

Expression of *en* and *wg* in the embryonic head and brain of *Drosophila* indicates a refolded band of seven segment remnants

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Summary

Based on the expression pattern of the segment polarity genes *engrailed* and *wingless* during the embryonic development of the larval head, we found evidence that the head of *Drosophila* consists of remnants of seven segments (4 pregnathal and 3 gnathal) all of which contribute cells to neuromeres in the central nervous system. Until completion of germ band retraction, the four pregnathal segment remnants and their corre-

sponding neuromeres become arranged in an S-shape. We discuss published evidence for seven head segments and morphogenetic movements during head formation in various insects (and crustaceans).

Key words: *engrailed*, *wingless*, brain development, head segmentation, *Drosophila*.

Introduction

In *Drosophila*, the embryonic development of the larval trunk is much better known than the development of the larval head. This is especially true for the corresponding regions of the central nervous system (CNS). Main reasons for this are that (1) the presumed underlying segmental pattern in the brain is highly derived compared to the ventral nerve cord (Hartenstein and Campos-Ortega, 1984), and (2) landmarks in the embryonic brain are scarce.

The number of segments that contribute to the head and brain of insects has been intensely discussed by morphologists (for reviews see Sharov, 1966; Rempel, 1975) using mainly coelomic cavities, neuromeres and appendages as criteria. Investigators of *Drosophila* generally prefer Rempel's view of six head segments (3 pregnathal and 3 gnathal; Jürgens et al. 1986; Cohen and Jürgens, 1991; Diederich et al. 1991) but the question to whether there is a fourth pregnathal segment seems to be unsettled (see below).

Fate mapping data show that until germ band retraction massive morphogenetic movements lead to an S-shaped deflection of epidermal head structures (Technau and Campos-Ortega, 1985; Fig. 1). These data also reveal that similar movements affect the formation of the brain, but they do not further elucidate the segmental organisation of the brain.

To obtain evidence concerning the position, orientation, and number of the brain neuromeres we studied the expression pattern of the segment polarity genes *engrailed* (*en*) and *wingless* (*wg*) during embryonic head development. In the trunk segments, *en* is expressed in posterior compartments and *wg* in anterior compartments, thus defin-

ing the polarity of segments on a molecular level (Baker, 1987; Martinez Arias et al. 1988; van den Heuvel et al. 1989). Our detailed description of the dynamic pattern of *en* and *wg* expression in the developing head argues for 4 pregnathal (and 3 gnathal) segments with their corresponding neuromeres. Until completion of germ band retraction these segments become arranged in a refolded band which is in good agreement with the fate mapping data (Technau and Campos-Ortega, 1985; Jürgens et al. 1986). Published data from a variety of insects (and crustaceans) give further support for 4 pregnathal segments and the occurrence of similar morphogenetic movements in other Mandibulata.

Materials and methods

Fly stocks

In our experiments we used a wild-type Oregon R stock. For some of the double labelling experiments we used the transformant line *ryxho25* (Hama et al. 1990), which carries an *engrailed-lacZ* fusion gene inserted at the *engrailed* locus, the stock *wg^{1L114}* (Nüsslein-Vollhard et al. 1984) which carries a temperature-sensitive allele affecting the secretion of the *wg* antigen (Gonzalez et al. 1991), and the transformant lines (*CyO*, *wg^{en11/sli}* and *wg^{17eni40cP1/CyO}*) with *lacZ* insertions in the *wingless* gene (for which we are indebted to N. Perrimon, Harvard).

Antibodies

We used the monoclonal antibody 4D9 (Patel et al. 1989) which recognizes the antigens of the closely related *engrailed* and *invected* genes (1/3 dilution; in the following we refer to both as *engrailed* staining), a polyclonal anti-*gal* (Cappel, 1/7000 dilution, preabsorbed), an anti-*wingless* antiserum (González et al. 1991; 1/2000 dilution, preabsorbed) and a monoclonal anti-BrdU antibody (Becton & Dickinson, 1/100 dilution). Peroxidase-

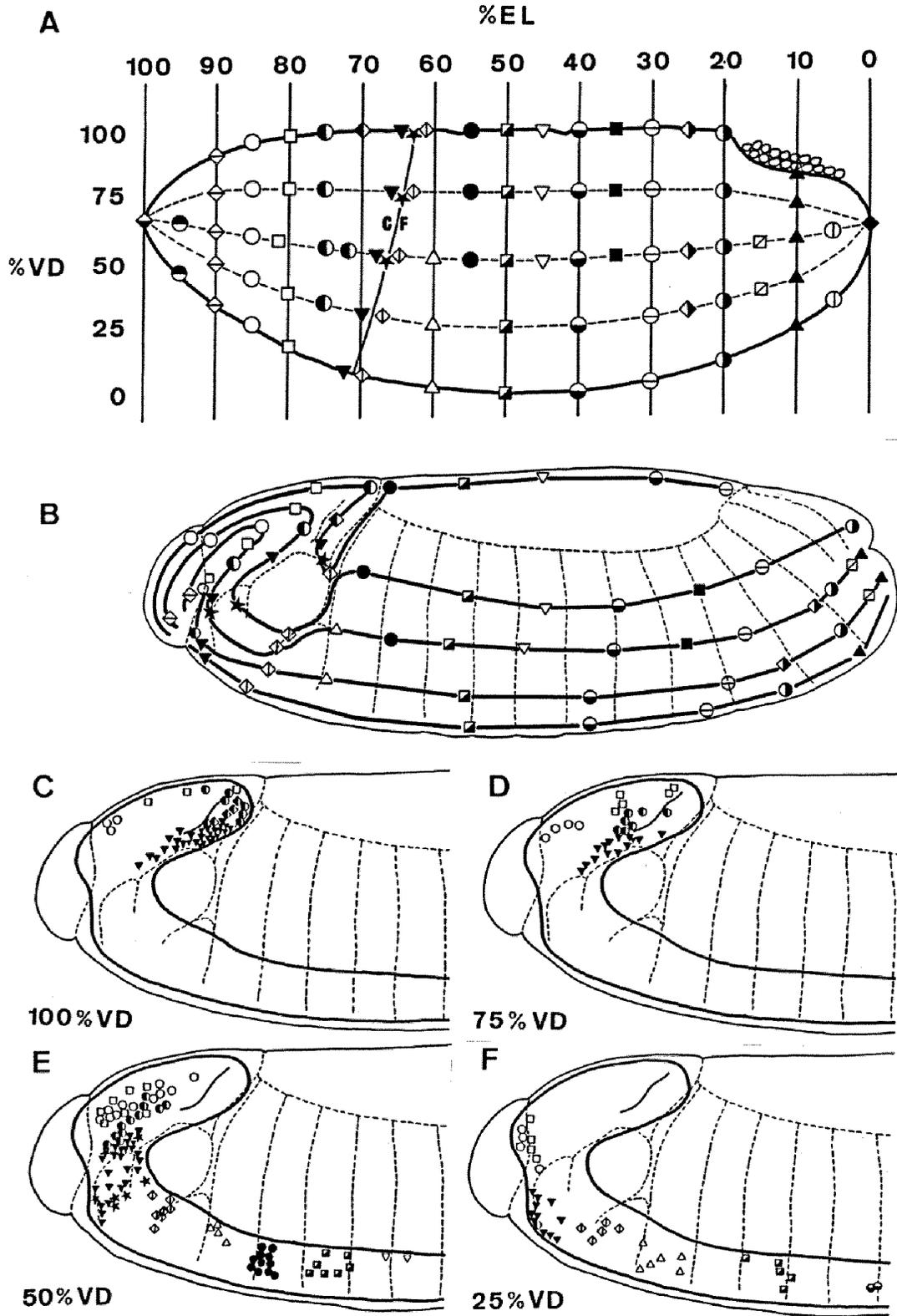


Fig. 1. Fate-mapping data after Technau and Campos-Ortega (1985). The symbols in (A) mark the sites of HRP injections at the beginning of gastrulation. (B) Summarizes the distribution of labeled cells after germ band retraction (stage 12/13) in the peripheral ectoderm, and (C-F) show the distribution of markings in the CNS. The lines in B ligate markings that derive from injections at 0, 25, 50, 75 and 100% VD, respectively. Note the S-shaped distribution of labelled cells at stage 12/13 that results from a linear row of injections. % EL, % egg length; % VD, % ventrodorsal diameter.

coupled goat-anti-mouse (IgG), goat-anti-rat (IgG), and goat-anti-rabbit (IgG) were used as secondary antibodies (Dianova, 1/500 dilution). For detection of the anti-wingless antibodies we used a biotinylated goat-anti-rat antibody with the Vectastain-ABC-Kit Elite (Cameron).

