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Original Article

Expression of *engrailed* can be lost and regained in cells of one clone in crustacean embryos

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ABSTRACT In three species of higher crustaceans (Malacostraca) the expression of *engrailed* has been analysed in relation to the development of the cell division pattern in the germ band. The species differ in the timing of initial *en* expression. Compared to *Cherax destructor* and *Neomysis integer* the onset of *en* expression in *Orchestia cavimana* is delayed and appears one cell cycle later. In *Cherax* and *Neomysis* cells of the posterior margin of early *en* stripes lose *en* expression. This phenomenon does not occur in *Orchestia*. In a second step the *en* stripes widen both by division of *en* positive cells and de novo expression at the posterior margin of the *en* stripes. The widening phase is similar among all investigated species. In *Cherax* and *Neomysis* the cells with de novo *en* expression are derivatives of cells, which have ceased to express *en* one cell cycle before. The results in higher crustaceans suggest that neither initiation nor maintenance of *en* expression is controlled by lineage restrictions and that early *en* expression is not clonally transmitted. Furthermore, some aspects of boundaries and fields in embryos are discussed.

KEY WORDS: *engrailed, Crustacea, cell lineage, segment formation*

Introduction

In *Drosophila*, the *engrailed (en)* gene is required in cells of the posterior part of a segment. *en* is expressed in cells of the embryo from the beginning of cellularisation (e.g. DiNardo *et al.*, 1985; Kornberg *et al.*, 1985). Though the exact lineage of *en*-expressing cells could not be established, it was commonly accepted that *en* expression and function is heritably transmitted to all progeny of a primary *en* expressing cell (see Lawrence, 1992; Lawrence and Morata, 1992).

This belief has been challenged and, in fact, has been shown to be incorrect by experiments of Vincent and O'Farrell (1992). They found clones of 4 or 8 cells in which only some of the cells express *en* while others do not. They demonstrate that clones straddling the posterior margin of a future segment include cells which have lost their *en* expression.

In embryos of other insects as well as of malacostracan crustaceans *en* also marks the posterior portion of segments (Patel *et al.*, 1989a; Fleig, 1990; Sommer and Tautz, 1991). In malacostracan crustaceans, the cells of the post-naupliar part of the germ band go through a sophisticated pattern of stereotyped cleavages so that the exact lineage and clonal relationships of most cells through several cell cycles are known (for review, see Dohle and Scholtz, 1988). This division pattern is quite similar throughout all investigated species. The mode of generation of the cells that undergo these divisions, however, shows remarkable variation (Dohle and Scholtz, 1988; Scholtz, 1992). The questions whether expression of *en* is lineage dependent and clonally restricted can therefore be tested in these animals.

We analysed the pattern of *en* expression in three different crustacean species: a decapod crayfish *(Cherax destructor)*, an amphipod *(Orchestia cavimana)*, and a mysid shrimp *(Neomysis integer)*. The exact cell lineage of these animals has been established by clonal analyses in previous studies (Scholtz, 1984, 1990, 1992). We found three interesting results. i) The first appearance of *en* expression is different in the three species – in *Cherax* and *Neomysis* it appears one cell cycle earlier than in *Orchestia*. ii) After cleavage of the initial *en* stripe in *Cherax* and *Neomysis*, posterior cells lose *en* expression. iii) Daughter cells of those cells that have ceased to express *en* can regain *en* expression one cell cycle later.

We conclude that neither initial *en* expression nor its transmission at the posterior margin of early *en* stripes, in particular, are determined by lineage but rather depend on cell-cell communication.

