Serially homologous *engrailed* stripes are generated via different cell lineages in the germ band of amphipod crustaceans (Malacostraca, Peracarida)

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ABSTRACT A monoclonal antibody (mAb 4D9) was used to analyze *engrailed* expression in amphipod embryos. As in other arthropods, *engrailed* is expressed in iterated transverse stripes in the germ band. In the anterior region these stripes are generated without a recognizable division pattern, and their appearance and formation show some irregularities. In the posterior region of the germ band, *engrailed* expression is correlated with a stereotyped cell division pattern resulting in a highly ordered formation and array of stripes. The *engrailed* positive cells mark the anterior border of genealogical units, which therefore can be compared with parasegments in *Drosophila*. Expression starts in the mandibular segment and proceeds first anteriorly and subsequently in a posterior direction. Initial stripes are one cell wide. The widening of stripes is caused by both division of *engrailed* positive cells and recruitment of new cells that did not previously express *engrailed*. The widening process is related to segment formation as the intersegmental furrows are established behind the *engrailed* expressing cells, which are restricted to the posterior portion of the forming segments. A comparison of the modes of *engrailed* expression in different segments suggests that initial *engrailed* expression is independent of a certain cell lineage or division pattern. The comparison of the development of the early *engrailed* stripes in different insects and crustaceans reveals some similarities which show that early *engrailed* expression is not necessarily clonally inherited.

KEY WORDS: *engrailed, segmentation, Crustacea, clonal analysis, homonomy*

Introduction

The serial repetition of morphological structures along the body axis is one of the basic features of segmented animals. Repeated characters of one organism are considered serially homologous, or homonomous, if the same criteria for homology can be applied as to similar structures of different organisms (Dohle, 1989; Minelli and Peruffo, 1991). The differentiation of segments into functionally different units, e.g. antennal, mandibular and maxillary segments, and the subdivision of the body into tagmata, such as head, thorax and abdomen, often obscure homonomy in the adult animals. However, it can still be detected in many structures during early development. Homonomy is not only manifest in morphological characters such as ganglia, appendages etc., but also prevails in the expression pattern of segmentation genes; pair-rule and in particular segment-polarity genes are expressed in iterated transverse (homonomous) stripes in the germ band of *Drosophila* (reviewed by Akam, 1987; Ingham, 1988).

It has been suggested that in animals with stereotyped cell lineages, these lineages might be associated with a precise cell fate specification and that serially homologous cell types and structures are generated via serially homologous genealogical pathways (Stent, 1985; Shankland, 1991).

The higher Crustacea (Malacostraca) are unique among the arthropods in showing stereotyped homonomous cell lineage patterns in the posterior (post-naupliar) part of the germ band (Dohle, 1970, 1972, 1976; Scholtz, 1984, 1990, 1992; Dohle and Scholtz, 1988). On the other hand, no such patterns can be found in the anterior region of the germ band. These differences between the cell division patterns of anterior and more posterior parts of the germ bands of malacostracans offer the possibility of addressing questions concerning the development of homonomy. For instance, it has been shown that despite different generation of cell rows in the malacostracan germ band, some morphological characters such as neuroblasts and appendage buds are differentiated in a homonomous manner (Dohle, 1976, 1989; Dohle and Scholtz, 1988; Scholtz, 1992).