Staining procedures

Embryos were dechorionated with 50% commercial bleach for 4 minutes and briefly washed in water. Excess liquid was blotted away and the embryos were subsequently fixed in a 1:1 mixture of n-heptane and 4% formaldehyde or paraformaldehyde in PEM buffer (0.1 M PIPES, 2 mM MgSO₄, 1 mM EGTA, pH 6.9) for 25 minutes on a shaker. The aqueous solution was then replaced by methanol. After 15 seconds of vigorous shaking the devitellinized embryos had sunk to the bottom. The embryos were washed three times with methanol, three times for 20 minutes with PBT (0.13 M NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, 0.1% Triton X-100) and blocked for 30 minutes with 10% goat or lamb serum in PBT. Incubation with the first antibody was carried out overnight at 4°C, followed by three washes with PBT and the incubation with the secondary antibody for 3 hours at room temperature. After three more washes (10 minutes each) the samples were stained for HRP with diaminobenzidine (0.1% DAB, 0.02% H₂O₂ in 0.2 M phosphate buffer) for 10 minutes, dehydrated in an ethanol series and mounted in Araldite (Serva). Following a suggestion of E. R. Macagno (Columbia University, New York), embryos and larval brains were sucked in borosilicate capillaries (Hilgenberg) with an inner diameter of 0.2 mm. This allowed the rotation of the samples under the compound microscope.

For the BrdU experiments, freshly hatched larvae were fed on a diet containing 0.1% BrdU (stock solution 33 mM in 40% ethanol; Truman and Bate, 1988) for 1-to-4 hours. The samples were fixed with paraformaldehyde and stained first with anti-engrailed or anti-*en* antibodies. After the DAB reaction and two washes in PBT, the larval brains were treated with 2 N HCl in PBT for 1 hour, blocked for 30 minutes and stained with the anti-BrdU antibody as described above. We added 0.08% NiCl₂ to the DAB-solution which resulted in an almost black staining that was easily distinguished from the normal brown stain.

Embryos of the *wg*^{L114} stock were raised at 27°C, fixed in paraformaldehyde, and stained first for *en* and second for *wg* antigens. Embryos of the transformant lines carrying a *lacZ* insertion at the *wg* locus were fixed in formaldehyde before double staining.

Note on figures

In all figures we used the following symbols to identify certain groups of *en*-expressing cells: ► en intercalary spot; ▷ en secondary head spot; ➡ en antennal stripe; ▶ en in anterior dorsal hemispheres; ▴ en antennal spot; ▷ en clypeolabral spot; ➡ en head spot; > en cells of uncertain origin.

Figs 2-10 each show different views of the same individual. Anterior is to the left or to the top. T1 marks the first thoracic segment or neuromere.

Results

engrailed (*en*) expression in the head

We found five “centers” of *en* expression anterior to the mandibular segment which appear in the following sequence from stage 8 on (stages after Campos-Ortega and Hartenstein, 1985): the “en antennal stripe”, the “en head spot”, the “en intercalary spot”, the “en expression in the anterior dorsal hemispheres” and the “en expression in the

clypeolabrum”. Subsequently, the en antennal stripe and the en head spot split in two groups of cells, thus generating in total 7 en spots (Figs 2-10; Table 1). In this way the “en antennal spot” separates from the en antennal stripe and the “en secondary head spot” from the en head spot. For the terminology of spots we followed Cohen and Jürgens (1990); new names were only introduced for spots not mentioned by these authors.

The **en antennal stripe** appears at stage 8 (Fig. 2). These cells remain largely in the peripheral ectoderm and can be traced until stage 17 (Figs 3-10). They migrate towards the dorsal organ and contribute axons to the antennal nerve (Fig. 11). The most anterior cells of this stripe (corresponding to the ventral cells of the trunk stripes) delaminate during stage 10/11 (Fig. 4, and Marcos González Gaitán, personal communication) to become part of the brain and can be traced until early larval stages (Fig. 11). We refer to these cells as **en antennal spot** (Fig. 4). At stage 12 these cells stain only faintly, but expression again becomes more prominent from stage 13 on (Figs 6-10). Thus, we cannot confirm the observation of Diederich et al. (1991) that “all of the *en*-expressing cell-clusters of the pre-oral head, with the exception of those associated with the clypeolabral lobe, appear to delaminate from the ectodermal surface”. Our data also suggest that the *en*-expressing cells of the en antennal spot are identical with those that Diederich et al. (1991, in their Fig. 1B) attributed to the labral neuromere (see also Discussion).

The **en head spot** (visible from stage 9 on; Fig. 3) becomes completely integrated into the CNS. At stage 13 it is a stripe that crosses each brain hemisphere transversely at its ventral side (Figs 5, 6). The two outermost cells of each stripe are larger than the inner ones. Until the end of embryogenesis *en* expression in the en head spot becomes restricted to only two cells (Fig. 10). At stage 13/14, a group of cells detaches from the en head spot and we refer to them as **en secondary head spot** (Figs 7, 8, 9). This splitting has not been noticed by Diederich et al. (1991).

The **en intercalary spot** (visible from stage 10 onwards; Fig. 3) also completely segregates into the CNS. At stage 12 these cells are located at the posterior margin of each connective between the supra- and the subesophageal ganglion. At stage 14 the en intercalary spot touches *en*-expressing cells of the mandibular neuromere and at stage 16 the cells of the en intercalary spot switch off expression of the *en* antigen.

From stage 11 onwards we noticed *en* expression in the area of the dorsal clypeolabral suture. This staining is “fuzzy” but becomes more distinct at later stages. We refer to this staining - which has not been mentioned by other authors - as ***en* expression in the anterior dorsal hemispheres** (Figs 4-9).

***en* expression in the clypeolabrum** starts at early stage 11 (Figs 4-10). These cells do not contribute to the CNS (see also Diederich et al. 1991). The clypeolabral expression is median and can be slightly dumb-bell shaped at stage 11 but is unpaired, in contrast to the patches of *en* expression mentioned above. At later stages it is found in the roof of the pharynx. We did not observe a bilateral splitting of this patch in two spots as mentioned by Diederich et al. (1991) for stage 15.

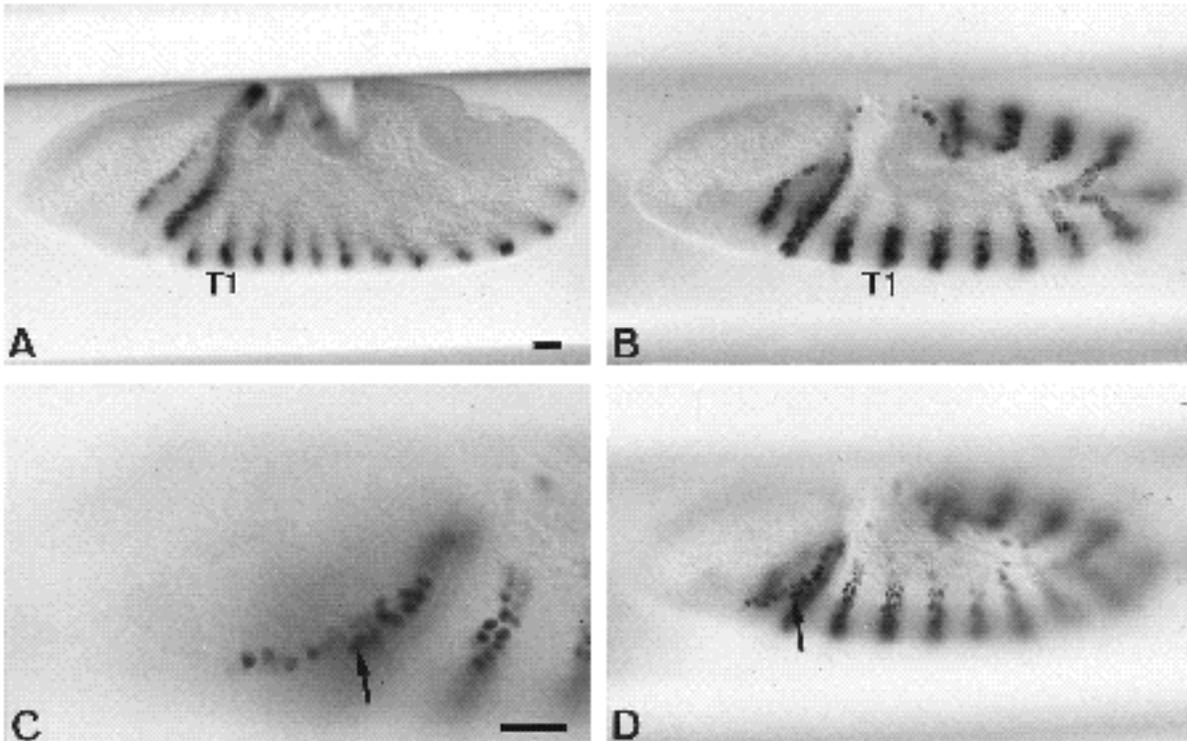


Fig. 2. Wild-type embryos at stage 6/7 (A) and 8 (B-D) stained with anti-en antibody. Anterior to the left, lateral views. At stage 6/7 the most anterior stripe corresponds to the mandibular segment. At stage 8 the en antennal stripe has formed (arrow in C and D) at the posterior margin of the procephalic lobe. T1 marks the first thoracic segment. Scale bar, 20 μ m.

All patches of *en* expression migrate and undergo changes in size (cell number) and shape (arrangement of stained cells) as documented in detail in Figs 2-10 and in Table 1.