Abbreviations used in this paper: en, engrailed; ET, ecto; *wg, wingless.*

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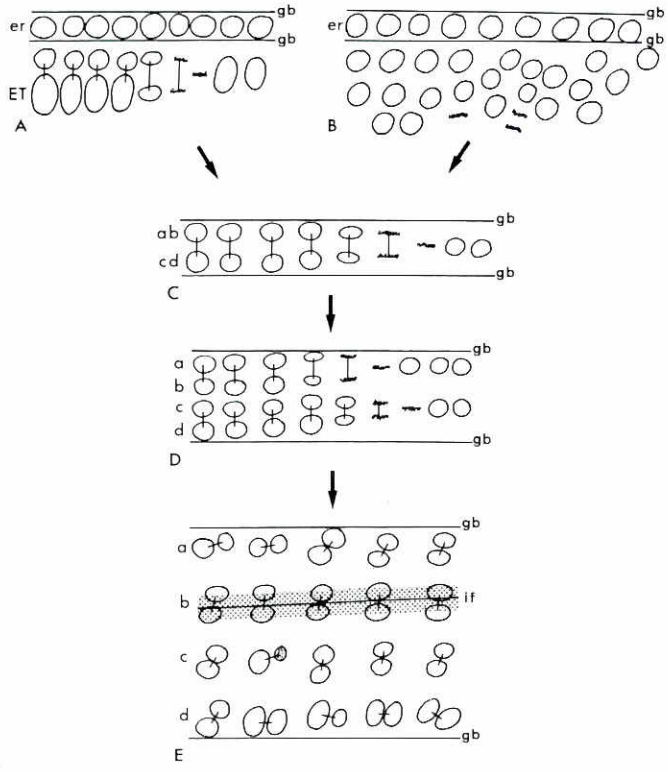


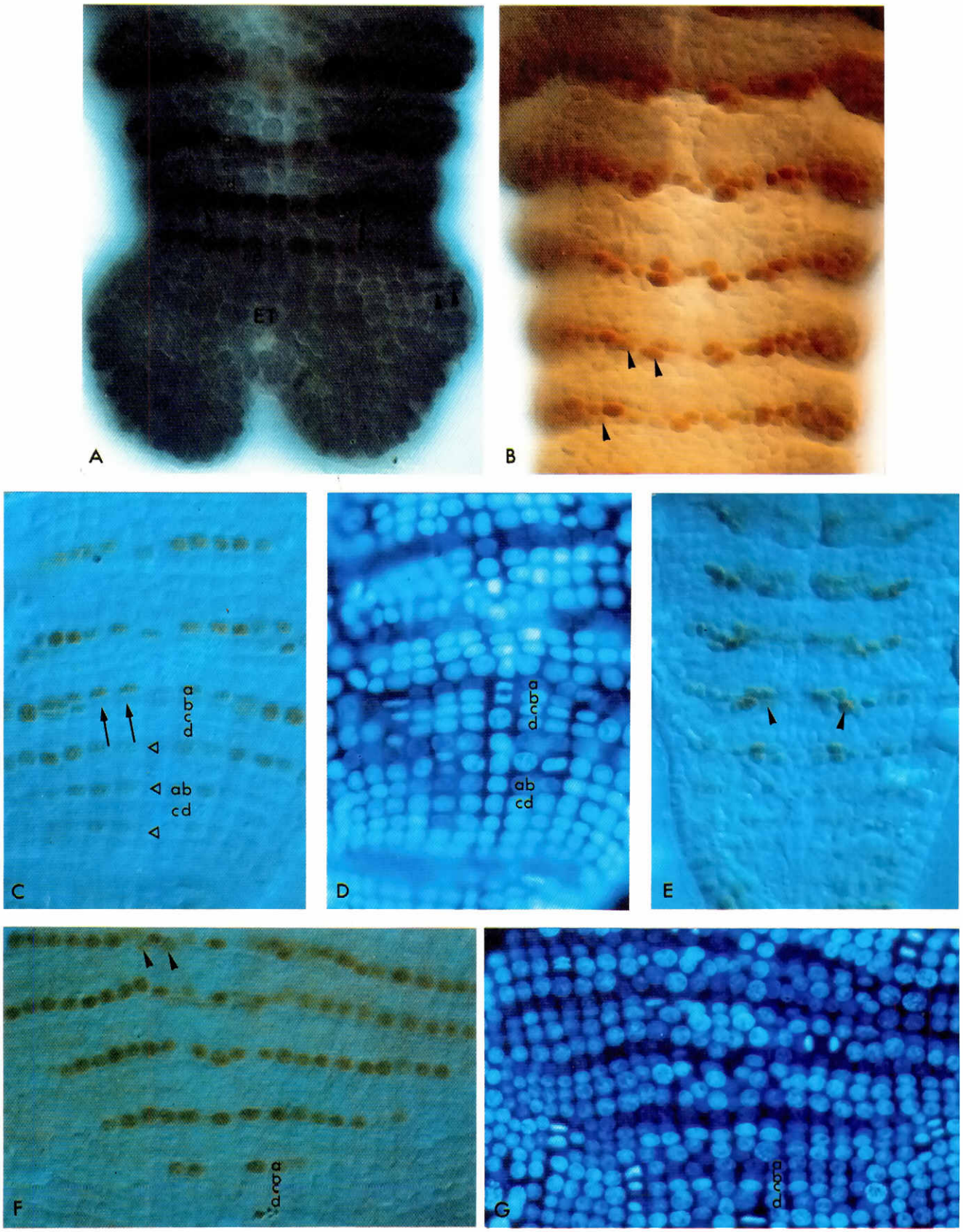
Fig. 1. Schematic summary of row formation and segmentation in the post-naupliar germ band of malacostracan crustaceans. *Only the animal's left side is shown. The midline of the germ band is on the left side. The transverse lines indicate the genealogical boundaries (gb) between the ectoderm rows. The transverse ectoderm rows are formed either by ectoteloblasts (ET) a condition found in Cherax and Neomysis (Scholtz, 1984, 1992)* **(A)** *or by scattered blastoderm cells* **(B)** *a condition found in Orchestia as in other amphipods (Dohle, 1976; Scholtz, 1990). After formation, each row (E(2) to E(17)) undergoes two mediolateral mitotic waves with only longitudinal-oriented and equal mitoses, resulting in four transverse descendant rows named a, b, c, d* **(C,D).** *Thereafter the differential cleavages begin. They show a stereotyped pattern of mitoses with regard to size and position of the division products.* **(E)** *Depicts a simplified schematic pattern of the first differential cleavage up to the fifth cells from the midline. Some characteristics of the individual mitoses differ among the investigated species, a phenomenon not shown here (for comparison see Dohle, 1976; Scholtz, 1984). With the differential cleavages, segmentation begins. The segment boundary (shaded area) marked by the intersegmental furrow (if) does not match the genealogical border (transverse lines). 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.*

