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# Structure of the insect head as revealed by the EN protein pattern in developing embryos

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## SUMMARY

**The structure of the insect head has long been a topic of enjoyable yet endless debate among entomologists. More recently geneticists and molecular biologists trying to better understand the structure of the head of the Dipteran *Drosophila melanogaster* have joined the discourse extrapolating from what they have learned about *Drosophila* to insects in general. Here we present the results of an investigation into the structure of the insect head as revealed by the distribution of *engrailed* related protein (Engrailed) in the insect orders Diptera, Siphonaptera, Orthoptera and Hemiptera. The results of this comparative embryology in conjunction with genetic experiments on *Drosophila melanogaster* lead us to conclude: (1) The insect head is composed of six Engrailed accumulating segments, four postoral and two preoral. The potential seventh and eighth segments (clypeus or labrum) do not accumulate Engrailed. (2) The structure known as the dorsal ridge is not specific to the Diptera but is homologous to structures found in other insect orders. (3) A part of this structure is a single segment-like entity composed of labial and maxillary segment derivatives which produce the most anterior cuticle capable of taking a dorsal fate. The segments anterior to the maxillary segment produce only ventral structures. (4) As in *Drosophila,* the process of segmentation of the insect head is fundamentally different from the process of segmentation in the trunk. (5) The pattern of Engrailed accumulation and its presumed role in the specification and development of head segments appears to be highly conserved while its role in other pattern formation events and tissue-specific expression is variable. An overview of the pattern of Engrailed accumulation in developing insect embryos provides a basis for discussion of the generality of the parasegment and the evolution of Engrailed patterns.**

Key words: insect head, evolution, *engrailed*, *Ultrabithorax*, dorsal ridge, Siphonaptera, Orthoptera, Diptera, Hemiptera

## INTRODUCTION

The structure and evolution of the insect head has long been a subject of somewhat heated debate among entomologists (Rempel, 1975). Recently, Drosophilists have joined the discourse and have attempted to extrapolate their findings from research done on *Drosophila melanogaster* to insects in general (Schmidt-Ott et al., 1994a; Schmidt-Ott and Technau, 1992; Cohen and Jurgens, 1991; Finkelstein and Perrimon, 1991; Diederich et al., 1991). Although some of what has been discovered is applicable to other insects, the highly derived state of the *Drosophila* head – particularly the larval head – make it a poor example of insects in general. The *Drosophila* segmentation gene *engrailed* produces a homeodomain-containing protein that is thought to be critical to the process of segmentation (DiNardo et al., 1985; Kornberg, 1981; Morata and Lawrence, 1975). Additionally Engrailed-like proteins (EN) are conserved among arthropods and are also believed to be involved in the segmentation of these animals (Patel, 1994a,b; Patel et al., 1989a,b). By observation of the distribution of EN to mark the boundaries of head segments, it has been possible to show that the expression pattern of this protein is highly conserved among insects (this work).

Most debate on the structure of the insect head has centered on its anterior-to-posterior segmental composition and the mode during evolution by which these segments were recruited from generalized trunk segments from a simpler less derived condition to the more complex one found in insects (Finkelstein and Perrimon, 1991; Rempel, 1975). In addition to shedding light on the anterior-to-posterior organization, the patterns of EN distribution have also revealed a conserved dorsal-ventral organization. Part of this conserved organization involves the formation of a specialized multipart structure, known as the dorsal ridge in *Drosophila* and *Calliphora* (Turner and Mahowald, 1979), which forms the junction between cephalic and thoracic domains and also organizes the posterior head.

We present the pattern of EN expression during the embryonic development of five species, each from a different order, focussing on the patterns in the head. These insects are *D. melanogaster* (Diptera), *Ctenophalides felis* (Siphonaptera), *Oncopeltus fasciatus* (Hemiptera) and *Acheta domestica* (Orthoptera). A comparison of the EN expression exhibited, combined with a reevaluation of previously described EN patterns for other insects has led us to conclude that the structure of the insect head is highly conserved and that the variation of head morphology among insect orders is largely due to differences in the development of similar embryonic

primordia. We have combined this comparative analysis with a genetic dissection of the *D. melanogaster* dorsal ridge. We have concluded that the dorsal ridge is a multipartite structure which, although variable in morphology among the insects, plays a similar role in the formation of the head. The ontogeny of the EN pattern in the head is also highly conserved, with each component added in a stereotypical order in development. This suggests that, unlike the trunk of insects, which uses varying mechanisms to produce a segmented trunk (Patel, 1994a,b), the mechanism of segmentation of the head may be more highly conserved. The apparent participation of EN in processes not involved in segmentation is also discussed.

## MATERIALS AND METHODS

### Insect stocks

Wild-type *Drosophila melanogaster* (Diptera) of the strain Oregon-R were raised in standard laboratory cultures at 22°C. Embryonic development was complete in approximately 1 day. Flies of the genotype P{$w^{+mC}$ $Ubx^{UAS}$=UAS::Ubx}/+;P{$w^{+mC}$ $Gal4^{prd}$=prd::Gal4} (hereafter referred to as prd=>UBX) were generated by crossing flies of the genotypes *y w*; P{prd::Gal4} (gift of C. Desplan) and *w*; P{UAS::Ubx} (Kalkbrenner, Hamilton, Miller, Yang, Huer and Kaufman, unpublished data). All the resulting progeny of this cross express UBX ectopically in a *paired*-like pattern.

*Oncopeltus fasciatus* (Hemiptera) were raised in staged cultures of 20-100 individuals. Milkweed bugs were provided water on moist towels and fed cracked sunflower seeds. Adult females laid eggs on cotton from which the eggs were collected daily. Embryonic development was complete in 8 (22°C) or 5 (31°C) days.

*Acheta domestica* (Orthoptera) were raised in staged cultures of 40-250 individuals at 22°C in high walled containers on a diet of Purina Cat Chow and lettuce. Adult crickets deposited their eggs in damp sand. Eggs were flooded from the sand with water and collected into Petri dishes containing damp filter paper. Eggs were incubated at 31°C until hatching. Embryonic development was complete in 12 days.

*Ctenophalides felis* (Siphonaptera) were collected from infested felines or swept from their bedding and incubated at 22°C. Some flea embryos were also provided by J. R. Georgi. Embryonic development was complete in 4 days.

### Fixation of embryos and in situ detection of EN and UBX with antibody

Fly embryos were fixed as described previously (Diederich et al., 1991). Flea embryos were fixed in a manner identical to flies except that after chemical dechorionation all embryos were collected. Although dechorionation was often incomplete, the shells were cracked sufficiently to allow the antibody access to the embryo. Milkweed bug embryos were prepared identically to *Drosophila* except that rocking embryos for 10-20 minutes in 50-60% bleach produced only partial dechorionation. Fixation was performed for 2 to 24 hours and the egg shells were removed after fixation but prior to exposure to antibody. Cricket embryos were fixed by manual dissection of the chorion away from the embryo in fixative. The dissected embryos were rocked for 20 minutes in a mixture of 50% fixative and 50% heptane.

The isolation of antibodies to the engrailed protein (EN) and the *Ultrabithorax* protein (UBX) have been described previously (Patel, 1989a; White and Wilcox, 1984). These were concentrated by a Centricon column from tissue culture medium. The immunochemical in situ detection of EN and UBX was done as previously described (Gorman and Kaufman, 1995). Cell numbers were estimated by counting nuclei immunochemically stained for EN. Expression of EN was considered de novo, not lineage related, if expression was initiated more than two to three cell diameters away from a preexisting EN-expressing cell.