For the following reasons we think that the traced movements of the *en*-expressing cells are real and do not simply reflect turning on and off of the *en* gene. (1) We analysed many more intermediate stages than those shown in Figs 2-10. (2) When cells turn off *en* expression towards the end of embryogenesis, weak staining can often still be recognized for some time. When using an *en-lacZ* strain we could follow cells that turned off *en* expression (and their descendants) even longer because of the perdurance of the *lacZ* product in the cellular cytoplasm (Monsma et al. 1988; Vincent and O'Farrell, 1992; Martinez Arias and Technau, unpublished data; see also Fig. 11 and Discussion). In both cases these cells followed the inferred movement of the *en*-expressing cells. (3) The traced morphogenetic movements are in good agreement with fate-mapping data (Fig. 1; Technau and Campos-Ortega, 1985; Jürgens et al. 1986).

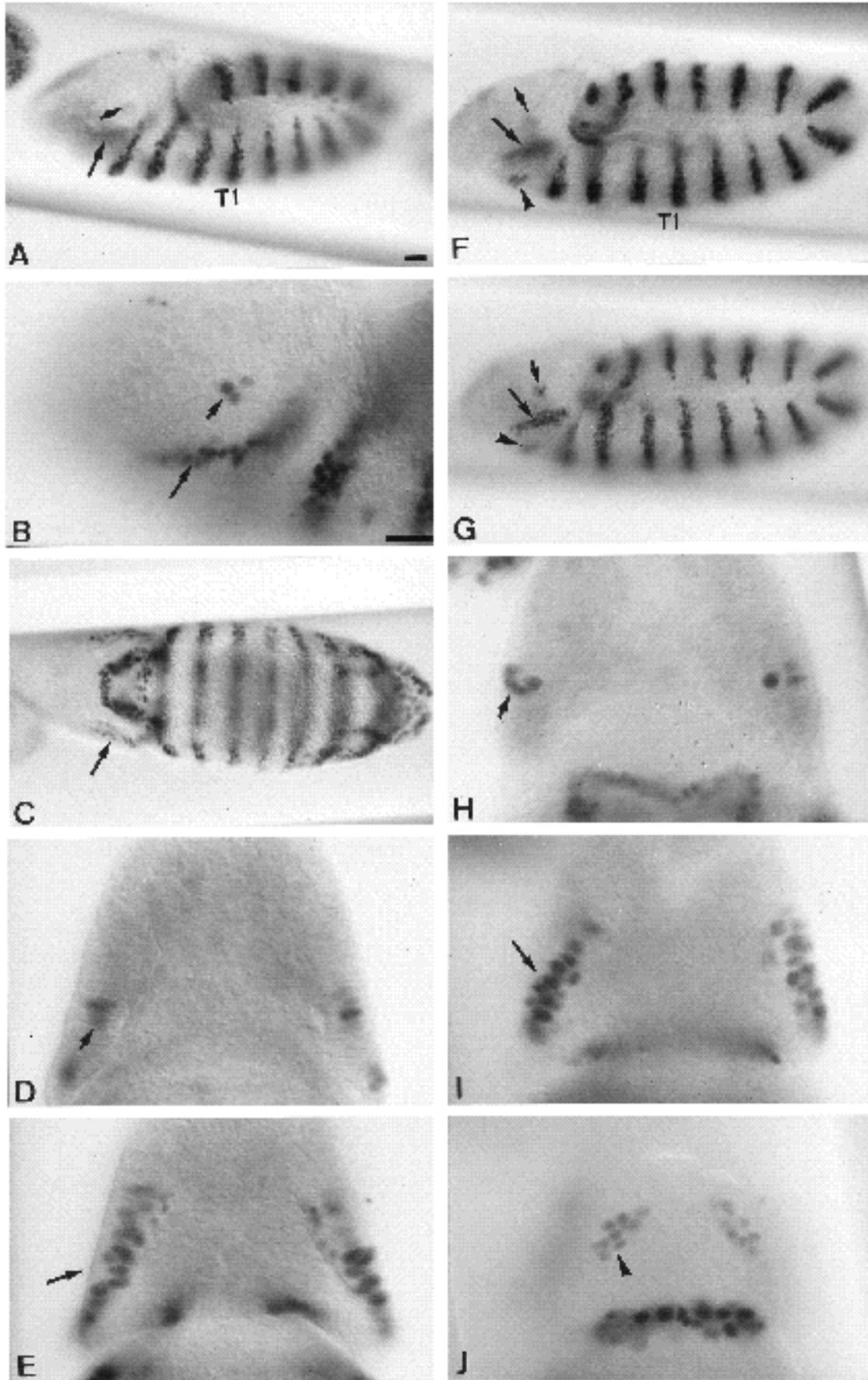
wingless (*wg*) expression in the head

To find out whether patches of *en* expression in the pre-natal head can be attributed to segments, and eventually to learn about their orientation, we double stained *wg^{LL114}* (Fig. 12) and transformant *wg lacZ (CyO, wg^{en11/sli} and CyO/wg^{17en40cP1}*; data not shown) embryos against *en* and *wg* or -galactosidase (*lacZ*-product). In heterozygous *wg^{LL114}* embryos raised at non-permissive temperatures (25°C or higher) the secretion of the *wg* protein is partly

interrupted (González et al. 1991). The accumulation of *wg* protein inside the cells thus allows for a stronger signal when staining with antiserum. The embryos shown in Fig. 12 are phenotypically wild-type, as can be seen from the normal *en* expression, but are probably heterozygous for the *wg^{LL114}* allele because of the stronger *wg* signal compared to homozygous wild-type embryos. Fig. 13 schematically shows *en* and *wg* expression at stages 10/11 and late stage 12.

At stage 10/11, the *en* intercalary spot, the *en* antennal stripe, the *en* head spot and the *en* expression in the anterior dorsal hemispheres, have their *wg* counterpart anterior to them (see Discussion) when using spatial vicinity as a criteria (Figs 12, 13; see also Cohen and Jürgens, 1991). The **wg labral spot** (*wg lr*) remains in the peripheral ectoderm of the clypeolabrum. The **wg head blob** (*wg hb*) becomes incorporated in the brain but cells of the optic Anlagen seem to be devoid of *wg* expression. The **wg antennal stripe** (*wg a s*) is limited to the peripheral ectoderm.

Fig. 3. Wild-type embryos at stage 9 (A-E) and 10 (F-J) stained with anti-en antibody. Anterior to the left or to the top (D, E, H, I, J). Lateral views (A, B, F, G) are focused on the peripheral ectoderm in (B) and (G). Dorsal views are shown at the level of the *en* head spot (D, H), the *en* antennal stripe (E, I) and the *en* intercalary spot (J). At stage 9 the *en* head spot (short arrow) shows up and at stage 10 the *en* intercalary spot (arrowhead). In (A) and (F) the arrows indicate the position of the *en* head spot and the *en* antennal stripe (long arrow) that are both out of focus. T1 marks the first thoracic segment. Scale bar, 20 μ m.



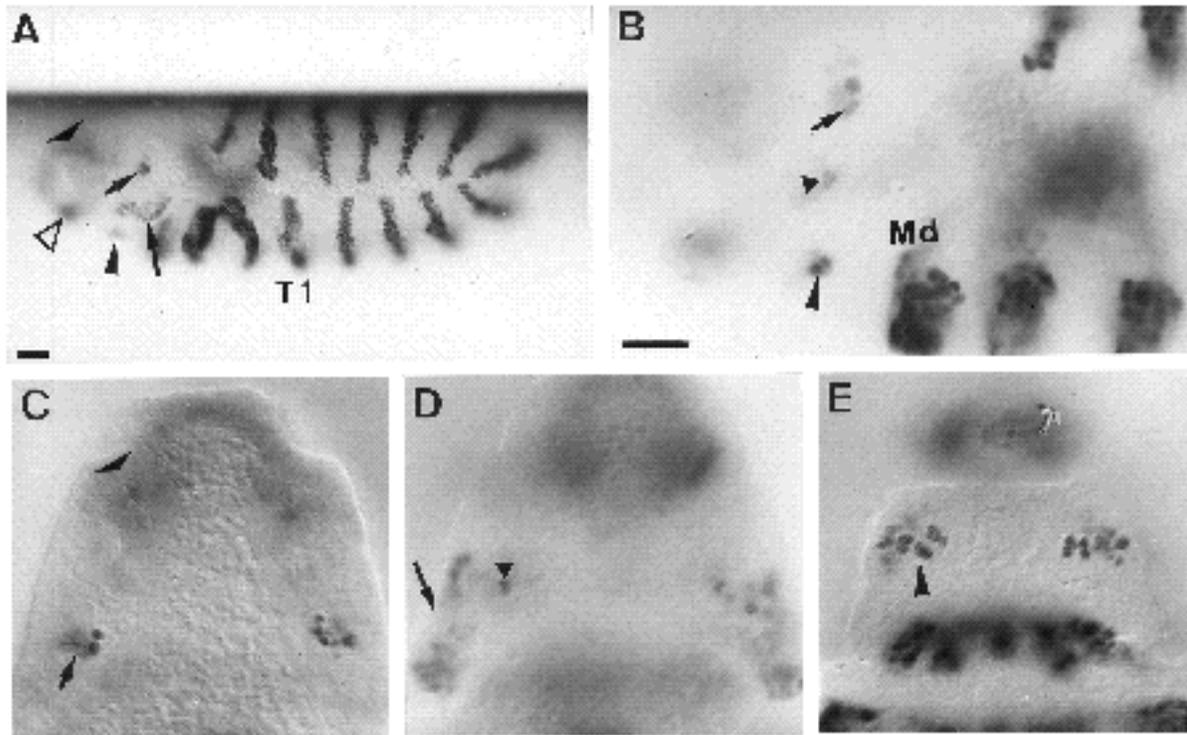


Fig. 4. Wild-type embryos at stage 11 stained with anti-*en* antibody. Anterior to the left or to the top (C-E). (A) and (B), lateral views; (C-D), dorsal views at the level of the *en* head spot (arrow in C), the *en* antennal spot (triangle in D), and *en* intercalary spot (arrowhead in E). *en* has become visible in the clypeolabrum (hollow triangle in A) and in the anterior dorsal hemispheres (filled triangle in C). Cells at the anterior end of the *en* antennal stripe have segregated (triangle in B, D). Note the position of the *en* head spot, the *en* antennal spot, and the *en* intercalary spot with respect to the mandibular segment (B). T1 marks the first thoracic segment, Md the mandibular neuromere. Scale bar, 20 μ m.