Results

Summary of the cell lineage in the post-naupliar germ band of higher Crustacea

The cell lineage in the post-naupliar germ bands of *Cherax destructor*, *Neomysis vulgaris*, and amphipods has been studied in detail (Scholtz, 1984, 1990, 1992). The main feature of all investigated malacostracans is the formation of exactly aligned transverse ectoderm rows in the post-naupliar germ band (Fig. 1). In *Neomysis* and *Cherax* most of these rows are generated by ectoteloblasts (Scholtz, 1984, 1992) (Fig. 1A). In *Orchestia*, as in other amphipods, the corresponding rows are formed by scattered blastoderm cells, which become arranged in rows (Dohle, 1976; Scholtz, 1990) (Fig. 1B). Independent of the different origin, the further fate of these rows is quite similar in all investigated species (Dohle and Scholtz, 1988). Each row undergoes two mediolateral mitotic waves with only longitudinal oriented and equal mitoses resulting in four transverse descendant rows named «a, b, c, d» (Fig. 1C,D). Thereafter, the differential cleavages begin. They show a stereotyped pattern of mitoses with regard to size and position of the division products (Fig. 1E). One striking feature of this cell division pattern is the fact that the segment borders do not match the genealogical borders. The intersegmental furrow runs transversely and slightly obliquely through the descendants of one row in

