leading tips of the AMG (stage 11) (D) Cross-section through the PMG behind its leading tips. Arrowheads point to morphologically distinguishable visceral mesoderm (stage 12). (E) Cross-section through the anterior half of the midgut after joining of AMG and PMG. The filled arrowhead marks the visceral mesoderm, the arrow points at the nuclei of the endodermal midgut epithelium and the open arrowhead indicates the small endodermal cells outlining the midgut at this stage in a second layer (stage 13). (F) Cross-section through the PMG of a *sna* null embryo. (G) Cross-section through the AMG (between arrows) of an early stage 11 embryo in which the expression of fasciclin III indicates the visceral mesoderm (between arrowheads; pr, proctodeum). (H) Cross-section through the PMG of an embryo as in G. All embryos carry the enhancer trap A490.2M3 and were immunostained for β-gal protein and, in the case of G and H, also for fasciclin III protein.

of the midgut are many cells thick during stages 10 to 12 and are not visibly organized in layers. The thickness of the arms slightly decreases towards the leading tips (Fig. 2A,B), but only at the very tip do they become merely one cell thick (Fig. 2C). Even the cells that are directly in contact with the visceral mesoderm and are arranged in a somewhat regular fashion remain small and round. Apparently throughout the time of migration the midgut cells stay mesenchymal. When AMG and PMG have joined in late stage 12, the midgut cells arrange into a columnar epithelium. Beginning from the

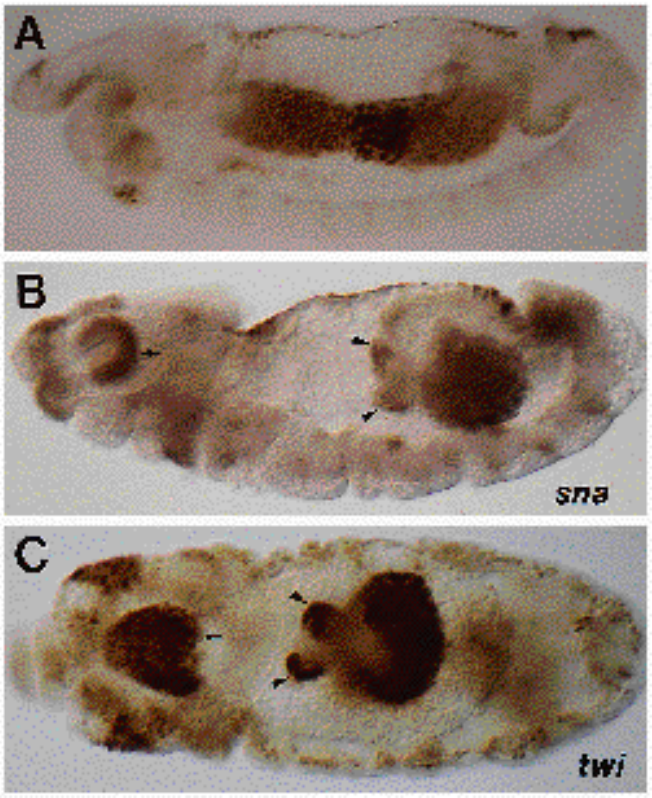

Fig. 3. Mutant embryos lacking the mesoderm do not form a midgut. (A) Wild-type (lateral view). (B) *sna* embryo (lateral view). The stomodeum/AMG remains an epithelium over a long time. The PMG stays as a multilayered cell mass and does not form the bilobate structure that wraps around the yolk. (C) *twi* null embryo (dorsal view). Both AMG and PMG remain compact cell masses, which are immersed in the yolk. Embryos are at stage 13 (A) or approximately at stage 15 (B,C). The mutant embryos retract their germ bands to variable degrees, but never completely. The arrows point to the AMG, the arrowheads to the Malpighian tubules, which remain as pouches in the mutant embryos. All embryos carry the enhancer trap A490.2M3 and were immunostained for β-gal protein.

anterior and the posterior end of the midgut, the cells in contact with the visceral mesoderm become elongated with their nuclei located towards the mesoderm (Fig. 2E). Concomitantly, the endoderm and the attached visceral mesoderm spread slightly dorsoventrally (compare the left and the right part of the midgut epithelium of the embryo in Fig. 2E which have developed slightly asynchronously). It should be pointed out that, in addition to the prominent layer of outer columnar cells, there remains a second layer of small round inner cells throughout the entire midgut (Figs 1H, 2E). These cells also express the enhancer trap A490.2M3, originate from AMG or PMG and presumably constitute the imaginal midgut precursors (Hartenstein and Jan, 1992). Furthermore, there is a cluster of large round PMG cells that had migrated just behind the leading tip and which also lie on the inside of the epithelium (Fig. 1G). It has been shown previously that both types of inner cells eventually become integrated into a single-layered midgut epithelium during later stages of development (Hartenstein and Jan, 1992; Reuter et al., 1990).