The segment polarity gene *engrailed* has been shown to be highly conserved among the arthropods. It is expressed
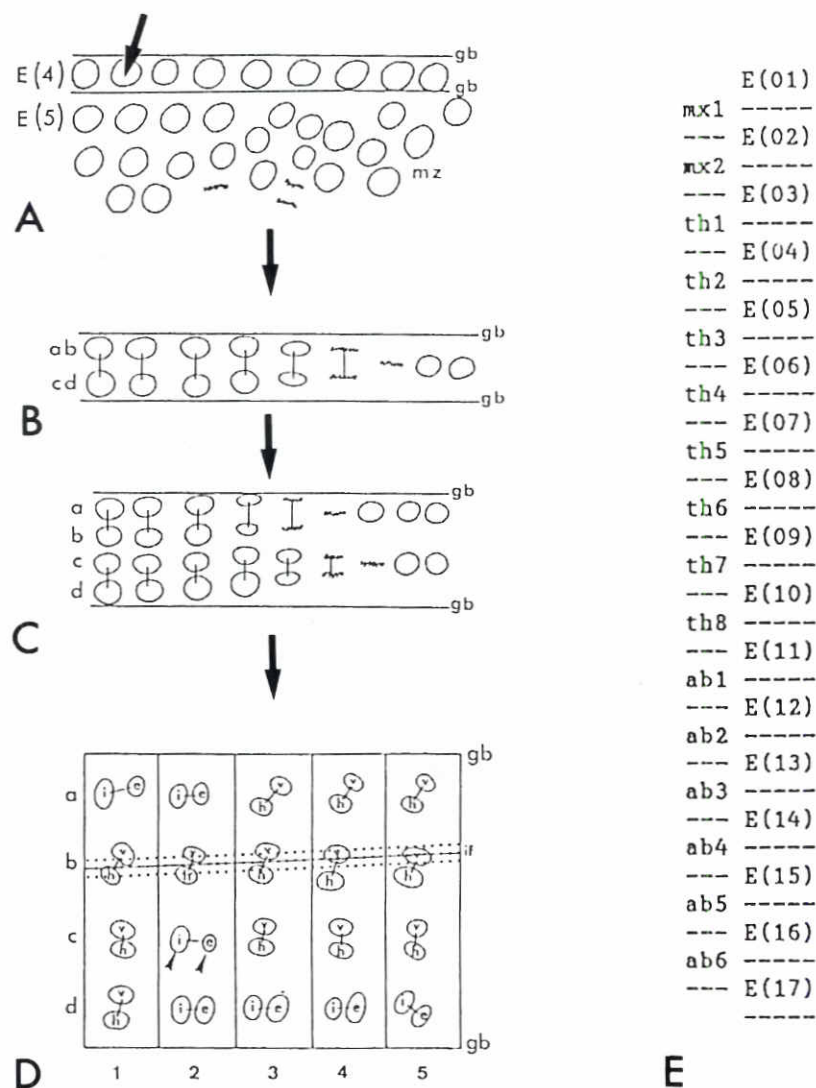
Fig. 1. Schematic summary of formation and segmentation of the post-naupliar amphipod germ band (for details see Scholtz, 1990). The naupliar region (1st and 2nd antennal segments and mandibular segment) shows no identifiable cell division pattern (see Fig. 2). (A-D) The post-naupliar segments (segments of 1st and 2nd maxillae, 8 thoracic, and 6 abdominal segments) are formed with an underlying stereotyped, metamerically iterated pattern. Only the animal's left side is shown with the symmetry axis to the left. (A) In the post-naupliar region 17 transverse rows (E(1) to E(17)) are generated in anteroposterior sequence by a posterior meristematic zone (mz) which is, in contrast to all other investigated malacostracans, not built up of ectoteloblasts. Each row is formed by a mediolateral alignment of scattered blastoderm cells. The cells of each row are designated in relation to their distance from the midline (e.g. E(4)$_2$ see arrow). After formation, each row (E(2) to E(17)) undergoes two mediolateral mitotic waves with only longitudinally-oriented and equal mitoses, resulting in four transverse descendant rows named a,b,c,d (B), (C). Thereafter the differential cleavages begin. They show a stereotyped pattern of mitoses with regard to size and position of the division products. Row E(1) shows a somewhat different division pattern during the differential cleavages, but a stage with four descendant rows occurs (see Fig. 3). (D) Depicts a simplified schematic pattern of the first differential cleavage (of rows E(2) to E(17)) up to the fifth cells from the midline. The cells are individually labeled, for example, c_2i and c_2e (arrowheads). With the differential cleavages, segmentation begins. The segment boundary marked by the intersegmental furrow (if) does not match the genealogical border (gb). The intersegmental furrow runs transversely and slightly obliquely through the descendants of one ectoderm row in the area of descendant rows a and b. Thus, the descendants of each ectoderm row contribute to two segments. For instance, row E(3) gives rise to the posterior part of the first thoracic segment and the anterior part of the second thoracic segment (compare also Figs. 1E, 6). The anterior descendants of row E(1) contribute to the mandibular segment. (E) The spatial relationship between the ectoderm rows (E(1) to E(17)) and the post-naupliar segments (mx1 to ab6). The lines between the ectoderm rows represent the genealogical borders which are out of register with the segment boundaries (lines between segment designations). mx, maxilla; th, thorax; ab, abdomen

homonomously in iterated transverse stripes in the posterior portion of segments in several insects (e.g. DiNardo et al., 1985; Kornberg et al., 1985; Patel et al., 1989a; Fleig, 1990; Sommer and Tautz, 1991, 1993; Patel, unpublished data) and crustacean species (Patel et al., 1989a,b; Manzanares et al., 1993; Scholtz et al., 1993).

In the present investigation we analyze the expression pattern of engrailed (en) in the germ band of amphipod crustaceans in which the cell lineage has formerly been established (Scholtz, 1990). We address the questions of whether a homonomous en stripe pattern occurs throughout the germ band despite the differences in the underlying cell division patterns and how en expression is related to cell lineage. We use the mAb 4D9 antibody (Patel et al., 1989b), which has been shown to recognize en in several crustacean species (Patel et al., 1989a; Scholtz et al., 1993). These earlier studies dealt with the relationship between en expression and cell lineage in the posterior germ bands only. An investigation of en expression in the whole germ band of crusta-

ceans and a more detailed analysis of the relation to cell lineage remained to be made.

We show that en is expressed in similar transverse stripes throughout the germ band of amphipods — independent of the preceding cell lineage: homonomy can arise via different pathways. The widening of en stripes is a combination of division of en-positive cells and recruitment of new cells. These results provide evidence that initial en expression is not closely linked to a certain lineage, not even in animals that display a stereotyped lineage. This corroborates the conclusions of Patel et al. (1989a), Manzanares et al. (1993), and Scholtz et al. (1993) in Crustacea and Vincent and O'Farrell (1992) in Drosophila.