Fig. 2. Development of the early pattern of *en* expression in *Cherax, Neomysis,* and *Orchestia*. *Compare Figs. 1 and 3. In all micrographs anterior is up.* **(A)** *en expression in the abdomen of Cherax. Haematoxylin was used for counterstaining. The degree of differentiation increases in the anterior direction. Immediately in front of the ectoteloblasts (ET) lies a yet undivided row. The next anterior row undergoes its first mitotic wave, recognizable by the mitotic figures on the animal's left side (arrowheads). The posteriormost en stripe (open triangle) occurs in a row that has divided once and the cells of descendant row ab are en-positive, whereas descendant row cd shows no label. The next anterior row is in the phase of the second mitotic wave. After division, the cells of descendant row b cease to express en (arrows) whereas descendant row a remains en-positive. In the next anterior stripe en expression is restricted to cells of descendant row a.* **(B)** *Abdomen of Cherax in an advanced stage. Arrowheads mark some anterior derivatives of descendant row b showing secondary en expression during the first differential cleavage. Their posterior sister cells remain en-negative. Note the intersegmental furrows behind the en expressing cells.* **(C)** *en expression in the posterior thoracic segments of Neomysis. Open triangles mark en expression in descendant rows ab during or after the first mitotic wave. Note that en expression starts close to the midline and proceeds laterally. Arrows point to cells of descendant row b that cease en expression during the second mitotic wave. In the anteriormost stripe en expression is restricted to descendant row a. Descendant rows b, c, and d show no label.* **(D)** *Same preparation counterstained with Bisbenzimid fluorescent dye to show the mitotic figures. Fluorescence in en-positive cells is quenched.* **(E)** *Advanced stage of a Neomysis germ band. Arrowheads point to derivatives of descendant row b, which regain en expression during the first differential cleavage.* **(F)** *en expression in the posterior thoracic region of Orchestia. Note that in contrast to Neomysis and Cherax, en expression starts during the second mitotic wave and therefore initially only the cells of descendant row a are en positive. Arrowheads point to derivatives of descendant row b with de novo en expression during the first differential cleavage.* **(G)** *Same preparation counterstained with Bisbenzimid. Fluorescence in en-positive cells is quenched.*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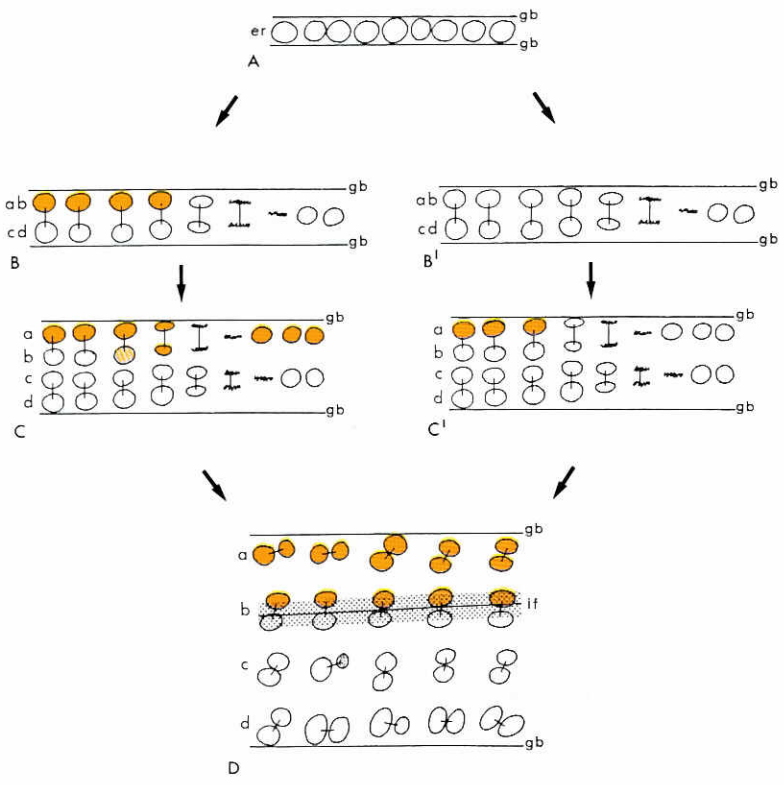

Fig. 3. Schematic representation of the differences and similarities in early *en* expression between *Cherax* and *Neomysis* on the one hand and *Orchestia* on the other hand. *Compare Fig. 2. In Cherax and Neomysis en expression starts in descendant row ab (brown nuclei) during the first mitotic wave of each transverse ectoderm row (er)* **(A, B).** *In Orchestia no en expression takes place at this stage* **(B')**. *During the second mitotic wave in the rows of Cherax and Neomysis, in descendant row b en expression is lost in a mediolateral sequence (represented by the shaded nucleus)* **(C)**. *In the corresponding stage of Orchestia en expression (brown nuclei) begins in descendant row a and propagates mediolaterally* **(C')**. *No loss of en expression occurs. During the first differential cleavage de novo en expression occurs in anterior derivatives of descendant row b in front of the intersegmental furrow (if)* **(D)**. *This pattern is very similar in all investigated species independent of the preceding differences. gb, genealogical border.*

the area of descendant rows a and b (Fig. 1E). This corresponds with the posterior margin of *en* stripes (Patel *et al.*, 1989b) (see also Fig. 3D).