Genetic analysis

The morphological studies above have shown that extending AMG and PMG are in close contact with the visceral mesoderm. If this spatial arrangement had a functional role for the extension of the midgut parts and their transition from mesenchyme to epithelium, one would expect that, in mutant embryos lacking the mesoderm, both processes should be affected. *twi* and *sna* embryos fail to develop a mesoderm completely but they undergo normal posterior midgut invagination and form the normal number of PMG cells with endodermal fate as indicated by the expression of the enhancer trap A490.2M3 (Fig. 3B,C). Apparently, neither *sna* nor *twi* are required in the developing posterior midgut. Also the AMG develops, but in *sna* embryos there are slight aberrations from the normal developmental path: the AMG remains a simple epithelium until later stages of embryonic development (Fig. 3B, Reuter and Leptin, unpublished data). In spite of the normal early development, in these mutant embryos neither part of the midgut shows any sign of extension, nor do AMG and PMG form bi-lobate structures. Instead they remain as large separate mesenchymal cell masses immersed in the yolk at the ends of the embryo (Fig. 3B,C). AMG and PMG do not join; they do not spread transversally over the yolk and are obviously unable to engulf it (Fig. 2F). At no time during development do their cells arrange into an epithelium or even acquire the columnar shape that is typical for the endodermal layer in contact with the visceral mesoderm around stage 13 (Fig. 2E). Thus the formation of the simple epithelium of the midgut appears to depend on the presence of mesoderm. We assume that epithelial differentiation including shape changes to columnar cells is induced in the endodermal cells upon the attachment of the midgut endoderm to the visceral mesoderm. In addition, the extension of the midgut parts also requires mesodermal structures. This corroborates our notion of the migration of midgut cells needing the visceral mesoderm as a substratum.

The specific requirement for the visceral mesoderm rather than for the entire mesoderm is apparent in weak *twi* mutant embryos (twi^{RY50}) which form a ventral furrow and initially also an apparently normal mesodermal germ layer (not shown). *twi* protein expression is normal during the early phases of development, in particular within the midgut primordia. Then, during germ band extension *twi* protein disappears from most of the mesoderm of the mutant embryos while in wild-type embryos it persists until after the onset of germ band retraction (Fig. 4A,B). Later, the mutant embryos partially differentiate somatic muscles (Fig. 4G) but no visceral muscles surrounding the midgut. The visceral mesoderm does not form a visibly discernible tissue. For example, fasciclin III is absent from the presumptive visceral region in the mutant embryos while all other aspects of its expression are undisturbed (Fig. 4E,F). In such embryos lacking the visceral mesoderm, the endoderm does not migrate (Fig. 4A) and AMG and PMG do not meet (Fig. 4C). They remain as compact, mesenchymal cell masses that insert into the yolk rather than enclose it. Both fail to form the typical bilobate structures, do not undergo the transition to an epithelium and ultimately no proper midgut is made.