Results

General features of en expression in amphipods

The prerequisite for the present study is the exact knowledge of the cell lineage in the amphipod germ band (Scholtz, 1990). A short
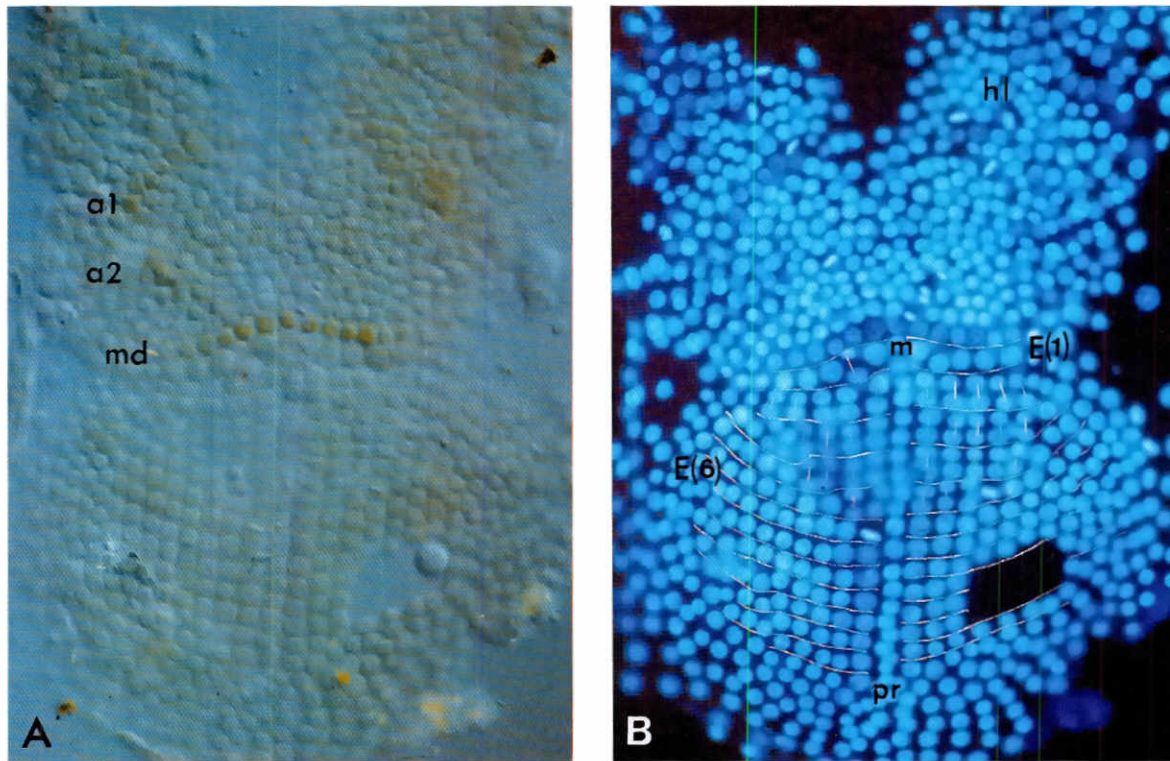
Fig. 2. The onset of *en* expression in the germ band *(Gammarus roeselii)*. *In the post-naupliar germ band about 11 or 12 ectoderm rows are formed, and rows E(1) to E(6) undergo their first wave of division. (A) The stripe marking the posterior portion of the mandibular segment (md) is clearly visible. The stripes of the first antennal segment (a1) and somewhat weaker of the second antennal segment (a2) are also recognizable. In all preparations they appear almost simultaneously. (B) Same preparation counterstained with fluorescent dye. Fluorescence in en positive cells is quenched. Genealogical borders (gb) between rows and divisions of first mitotic waves in the post-naupliar germ band are indicated by white lines (comp. Fig. 1 and Scholtz, 1990). In the post-naupliar region rows E(11) and E(12) are formed. Rows E(1) to E(6) undergo their first mitotic wave. Note the difference in the cellular arrangement between the naupliar and post-naupliar regions. hl, headlobes; m, median longitudinal cell row; pr, proctodaeum.*

summary is given in Fig. 1. The cell lineage and the pattern of *en* expression are nearly identical in all species examined. Therefore, the principles of *en* expression in amphipods are described with no reference to the individual species. *en* expression is exclusively restricted to the ectoderm in the stages examined. Staining is nuclear. This is particularly evident in interphase nuclei. During karyokinesis, the staining is first concentrated around the chromosomes and then fades away. It reappears strongly after telophase. The first indications of *en* expression are detectable in the posterior region of the prospective mandibular segment (Fig. 2). From the mandibular segment, *en* expression spreads out over the germ band, first in the anterior and then in the posterior direction (Figs. 2,3).

en stripes are generated by different cell division patterns in the naupliar and post-naupliar regions

In the naupliar region, the development of *en* stripes follows a mediolateral gradient, with staining first appearing in more median cells. The appearance of *en* stripes varies between the naupliar segment primordia. In the first antenna, two hemisegmental stripes are formed with no median connection. The second antennal and the mandibular segments clearly show continuous transverse *en* stripes. From the onset, the stripes of the antennae are irregularly

formed and in part more than one cell wide (Figs. 2,3). In contrast, the one-cell-wide *en* stripe of the mandibular segment shows a proper array of aligned cells (Fig. 2). The initial ratio of *en* positive to *en* negative cells in the naupliar region varies; but there is approximately one *en* expressing cell in a longitudinal row of five cells (Fig. 3).