However, until stage 10 it is in contact with the *wg* head blob (Baker, 1988, van den Heuvel et al. 1989). Expression in the ***wg* intercalary spot** (*wg* Ic) is turned off at stage 11 (Baker, 1988; van den Heuvel et al. 1989), but staining embryos of transformant lines carrying a *lacZ* insertion at the *wg* locus (*CyO*, *wg^{en11/sli}* and *wg^{17en40cP1/CyO}*) reveals that these cells segregate into the CNS (data not shown). There is also a median spot of ***wg* expression in the foregut** (Baker, 1988; van den Heuvel et al. 1989) which probably corresponds to the median *en* clypeolabral spot (Figs 12, 13). The gap between this *wg* expression and the median *en* clypeolabral spot increases during development. This might be due to cell proliferation and intercalation in the course of morphogenetic movements during stomodeum formation (Hartenstein and Campos-Ortega, 1985; Technau and Campos-Ortega, 1985).

Cells at the ventro-anterior side of the en head spot contribute to the antennal lobe cortex

In the brain of the freshly hatched larvae, five proliferating cells can be marked by BrdU. The fates of these five neuroblasts and their descendants are known (Ito and Hotta, 1992; Prokop et al., 1991). Four of them contribute to the mushroom body cortex. The fifth neuroblast and its descendants which lie laterally become incorporated in the cortex of the antennal lobe and hence belong to the deutocerebrum. This group of cells lies in the area of the *en* head

spot. To find out if it is (partly) identical with the *en* head spot, we fed freshly hatched larvae for 1-to-4 hours on a diet containing BrdU, which becomes incorporated in the DNA of the five proliferating neuroblasts and their descendants in each brain hemisphere. We then double stained larval brains against *en* and BrdU (wild-type) or anti-*-gal* and BrdU (*ryxho25*). This procedure revealed that the fifth (lateral) BrdU-labeled neuroblast is not identical with the cells of the *en* head spot or their descendants but is in direct contact with these cells (Fig. 14; $n = 10$, no exceptions). The descendants of *en*-expressing cells are recognizable due to the perdurance of the *lacZ* product in the transformant *en-lacZ* line (Monsma et al. 1988; Vincent and O'Farrell, 1992; Martinez Arias and Technau, unpublished data). As expected, the patterns of *en* expression in the *en* head spot as revealed by anti-*-gal* and anti-*en* are very similar until stage 13, because only then do some cells of the *en* head spot turn off *en* expression so that more cells are stained by anti-*-gal* in *ryxho25* than by 4D9 in wild-type embryos at older stages.

The position of the BrdU-marked cells is always at the anterior ventral side of the *en* head spot cells (Fig. 14). In embryos stained with anti-*-gal* and anti-BrdU (Fig. 14B) the BrdU-marked cells exhibit a halo that separates them from the *en* head spot. This suggests that the BrdU-marked cells do not express *lacZ* and, therefore, might not derive from the *en* head spot cells.

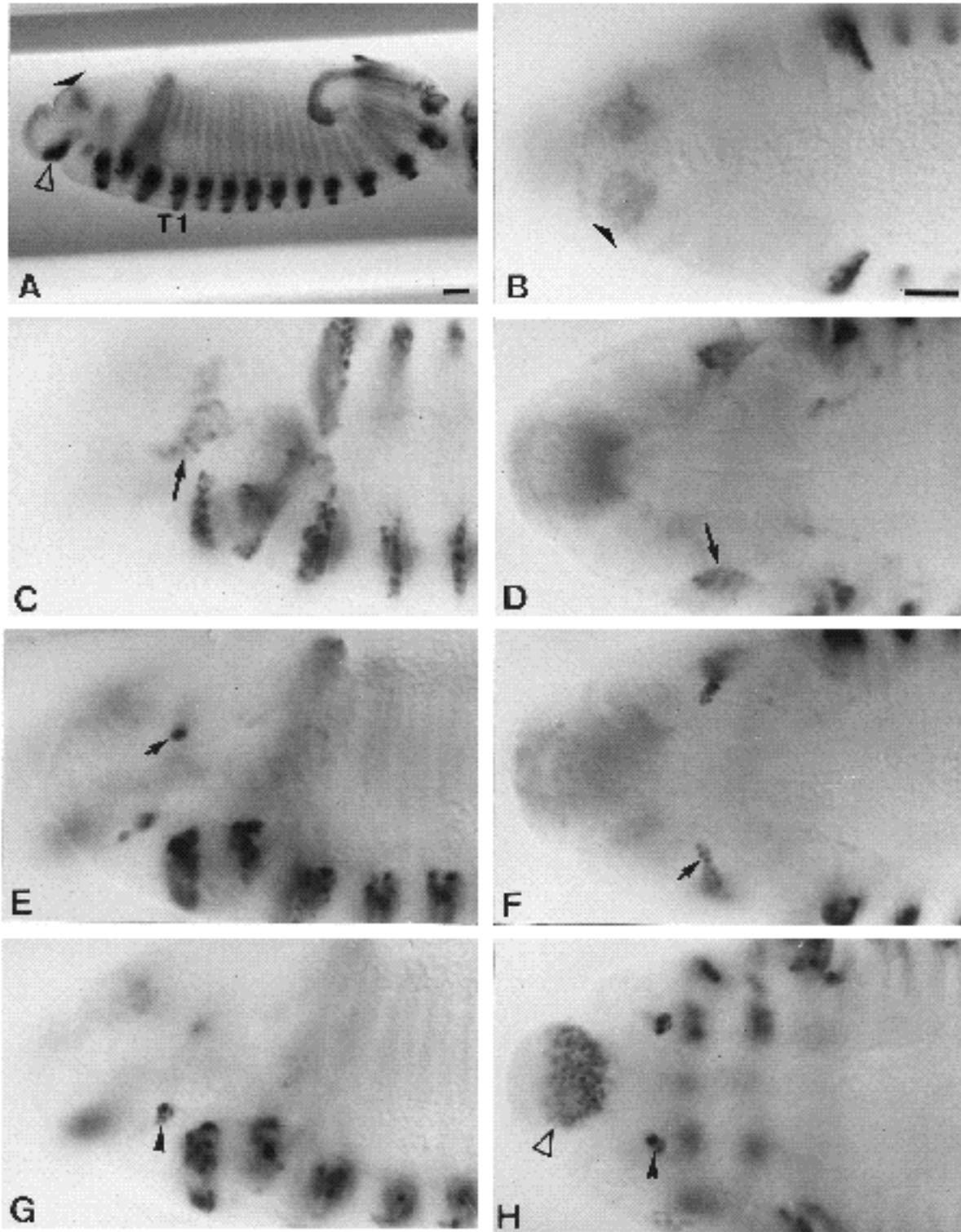


Fig. 5. Wild-type embryo at stage 12 stained with anti-en antibody. Anterior to the left. (B), (D), (F) and (H) show dorsal views at the level of en expression in the dorsal hemispheres (triangle), the en antennal stripe (long arrow), the en head spot (short arrow), and the en intercalary spot (arrowhead), respectively. The same symbols are used in the lateral views (A, C, E, G). The hollow triangle in (A) points to the en expression in the clypeolabrum. Note that the en head spot lies at the ventral side of the brain hemisphere and that the en intercalary spot now lies at the posterior margin of both connectives between the supraesophageal and the subesophageal ganglia. T1 marks the first thoracic neuromere. Scale bar, 20 μ m.