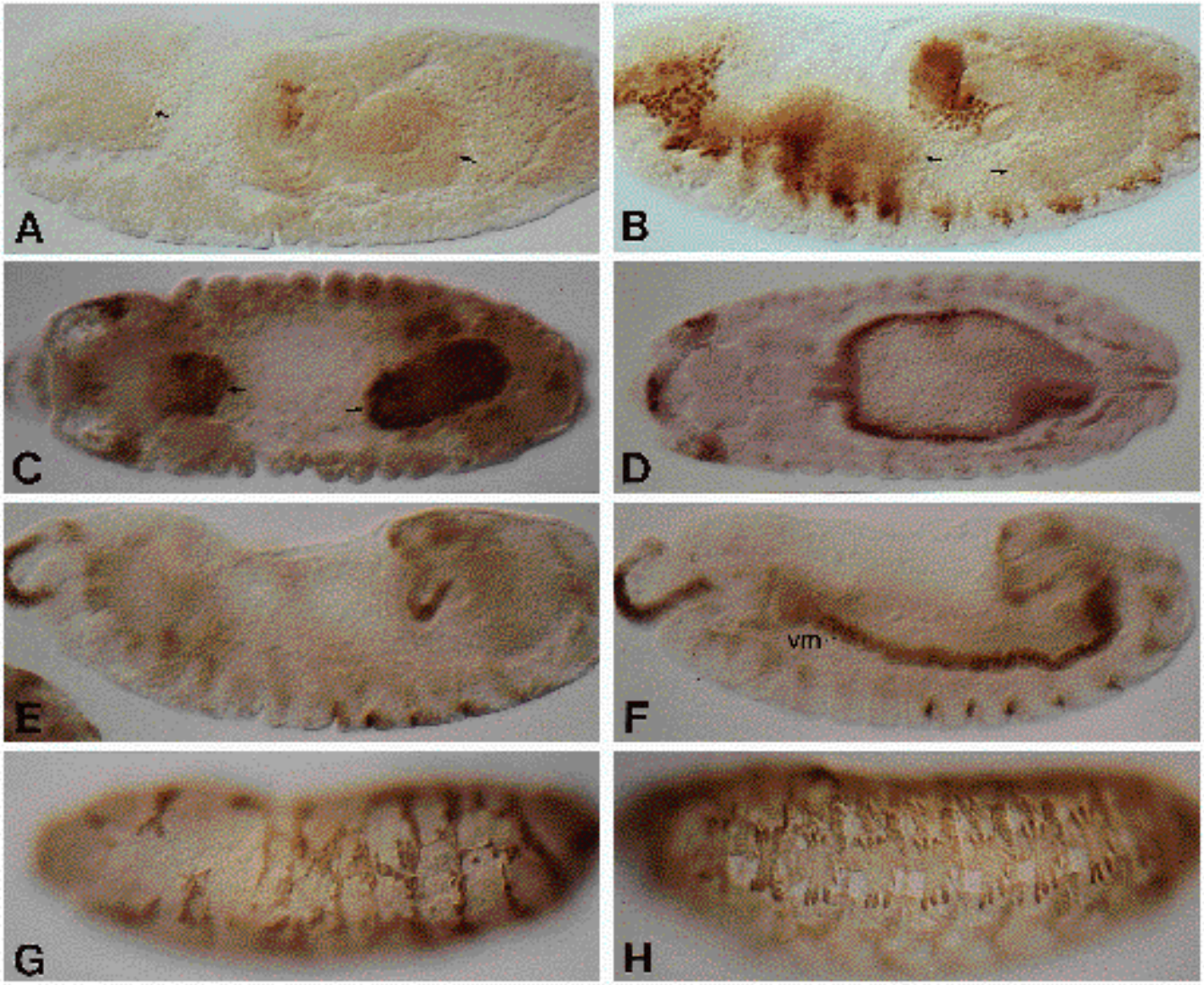

Fig. 4. Mutant embryos with a failure in visceral mesoderm differentiation do not form a midgut. (A,C,E,G) *twi*^{RY50} embryos, (B,D,F,H) wild-type embryos. (A) The late *twi* protein expression is affected in *twi*^{RY50} embryos (stage 11). Arrowheads indicate the leading edge of anterior and posterior midgut in wild-type and mutant embryo. (C) In *twi*^{RY50} embryos, AMG and PMG remain compact cell masses and do not enclose the yolk (dorsal view, stage 14). The visceral mesoderm (vm), visualized by immunostaining of the fasciclin III protein in a wild-type embryo (F), does not differentiate in *twi*^{RY50} embryos (E) (stage 11). (G) In contrast, *twi*^{RY50} embryos make somatic mesoderm as shown by staining for muscle myosin heavy chain (stage 17). (H) Wild-type embryo. All embryos in lateral view except in C,D (dorsal view). The embryos shown in C and D carry the enhancer trap A490.2M3 and were immunostained for β-gal protein.

Further support for our notion that the visceral mesoderm is required for endoderm migration and the transition of mesenchyme to epithelium comes from the phenotype of embryos deficient for the homoeobox gene *tin. tin* is expressed in the mesoderm in a *twi*-dependent fashion but is neither expressed in the primordia of the AMG nor of the PMG (Bodmer et al., 1990). It is required for the differentiation of the visceral mesoderm as embryos deficient for *tin* do not express fasciclin III within the mesoderm, and the mesodermal layer surrounding the yolk does not develop (Fig. 5E; Bodmer, 1993; Azpiazu and Frasch, 1993). Consistent with the postulated role of the visceral mesoderm, AMG and PMG of the *tin*-deficient embryos do not develop properly. They fail to form bilobate structures, do not migrate properly and are still at a considerable distance from each other when the germ band has been fully retracted (Fig. 5B). Later, AMG and PMG eventually join and appear to spread transversally as they often manage to wrap around the yolk ventrally (Fig. 5C). Only occasionally the midgut covers the yolk dorsally in a very irregular fashion at the end of embryogenesis. Since *tin* does not function directly in the midgut primordia (it is not expressed there), the failure of the midgut parts to migrate properly towards each other in the *tin*-deficient embryos strongly indicates that a differentiated visceral mesoderm is needed for this step in midgut organogenesis.

What are the molecules involved in migration and mesenchyme-to-epithelium transition?