Initial activation of en occurs in different stages

In the crayfish *Cherax destructor* and the mysid shrimp *Neomysis integer*, the onset of *en* expression in each row occurs during the first mitotic wave (Figs. 2A,C, 3B). The turn on of *en* expression proceeds mediolaterally (Figs. 2C, 3B) showing a higher velocity in *Cherax*. The cells of the anterior descendant row ab express *en* whereas the cells of the posterior descendant row cd remain *en* negative (Figs. 2A,C,D, 3B). This situation is found in *Cherax* only in the posteriormost *en*-expressing row of each developmental stage (Fig. 2A). In *Neomysis* it comprises up to three subsequent rows (Fig. 2C). In contrast to the events in *Cherax* and *Neomysis*, in *Orchestia en* expression starts not earlier than during the second mitotic wave of an ectoderm row (Figs. 2F, 3C'). It is restricted to the cells of descendant row a. Descendant rows b, c, and d show no *en* expression (Figs. 2F,G, 3C'). Again *en* expression propagates laterally, starting close to the midline (Figs. 2F, 3C').

Cells at the posterior margin of en stripes lose en expression

After the second mitotic wave in the ectoderm rows of *Cherax* and *Neomysis*, the cells of descendant row b show a decay of *en* expression (Figs. 2A,C, 3C). Their sister cells, the cells of descendant row a, on the other hand, maintain their state of *en* expression (Figs. 2A,C, 3C). Therefore, the progeny of descendant row ab (rows a and b) forms clones straddling the posterior edge of the *en* stripe. The cells of the descendant rows c and d are *en* negative. Thus, the resulting pattern is the same as the initial pattern of *en* expression in *Orchestia* (compare Figs. 2F, 3C'). In *Orchestia*, as opposed to *Cherax* and *Neomysis*, loss of *en* expression does not occur in these early stages.

Cells adjacent to the posterior margin of initial en stripes show de novo en expression

The subsequent pattern of *en* expression is very similar in all three investigated species. During the first differential cleavage all derivatives of *en* expressing cells of descendant row a are *en* positive (Figs. 2B,E,F, 3D). Additionally, anterior derivatives of descendant row b show *de novo en* expression after mitosis, whereas their posterior sister cells remain *en* negative (Figs. 2B,E,F, 3D). Thus, the recruitment of new *en*-expressing cells again produces mixed clones (the progeny of descendant row b) that straddle the posterior edge of the *en* stripe. With the differential cleavages the intersegmental furrows are formed. They mark the segment borders and match the posterior boundaries of *en* stripes (Figs. 2B, 3D).

Discussion

Control of en expression by cell-cell communication

In all three investigated crustacean species the initial *en* stripes are one cell wide and appear at the anterior margin of the descendants of the ectoderm rows. Corresponding to the findings in *Drosophila* (Vincent and O'Farrell, 1992), the anterior edge seems to mark a boundary of lineage restriction (at least in the investigated stages) and shows a sharp and stable appearance from the onset. It must be stressed, however, that in crustaceans this lineage boundary is already well established before *en* expression

starts, and that therefore *en* does not seem to play an important role for the establishment of this lineage boundary. Initial *en* expression occurs in different developmental stages – in *Cherax destructor* and *Neomysis integer* during the first, in *Orchestia cavimana* during the second mitotic wave of the ectoderm rows. Furthermore, the cells that express *en* are generated differently in the posterior part of the germ band; in *Cherax* and *Neomysis* they are derivatives of ectoteloblasts, in *Orchestia*, on the other hand, they are derivatives of normal blastoderm cells.

These differences lead to the conclusion that initial *en* expression is not closely linked to a certain cell lineage or to a certain stage of that lineage. Cells apparently do not count their mitoses before they express a certain gene or become shifted to a certain fate, as assumed by Shankland (1991). Against this background we would expect the possibility of an even later onset of *en* expression. Some results in amphipods where a somewhat delayed *en* expression occurs in anterior ectoderm rows point to that possibility (Scholtz *et al.*, in preparation). The possibility of a more advanced *en* expression than seen in *Cherax* and *Neomysis* seems to be less likely because it would result in adjacent *en* stripes with no cells between them in which other segment polarity genes such as *wingless* could be expressed.