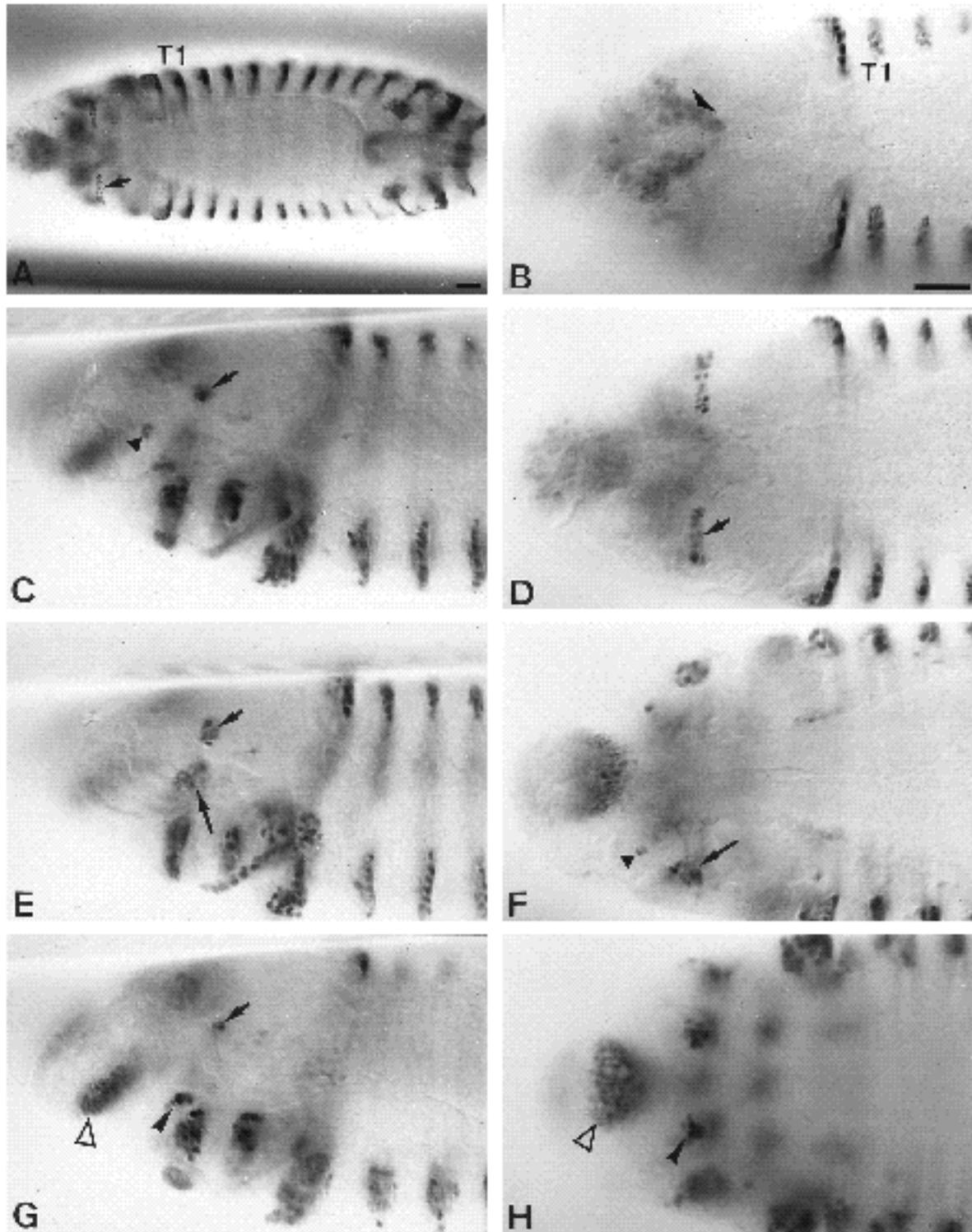


Fig. 6. Wild-type embryo at stage 13 stained with anti-en antibody. Anterior to the left. (B), (D), (F) and (H) show dorsal views. en head spot: small arrow in (A), (C), (D), (E) and (G); en expression in the anterior dorsal hemispheres: triangle in (B); en antennal spot: small triangle in (C) and (F); en antennal stripe: long arrow in (E) and (F); en expression in the clypeolabrum: open triangle in (G) and (H); en intercalary spot: arrowhead in (G) and (H). Note that the en head spot now forms a stripe crossing each hemisphere at its ventral side. The two outermost cells of each stripe are larger than the inner ones. T1 marks the first thoracic segment. Scale bar, 20 μ m.

Discussion

Our detailed description of the dynamic pattern of expression of *en* and *wg* contributes towards clarification of the segmental organization of the head. Furthermore, it provides useful reference points for future investigations of brain development.

The number and arrangement of neuromere remnants in the supraesophageal ganglion of Drosophila

We studied the spatial and temporal expression pattern of *en* (see also DiNardo et al. 1985 and Diederich et al. 1991) and *wg* (see also Baker, 1988 and van den Heuvel, 1989) in relation to each other during embryonic development of the head to obtain evidence concerning the number, location and orientation of brain neuromeres and their corresponding segments in the peripheral ectoderm. As shown below, our data support the theory that there are four pregnathal head segments which are aligned in an S-shape. These conclusions differ from those drawn by Diederich et al. (1991) who, based on the expression of *en*, argue for only three linearly aligned pregnathal segments (for discrepancies between their observations and ours, see first chapter of Results).

Four of five "centers" of *en* expression in the pregnathal head contribute cells to the brain. These are: the *en* inter-

calary spot, the *en* antennal stripe which contributes the cells of the *en* antennal spot, the *en* head spot, and the *en* expression in the anterior dorsal hemispheres (Fig. 13). These patches are good candidates for segmental markers because normally, a segment contributes a neuromere to the central nervous system.

We attribute these patches of *en* expression to four pregnathal neuromeres as follows.

The *en* intercalary spot is attributed to the intercalary neuromere because of its position just anterior to the mandibular neuromere.

The *en* antennal spot is attributed to the antennal segment for two reasons. (1) At stage 10/11, these cells segregate from the anterior end (corresponding to the ventral side of trunk stripes) of the *en* antennal stripe which contributes cells to the dorsal organ. (2) In *buttonhead* mutants (*btd XG* and *btd XO*; Jürgens et al. 1984) which lack *wg* and *en* expression in the mandibular, the intercalary, and the antennal segments (Cohen and Jürgens, 1990), the *en* antennal spot does not show up, either (Schmidt-Ott and González Gaitán, unpublished observation).

The *en* head spot is attributed to the ocular segment (to which the optic lobe fuses secondarily; Campos-Ortega and Hartenstein, 1985; own observations). We believe that the cells of the *en* secondary head spot also belong to the ocular segment because they detach from the *en* head spot at stage 13/14.

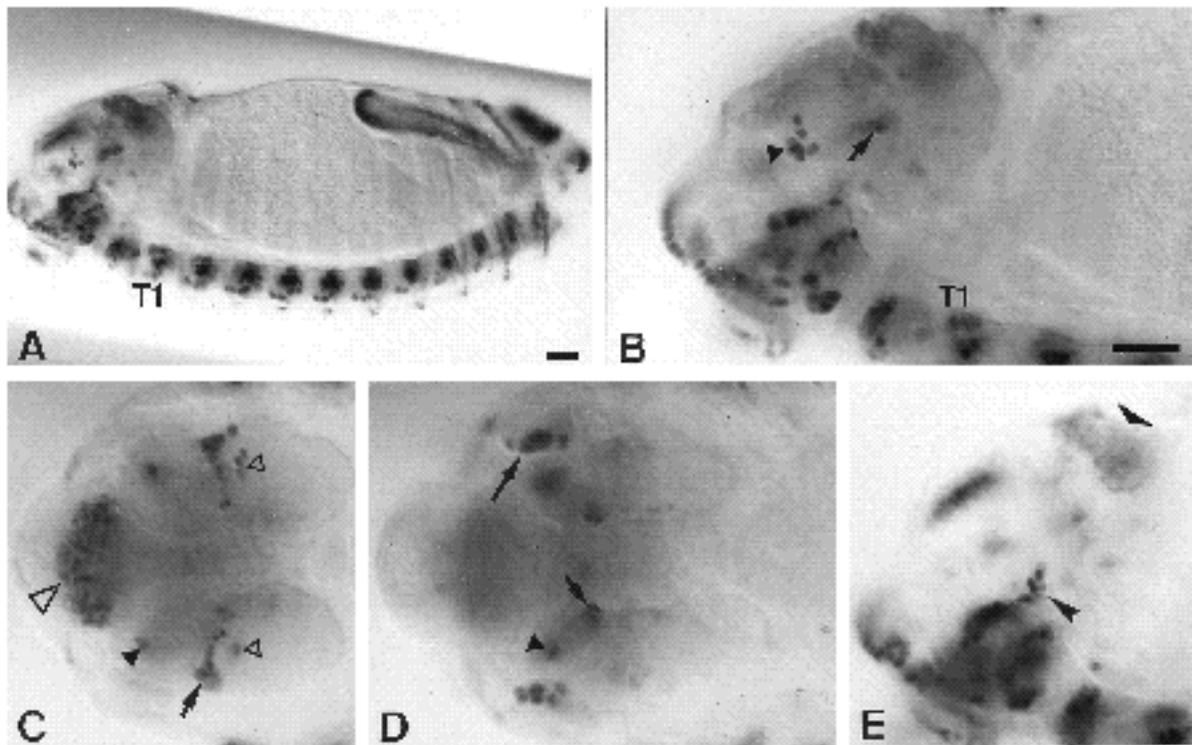


Fig. 7. Wild-type embryo at stage 14 stained with anti-*en* antibody. Anterior to the left. (C) and (D) show dorsal views at the level of the *en* head spot and of the *en* antennal stripe, respectively. The small hollow triangles in (C) point to cells of the *en* secondary head spot which separate from the *en* head spot cells at stage 13/14 and are not visible as a separate spot at earlier stages. Other symbols as in Fig. 6. Note that the cells of the *en* intercalary spot are now in contact with *en*-expressing cells of the mandibular neuromere which makes it difficult to distinguish between both groups. The *en* antennal spot has enlarged compared to the preceding stage. T1 marks the first thoracic neuromere. Scale bar, 20 μ m.