It is reasonable to assume that cell adhesion molecules

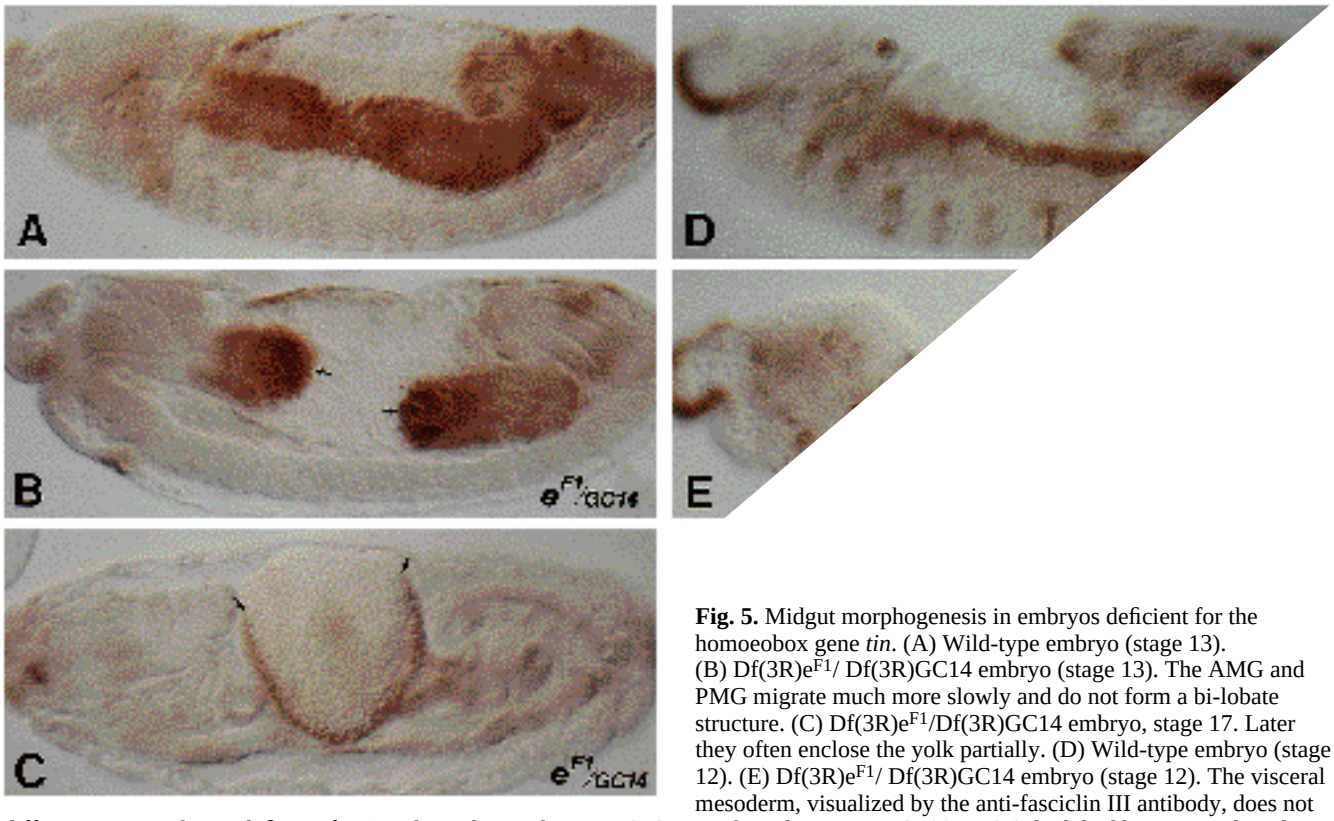

Fig. 5. Midgut morphogenesis in embryos deficient for the homoeobox gene *tin*. (A) Wild-type embryo (stage 13). (B) Df(3R)e^{F1}/ Df(3R)GC14 embryo (stage 13). The AMG and PMG migrate much more slowly and do not form a bi-lobate structure. (C) Df(3R)e^{F1}/Df(3R)GC14 embryo, stage 17. Later they often enclose the yolk partially. (D) Wild-type embryo (stage 12). (E) Df(3R)e^{F1}/ Df(3R)GC14 embryo (stage 12). The visceral mesoderm, visualized by the anti-fasciclin III antibody, does not differentiate in embryos deficient for *tin*. The embryos shown in A-C carry the enhancer trap A490.2M3 (which had been recombined onto the Df(3R)GC14 chromosome) and they were immunostained for β-gal protein.

expressed on the visceral mesoderm and the midgut endoderm mediate the interaction between these tissues. So far, however, we have been unable to identify any molecules that are stringently and specifically required. For example, embryos lacking fasciclin III, a cell adhesion molecule with immunoglobulin-type extracellular domains (Snow et al., 1989), do not show any disturbances of midgut development (data not shown). Fasciclin III mediates homophilic cell adhesion (Snow et al., 1989), is expressed on the entire surface of the visceral mesoderm cells and therefore might be involved in keeping the visceral mesoderm together rather than in mediating attachment to the endoderm. The integrin subunit α_{PS2} is localized at the interface between visceral mesoderm and midgut endoderm (Bogaert et al., 1987), is expressed in the visceral mesoderm and is believed to heterodimerize with the ubiquitous β1 chain (PS3) to a functional integrin. However, in mutants for *l(1)mys*, the gene encoding β1, the endoderm migrates properly on the visceral mesoderm. This is even the case in embryos lacking maternal and zygotic contribution of β1 or in the additional absence of fasciclin III (data not shown). Also embryos transheterozygous for deficiencies that remove an endoderm-specific β integrin (β_v; Yee and Hynes, 1993) develop a normal midgut (data not shown).

Of the cell surface molecules tested only *Notch* (*N*) and *Delta* (*Dl*) are required for migration of the endoderm and its transition to an epithelium. In *N* and *Dl* embryos, AMG and PMG express midgut-specific genes but remain as large mesenchymal cell masses immersed in the yolk as they do in *twi* embryos (shown in Fig. 6B,D for *Dl*; see also Hartenstein et al., 1992). They do not assume a bilobate structure and do not attach to the visceral mesoderm (Fig. 6B). In contrast, *N* and *Dl* do not seem to be required for the development of the visceral mesoderm. In *N* and *Dl* embryos, the mesoderm expresses fasciclin III and forms an apparently normal, continuous layer of cells along the germ band (Fig. 6F). It remains to be seen how *N* and *Dl* are involved in the process of migration.