In contrast to the naupliar region, *en* expression in the ectoderm of the post-naupliar germ band follows a stereotyped pattern. This is correlated with the orderly array of the ectoderm rows and the stereotyped cell division patterns. In rows E(1) (see below) and E(2), the onset of *en* expression is delayed compared to more posterior rows and some irregularities can be seen in the sequence of *en* expression (Fig. 3). During the second mitotic wave of each ectoderm row, the cells of the most anterior descendant row a begin to express *en*. Expression starts in cell a_1 when 3 to 4 cells per hemisegment of descendant row ab have divided (Fig. 3). The *en*-positive reaction proceeds laterally cell by cell lagging behind the mitotic wave by 2 cells (Fig. 4C). The unpaired median cell of each transverse stripe shows staining when 1 to 3 cells per side are already labeled (Fig. 4C). In row E(2) this occurs even later (Fig. 3). The initial ratio of *en* expressing rows to non-expressing rows is one to a total of four (row a expressing, rows b,c, and d non-expressing). The only exception is seen in row E(1). This row
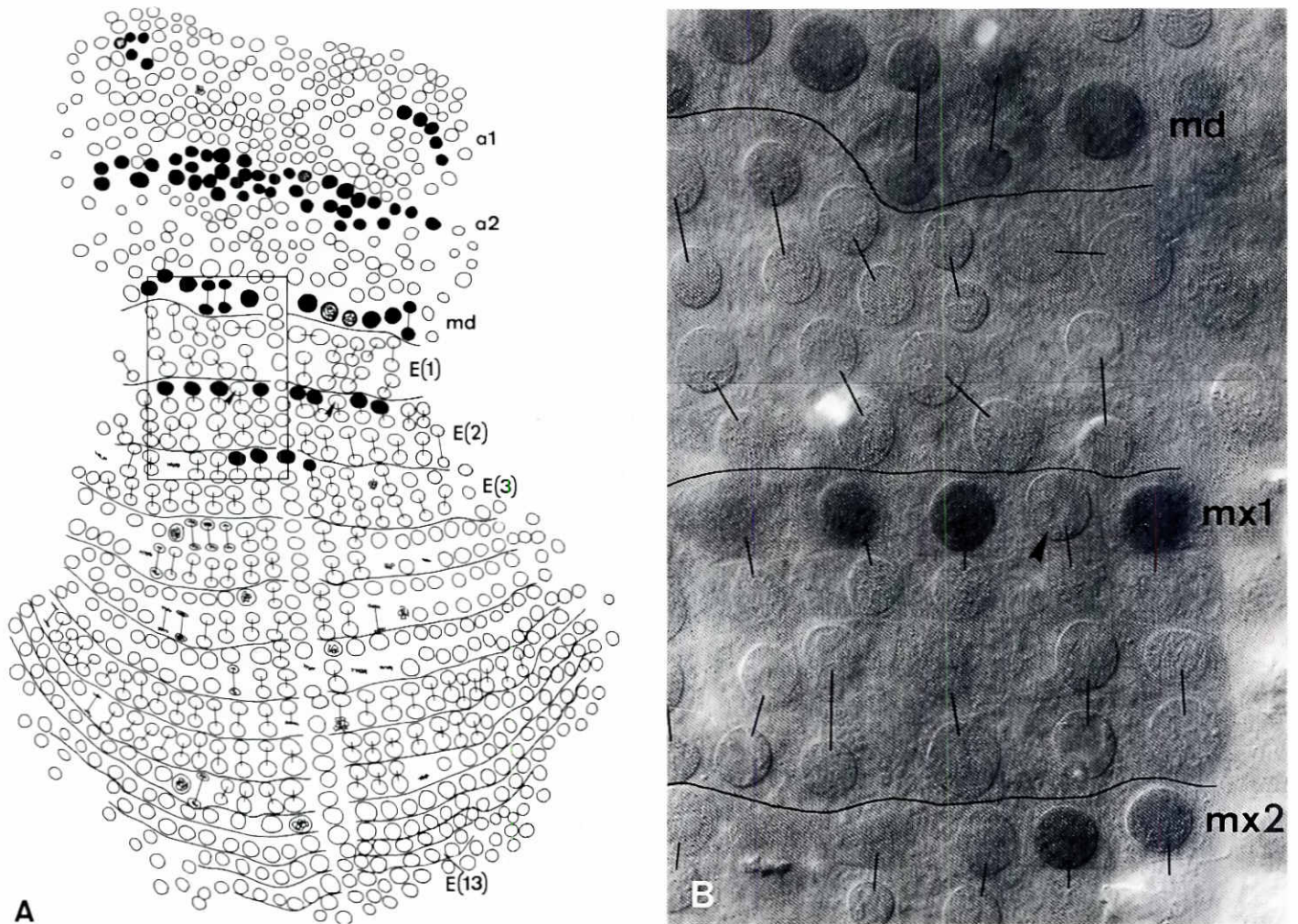
Fig. 3. Onset of *en* expression in the post-naupliar germ band in rows E(2)a and E(3)a *(Orchestia cavimana).* (A) *Camera lucida drawing of a whole germ band. Row E(13) is almost completely arranged. Widening of stripes in the naupliar region has begun. Row E(1) does not show any en expression in that stage. Some irregularities in the mediolateral sequence of en expression can be seen in row E(2)a. The cells a_2 (animal's right side) and a_3 (animal's left side) show delayed expression (arrowheads). Note the different degree of differentiation between the lateral halves of the germ band in the naupliar and post- naupliar regions. This concerns the number of divisions and the number of en-positive cells. In contrast to the naupliar region (Fig. 2), the stripes in the post-naupliar region appear in a strictly anteroposterior sequence (compare Fig. 4). (B) Photomicrograph of the same preparation (animal's right side) showing the widening of the mandibular stripe and the formation of the stripes of first and second maxilla (rows E(2)a and E(3)a).*