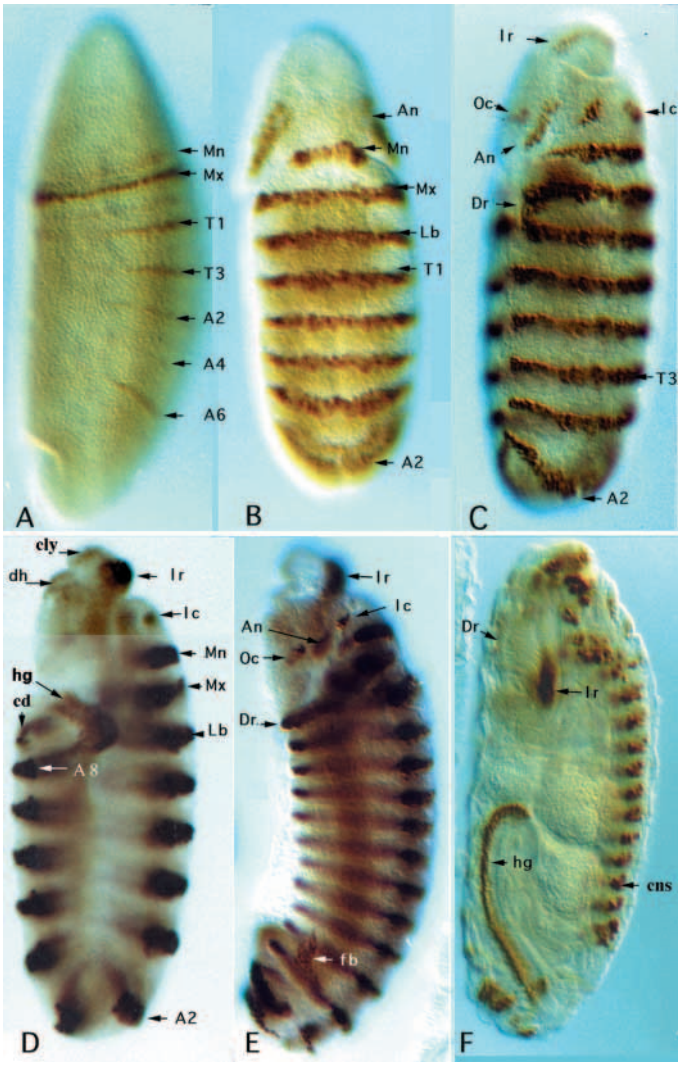



**Fig. 1.** EN expression in the embryo of *Drosophila melanogaster* (fly). Arrows mark the accumulation of EN (shown in brown). Embryos are presented in chronological order. (A) A blastoderm stage embryo has pair-rule modulated accumulation of EN in the mandibular (Mn) through eighth abdominal (A8) segments. (B) During germ-band extension, EN accumulates in the antennal (An) segment. (C) EN then accumulates in the intercalary (Ic), ocular (Oc) and labral (lr) segments and the dorsal ridge (Dr) of a germ-band-extended embryo. (D) EN accumulates in the hindgut (hg), clypeus (cly) and dorsal hemispheres (dh) and caudal (cd) region as seen in a germ-band-retracted embryo. (E) An embryo undergoing head involution and dorsal closure accumulates EN in the fatbody (fb). (F) At the completion of dorsal closure, EN expression has diminished in the Dr and epidermis except in structures of the pseudocephalic and caudal regions, central nervous system (cns), the hg and lr region of the pharynx. Embryos are shown in a lateral view with the anterior pointing up and dorsal to the left, except (B) which is a ventral and (C) which is ventrolateral view.

### Slides and photography

After fixation, animal tissues and embryos were dissected in PBS, primarily to remove yolk and were then mounted on microscope slides using Aqua-Polymount (PolySciences Inc.) or methyl salicylate as the

mounting media. Slides were examined on a Zeiss axiophot and photographed with Kodak ASA100 print film at 50-200× magnification.

### Cuticle preparations

Preparations of cleared cuticles of *Drosophila* larvae were produced as described previously (Gorman and Kaufman, 1995).

## RESULTS

An overview of the embryonic development of the fly (Diptera), the flea (Siphonaptera), the milkweed bug (Hemiptera) and the cricket (Orthoptera) have been described previously (Campos-Ortega and Hartenstein, 1985; Kessel, 1939; Newcomer, 1948; Mahr, 1960). Rather than attempt to merge the existing disparate staging systems, we will use descriptive terms to focus on key landmarks during embryonic development (Anderson, 1973; Counce and Waddington, 1972). These terms, presented in approximate chronological order are: (1) blastoderm formation, migration of syncytial nuclei to the outer surface of the embryo; (2) formation of the germ rudiment, coalescence of embryonic cells to a ventral and posterior position in the egg; (3) anatrepsis, invagination of the germ rudiment into the egg; (4) gastrulation, formation of a two-layered embryo composed of mesoderm and ectoderm; (5) germ-band extension, formation of a segmented embryo with a full compliment of segments; (6) neurogenesis, delamination of neuroblasts from the ventral ectoderm; (7) germ-band retraction and katatrepsis, shortening of the embryo followed by emergence of the embryo from the yolk to the inner surface of the egg; (8) dorsal closure, dorsal growth and fusion of the left and right portions of the thoracic and abdominal ectoderm engulfing the remaining yolk. Although development does not end at dorsal closure, we end our description at that point. We also use the term 'head compaction' to refer to the migration of the gnathal and pregnathal segments from their relatively linear position along the embryo to their appropriate position around the mouth and brain. Head compaction begins during germ-band retraction and ends after dorsal closure.

The pattern of EN in the developing embryos was revealed by immunohistochemistry. Timed collections of insect embryos were fixed and then probed with 4D9 (Patel et al.,1989a), a monoclonal antibody to EN. The in-situ position of the 4D9 antibody was determined by the localized accumu-

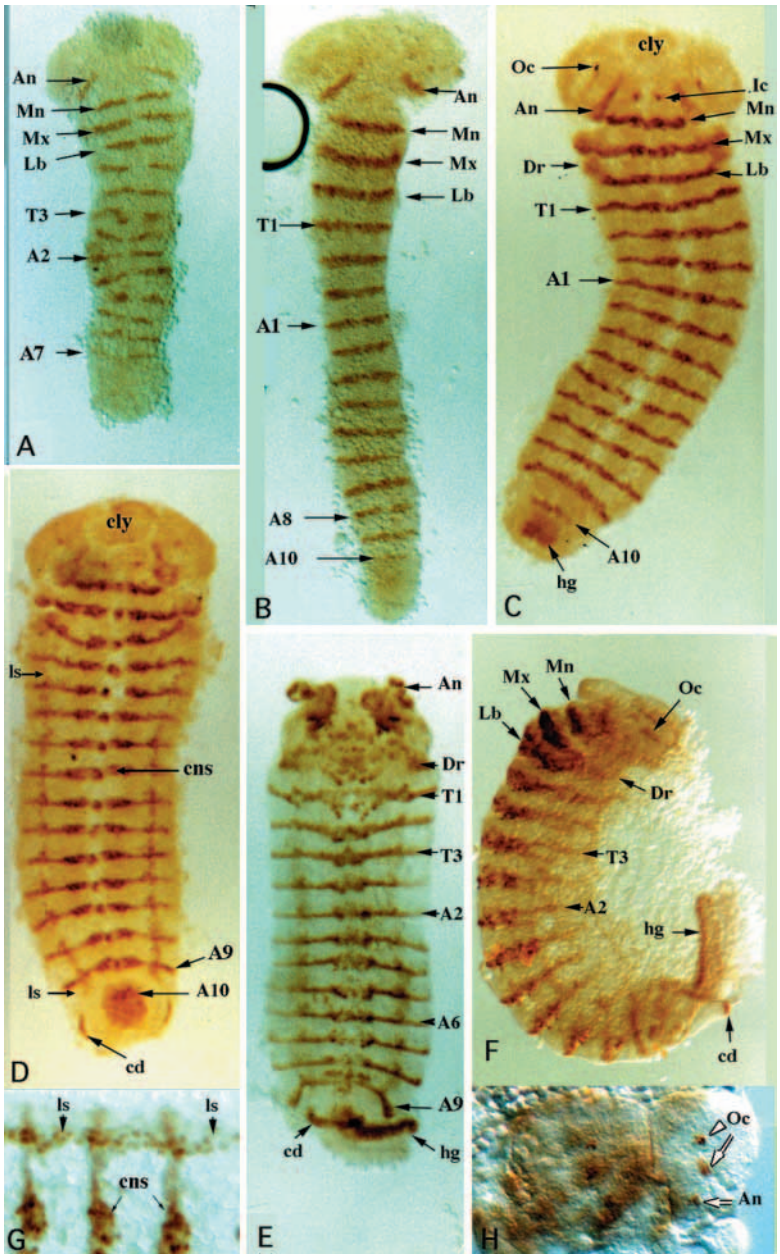



**Fig. 2.** EN expression in the embryo of *Ctenocephalides felis* (flea). Arrows mark the accumulation of EN (shown in brown). A-F are presented in chronological order. (A) The condensed germ rudiment of a flea embryo at the onset of gastrulation accumulates EN in the An, Mn, Mx, Lb, T1-3 and A1-A7 segments. (B) At the completion of gastrulation, the three remaining abdominal segments A8-A10 have been added. (C) At the onset of neurogenesis, EN accumulates in the Oc and Ic segments, the Dr and the hg. No accumulation of EN is detected in the clypeus (cly). (D) During germ-band shortening, flea-specific patterns of EN accumulation can be seen. Expression of EN can be detected in a lateral stripe (ls), which runs longitudinally through the lateral region of the embryo from posterior T2 through the anterior of A10. Caudal (cd) accumulation of EN begins as a lateral stripe posterior to A10. EN accumulates in delaminating neuroblasts of the cns. The EN-expressing cells of A10 become completely restricted to the cns. (E) During head compaction, EN accumulates in the Dr as a single patch dorsal to both the Mx and Lb segments. The cd stripes join together to make an open collar around the hg and proctodeal opening. (F) As seen in this lateral view of dorsal closure, a single EN-expressing stripe grows dorsally from each of the abdominal and thoracic segments and the Dr. (G) In an extended germ band, the ls is 2 to 4 cells wide. (H) The cells of the An and Oc region segregate into multiple secondary Oc spots and a single An spot in the brain (white arrows). Embryos are shown in a ventral view with the anterior pointing up except (F) which is a lateral view. The dorsal portion of the embryo corresponds to the edges of the embryo in these preparations while the ventral region corresponds to the midline.

lation of horseradish peroxidase (hrp) products produced after probing with a commercially available anti-mouse antibody conjugated to hrp, and providing the appropriate substrates. Embryos were mounted on slides and examined under a microscope using Nomarski and unphased optics.