How is the direction of endodermal migration determined?

It is conceivable that specific cues might be present on the anterior visceral mesoderm to direct the AMG posteriorly and on the posterior visceral mesoderm to direct the PMG anteriorly. We believe that this is not the case. Embryos from *torso* (*tor*) mothers develop an apparently normal AMG primordium and differentiate the visceral mesoderm, but no PMG is formed (Fig. 7A and data not shown). The AMG migrates far into the posterior region of the embryo, makes its transition to an epithelium, and later spreads transversally to enclose most of the yolk even in the absence of the PMG (Fig. 7B). In a wild-type embryo at stage 13, the junction between cells derived from AMG and cells derived from PMG is well visible (Fig. 1E). The visceral mesoderm just posterior to this junction, i.e. visceral mesoderm that touches the most anterior part of the PMG, expresses *Ultra - bithorax* (*Ubx*) over the length of one parasegment (Bienz and Tremml, 1988; Tremml and Bienz, 1989; Reuter and Scott, 1990; data not shown). When we use *Ubx* expression as a landmark in the visceral mesoderm of embryos from *tor*

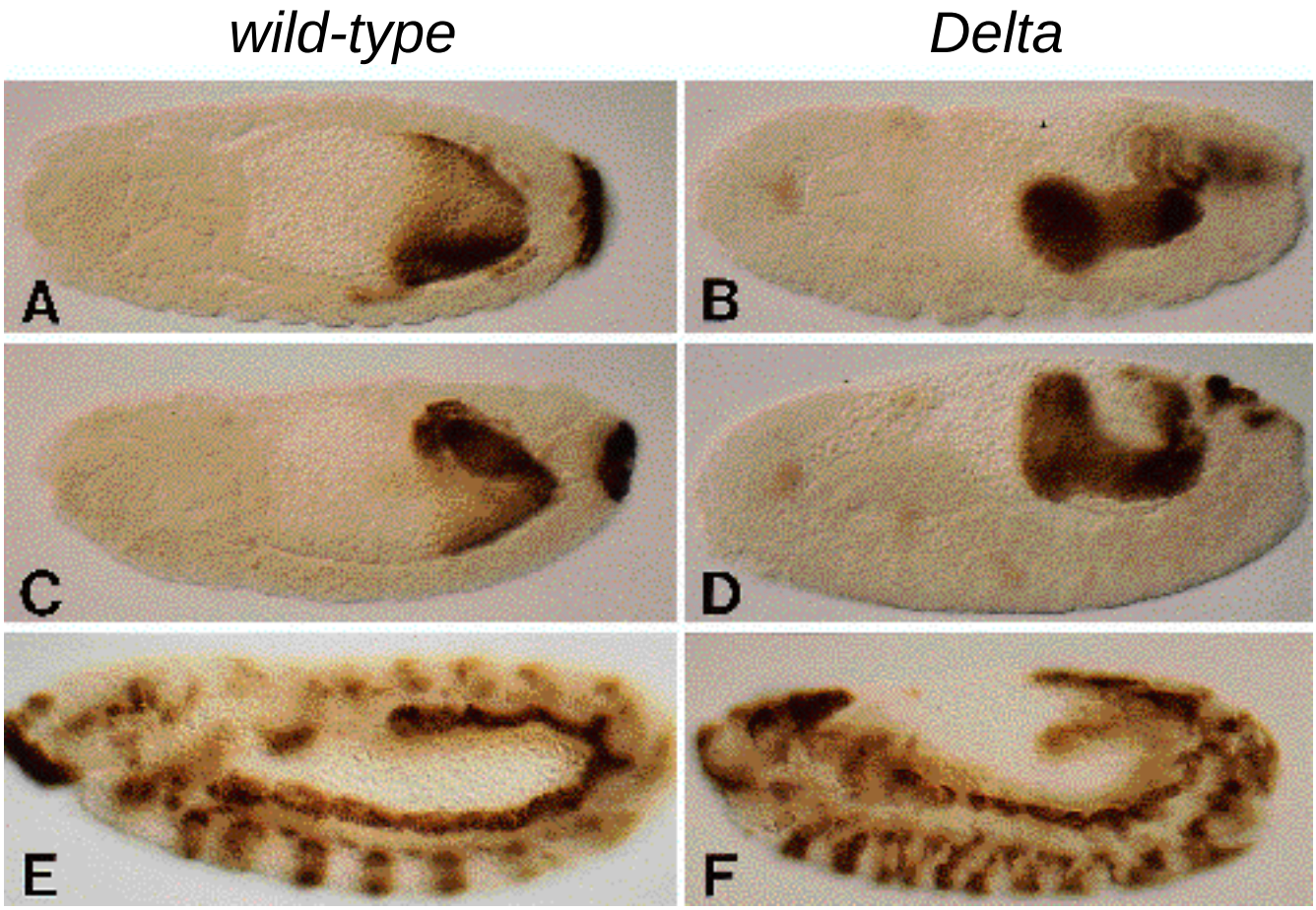

Fig. 6. Various cell surface proteins might be involved in midgut cell migration. (A,C,E) Wild-type embryos. (B,D,F) *Delta* embryos. In *Delta* embryos (B,D), the PMG expresses specific genes like *caudal* but remains a compact cell mass and does not enclose the yolk (B, dorsal view; D, lateral view of a stage 14 embryo). The visceral mesoderm is present in the mutant embryos (F) and expresses fasciclin III protein (stage 12). Embryos have been immunostained for *caudal* protein (A-D) or for fasciclin III protein (E,F).