exhibits a somewhat different division pattern compared to the more posterior rows (Scholtz, 1990). When four descendent rows are formed, none of these shows *en* expression. Thus, there are four unlabeled rows behind the mandibular *en* stripe. Therefore, the initial ratio in the 1st maxillary segment is one *en* expressing cell in a longitudinal row of five cells (Fig. 3).

en stripes widen by a combination of division and recruitment

With advanced development, the *en* stripes widen in the longitudinal direction. In the antennal segments and the ocular region the analysis of the mode of widening is hampered by the irregular division pattern. In all other segments, division as well as recruitment are responsible for the widening of *en* stripes. In the stripe of the mandible segment a mediolateral wave of divisions with longitudinal spindle axes can be seen resulting in a two-cell-wide stripe of *en* expression (Fig. 3). Only the cell closest to the midline

divides obliquely, and its mitosis is somewhat delayed. Additionally, anterior descendants of post-naupliar row E(1) are recruited in later stages to express *en* (Fig. 4A).

The widening of the stripes in most rows of the post-naupliar region follows a stereotyped pattern. Addition of *en* expressing cells is clearly a combination of division and recruitment. The process of widening is correlated with the differential cleavages of the ectoderm row progeny, and it proceeds in a lateral direction. After the first differential cleavage, all descendants of cells of row a which previously expressed *en* also express *en*, independent of the orientation of the spindle axes of the mitoses (Figs. 5,6). Additionally, anterior daughter cells of the divisions of row b start to express *en* shortly after mitosis (Figs. 5,6). Their posterior sister cells remain *en*-negative. After the first differential cleavage, only the inner five or six anterior descendants of row b express *en* (up to cell b_5v or b_6v) (Fig. 6). After the first differential cleavage, the

median part of the *en* stripe is about three cells wide as most mitoses in rows a and b have longitudinal spindle axes. During the next differential cleavage (as far as analyzed), only the progeny of *en* expressing cells shows *en* labeling again (Fig. 6). *en* expression does not occur in the progeny of descendant rows c and d.

With widening of *en* stripes during differential cleavages, the ratio between *en*-positive and *en*-negative rows is shifted towards relatively more *en* expressing rows. This shift is due to the transversely oriented mitoses in the rows in front of the anterior margin of the *en* expressing areas (in the post-naupliar germ band row d) (Figs. 4,6). A corresponding phenomenon can be seen in the naupliar region. The anterior border of the *en* expressing regions is a sharp transverse line in naupliar and post-naupliar segment primordia (Figs. 4,5).

Segment boundaries match the posterior margin of en stripes

After the first differential cleavage, the segmental borders become established. These are marked by intersegmental furrows. In the post-naupliar region, the furrows run transversely and slightly obliquely through the descendants of row b and more laterally behind row a, that means within the descendants of one initial cell of an ectoderm row. *en* is expressed in the posterior portion of forming segments and the position of the intersegmental furrow corresponds to the posterior margin of the area of *en* expression (Fig. 6). This is also true for the naupliar segments, where no differential cleavage patterns occur (Fig. 4). During further development the posterior margins of limb buds and ganglion primordia also express *en* in naupliar as well as in post-naupliar segments (Fig. 6).

Discussion

Appearance of en stripes

The present investigation shows that most features of initial segmental *en* stripes are similar within individual germ bands of amphipods. These similarities include the mediolateral propagation of each *en* stripe, the one-cell width of the initial stripes, and the widening by both recruitment of new *en* expressing cells and division of *en*-positive cells. Furthermore, *en* is expressed in the posterior region of all segments immediately in front of the intersegmental furrows as in other crustaceans and insects. These combined similarities allow us to homologize the individual *en* stripes. Despite these overall similarities, the underlying cell division pattern varies considerably between the naupliar and the post-naupliar regions of the amphipod germ band. In the naupliar segments there are scattered cells with no recognizable division pattern, whereas in the post-naupliar segments there are stereotyped iterated cell lineages. This clearly demonstrates that homonomous patterns and cell fates can arise via different developmental pathways. How can these differences in the underlying cell division patterns be explained? One possibility is that the different lineages represent an early "tagmatisation" and that homeotic genes may specify the variety of cell division patterns on the germ band. At least six homeotic genes have been identified in the crustacean *Artemia* (Averof and Akam, 1993).