### EN expression in *Drosophila*

Many aspects of the EN expression pattern in the developing *Drosophila* embryo have been described previously (Martinez Arias, 1993; Schmidt-Ott and Technau, 1992; Diederich et al., 1991; Kassis, 1990; Dinardo et al., 1985). Here we present a brief verification and expansion of these descriptions. The development of EN accumulation patterns are illustrated in Fig. 1. In short, EN is expressed in both segmentally repeated and non-segmental patterns. As shown in Fig. 1A-D from anterior gnathos to posterior abdomen, EN accumulates in stripes, which mark the approximate borders of the posterior compartment (Vincent and O'Farrell, 1992; Patel et al., 1989a; Kornberg, 1981; Morata and Lawrence, 1975). EN is expressed in segmentally repeated derivatives of the ectoderm including elements of the central nervous system (cns) (Fig. 1F) and peripheral nervous system (pns). EN is also expressed in non-segmental patterns. These are the caudal (cd) region (Fig. 1D), the hindgut primordia (hg) (Fig. 1D,F), fat body (fb) (Fig. 1E) and the dorsal ridge (Dr) (Fig. 1C,E), which develops into the frontal sac (Fig. 1F).

EN accumulation in the developing head of *Drosophila* has most recently been described by Schmidt-Ott and Technau (1992). They detected the expression of EN in three pregnathal regions, which they called the ocular (Oc), antennal (An) and intercalary (Ic) segments. We have verified EN expression in these regions of *Drosophila* (Fig. 1C) and have also found homologous EN expression in the other insects that we investigated (see below). Schmidt-Ott and Technau (1992) also detected expression in the dorsal hemispheres (dh) of the brain and the labrum (lr). These authors called this labral (lr) accumulation clypeolabral and they attributed the dh to the labral segment. We refer to this element as the lr and dh to distinguish these EN accumulations from that which is also detected in the clypeus (cly) (Fig. 1D,E; Figs. 5A, 6G in Schmidt-Ott and Technau, 1992). This latter cly expression was not previously described. Although the dh and lr expression of EN is weak, we did not find it to be 'fuzzy'; it accumulated in distinct nuclei. The dh, lr and cly accumulation of EN as well as the fb expression of EN were found to be unique to *Drosophila* among the insects investigated.

The appearance of each EN pattern element occurs at a stereotypical time during development. The gnathal, thoracic and abdominal EN stripes are added in a pair-rule fashion during blastoderm formation and are present by the onset of gastrulation (Fig. 1A). The cephalic pattern of EN accumulation develops slightly later. Our observation of the order of appearance of the cephalic EN elements agrees with what has been reported by Schmidt-Ott and Technau (1992). The antennal stripe (Fig. 1B) is added at the germ-band-extended stage prior to EN accumulation in the Ic and Oc segments, the lr region and the Dr (Fig. 1C). In slightly later embryos, just prior to germ-band retraction, expression in the hg and cly can be seen (Fig. 1D). Finally, EN can be detected in the fb of germ-band-retracted animals. Schmidt-Ott and Technau (1992) also describe the segregation of EN-expressing cells of the An and Oc segments into secondary clusters or 'spots'. We have verified the presence of a single An and two Oc spots within the embryonic brain and have identified homologs in the other insects examined (see below).

### EN expression in fleas

Consistent with the close relationship of the Diptera and Siphonaptera, the flea exhibits a pattern of EN accumulation, shown in Fig. 2, most similar to that of *Drosophila*. Part of this similarity also derives from the fact that the flea and fly are long germ-band insects (Anderson, 1973), which establish most of their segmental pattern in the blastoderm. EN was first detected in the blastoderm (not shown). At the onset of gastrulation it is present in fourteen stripes, in the antennal, mandibular (Mn), maxillary (Mx), labial (Lb), first through third thoracic (T1-3), and first through seventh abdominal segments (A1-A7) (Fig. 2A). EN is not detected over the most ventral region of the embryo containing the presumptive mesoderm. The three remaining abdominal segments, A8-A10, express EN at the completion of gastrulation (Fig. 2B). These seventeen stripes appear to mark the posterior region or compartment of each segment. Later at the onset of neurogenesis EN accumulation can be detected in the Ic and Oc segments (Fig. 2C). EN can also be detected in a cluster of cells in the dorsal region of the embryo between the Lb and Mx stripes (Fig. 2C). We propose that this region is homologous to the Dipteran dorsal ridge (Dr) and that EN accumulates in the same region of the Dr in both fleas and flies (compare Figs 1C and 2C). EN can also be detected in a caudal region that corresponds to the hg primordia (Fig. 2C). The expression of EN in the Ic, Oc and A10 segments is restricted to a relatively small set of cells (Fig. 2C,D). As in *Drosophila*, the cells of the An segment produce a secondary spot within the developing brain while the Oc segment produces two such spots (Fig. 2H). No expression of EN is ever detected in the cly or lr region of the embryo.

Two novel aspects of the flea EN expression pattern are the lateral stripes (ls) and the caudal (cd) stripes. The ls forms as a 2-cell-wide stripe running longitudinally through the lateral portion of the embryo and extends posteriorly from T2 through A10 (Fig. 2D,G). The ls subsequently widens to 4 cells and finally fades from all but a few cells in each segment (Fig. 2E). The cd stripes appear at a dorsolateral position on the embryo posterior to A10. The lateral components of the cd stripes migrate together to form a single patch of EN-expressing cells posterior to the hindgut (Fig. 2E,F). It was not determined as to what structures the ls and cd stripes contribute.

### EN expression in milkweed bugs

The pattern of EN accumulation during the development of the milkweed bug, *Oncopeltus fasciatus*, is shown in Fig. 3. Unlike fleas and flies, the milkweed bug is a hemimetabolous, short germ insect, which produces fully appendaged first instar larvae. As is typical of short germ-band development, only some of the segments are defined in the blastoderm and the remaining segments are added during germ-band extension (Sander, 1976). Accumulation of EN is first detected in the blastoderm (not shown) in at least six pairs of stripes, which may be as narrow as a single cell. The EN stripes are laterally situated on the embryo and are not continuous over the most dorsal or ventral (presumptive mesoderm) region of the

embryo. These stripes widen to three cells before the two bands of stripes zipper together as the embryo invaginates into the yolk (Fig. 3A). At the completion of anatrepsis, (Fig. 3B) six bands of EN-expressing cells, each about five cells wide, are detected across the main body of the germ rudiment. One stripe is present in each of the three thoracic and three gnathal segments. There is a seventh, more loosely folded stripe of cells across the rudiment of the antennal segment. No expression is detected in the posterior growth zone or in the elaborately folded region anterior to the antennal rudiment. These EN stripes appear to mark the posterior compartment of each segment.

Shortly after anatrepsis when the EN stripes are four cells wide, the embryo makes the first overt signs of segmentation. Grooves form at the anterior and posterior boundaries of the EN accumulation within each segment (Fig. 3B). These are apparently homologous to the segmental (posterior to EN) and compartmental (anterior to EN) boundaries of *Drosophila*. There is no obvious difference between the 'segmental' and 'compartmental' grooves. At the anterior end of the posterior growth zone, narrow stripes are added one at a time (Fig. 3B). These EN-expressing cells mark the posterior compartments of the first through tenth abdominal segments (A1-A10) as they are added to the germ band. In the abdomen, grooves first form just anterior to the EN stripe producing a transient parasegmental pattern before also forming grooves at the segmental boundary. This pattern is reiterated in each abdominal segment as it forms (Fig. 3C). The compartmental grooves of the gnathos and thorax are transient and are replaced by a segmental pattern of constriction (Fig. 3C).

In the gnathal, thoracic and An segments, the EN-expressing cells compose the posterior third of each segment. The segmentation of the embryo continues until a full compliment of ten abdominal segments is formed (Fig. 3D). EN accumulates along the posterior region of all appendages (Fig. 3D) including the Mx and Mn seta (Fig. 3I). EN is also expressed in the salivary gland rudiment. The invagination of the salivary gland occurs in the posterior region of the Lb segment where EN can be detected (Fig. 5F). The body of the gland can be detected by other antibodies (not shown). After the completion of segmentation, EN accumulation can be detected in the dorsal region of the embryo between the Lb and Mx segments (Fig. 5G) and marks a part of the proposed Dr homolog.

In a germ-band-extending embryo, a small, roughly circular, cluster of EN-expressing cells can be found just anterior to the constriction separating the An segment from the Oc segment (Fig. 3C,G) . Also at this time, the rudiment of the stomodeum (st) forms within the EN-expressing stripe of An cells (Fig. 3C,G). After the stomodeum is formed, the level of EN drops in these cells and EN begins to accumulate within the Ic segment (Fig. 3D,H).