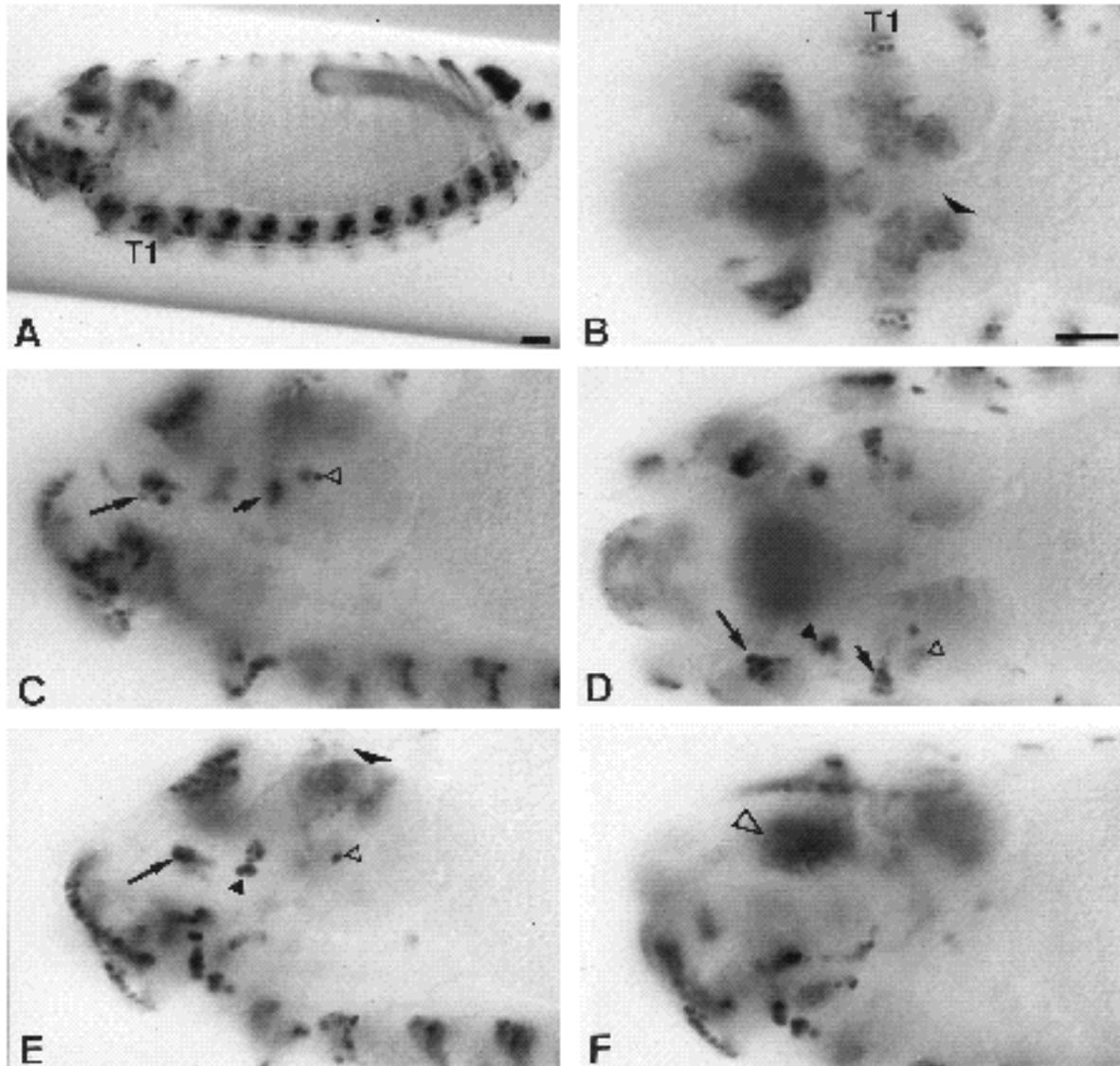


Fig. 8. Wild-type embryo at stage 15 stained with anti-en antibody. Anterior to the left. (B) and (D) show dorsal views at the level of the dorsal hemispheres and the en head spot, respectively. Symbols as in Figs 6 and 7. T1 marks the first thoracic neuromere (A) or segment (B). Scale bar, 20 μ m.

en expression in the anterior dorsal hemispheres is attributed to the labral neuromere as it has its *wg* counterpart on the clypeolabrum.

At stage 12/13 the four pregnathal neuromeres and their epidermal counterparts are arranged in an S-shape (Fig. 13). Several lines of evidence support this assumption. (1) The suggested alignment of segments is in excellent agreement with published fate-mapping data (Fig. 1; Technau and Campos-Ortega, 1985; Jürgens et al. 1986). Fig. 1A shows sites of HRP injection at the onset of gastrulation. After staining these embryos at stage 12/13, the markings were found in the peripheral ectoderm (Fig. 1B) and/or in the brain (Fig. 1C-F), revealing the S-shaped arrangement of cells that had been in a line at the onset of gastrulation (Technau and Campos-Ortega, 1985). Our observation that cells of the en head spot (ocular neuromere) lie in the imme-

mediate vicinity of cells that contribute to the cortex of the antennal lobe (Fig. 14) is also consistent with an S-shaped arrangement of the brain neuromeres: these presumptive cells of the cortex of the antennal lobe lie ventro-anterior (i.e. ventro-posterior with respect to the polarity of the ocular neuromere!) to the en head spot cells, and they do not express *lacZ* which suggests that these cells do not derive from the en head spot cells (Fig. 14B). We suggest that the segmental border runs just between both groups. In fact, in the adult brain the BrdU-marked cells shown in Fig. 14 lie laterally in the antennal lobe cortex towards the cortex of the optic lobe (Ito and Hotta, 1992; Prokop and Technau, unpublished data). (2) At stage 13 the en head spot forms a horizontal stripe which transversely crosses each hemisphere on its ventral side (Figs 6A, D; 5E). Analogous to the situation in the trunc, we would expect that

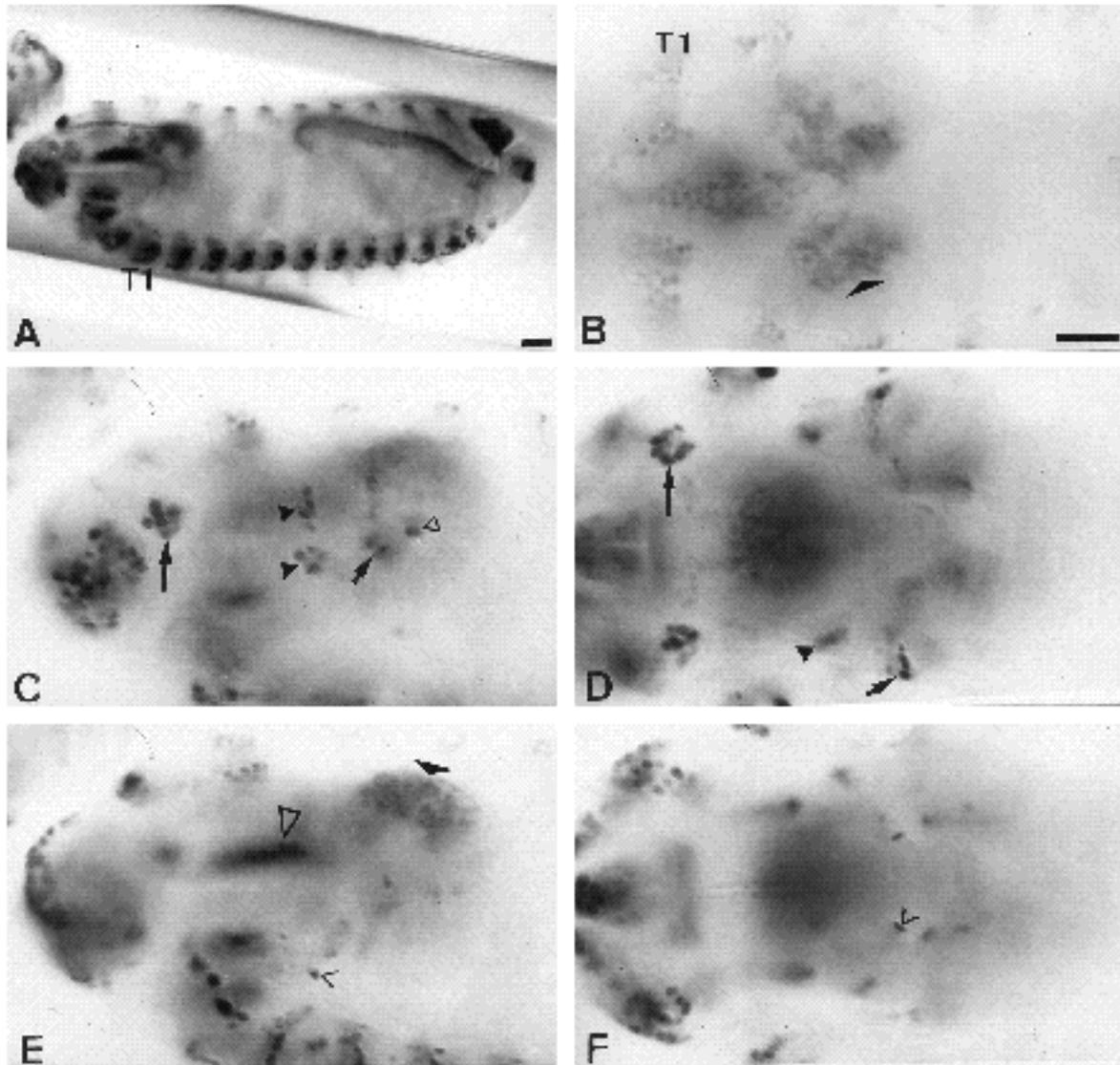


Fig. 9. Wild-type embryo at stage 16 stained with anti-*en* antibody. Anterior to the left. Dorsal views are shown at the level of the dorsal hemispheres (B), the *en* head spot (D) and the connectives (F). The angle in (E) and (F) marks an *en*-expressing cell of unknown origin at the posterior margin of each connective. Other symbols as in Figs 6 and 7. T1 marks the first thoracic neuromere (A) or segment (B). In several cases we observed a splitting of the *en* antennal spot in two groups of cells at this stage as shown in (C). Scale bar, 20 μ m.