The correlation between *en* expression and the stereotyped cell division pattern in the posterior germ band of amphipods and other malacostracans suggests that cell lineage might play a major role in controlling the *en* gene expression and segmentation in general. However, the comparison between the naupliar and post-naupliar
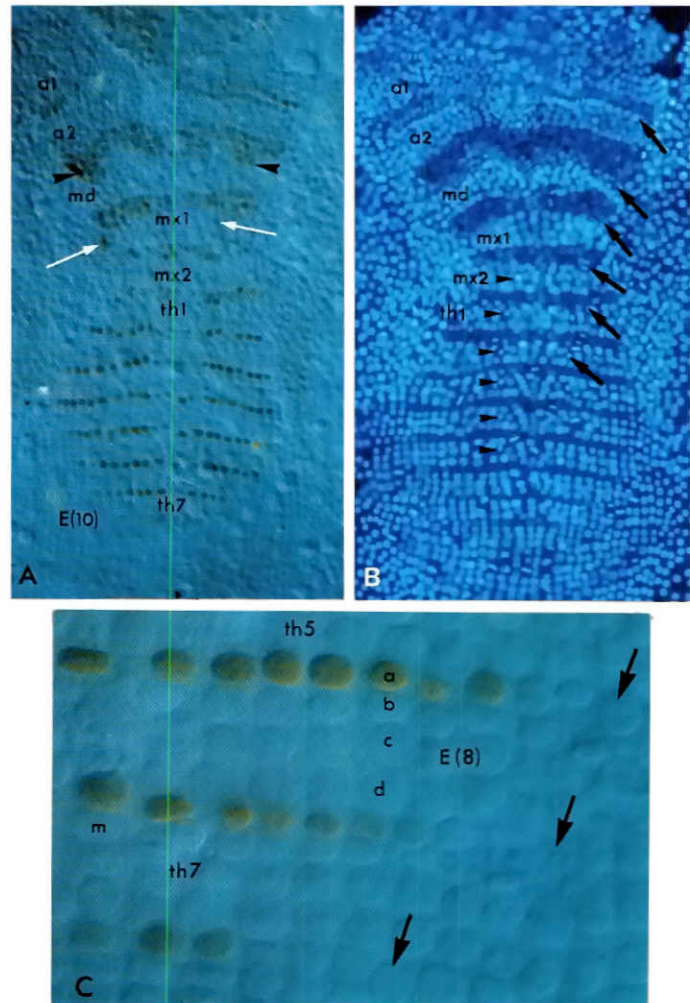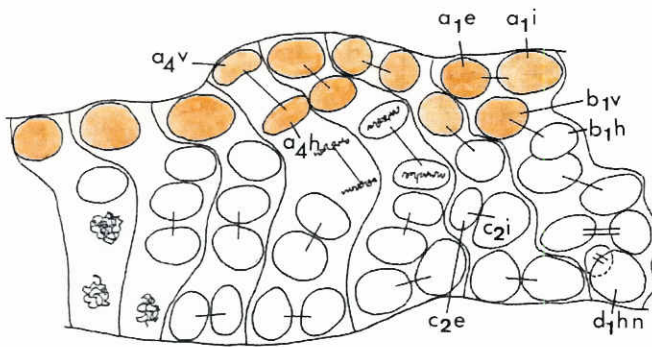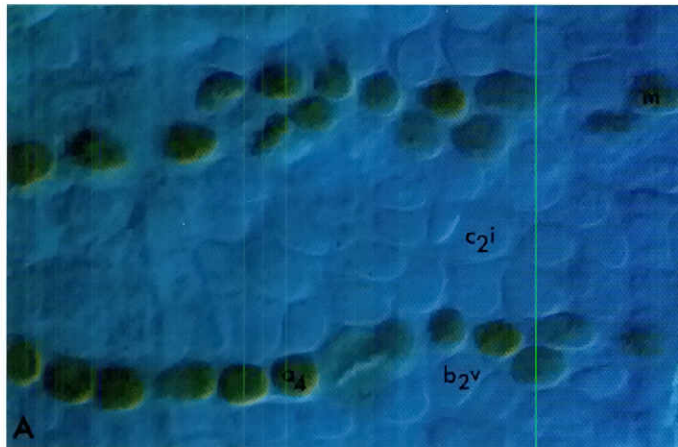

Fig. 4. Advanced stage of *en* expression in the whole germ band (*Orchestia cavimana*). (A) Row E(16) is formed and en expression has reached row E(10) (7th thoracic segment (th7), see Tab. 1). The widening process takes place up to row E(7) (4th thoracic segment). The stripes in the naupliar region show the most advanced development. The stripes of the maxillary segments are the shortest (rows E(2)a and E(3)a). Posteriorly, the number of en-positive cells increases from stripe to stripe until a maximum of about 10 to 11 en cells per hemisegment is reached in row E(6) and subsequent rows. The most lateral cells of each row do not express en. Arrows point to single randomly distributed en expressing cells. Their fate is unknown. Arrowheads indicate anterior en-positive cells of row E(1). (B) Counterstaining with fluorescent dye to show the nuclei and mitotic figures. Fluorescence in en positive cells is quenched. Intersegmental furrows occur in the anterior region of the germ band (from the first antennal segment to the first thoracic segment). They are recognizable by the deeper-lying nuclei (arrows) posterior to the en positive cells. For orientation, arrowheads point to the derivatives of d₂ in different rows. (C) Higher magnification of the posterior region (5th to 7th thoracic segments) of the same preparation to show the formation of en stripes in the post-naupliar germ band. (Compare Figs. 1C, 3). Anterior is up, midline to the left. en expression follows an anteroposterior and a mediolateral gradient. The second mitotic wave in each row is about three cells ahead of en expression (arrows). a1, first antenna; a2, second antenna; m, median cell row; md, mandible; mx1, first maxilla; mx2, second maxilla; th1, first thoracic segment.