As in flies and fleas, the cells at the ventral end of the An stripe segregate into a separate cluster (Fig. 3H). Unlike flies and fleas, which have essentially eyeless larvae, the EN-expressing cells in the Oc segment of the milkweed bug first segregate into two separate clusters: a set of cells expressing a high level of EN overlaid by a larger, circular cluster of about twenty cells with a lower level of expression (Fig. 3H). The larger cluster of epidermal EN-expressing cells are in the developing eye (Fig. 3H,I). This segregation is coincident with the onset of neurogenesis and accumulation of EN within the cns, and thus probably represents the formation of neuroblasts from the An and Oc segments. The cells expressing high levels of EN in the Oc segment are apparently homologous to the small set of cells seen in the Oc segment of both flies and fleas which end up completely within the embryonic brain. As in fleas and flies, the secondary cluster of EN-expressing cells derived from the Oc segment divides further to produce at least two groups of EN-expressing cells in the embryonic brain (Fig. 3I).

During head compaction the left and right Lb appendages migrate ventrally, fuse together (Fig. 3E,J) and position themselves under the mouth (Fig. 3E). As the Lb appendages fuse they appear to orient themselves according to the pattern of EN accumulation. The EN-expressing domain of each appendage broadens slightly and the appendages rotate until the EN-expressing side of the appendage faces the embryo, while the EN non-expressing side of the appendage is oriented away from the embryo. As the appendages fuse, the EN-expressing domain of the appendages forms a single sheet cells facing the embryo (Fig. 3I). During this time, the Mn and Mx segments give rise to stylets that appear to be derived completely from the posterior compartment and accumulate EN in all their cells (Fig. 3F,I)

Just prior to katatrepsis, EN accumulation can be detected in stripes, two cells wide, in the amnion (double arrow in Fig. 3I). Although no EN was detected in the hg, even after the completion of dorsal closure, EN can be detected in the posterior midgut (pmg) at a low level (Fig. 3K). No EN accumulation was detected in the cly region of the embryo, although a very low level of transient EN accumulation can be detected when the labrum connects to the stomodeum.

### EN expression in crickets

The pattern of EN accumulation for another orthopteran, *Schistocerca americana* (grasshopper), has been reported previously (Patel et al., 1989a,b). The development and pattern of EN accumulation in the cricket, *Acheta domestica*, is similar to this pattern with a few differences. The cricket defines its thoracic as well as gnathal segments in the blastoderm and produces a condensed germ rudiment with seven EN stripes (Fig. 4A): three thoracic, three gnathal and an An segment. The first overt signs of segmentation visible for the cricket are constrictions at the boundaries of the thoracic and gnathal segments posterior to the EN stripes (Fig. 4B). The abdominal segments are added one at a time, anterior to posterior, as indicated by the presence of EN-expressing stripes (Fig. 4B) until a full compliment of ten abdominal segments is reached (Fig. 4D). As they extend laterally from their respective segments, EN accumulation can be detected in the posterior region along the length of all appendages (Fig. 4C,E). Additionally, EN accumulates in a single cluster of cells dorsal to both the Mx and Lb segments and represents part of the Dr homolog (Fig. 4D).

Just as in the milkweed bug, EN accumulation in the Oc segment occurs anterior to a constriction separating the An and Oc segments (Fig. 4B). The Ic expression of EN is absent from the embryo until after the formation of the stomodeum (Fig. 4C,F). The Oc accumulation of EN occurs in a roughly circular patch of cells. As in the milkweed bug, the An stripe and Oc patch of EN-expressing cells segregate into secondary spots containing presumptive neuroblasts (Fig. 4F). At approximately the same time, the neuroblasts of the remaining

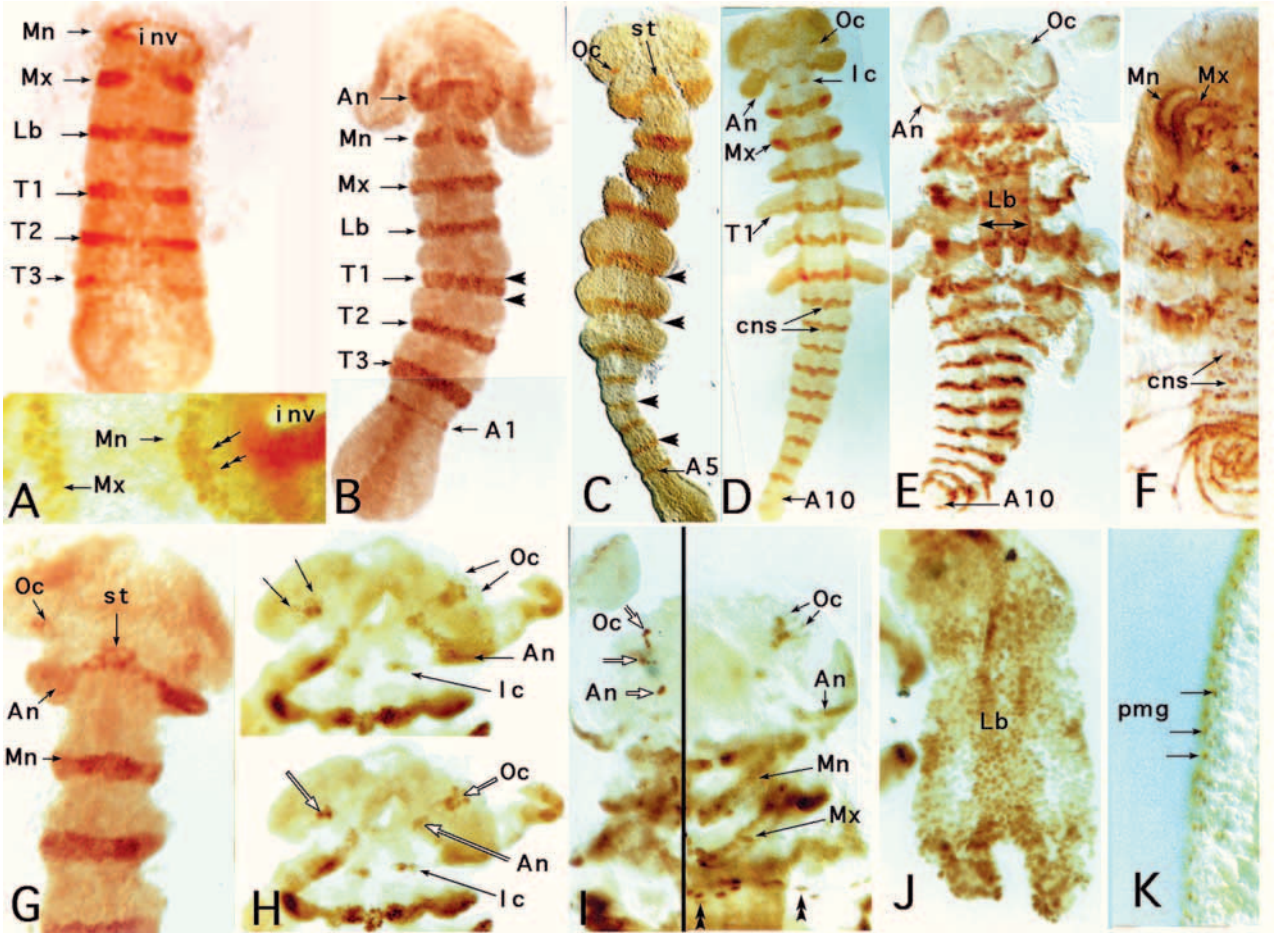



**Fig. 3.** EN expression in the embryo of *Oncopeltus fasciatus* (milkweed bug). Arrows mark the accumulation of EN (shown in brown). A-F are presented in chronological order. (A) Stripes initiate in one to two cell widths (not shown) which become three cells wide prior to invagination (see inset). The anterior border (single arrow) is slightly better defined than the posterior border (double arrows) of each stripe (inset). 'inv' marks the site of invagination at the surface of the blastoderm. A condensing germ rudiment has seven EN stripes (An-T3). (B) At the beginning of the germ-band extension, the initial seven stripes have become 4 cells wide and an eighth (A1) stripe is added at the anterior end of the growth zone. The embryo forms grooves (marked with arrow heads) at the borders of EN expression and demarcates the presumptive compartments. (C) During germ-band extension, the gnathal and thoracic segments lose their compartmental grooves and adopt a segmental appearance. As the second through fifth abdominal EN stripes are added, they reiterate the formation of compartmental grooves (arrowheads) but initiate the parasegmental groove (anterior to EN) before the segmental groove (posterior to EN). (C,G) EN accumulates in a small circular cluster of 8-10 cells in the Oc region. These Oc cells lie just anterior to a constriction and fold within the procephalon. The stomodeum (st) forms within the EN-expressing cells of the An segment. At this point in development, no accumulation of EN can be observed in the Ic segment. (D) EN accumulates in the posterior of all ten abdominal segments of a fully segmented embryo. EN accumulates throughout the posterior compartment of the thoracic and gnathal appendages. (D,H) Expression of EN in the Ic is limited to a small cluster of cells. (E) During germ-band shortening and head compaction the Lb appendages migrate ventrally under the mouth, and fuse together to form the labium (double headed arrow). (E,I) The Mx and Mn lobes position themselves at the sides of the mouth. (F) A dorsal view of structures forming in the interior of half of an embryo after the completion of dorsal closure. The embryo has been opened dorsally, and the gut and yolk removed and examined separately (K). EN accumulates in the cns and stylets of the Mn and Mx segments. (H) Two focal planes of a germ-band-extended embryo. The EN-expressing cells of the An segment segregate into an epidermal stripe (upper panel, solid arrow) and a spot (lower panel, white arrow), which will become part of the cns. Similarly, the cells of the Oc also segregate into two separate populations, an epidermal group of about 20 cells corresponds to the developing eye primordia and a spot (white arrow), which becomes incorporated into the cns. No EN accumulation is detected in the cly or lr, except transiently when the labrum connects with the stomodeum (not shown). (I) Two focal planes of the embryo in E showing the epidermal (right panel, solid arrow) and cns components of the Oc and An segments (left panel, white arrow). The cells of the Oc spot have undergone division and segregation producing secondary spots (white arrows) while the weaker staining epidermal cells remain associated with the developing eye. Stripes of cells expressing EN (double arrowheads) are seen in the amnion. (J) The fused labium of a germ-band-shortened embryo undergoing head compaction, distal is downward. As the two lateral appendages fuse at the ventral midline, they orient themselves according to the pattern of EN accumulation. The EN-expressing cells from both appendages form a single sheet of EN-expressing cells on the side of the appendage now facing the rest of the embryo. The cells not expressing EN are on the side of the appendage facing away from the embryo. (K) Weak accumulation of EN is detected in the posterior midgut (pmg) of the milkweed bug shown in (F). Embryos in A-E are shown in a ventral view with the anterior pointing up. Dorsal is toward the edge, ventral to the midline. Embryos in A-I are shown in a ventral view with the anterior pointing up. Dorsal is toward the edge, ventral to the midline.