In a manner comparable to *Drosophila* (Vincent and O'Farrell, 1992) regulative loss of *en* expression and formation of mixed clones occur in cells at the posterior edge of the early *en* stripe. The loss of *en* expression seems linked to a precocious onset of *en* expression and therefore takes place only in the embryos of *Cherax* and *Neomysis* but not in *Orchestia*, where at this stage no mixed clones occur. The resulting pattern and further fate of *en* stripes is the same in all three species, independent of the occurrence of early regulative loss of *en* expression. For *Drosophila* the loss of *en* expression is considered a maturation process of the *en* stripe (Lawrence and Morata, 1992; Vincent and O'Farrell, 1992). Our findings might support this view, because after the initial differences all three investigated species pass through similar *en* expression patterns. However, the different data between *Cherax* and *Neomysis* on the one hand and *Orchestia* on the other hand, suggest that an initial regulatory phase is not necessary to establish a stable state of *en* expression. The same result can be achieved by a delayed onset of *en* expression.

In contrast to what is reported for *Drosophila* (Vincent and O'Farrell, 1992) but in accordance with other insects and Crustacea (Patel *et al.*, 1989a; Fleig, 1990; Scholtz *et al.*, in preparation), *de novo en* expression takes place in the posterior part of the *en* stripes in all three investigated crustacean species. Apart from the temporal sequence, the recruitment of new *en* expressing cells follows the same pattern in *Cherax*, *Neomysis* and *Orchestia*. This event leads again to mixed clones at the posterior edge of the *en* stripe. The *de novo en* expression seems correlated with the formation of the segment border. Interestingly, this new *en* expression occurs in *Cherax* and *Neomysis* in the anterior derivatives of cells that lost *en* expression two mitoses before.

In summary, the onset of *en* expression in different stages, the loss and *de novo* expression, and the turn off and turn on events all suggest a control of *en* expression by positional rather than lineage-specific cues. This seems true at least for early *en* stripes prior to the occurrence of intersegmental furrows. Whether this also holds true for later stages or whether *en* expression in crustaceans is clonally transmitted from the establishment of segment borders up to the adult stage must remain an open question at present.

The establishment of boundaries and polarity

Embryonic fields are characterized by boundaries and polarity. These two properties are suggested to be inextricably linked (Ingham and Martinez Arias, 1992). To achieve both polarity and boundaries, the repetition of at least three different cell states is required – two alternating cell states produce boundaries but no polarity (Meinhardt, 1986; Martinez Arias *et al.*, 1988; Ingham and Martinez Arias, 1992). The ectoderm rows of the germ bands of *Cherax* and of *Neomysis* pass through a stage where only two alternating cell states seem to occur. This can be deduced from the fact that *en* positive transverse cell rows (ab) alternate with *en* negative transverse cell rows (cd). Furthermore, it seems likely that pair-rule genes are not involved in segmentation in short-germ insects and crustaceans (Patel *et al.*, 1989a, 1992). Assuming that most of the basic processes of segmentation are similar throughout arthropods, we would expect that the *en*-negative cells might express a crustacean homologue of the *wingless (wg)* gene. The initial pattern in these crustaceans would therefore correspond to the pattern in *Drosophila* embryos mutant for the *patched* and *naked* genes where only *wg* and *en* are expressed in alternating areas (Ingham and Martinez Arias, 1992). The possible occurrence of a two cell state phase in Crustacea might provide evidence that boundaries and polarity may not be so closely linked as suggested. The data point rather to a temporal sequence where a sort of parasegmental boundary is formed first. Subsequently metamerical polarity is established and in a third step the segment border appears.

Materials and Methods

For collecting and maintenance of the animals see Scholtz (1984), Sandeman and Sandeman (1991), and Scholtz *et al.* (in preparation). The antibody labeling procedure followed the protocols of Patel *et al.* (1989b). The staining techniques using Bisbenzimid H 33285 fluorescent dye and Haematoxylin (Ehrlich's) are described in Scholtz (1992).

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