Comparisons between different segments of the amphipod germ band as well as between corresponding segments in different crustacean species allow the conclusion that initial *en* expression in crustaceans does not seem to be controlled by cell lineage. The stereotyped cell division pattern in the post-naupliar germ band of malacostracans is a new evolutionary acquisition of or within this taxon (Scholtz, 1992), whereas the segmental *en* expression is phylogenetically much older (Patel *et al.*, 1989b). Therefore, the cell lineage pattern of malacostracans may just be a complicated invariant way of generating competent material for subsequent segmentation, as suggested by Dohle and Scholtz (1988) and Patel *et al.* (1989a).

Widening of en stripes

The initial one-cell-wide *en* stripes on the amphipod germ band widen by a combination of division of *en*-positive cells and the recruitment of cells which previously did not express *en*. The *de novo en* expression was exclusively observed at the posterior margin of the stripes. Corresponding events have been reported from other crustacean species (Patel *et al.*, 1989a; Manzanares *et al.*, 1993; Scholtz *et al.*, 1993) and within the insects from the locust (Patel *et al.*, 1989a) and the honey bee (Fleig, 1990). In contrast, the initial widening of the *en* stripes in *Drosophila* (from one- to two-cell-width) seems to be due to cell rearrangement during early germ band extension; during subsequent mitotic activity, only the decay of *en* expression at the posterior margin of the stripes has been detected (Vincent and O'Farrell, 1992). What are the possible explanations for these differences between *Drosophila* and the other arthropod species?
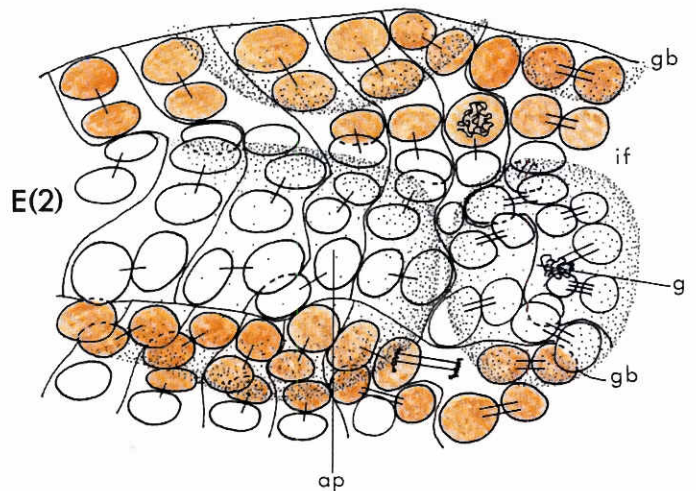
Fig. 5. Widening of stripes in the thoracic region during first differential cleavage *(Orchestia cavimana)* *(for orientation and nomenclature compare Fig. 1D).* **(A)** *Micrograph using differential interference contrast. Anterior is up, midline to the right. Widening is due partly to divisions of en-positive cells of row a and partly to recruitment of new cells which are anterior derivatives of row b. The latter become en positive shortly after division. Cell a₁i is always more weakly stained than other cells. The weak expression of en in cells at the anterior margin of more advanced stripes is also reported from Drosophila (Vincent and O'Farrell, 1992) and other insects (Patel unp. data). The next posterior row is less advanced with regard to the number of divisions. m, median cell row.* **(B)** *Camera lucida drawing of the same preparation showing the genealogical relationships between the descendant cells (only nuclei are drawn) of one row. One line connects sister cells after the first differential cleavage; two lines connect cells after the second differential cleavage.*

Fig. 6. Onset of second differential cleavage and segmentation in row E(2) (post-naupliar) *(Orchestia cavimana).* *One line connects sister cells after the first differential cleavage; two lines connect cells after the second differential cleavage. Anterior is up, midline to the right. en-positive cells are shaded brown. The genealogical border (gb) runs through the anlagen of the appendage bud (ap) and the ganglionic primordium (g) of the second maxilla, which are thus composed of descendants of two adjacent rows. In both, the posterior portion is formed by en-positive cells. The intersegmental furrow (if) (in this case between the first and second maxillae) lies within the progeny of a given row.*

segments and the irregularities during formation of individual post-naupliar *en* stripes show that *en* expression is not strictly linked to a certain cell division pattern in malacostracans. Corresponding results have been obtained from comparisons between different crustacean species. It has been shown that in the post-naupliar germ bands of other malacostracans such as the crayfish *Cherax destructor* and the mysid *Neomysis integer*, initial *en* expression appears one cell cycle earlier than in amphipods (Scholtz *et al.*, 1993). Moreover, in the anostracan *Artemia franciscana*, an *en* homolog is expressed in a similar pattern as in malacostracans (Manzanares *et al.*, 1993) although a corresponding cell division pattern does not occur (Freeman, 1989; Manzanares *et al.*, 1993).