this stripe of cells marks the posterior segment border of the neuromere. This is only the case if the neuromeres are arranged as shown in Fig. 13B. (3) When assuming an S-shaped arrangement of head segment remnants (Fig. 13), *wg* counterparts to the *en* intercalary spot, the *en* antennal stripe and the *en* head spot, and *en* expression in the anterior dorsal hemispheres are located anterior to each *en* patch analogous to the situation in the trunk (Baker, 1987; Martínez Arias et al. 1988; van den Heuvel et al. 1989). The corresponding *wg* expression is also a good confirmation that the mentioned *en* spots mark different segments (neuromeres). (4) Four pregnathal head segments and their S-shaped arrangement are also in very good agreement with ectopic *en* expression in the head of *patched* null mutants (González Gaitán and Schmidt-Ott, unpublished data).

Expression of en and wg in the foregut

en expression in the clypeolabrum is median and unpaired. During further embryonic development this patch becomes part of the dorsal pharynx. *wg* expression is found in the invaginating stomodeum (Baker, 1988; van den Heuvel et al. 1989). We think that this “pair” of *en* and *wg* expression does not mark a true segment because these cells do not contribute to the CNS and because *en* and *wg* are expressed in an analogous manner in the non segmental hindgut (DiNardo et al. 1985; Baker, 1988; van den Heuvel et al. 1989).

The number of segments in the insect head

Our data suggest that 4 pregnathal segment remnants exist in the *Drosophila* head. How does this assumption fit with

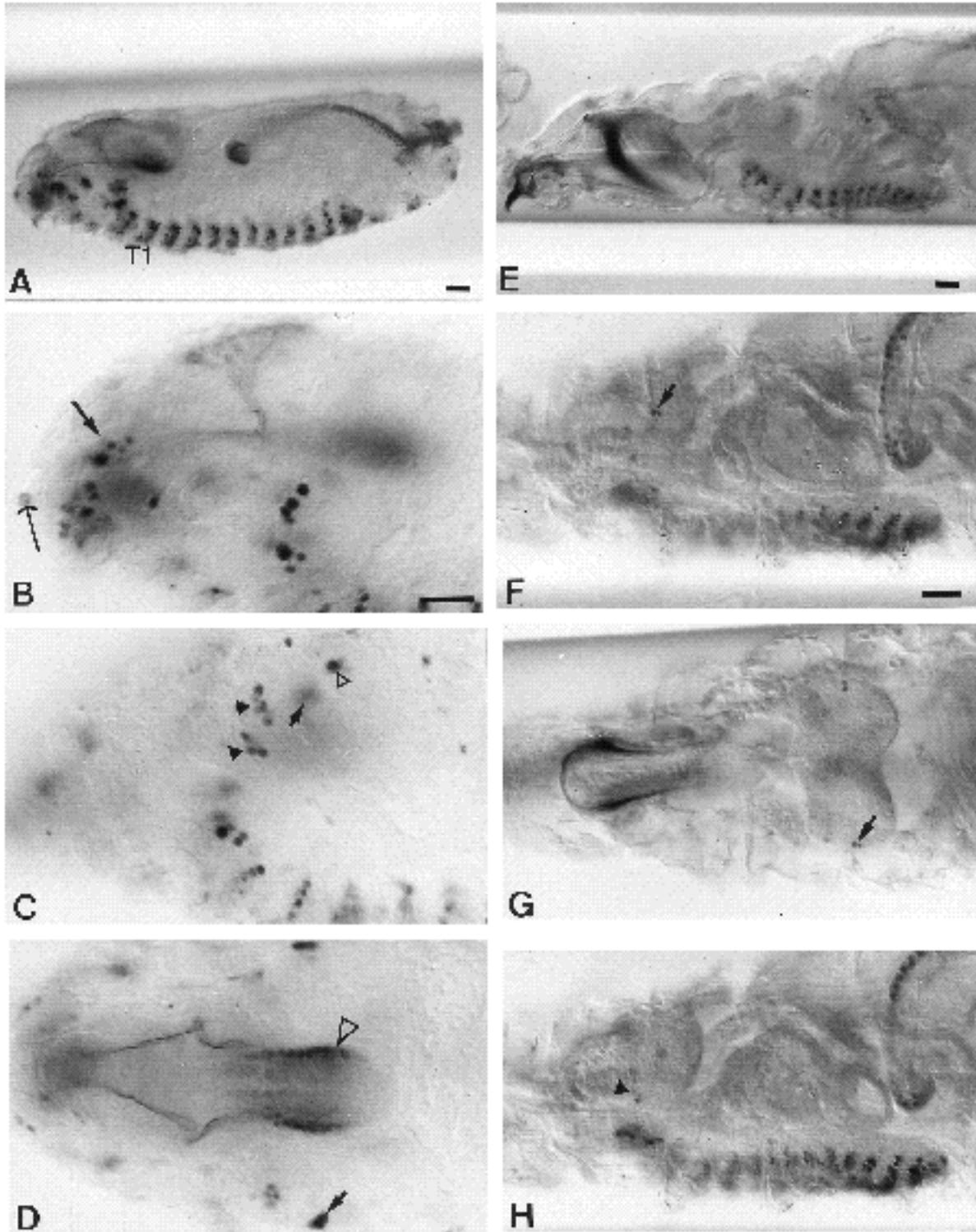


Fig. 10. Wild-type embryo at stage 17 (A-D) and hatching larva (E-H) stained with anti-*en* antibody. Anterior to the left. Lateral views and dorsal views (D, G) at the level of the *en* head spot. The thin arrow in (B) points to the dorsal organ that stains unspecifically. Other symbols as in Figs 6 and 7. Note that the *en* headspot reduces to two cells on each side (F, G) in the hatching larva. The cells of the *en* antennal stripe contribute to the dorsal organ (arrow in B, see also Figs 8C and 11). T1 marks the first thoracic neuromere. Scale bar, 20 μ m.

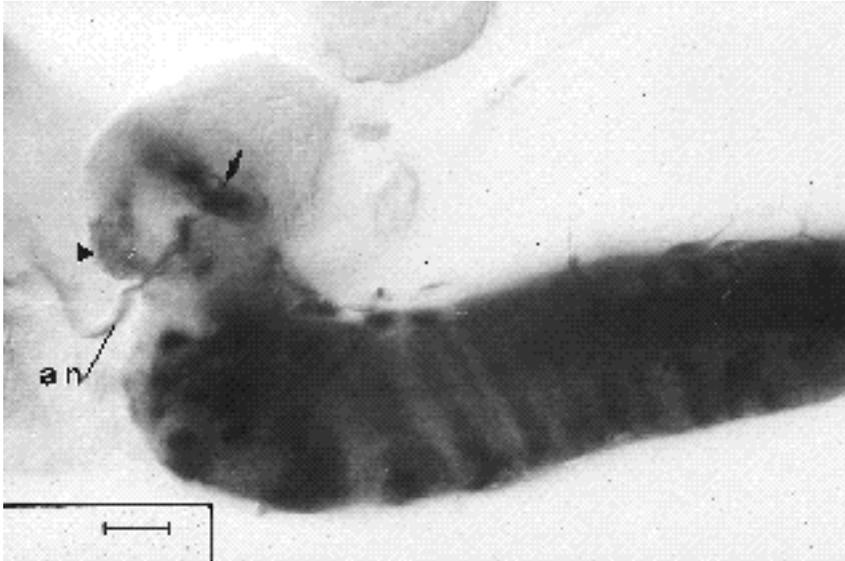


Fig. 11. Brain of transformant *en-lacZ* larva (*ryxho25*) two hours after hatching, stained with anti-*gal* antibody. Lateral view, anterior to the left. The arrow points to the en head spot cells and their descendants and the triangle to the cells of the en antennal spot and their descendants. Note staining of the antennal nerve (an) due to en-positive cells in the dorsal organ. Scale bar, 13 μ m.