mothers, we unambiguously see that the AMG migrates posteriorly beyond this point (Fig. 7C,D). Thus, the AMG migrates over visceral mesoderm that normally would contact PMG, and it migrates over this mesoderm in the direction opposite to the one that the PMG would normally take. This shows that the mesoderm does not contain any directional cues pointing towards the position where AMG and PMG normally meet; there is also no specific signal at this point that stops the migration. We propose that AMG and PMG merely spread over the available surface of the visceral mesoderm starting from their positions at opposite ends of the germ band.

DISCUSSION

The requirement of the visceral mesoderm for midgut morphogenesis

Our data on midgut development in wild-type and mutant embryos indicate that the visceral mesoderm is needed (i) for the migration of AMG and PMG towards each other and (ii) for the establishment of the midgut epithelium. We assume that the visceral mesoderm guides the migrating endoderm and that the contact to the visceral mesoderm is required for the endoderm to become an epithelium. We can only draw these conclusions if neither *sna*, *twi* nor *tin* have a direct function in the development of the midgut primordia and the observed midgut defects therefore have to be of indirect nature and due to the missing mesoderm. Certainly for *tin* this is the case. The midgut primordia never express *tin* (Bodmer et al., 1990) and their development proceeds normally until the time when migration should begin. Since *tin* codes for a transcription factor, like *sna* and *twi*, and therefore acts cell-autonomously, the defects seen in midgut development of *tin* embryos must be of indirect nature. We consider it unlikely that the transheterozygous deficient embryos that we used for our studies are lacking other genes directly involved in endodermal development since the same phenotype in midgut development has been observed in embryos mutant exclusively for *tin* (Bodmer, 1993; Azpiazu and Frasch, 1993).

twi protein is expressed in the primordia of AMG and PMG for a short period of time. We do not believe that this expression has a function for midgut development, although we have not proven this directly. However, a number of findings support our view. First, in *twi* embryos, early midgut development proceeds normally, up to the stage when migration should start. Secondly, weak *twi* embryos

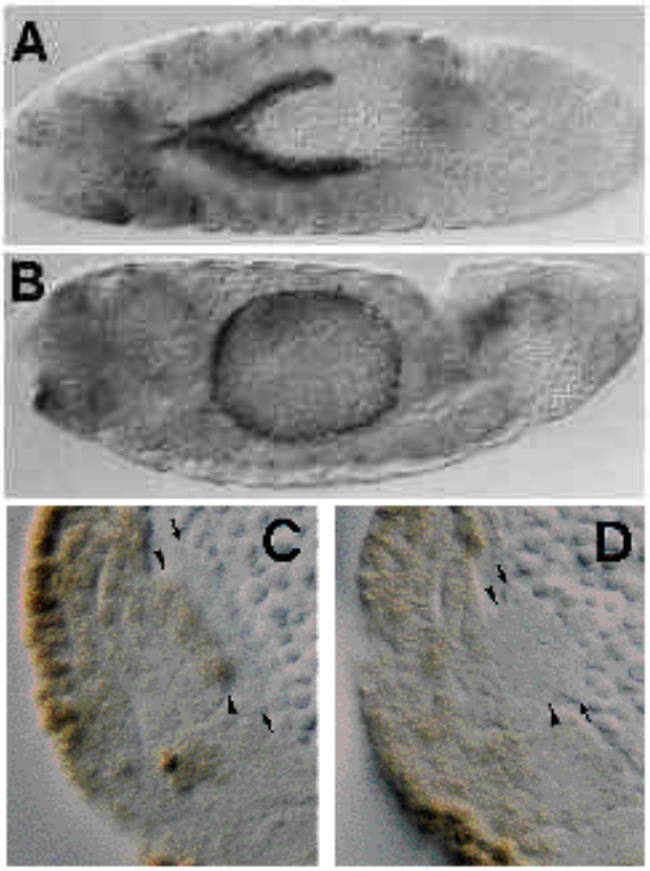

Fig. 7. Midgut development in mutant embryos lacking maternal terminal input. (A) Only the AMG develops in embryos from *tor* mothers. The lobes of the AMG reach far into the posterior of the embryo at stage 13 (dorsal view). (B) The AMG is sufficient to wrap around the yolk and to form a rudimentary midgut (about stage 16, lateral view). (C,D) Cross-sections through an embryo from a *tor* mother. The sections are 10 μm thick and 10 μm apart. (C) The AMG (arrow) not only reaches the visceral mesoderm, which expresses *Ubx* (arrowheads), (D) but even more posterior regions. Normally, the AMG is not in contact with visceral mesoderm expressing *Ubx* (compare Fig.1E). All embryos are derived from *tor* mothers that had been mated with males carrying the enhancer trap A490.2M3. The embryos in A and B have been immunostained for the β-gal protein, the embryo in C and D has been immunostained for the *Ubx* protein and cross-sectioned.