**Fig. 4.** EN expression in the embryo of *Acheta domestica* (cricket). Embryos are shown in a ventral view with anterior pointing upward. (A) A condensed germ rudiment has stripes of EN in seven segments (An, Mn-T3). (B) The An, thorax and gnathal segments have developed segmental grooves and the first abdominal stripe is being added to the growing germ band of an extending embryo. EN accumulation is detected in the Oc segment anterior to a constriction and fold in the procephalon. (C) Accumulation of EN can now be detected in the Ic segment of an embryo undergoing germ-band extension. The Oc EN-expressing cluster is about 10-12 cells. No EN ever accumulates in the cly or lr region of the embryos. (D) EN accumulates in the developing cns of an extended germ-band embryo and in the full complement of ten abdominal segments. EN accumulates in the Dr in a single patch dorsal to both the Lb and Mx segments. EN accumulates only on the ventral side of the cerci (cr). No EN accumulation is detected in the hg. (E) EN accumulates in the posterior region of all appendages (arrows). (F) In the head of a cricket embryo at the onset of neurogenesis, some EN-expressing cells of the An and Oc segments segregate and form secondary spots. These spots appear to become part of the brain. (G) The Oc spot within the brain splits, forming additional secondary spots. (H,I) The EN accumulation in the cerci begins as a stripe at the edge of the embryo that becomes a wedge during the development of the cerci and eventually covers the entire ventral surface, shown in D.

segments delaminate and EN can be detected in the developing cns (Fig. 4D). The secondary Oc spot further divides into two separate clusters of EN-expressing cells within the brain (Fig. 4G). The remaining epidermal cells of the Oc segment are associated with a portion of the developing eye.

The EN accumulation in the caudal region of the cricket begins after all ten abdominal segments have been added. Like the cd stripes of the flea, this expression begins as two separate stripes at the edge of the embryo posterior to A10 (Fig. 4H). Unlike the flea, the two sets of EN-expressing cells remain separate and become part of the developing cerci (cr) (Fig. 4I,D). The similarity in the onset of the cd and cr patterns may suggest that the two are homologous. In the formed cerci, the EN-expressing cells cover the ventral surface, while non-expressing cells form the dorsal surface (Fig. 4D). No EN accumulation was detected in the cly or hg.

## A homolog of the Dipteran dorsal ridge is conserved among insects

In the Dipterans, *Drosophila* and *Calliphora*, a cluster of cells forms a structure easily observable by light or scanning electron microscopy, which has been called the dorsal ridge (Dr) (Turner and Mahowald, 1979). The Dr of *Drosophila* is thought to be composed of the dorsal portions of all the gnathal and cephalic segments (Younossi-Hartenstein et al., 1993) and at least part of this structure is made up by EN-expressing cells (Fig. 1C; Diederich et al., 1991). During head involution, the Dr matures into the dorsal pouch which is intimately associated with the imaginal discs of the head (Younossi-Hartenstein et al., 1993; Campos-Ortega and Hartenstein, 1985). Based on these observations, it seemed possible that the Dr was a specialized structure necessary for the production of the pseudocephalic head of the maggot, or the imaginal discs of holometabolous insects. However, as noted above, we have observed EN-expressing cells in a similar position to *Drosophila* in the dorsal gnathal region of the species examined. Additionally, EN-expressing cells in a position homologous to the dorsal ridge have been previously reported in Coleoptera (Brown et al., 1994; Fleig, 1994; Schmidt-Ott et al., 1994b), Diptera (Schmidt-Ott et al., 1994b) and a single Orthopteran (Patel et al., 1989a). These latter observations, coupled with our determination of the fate of these EN-expressing cells, lead us to conclude that the Dr is not specific to Diptera or to Holometabola, but is a general feature of the insect head.

In both the fly and the flea, EN accumulation in the dorsal ridge begins with de novo expression of EN in cells derived from the anterior of the dorsal-most region of the Lb segment (Fig. 5A,C). This expression is considered de novo because it begins in single cells that are separated from all other EN positive cells by several cell diameters (not shown). This cluster increases in cell number and eventually fills the dorsal region between the Lb and Mx EN stripes (Fig. 5B,D).

Although the initiation of EN expression in the Dr of crickets and milkweed bugs may not be identical to that of flies and fleas, the result is the same. In the milkweed bug and cricket, the expression of EN appears uniformly across the dorsal region of the anterior compartment of the Lb segment

(Fig. 5G,H). The result is a continuous band of EN-expressing cells connecting the posterior Mx compartment with the posterior Lb compartment. The structure of the dorsal region of the milkweed bug is particularly informative. The milkweed bug embryo forms a dorsal plate (pl) of cells along the length of the embryo from the Mx through A10 segments (Fig. 5G). As deduced from EN stripes, each segment of the thorax and abdomen has both a posterior and anterior region of the plate (Fig. 5G). The gnathal region, represented in the plate by the Mx and Lb segments has a single continuous stretch of EN positive cells (Fig. 5G). The pattern of EN expression ventral to the plate has normal segmental periodicity (Fig. 5F). All the cells that grow dorsally during dorsal closure are derived from this plate. A structure similar to this plate, although not as distinctive, can also be seen in cricket embryos.

During dorsal closure, cells from each side of the thorax and abdomen grow dorsally and fuse with their counterpart at the dorsal midpoint. Again, based on EN stripes, each segment produces a posterior and anterior region. Unlike *Drosophila* larvae with their rather reduced and internalized head, the first instars of fleas, milkweed bugs, and crickets have complete and fully formed heads. In these insects, a single segment-like entity dorsal to the gnathal region also grows dorsally and fuses with its counterpart at the dorsal midpoint (Figs 2F, 5E,I). This segment-like entity has a posterior EN expressing, and anterior non-EN-expressing, region. The EN-expressing region derives from cells connecting the Mx and Lb segments on their dorsal side while the non-EN-expressing cells are derived from the anterior Mx segment. We propose that this segment-like entity is part of the Dr homolog.

### Analysis of the dorsal ridge by ectopic expression of UBX

To elucidate the structure and function of the Dr of *Drosophila*, we have examined the effects of ectopic expression of the protein product of the homeotic gene *Ultrabithorax* (*Ubx*). *Ubx* is required for the proper development of the thorax and abdomen and the protein product (UBX) of the gene does not normally accumulate in the embryonic head (White and Wilcox, 1984). Using a two component expression system that allows regulated ectopic expression of a gene (Brand and Perrimon, 1993), we produced embryos of the genotype P{UAS::Ubx}/+;P{prd::Gal4}, which we call prd=>UBX. The prd=>UBX embryos are inviable and die as unhatched larvae. A complete description of the effect of ectopic UBX expression in prd=>UBX embryos will be given elsewhere (Rogers, Kalkbrenner and Kaufman, unpublished data). An examination of cuticle preparations of unhatched prd=>UBX larvae reveals that the head segments are transformed toward an abdominal fate as determined by the production of ventral abdominal denticles (Fig. 6B).