Table 1. Expression of *en/inv*

Stage	Intercalary spot (n)	Antennal stripe (e)	Antennal spot (n)	Head spot (n)	Secondary head spot (n)	Dorsal anterior hemisphere (n)	Clypeolabrum (e)
8	-	27		-		-	-
9	-	26-27		2-3		-	-
10	8	26		3-4		-	-
11	16	26	3	9		+	+
12	10	+	(2)	16		+	+
13	10	+	1-2	16-19		+	+
14	+	10	7	16(?)	3	+	+
15	-	10-12	9	10	6	+	+
16	-	ca. 12	11	10	7	+	+
17	-	11	9	3	4-5	(+)	+
L ₁	-	+	(+)	2	-	(+)	-

The table lists numbers of *en*-expressing cells for every *en* spot in the head (see text), as counted for the individuals shown in Figs 2-10 in function of the developmental stage. The spots are attributed to segments in the following manner (see also text): intercalary spot, intercalary segment (tritocerebrum); antennal stripe and antennal spot, antennal segment; (deutocerebrum) head spot and secondary head spot, ocular segment (protocerebrum); expression in dorsal anterior hemispheres, labral segment (protocerebrum); expression in clypeolabrum, non-segmental. "+" is used when the precise number of cells is not known,

"-" indicates the absence of staining. n, neuronal; e, peripheral ectoderm.

comparative data? Two lines of evidence support the assumption of 4 pregnathal segments in the Mandibulata. (1) In several species of insects and in a crustacean, vestiges of two pairs of coelomic cavities have been found anterior to the antennal segment (Wiesmann, 1926¹; Miller, 1940; Nair, 1949; Sharov, 1966; Rohrschneider, 1968). (2) Wada (1966a,b,c) found seven "morphogenetic units" in addition to the stomodeum in the head of the *Tachycines* embryo (Saltatoria) that he revealed by injuring the early embryos and analysing the malformations (doubling or absence of defined structures) of the hatching larvae. His first (i.e. most anterior in the fate map) unit contains the clypeolabrum and the frons, the second is formed by the eyes and neighbouring epidermal structures, the mushroom

bodies, the central body and the lobi optici of the brain. The following five units refer to neuronal and epidermal structures of the antennal, the intercalary, and the three gnathal segments, respectively. Wada (1966a,b) did not find a part of the brain affected together with the first unit but this might be due to the lack of a known morphological marker. Based on these arguments (*en* and *wg* in *Drosophila*, teratology and coeloms in different insects and a crustacean) we suggest seven head segments in the basic body plan of insects (and other Mandibulata): the labral, the ocular, the antennal, the intercalary and the three gnathal segments.

Morphogenetic movements in the head of insects and crustaceans

Morphogenetic movements described for the *Drosophila* head might exist in a very similar manner in other insects and in crustaceans (Sharov, 1966). In *Lepisma* (Apterygota, Insecta) and *Gammarus* (Amphipoda, Crustacea) embryos,

¹In contrast to Wiesmann, Scholl (1969) believes that in *Carausius* (Phasmida) the second (optic) pair of coelomic cavities in front of the antennal segment derives from the intercalary coelom.

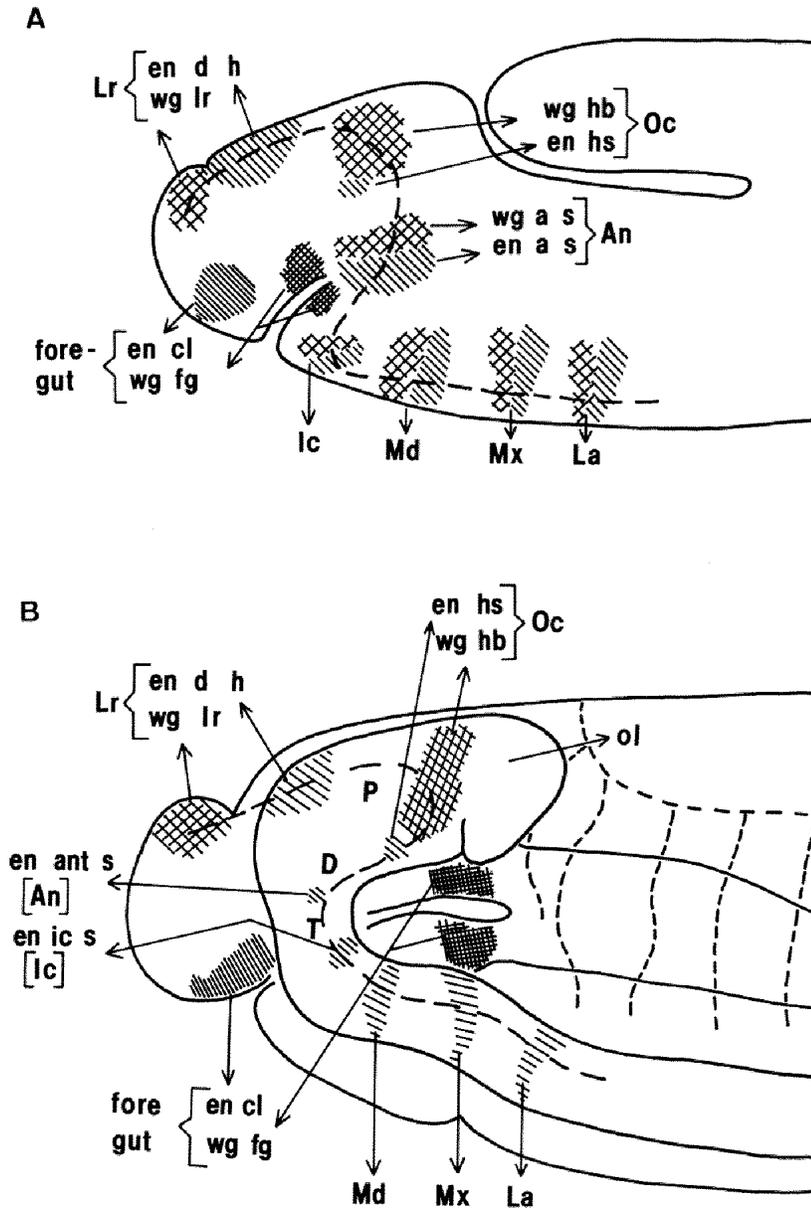


Fig. 13. Scheme of the *Drosophila* head indicating expression of *en* (hatched) and *wg* (crosshatched) at embryonic stage 10/11 (A) and late stage 12 (B). The dotted line indicates the alignment of segments. Note that at stage 10/11 every patch of *en* expression has its *wg* counterpart. In (B) expression of *en* and *wg* in the peripheral ectoderm of the antennal and gnathal segments has been omitted. An, to antennal segment; ant s, antennal spot; a s, antennal stripe; cl, clypeolabral; D, deutocerebrum; d h, dorsal hemisphere; hb, head blob; hs, head spot; Ic, to intercalary segment; ic s, intercalary spot; La, to labial segment; Lr, to labral segment; Mx, maxillary segment; Oc, to ocular segment; ol, optic lobe; P, protocerebrum; T, tritocerebrum.

the labral segment shifts ventrally towards posterior during development (Weygoldt, 1958; Sharov, 1966). This leads to a recess in the segmental band (Sharov, 1966, his Fig. 63) of these hypognathous Mandibulata. In most insects and crustaceans the hypognathous state independently changed into a prognathous one which in insects brought the eyes into a more posterodorsal position (for review see Sharov, 1966). The resulting S-shaped arrangement of head segments seems to match well with the situation in the *Drosophila* embryo at the stage of germ band retraction. Therefore, this arrangement of head segments might be typical for embryos of prognathous mandibulates in general.

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Fig. 12. Embryos heterozygous for the mutation *wg^{LL14}* (see text) double stained against *en* and *wg*. Anterior to the left. (A) Stage 10, only the head is shown from the latero-ventral side. *en* and *wg* expression in the ocular segment are out of focus. Mx, Md, Ic, An, Oc, and Lr: *en* and *wg* to maxillar, mandibular, intercalary, antennal, ocular, and labral segment, respectively. The arrowhead points to the *en* intercalary spot. The *en* expression in the anterior dorsal hemispheres is not yet visible at this stage (see text). Bar, 20 μ m. (B, C) Early stage 12, lateral and dorsal view. Note the weak *en* expression in the anterior dorsal hemispheres which becomes more prominent in following stages (see also Figs 4, 5, 7, 8). The large arrow points to the *wg* head blob, other symbols as in Figs 6 and 7. Bar, 50 μ m.

Fig. 14. CNS of larvae fed on a BrdU diet for 3.5 and 1.5 hours after hatching. Lateral view, anterior to the left. (A) Wild-type 3.5 hours after hatching, double stained against *en* (brown) and BrdU (black); (B) *en-lacZ ryxho25*, 1.5 hours after hatching stained against β -gal (brown) and BrdU (black). The BrdU-marked lateral neuroblast (asterisk) and its descendants are later found in the cortex of the antennal lobe (Ito and Hotta, 1992; unpublished data). The arrow points to the *en* head spot. It is larger in (B) because of the perdurance of the *lacZ* product. Note the halo (white arrows) separating the BrdU-marked cells from the *en* head spot cells and their descendants (black arrow). br, brain; vn, ventral nerve cord. Scale bar, 20 μ m.