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

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0214-6282/94/$03.00

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Printed in Spain
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 crustaceans 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 rosenii). 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, head lobes; m, median longitudinal cell row; pr, proctodaeum.

A 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 homosegmental 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, 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...
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_2v or b_3v) (Fig. 6). After the first differential cleavage, the
median part of the \textit{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 \textit{en} expressing cells shows \textit{en} labeling again (Fig. 6). \textit{en} expression does not occur in the progeny of descendant rows c and d.

With widening of \textit{en} stripes during differential cleavages, the ratio between \textit{en}-positive and \textit{en}-negative rows is shifted towards relatively more \textit{en} expressing rows. This shift is due to the transversely oriented mitoses in the rows in front of the anterior margin of the \textit{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 \textit{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 \textit{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. \textit{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 \textit{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 \textit{en} in naupliar as well as in post-naupliar segments (Fig. 6).

**Discussion**

**Appearance of \textit{en} stripes**

The present investigation shows that most features of initial segmental \textit{en} stripes are similar within individual germ bands of amphipods. These similarities include the mediolateral propagation of each \textit{en} stripe, the one-cell-width of the initial stripes, and the widening by both recruitment of new \textit{en} expressing cells and division of \textit{en}-positive cells. Furthermore, \textit{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 \textit{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 homonomus 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 homoeotic genes may specify the variety of cell division patterns on the germ band. At least six homoeotic genes have been identified in the crustacean \textit{Artemia} (Averyf and Akam, 1993).

The correlation between \textit{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 \textit{en} gene expression and segmentation in general. However, the comparison between the naupliar and post-naupliar
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 ai 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 et al., 1989b). 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.

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. 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 descendents 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.
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). en 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 gammarellus*) 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. gammarellus*) (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 6.05, 2.0 mM EGTA, 1.0 mM MgSO4, 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 PBST (PBS, 0.2% SAA, 0.1% Triton X-100) and then incubated for 30 min in PBST-N (PBST 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 PBST and again incubated in PBST-N for 30 min. Goat anti-mouse IgG (Jackson Immunoresearch) was added to a dilution of 1:200 in PBST-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 PBST and then placed in a solution of 0.3 mg/ml DAB in PBST for 15 min. Then H2O2 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 33258) for 15 min, then washed in distilled water and mounted in glycerol. Additionally, some preparations were stained with Dapi’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 Axioskop.

**Acknowledgments**

We thank P. Whittington and D. Sandeman for critically reading the manuscript.

**References**


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Accepted for publication: May 1994