Immunochemical detection of UBX in prd=>UBX animals confirmed ectopic UBX accumulation in the heads of developing embryos (Fig. 6G). The major effect of this accumulation is to transform head to abdomen. In wild type, a set of ventral denticles, dorsal denticles and dorsal hairs is associated with each thoracic and abdominal segment, but no denticles or hairs are associated with the head segments (Fig. 6A). In prd=>UBX cuticles, a set of ventral denticles can be assigned to all the head segments: Oc, An, Ic, Mn, Mx, and Lb (Fig. 6B). In contrast, only a single set of dorsal hairs and denticles is formed (Fig. 6B). These hairs and denticles all form within the Dr (Fig. 6B). We conclude from this observation that the Dr is the most anterior structure capable of adopting a dorsal fate and that the more anterior components of the head can only produce ventral structures. Our conclusion about the dorsal-ventral competency of the head segments is consistent with the conclusions drawn from a similar experiment that also utilized ectopic UBX (Gonzalez-Reyes and Morata, 1991). However, these authors did not follow the development of each segment with a marker like EN and so they were unable to determine segment identity and number (see below). The transformation of the Dr by UBX into a cuticle with both a naked posterior and haired anterior (Fig. 6B) typical of abdominal segments also demonstrates the segment-like nature of the Dr.

The relative positions and boundaries of each segment were determined by examining the pattern of EN accumulation in both wild-type (wt) and prd=>UBX embryos. After germ-band shortening, EN accumulation can be seen in stripes that mark the segment boundaries at their posterior edge (Fig. 6C,D; Ingham and Martinez Arias, 1992). Previous work has shown that the most posterior EN-expressing cell underlies the most anterior denticles in both the dorsal and ventral cuticle of the abdomen (Heemskerk and Dinardo, 1994; Dougan and Dinardo, 1992). Using the relationship between the EN expression pattern and the cuticular denticles we were able to determine the approximate segment borders in cuticle preparation, as shown in Fig. 6A,B. In addition to relative position, the identity of the Mn and Mx segments were confirmed by the presence of a mouth hook base (mh) and cirri (cr), respectively.

The perturbations of the WT *Drosophila* EN pattern by ectopic UBX are intriguing because of their similarity to the EN pattern in other insects. In WT *Drosophila*, the EN-expressing cells of the Dr forms a broad stripe which continues to have detectable EN accumulation even after formation of the dorsal pouch. In prd=>UBX embryos, the EN pattern in the Dr takes on abdominal characteristics, narrowing to a stripe 1 to 2 cells wide (Fig. 6H) that grows dorsally during dorsal closure. In WT, the Mx and Lb lobes move away from the ventral tip of the Dr and only a thin 2-cell-wide stripe of cells is left ventral to the Dr (Fig. 6E, arrow). The exact origin of these cells is unknown, but they derive either from the Dr or the Lb segment. In prd=>UBX embryos, the Mx and Lb lobes remain attached to the dorsal ridge (Fig. 6F) and give the fused segment appearance of the Mx and Lb segments of fleas, crickets and milkweed bugs. In WT, no EN accumulates in the epidermis of the optic lobe. In prd=>UBX embryos, accumulation of EN occurs in a circular cluster of cells reminiscent of the EN expression in the Oc segment of milkweed bugs and crickets. The WT pattern of EN in the An segment is dynamic, beginning as a stripe, fading from all but a few cells and then returning to a larger cluster of cells (Schmidt-Ott and Technau, 1992). In prd=>UBX embryos, the An accumulation is not dynamic and remains a strong stripe throughout development. It is not clear whether the apparent transformations of these segments towards a more ancestral state, as observed by EN pattern, is the result of coincidence or represents a disengagement of the derived developmental program, which produces the highly specialized structures of the maggot head, to allow a more general pattern of segment development to occur. However, the striking similarity of the *Drosophila* mutant

patterns and that seen in the less derived insects lends credence to the latter conclusion.

## DISCUSSION

### EN expression in the embryonic heads of insects reveals six segments, including the primordia of the eye

The structure of the insect head has puzzled and intrigued researchers for many years. The number of segments comprising it has been estimated from as few as three to as many as seven (Rempel, 1975). Recently using *Drosophila* as a model to investigate the structure of the head, Schmidt-Ott and Technau (1992; Schmidt-Ott et al., 1994a) have argued for the presence of a seventh, clypeal or labral, segment in addition to the more posterior Lb, Mx, Mn, Ic, An and Oc segments. These researchers used paired patterns of EN and *wingless* (*wg*) expression anterior to the Oc segment (Schmidt-Ott and Technau, 1992) and the existence of sensory organs and nerves (Schmidt-Ott et al., 1994a) that derive from regions anterior to the Oc segment as evidence for a seventh segment. While these data are consistent with the existence of a seventh segment, they do not definitively demonstrate its existence.

First, we have shown that the pattern of EN expression in the *Drosophila* embryo is unique in its complexity among the four insect orders studied here. Additionally, examination of the reported EN expression pattern in other insects from the orders Orthoptera (Patel et al., 1989a), Hymenoptera (Fleig, 1990), Coleoptera (Brown et al., 1994; Fleig, 1994) and Diptera (Schmidt-Ott et al., 1994b) demonstrates that, although some other Diptera also have the lr EN expression, accumulation in the cly and dh of *Drosophila* is unique among these six insect orders. This failure to detect EN expression is not simply a reflection of sensitivity as we are able to detect low levels of EN expression in other tissues such as the pmg of milkweed bugs. Although Schmidt-Ott and Technau argued for the presence of a seventh segment based on the pairing of *wg* and EN expression patterns in each segment, the expression of *wg* in the clypeolabrum appears unconnected to the dh EN expression and overlaps the cly expression of EN (Fig. 12 of Schmidt-Ott and Technau, 1992). As the dh is claimed to be the EN-expressing component of the labral segment (Schmidt-Ott and Technau, 1992), expression in this region of *Drosophila* alone cannot be used to argue for a seven segment head in all insects and the common insect ancestor.

Secondly, although sensory neurons and cuticular structures such as sensory organs form in the region of the *Drosophila* embryo anterior to the Oc segment, this is not itself evidence for additional segments because the existence of the clypeolabrum is not in question, only its standing as a segment. The term segment implies a unit of serial homology. Serial homology can be established using many criteria including position, fate and homeotic transformation. Our claim that the other segments, including the Oc, are serial homologs is in part evidenced by the identification of cells in similar positions in each segment adopting similar fates. Cells within the ventral posterior region of each segment, the ventral edge of EN-expressing stripe, become neuroblasts and migrate to the cns. Furthermore, homeotic transformations have been observed for the epidermis of every segment, including Oc (Lindsley and Zimm, 1990), but not the clypeolabral region. Homeotic transformations change a structure of one segment to the homologous structure of another due to gain or loss of gene function. Failure of ectopic UBX to transform the labrum to abdominal identity (this work; Gonzalez-Reyes and Morata, 1991) further strengthens the argument that the labrum is not serially homologous to the other segments. Additional information about the sensory organs and nerves of the clypeolabrum is therefore necessary to establish the identity of serial homologs of these structures in other segments.

It is possible, and perhaps likely, that the anterior termini, including the clypeolabrum, and the posterior end, including cerci, are patterned in a way that is not homologous to the other segments of the body. It is clear that, if the seventh head segment does indeed exist, it is unique in that it is missing a large component of both the epidermal and neural cells present in all other segments and therefore could only be a partial segment. Additionally the activities of *wg* and *en* are required for the formation of the anterior-posterior compartment (parasegment) boundary, which is critical for proper patterning of every segment (see below). The patterning of this potential seventh segment would have to be accomplished by a novel mechanism.

In contrast to the clypeolabrum, the six other segments identified by Schmidt-Ott and Technau (1992) have highly conserved patterns of EN accumulation. The behavior of these EN-expressing cells is also conserved. For most segments, this is evidenced by the conservation of EN accumulation in a stripe of posterior ectodermal cells in each segment and appendage, and in the neuroblasts of the cns. In the Oc segment, the accumulation of EN in a circular subset of epidermal cells and the formation of two clusters of neuroblasts, which occupy stereotypical position within the insect brain, is conserved. We interpret the conservation of these patterns to mean that the EN accumulation in these regions marks the six segments or segment remnants present in all insects and probably the insect ancestor. We do not interpret the novel accumulation of EN in the head of *Drosophila* or other Diptera as the result of an evolutionary increase in segment number because these ‘stripes’ are not correlated with any novel anatomical structures, but instead are expressed in structures common to all insects.

Some researchers have been reluctant to use the term ocular to describe the segment anterior to the An segment preferring to use terms such as preantennal, procephalic, or third cephalic segment. Some of this uncertainty comes from the inability to correlate this segment with the developing eye in an essentially eye-less larva. Here we have demonstrated that EN accumulation in the Oc segment does correlate with the developing embryonic eye in both crickets and milkweed bugs. We therefore endorse the use of the term ocular as proposed by Schmidt-Ott and Technau (1992). The segmental organization of the insect head is diagrammed in Fig. 7A.