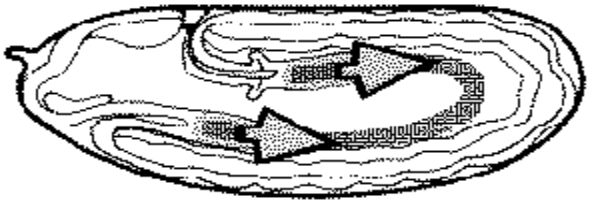

Fig. 8. We suggest that the visceral mesoderm (checkered) serves as a guiding tract for the migrating endoderm of anterior and posterior midgut (shaded). There are two possible mechanisms for such a movement. The endoderm could glide on the visceral mesoderm like a slug. Then the endodermal cells would constantly change their contact points to the mesoderm until the gap between anterior and posterior midgut is closed. Alternatively, motility could be confined to the inner endodermal cells, which are not in contact with the mesoderm while the endodermal cells contacting the visceral mesoderm stay in place. The extension would then occur by new endodermal cells being placed onto the visceral mesoderm at the leading tip.

initially express *twi* in the normal pattern and perform early *twi*-dependent events, like ventral furrow formation, only slightly slower than normal. Thus, the *twi* protein is apparently functional in these mutants. However, its later expression is impaired and the defects in the differentiation of somatic and visceral mesoderm can be attributed to the disappearance of the *twi* protein from the mesodermal cell layer after the onset of germ band extension (Fig. 4E,G). In these weak *twi* mutant embryos, *twi* protein is expressed normally in the midgut primordia. If *twi* had a function in these primordia it should fulfill it, as it fulfills its function in ventral furrow formation in these mutants. Any later defects in midgut development seen in these embryos should be indirect in nature. Since, with respect to gut development, the phenotypes of *twi* embryos homozygous for weak (Fig. 4C) or for amorphic alleles (Fig. 3C) are indistinguishable, we assume that also *twi* does not have a direct function in the midgut primordia.

sna protein is only very weakly expressed in the PMG primordium during cycle 13 (Alberga et al., 1991; Ray et al., 1991) before *sna* transcription is repressed by the activity of the terminal system in this region (Casanova, 1991; Leptin, 1991; Ray et al., 1991). Therefore it appears very unlikely that *sna* itself functions directly in posterior midgut development. Only the proper development of the AMG requires *sna* function (Reuter and Leptin, unpublished data). Therefore, the prerequisite for our conclusions, the indirect nature of the defects in midgut development, is fulfilled for *tin* embryos and most likely for the PMG of *sna* embryos and for AMG and PMG of *twi* embryos.

twi and *sna* embryos often fail to retract their germ band and then form a U-shaped cuticle (compare Fig. 3B and C). It has been speculated that germ band retraction plays a key role in bringing together the midgut parts (Campos-Ortega and Hartenstein, 1985). Thus, the failure of germ band retraction in *sna* or *twi* embryos might be responsible for the defects in midgut development. However, this is not the case. First, in wild-type development the extension of the midgut is already very far advanced when the germ band starts to retract (Fig. 1C). And secondly, in embryos with defective germ band retraction but normal mesoderm differentiation like *u-shaped* (*ush*) and *tail-up* (*tup*) embryos (Nüsslein-Volhard et al., 1984), the midgut primordia nevertheless extend normally along the visceral mesoderm and the yolk is completely engulfed by the endoderm (data not shown). Therefore, it is not germ band retraction alone which brings AMG and PMG closer to each other. In this context, it is worth mentioning that in *hkb* embryos, which do not form endodermal derivatives (Weigel et al., 1990), germ band retraction is properly executed (data not shown). Thus, the two processes, germ band retraction and endoderm migration, occur completely independent from each other. Other mechanisms that have been proposed to cause the movement of the AMG are its growth by cell division and pressure from the ingrowing foregut (Campos-Ortega and

Hartenstein, 1985). While these may contribute, they are neither sufficient (mitoses and pressure from the foregut) nor necessary (mitoses). For example, in *twi*s, the AMG proliferates and the foregut forms normally (Figs 3C, 4C), but the AMG does not extend; in *string* mutants, the AMG extends even in the absence of mitoses after cycle 14 (Edgar and O'Farrell, 1989; our data not shown).