On the one hand, recruitment could also take place in early *Drosophila en* stripes but it has not been detected so far. In their study, Vincent and O'Farrell (1992) analyzed the outcome of two to three cell cycles after the blastoderm stage (one-cell-wide stripes) and found only decay of *en* expression. They did not analyze the situation after the first post-blastoderm division. However, in amphipods as in crayfish, locust (Patel *et al.,* 1989a) and honey bee (Fleig, 1990) recruitment only occurs during the first division, after the stage of the initial one-cell-wide *en* stripes.

On the other hand, recruitment of new *en* expressing cells may not occur in a corresponding way in *Drosophila* and the observed differences may reflect the different modes of germ band development. One might speculate whether the initial widening of *en* stripes in *Drosophila* caused by cell rearrangement might be equivalent to the recruitment phase in other arthropods where these cell rearrangements do not occur. It is well established that early *en* expression in *Drosophila* is regulated by the activity of the *wingless (wg)* gene product (Bejsovec and Martinez-Arias, 1991; Ingham and Martinez-Arias, 1992), and there is some evidence that this is also true for short-germ insects such as the beetle *Tribolium castaneum* (Nagy and Carroll, 1994). *wg* is expressed in transverse stripes which lie anterior and adjacent to the *en* stripes. The wg protein can be found up to a distance of about three cell diameters from the synthesizing cells (Bejsovec and Martinez-Arias, 1991). It has been suggested that only cells which remain in the *wg* domain continue to express *en* after post-blastoderm divisions and that cells which lie posterior to the *wg* influence secondarily lose *en* expression (Vincent and O'Farrell, 1992). Since the *en* stripes are already two cells wide with the onset of the post-blastoderm mitoses the first round of post-blastoderm divisions must already lead to a decay of *en* expression at the posterior margin of the *en* stripes.

Our findings in amphipods and other crustaceans (Patel *et al.,* 1989a; Scholtz *et al.,*1993) suggest that a crustacean homologue of the *Drosophila wg* gene might be involved in the regulation of early *en* expression in crustaceans. In amphipods, it would be expressed during the second wave of division in the descendant row d which lies anterior to descendant row a (*en* positive row) of the next posterior ectoderm row. During the first differential cleavage, the wg protein would then spread out and the anterior descendants of row b eventually lie in the domain of the wg protein and start to express *en* in addition to the descendants of row a.

In summary: despite the differences between *Drosophila* and other arthropods with regard to some details in initial *en* stripe formation, the observed recruitment of new *en* expressing cells leads to the same conclusions as the reported loss of *en* expression in *Drosophila* (Vincent and O'Farrell, 1992) and the early regulation of *en* stripes in some higher crustaceans (Scholtz *et al.,* 1993): namely that *en* expression is not strictly clonally inherited from the onset but that cell-cell interactions determine and modulate *en* patterns.

Materials and Methods

Embryos of 3 amphipod crustacean species *(Gammarus pulex, Gammarus roeselii* and *Orchestia cavimana)* were investigated. These were collected from a brook in the north of Berlin *(G. pulex* and *G. roeselii)* and from the banks of the river Weser near Oldenburg *(O. cavimana)* (Germany). Females with developing eggs in the marsupium were processed immediately. Pairs in pre-copula were isolated and kept in vials at 18°C. In this way, the time of egg-laying can be exactly determined.

The antibody labeling procedure mainly followed Patel *et al.* (1989b). Eggs and embryos were removed from the marsupium of the females and transferred to the PEM-FA fixative (0.1 M PIPES (pH 6.95), 2.0 mM EGTA, 1.0 mM $MgSO_4$, 3.7% formaldehyde). The chorion and most of the yolk were then removed with insect pins under a dissecting microscope. The germ bands were fixed for 30 min. After fixation they were washed for 5 min in PBS, three times for 5 min and twice for 30 min in PBT (PBS, 0.2% BSA, 0.1% Triton X-100) and then incubated for 30 min in PBT+N (PBT plus 5% normal goat serum). An equal volume of mAb 4D9 was added and the germ bands were incubated overnight at 4°C. After incubation they were washed three times for 5 min and four times for 30 min in PBT and again incubated in PBT+N for 30 min. Goat anti-mouse IgG (Jackson Immunoresearch) was added to a dilution of 1:200 in PBT+N and the germ bands were incubated overnight at 4°C. After incubation they were washed three times for 5 min and four times for 30 min in PBT and then placed in a solution of 0.3 mg/ml DAB in PBT for 15 min. Then H_2O_2 was added to a concentration of 0.03% and the reaction was allowed to proceed for about 10 min. The stained germ bands were washed in PBS for 10 min and counterstained with fluorescent dye (0.2% solution of Bisbenzimid H 33258) for 15 min, then washed in distilled water and mounted in glycerol. Additionally, some preparations were stained with Delafield's haematoxyline (Romeis, 1968) instead of Bisbenzimid and mounted in Euparal after dehydration with ethanol.

Analysis, camera lucida drawings, and photography were done with brightfield, differential interference contrast, and epifluorescence microscopy using a Zeiss Axiophot.

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