### The dorsal ridge is a general component of the insect head

In addition to shedding light on anterior-to-posterior organization, the pattern of EN accumulation reveals a stark contrast in the organization between the dorsal and ventral regions of the insect head. Although in *Drosophila* the Dr develops into the dorsal pouch and is intimately associated with the eye-

antennal imaginal discs (Younossi-Hartenstein et al., 1993), it is not a structure specialized exclusively for this function but represents a general feature of the head which is identifiable in six insect orders (this work; Brown et al., 1994; Fleig, 1990; Schmidt-Ott et al., 1994b). The result of the broad EN accumulation in the dorsal region of the Lb and Mx segments is to produce a segment-like entity (Dr-I in Fig. 7A,B), positioned at the junction between head and thorax, which is the most anterior structure capable of producing dorsal cuticular structures. Arguments for the eye being a dorsal structure by virtue of its homeotic transformation to wing have been countered by recent evidence showing that the wing primordia is actually a more ventrolateral structure that co-localizes to the leg primordia (Cohen et al., 1993). The apparent dorsal location of the eye and other head structures is accomplished by the folding of anterior head segments.

It has been thought that the Dr is a segmentally composite structure of all gnathal and pregnathal segments (Younossi-Hartenstein et al., 1993). Although this assumption appears to be largely true, the organization of the Dr is complex. The Dr can be divided into two parts (Dr-I, II), which behave quite differently during dorsal closure. Dr-I is the segment-like entity derived from the Lb and Mx segments (Fig. 7A). The posterior region of Dr-I is marked by EN accumulation and is derived from both the Lb and Mx segments while its anterior region is derived entirely from the Mx segment. This is consistent with the observation that the products of the *Sex combs reduced* (*Scr*) gene co-localize with EN, while the products of the *Deformed* (*Dfd*) gene accumulate anterior to the EN-expressing cells of the Dr in both *Drosophila* (Gorman and Kaufman, 1995; Rogers, Kalkbrenner and Kaufman unpublished data) and the milkweed bug (Rogers and Kaufman unpublished data). Dr-II (Fig. 7A,B) is derived from the dorsal-most portions of the Mx, Mn, Ic and An segments. This portion of the Dr is marked with

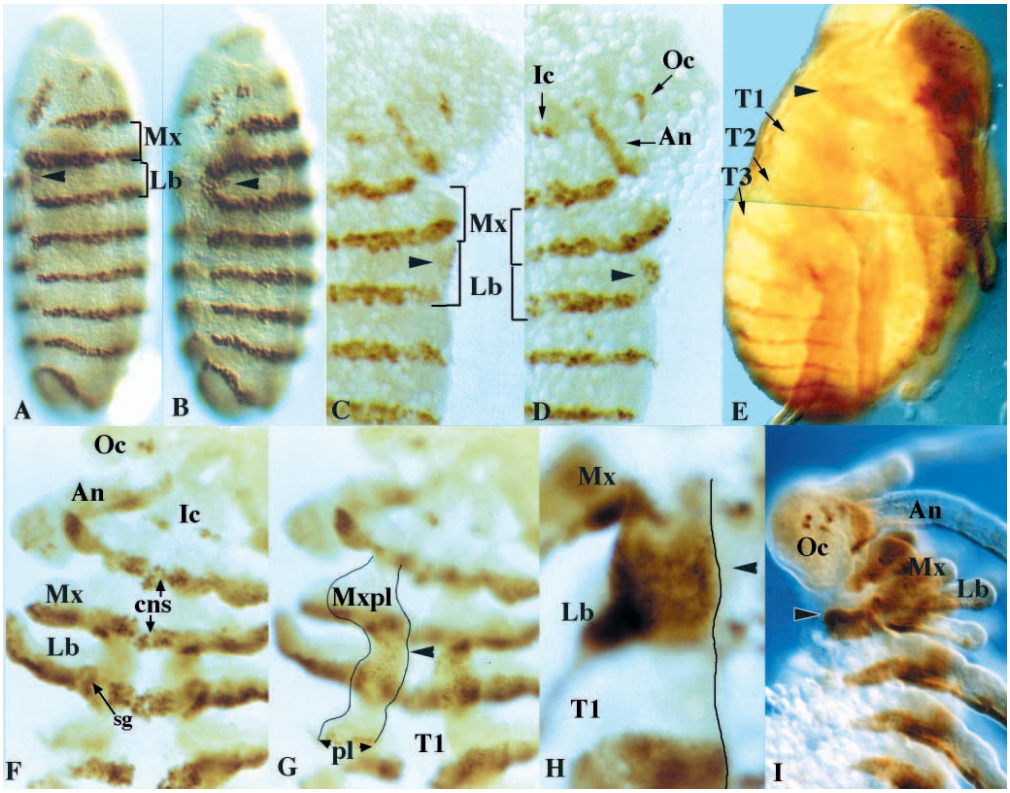



**Fig. 5.** Development of the EN expression pattern in the dorsal ridge. Arrows mark EN accumulation and the large arrowhead marks the Dr accumulation of EN. (A,B) Fruit fly embryos are shown with anterior pointing up and ventral to the right. (A) A germ-band-extending embryo accumulates EN in cells of the anterior compartment of the labial (Lb) segment. These cells become part of the dorsal ridge (Dr) and later the dorsal pouch. (B) The number of cells expressing EN increases until EN accumulates in cells connecting the posterior labial compartment with the posterior maxillary (Mx) compartment at the dorsal tip of each lobe. (C, D) Half of a germ-band-extending flea embryo is shown split down the ventral midline: anterior is up, ventral is left to the midline and dorsal is right to the edge. (C) EN accumulates in cells of the anterior Lb compartment of the embryo. These cells are part of the Dr homolog. (D) In a slightly older embryo than C, EN is expressed in cells that traverse the anterior compartment of the Lb segment and connect the posterior compartments of the Lb and Mx segments. (E) A milkweed bug embryo undergoing dorsal closure. The cells of the dorsal plate grow, either by division or by stretching, around the yolk mass. There is an identifiable EN-expressing stripe of cells for every abdominal and thoracic segment (T1-3) but only a single stripe associated with the gnathos (arrowhead). These cells derive from the Mx and Lb components of the dorsal plate and are part of the Dr homolog. (F,G) The head of a fully segmented milkweed bug embryo is shown in both a ventral (F) and dorsal (G) plane of focus with anterior pointing up. (F) EN accumulates in stripes across the ventral ectoderm and appendages. EN also accumulates in the cns and salivary gland (sg). (G) The image is focussed at the level of the dorsal plate. The drawn lines outline the structure of the dorsal plate (pl), which runs on the dorsal side of the embryo from abdomen through the Mx segment. An outgrowth of the maxillary plate (Mxpl) composed of both posterior, EN-expressing cells, and anterior cells of the maxillary segment, marks the anterior end of the dorsal plate. The thoracic and abdominal regions of the plate have the typical EN stripe pattern while the gnathos produces a single-cell-wide patch of EN accumulation (arrowhead). The labial region of the plate is completely filled with EN-expressing cells. The posterior of the Mx and Lb segments are connected by the EN-expressing cells of the dorsal plate. (H) Dorsal view of a fully segmented cricket embryo showing the accumulation of EN in the Dr (arrowhead). Anterior is up, distal to the left, and the drawn line marks the dorsal edge of the embryo. EN-expressing cells fill this dorsal region of the Lb segment. This connects the posterior compartments of the Mx and Lb segments with EN-expressing cells at their dorsal edge. (I) A germ-band-shortened cricket embryo. The EN-expressing cells of the Dr (arrowhead) have fused to the anterior of T1 where they will produce a dorsal stripe of cells during dorsal closure.