The phenotype of *tin*-deficient embryos in midgut development is less severe than the one observed in *twi* or *sna* embryos. While bilobate structures are not formed and migration of the endoderm is not supported by visceral mesoderm, the two parts of the midgut nevertheless eventually join after stage 14, and the midgut partially encloses the yolk at the end of embryogenesis (Fig. 5B,C). We do not have an explanation for this phenomenon but we speculate that the same movement of the endoderm that normally leads to the transversal spreading also closes the gap in the mutant. The endoderm of the deficient embryos is in fact capable of the transversal spreading (Fig. 5C), and we assume that this movement is not directional by itself but merely involves spreading over the available surface of the yolk. During their spreading the cells never assume the organization of a columnar epithelium, and the resulting layer of endodermal cells is irregular unlike the wild-type epithelium. It is not clear whether the formation of this layer is aided by some residual function of rudimentary visceral mesoderm in *tin*-deficient embryos or whether it is completely independent of mesoderm.

The midgut does not seem to need its mesodermal component for the transversal spreading. However, the endodermal component is clearly required. Normally both the midgut endoderm and the adhering visceral mesoderm spread dorsoventrally so that the yolk is enclosed by the two tissues simultaneously. However, in *hkb* embryos that lack the entire endoderm the yolk does not become enclosed by the visceral mesoderm. Instead, a rudimentary net of muscles spreads over part of the yolk sac and partitions it into several irregular lobes (data not shown). We conclude that of the two tissues only the midgut endoderm can migrate on the yolk membrane in *Drosophila*.

How does the directional migration occur?

We propose that the visceral mesoderm serves as an adhesive guiding tract for the migration of AMG and PMG towards each other (Fig. 8). At the moment we can only speculate on the cellular mechanism of this migration. We assume that midgut cells have an affinity for cells of the visceral mesoderm and, as motile cells in their mesenchymal state, distribute over the surface of the visceral mesoderm. At the same time adhesion among the endodermal cells and among the mesodermal cells ensures the integrity of the respective tissues. The direction of migration would then result from the initial positions of AMG and PMG at opposite ends of the centrally located midgut visceral mesoderm and the availability of free mesoderm surface to migrate on. Thus, the migration would be only virtually directional and would not require specific cues for the AMG to migrate posteriorly or the PMG anteriorly. The finding that embryos from *tor* mothers extend their AMG far into the posterior region of the embryo supports this view (Fig. 7).

Molecules involved in endodermal migration or the transition from mesenchyme to epithelium

Of the cell adhesion molecules tested, only *N* and *Dl* appear to have a function in endodermal migration and/or the transition from mesenchyme to epithelium. The others (fasciclin III, PS3, β_ν integrin) might participate in these processes only in a redundant fashion. In *N* and *Dl* embryos AMG and PMG do not contact the apparently intact visceral mesoderm, remain mesenchymal and do not enclose the yolk (Fig. 6). The latter two aspects strikingly resemble the phenotype observed in mutants lacking the visceral mesoderm. However, *N* and *Dl* are nearly ubiquitously expressed (Haenlin et al., 1990; Kooh et al., 1993) and involved in so many processes during development (for a discussion see Hartenstein et al., 1992) that we cannot exclude an indirect effect on endodermal migration. In particular, it is difficult to decide whether *N* and *Dl* might not merely be required for a developmental decision within the endoderm, which would lead to the mesenchyme-to-epithelium transition (Hartenstein et al., 1992). For example, the expression of genes mediating the contact to the visceral mesoderm could depend on *N* and *Dl*. Without the attachment, the migration and the transition to an epithelium would not occur (like in the mutant embryos lacking the visceral mesoderm). Alternatively, it is possible that *Dl* and *N* directly mediate cell adhesion between visceral mesoderm and endoderm. It is tempting to speculate that *N* and *Dl* could even be involved in transmitting the signal to the endoderm, which induces the transition to a columnar epithelium upon adhesion to the visceral mesoderm.

The midgut as a model system for the interaction between germ layers during organogenesis

During the development of the midgut, two tissues derived from different germ layers, the visceral mesoderm and the midgut endoderm, interact at various times and in several ways so that different steps in morphogenesis are taken or regional specification is obtained. (i) We have shown that the layer of visceral mesoderm cells serves as a substratum for the migration of the endodermal midgut cells, the most prominent example of cell migration in *Drosophila* that leads to the fusion of the two previously separate primordia. We assume that such a mechanism is used in a wide range of insect species since the outgrowth of bilobate midgut primordia from cells close to stomodeum and proctodeum is commonly observed (for a review see Siewing, 1969). (ii) The formation of the endodermal columnar epithelium immediately after the migration is also dependent on the contact to the visceral mesoderm. (iii) Later the midgut develops several well-defined constrictions, which transiently partition the organ. Their formation is not only regulated by homoeotic genes expressed in the visceral mesoderm (Bienz and Tremml, 1988; Tremml and Bienz, 1989; Reuter and Scott, 1990), but they also are imposed by this tissue on the underlying endoderm (Reuter and Scott, 1990). (iv) The mesoderm also directs gene expression in the endoderm through appositional induction (Immerglück et al., 1990; Panganiban et al., 1990; Affolter et al., 1993). The genetic and molecular tools available for *Drosophila* therefore make midgut development a suitable model to

study developmental mechanisms that are widely used in morphogenesis and differentiation of various organs in *Drosophila* and in other species.

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