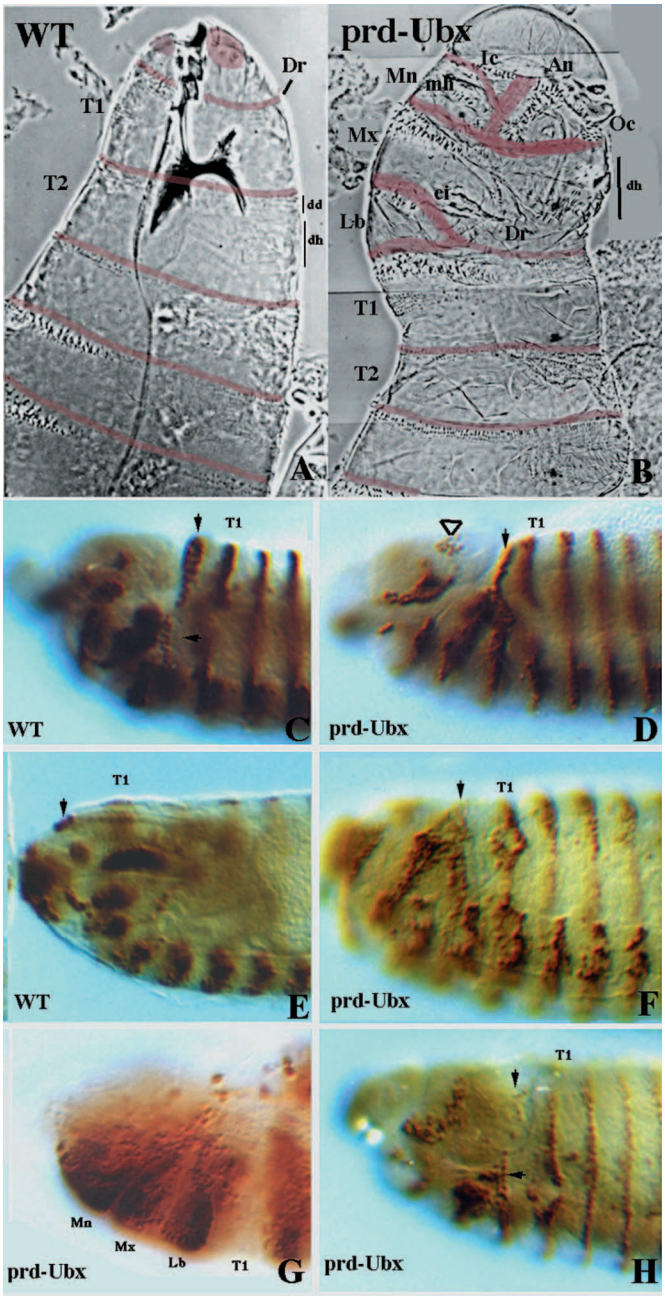
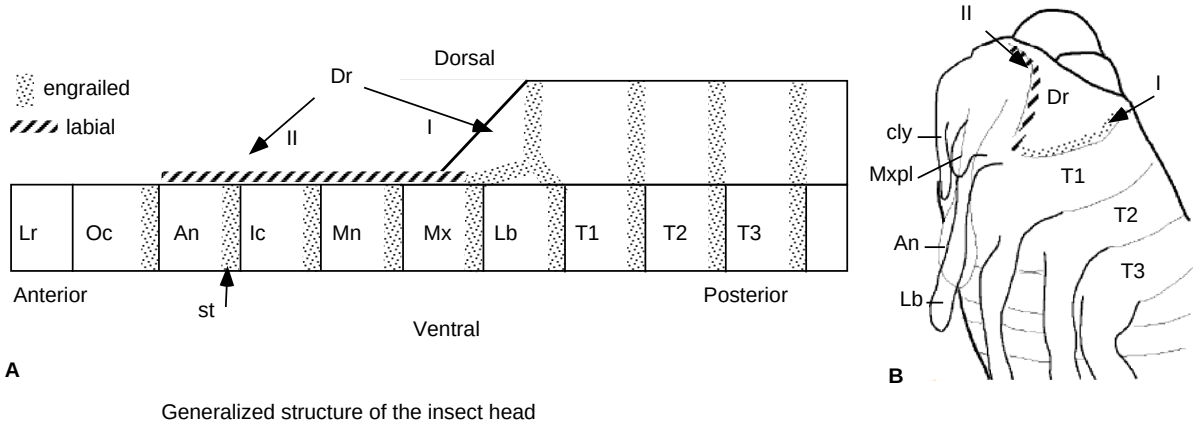



**Fig. 6.** The effect of ectopic *Ultrabithorax* protein (UBX) in the dorsal ridge of *Drosophila*. (A) A cuticle preparation of a wild-type (OreR) *Drosophila* first instar larva shortly after hatching. The brown lines show approximate positions of EN accumulation prior to cuticle deposition. The lines are shown for instructive purposes and are not intended to show EN accumulation at a cellular resolution. The EN stripes mark the approximate segment border of thoracic and abdominal segments. The pattern of EN is derived from the pattern of dorsally closed embryos such as the one shown in E. EN accumulation in the pseudocephalic head of *Drosophila* is shown as brown blobs, as it is complicated and not easily separated into segments. The dorsal cuticle of thoracic segments produces unique cuticular structures known as dorsal denticles (dd) and dorsal hairs (dh) as indicated for T1. The Dr and a ventral stripe derived from the Lb segment (horizontal arrow in C) combine to produce an EN stripe, which borders the T1 segment. The majority of the Dr is not visible because it has been internalized as the dorsal pouch. (B) A cuticle preparation of an unhatched prd=>UBX larva. The brown lines show approximate positions of EN accumulation just prior to cuticle deposition and mark the approximate segment border. The pattern of EN is derived from the pattern of dorsally closed embryos such as the one shown in (F,H). The head segments of these embryos are partially transformed toward an abdominal identity. The Mn segment and Mx segment can be identified by the presence of mouth hook base (mh) and maxillary cirri (ci), respectively. The region of the Dr is no longer internalized but develops as the dorsal region of an abdominal segment complete with dd and dh. The EN-expressing cells of the Mn, Ic, An and Oc segments often touch, resulting in an odd pattern of ventral denticles. Although the Mn, Ic, An and Oc segments produce ventral denticles none of these segments produce dd or dh. (C) The EN-expressing portion of the Dr is marked with a vertical arrow in a wild-type germ-band-shortened embryo. A ventral EN-expressing stripe probably derived from the Lb segment marked with a horizontal arrow. Note the discontinuity of the dorsal and ventral portions of this stripe. The embryo in E has completed dorsal closure. The arrow marks the EN-expressing portion of the Dr in the cuticle. The dorsal pouch extends posteriorly inside the embryo from the arrow. (D,F,H) EN accumulation in prd=>UBX embryos. (D) A germ-band-shortened embryo. The EN-expressing portion of the Dr is marked with a vertical arrow and ectopic accumulation of EN in the Oc segment is marked with an open arrowhead. (F,H) The same embryo from two different angles. (F) A ventrolateral view of the embryo shows that the Mx and Lb segments have failed to detach from the Dr. The dorsal EN-expressing cells of the Mx and Lb segments form a single cluster of cells uninterrupted by EN non-expressing cells. (H) A lateral view of the embryo shows the position of the EN-expressing cells (vertical arrow) of the Dr. The ectopic EN-expressing cells of the Oc segment become continuous with the EN-expressing cells of the An segment. (G) A prd=>UBX embryo showing the accumulation of UBX. UBX is detected in stripes within the thorax and abdomen, but is expressed widely within the gnathos and head. UBX does not accumulate in the T1 segment, but does accumulate in the Oc, An, Ic, Mn, Mx and Lb segments and Dr.

the *labial* gene product in the milkweed bug (Fig. 7B) (Rogers and Kaufman, unpublished data). The two parts of the Dr function differently. During dorsal closure Dr-I behaves like the thorax, growing up and around the yolk, producing dorsal cuticle. Dr-II moves anteriorly and forms a suture over the dorsal cephalic region. In contrast to Dr-I, no dorsal cuticle is formed and the yolk is excluded by the movements of Dr-II. As of yet, no part of the Dr can definitely be attributed to the Oc segment. As with other aspects of head structure, the Dr of *Drosophila* appears to be a highly derived structure when compared with other insects.

## Evolution of EN function

Although the pattern of EN expression is highly conserved in the posterior compartments of each segment, additional accumulation is not conserved and these can be considered specializations of the expression pattern for each insect or insect group. The expression of EN in the hg of fleas and flies is nearly identical, and there is a ring of expression in the hg of *Tribolium* (Schmidt-Ott et al., 1994b) that is consistent with the close relationship between the Siphonaptera and Diptera and may reflect the presence of hg accumulation in the common ancestor of all three orders. Expression in the pmg of milkweed bugs is unique and is probably unrelated to the hg expression of flies and fleas. While the role of EN in these tissues is not known, it is reasonable to assume that EN




Generalized structure of the insect head

**Fig. 7.** Epidermal fate map of a generalized insect. (A) In this diagram, dorsal is up and anterior is to the left. Anterior of the thorax (T1-3), the insect head can be divided into six segments, which are marked by EN accumulation. These are the labial (Lb), maxillary (Mx), mandibular (Mn), intercalary (Ic), antennal (An), and ocular (Oc). The status of the labral region as a potential seventh segment can not be confirmed by EN accumulation. We have split the embryo arbitrarily into two portions along the dorsal-ventral axis. The cells in the dorsal portion grow dorsally and engulf the yolk during dorsal closure and produce dorsal cuticle. The cells in the ventral portion do not grow dorsally. The dorsal ridge is composed of at least two parts (I, II). One (I) is derived from the Lb and Mx segments and grows dorsally during dorsal closure engulfing the yolk and producing dorsal cuticle. The other part (II) of the dorsal ridge forms from the most dorsal cells of the An, Ic, Mn and Mx segments. These cells migrate to close the cephalic region of the embryo without growth, exclude the yolk and do not produce dorsal cuticle. The head of the insect is formed by the growth of region Dr-I and the folding of region Dr-II. (B) Drawing of a milkweed bug embryo undergoing dorsal closure. The boundaries of the Dr are shown as marked by EN (stipple) and the product of the *labial* gene (stripes). st, stomodeum.

functions as a transcription factor if it functions at all. However, since EN accumulation in the integument is often associated with pattern formation or morphogenetic events, conjecture about its possible role in variant epidermal domains may have a firmer basis. Accumulation in the caudal and lateral stripes of fleas, and in the amnion of milkweed bugs, occurs in stripes, while the epidermal expression in the cerci of crickets and in the fused labium of milkweed bugs is surface specific. For example, EN accumulates only in the cells that form the apparent ventral surface of the cerci. Thus it is possible to invoke a role for the other components of the EN dependent cell-cell communication pathway (Martinez-Arias, 1994), such as *wingless* and *naked*, in organizing cell movement and morphogenesis in these processes.

## The generality of the parasegment as a fundamental unit of pattern?

Since it was first proposed the existence of the parasegment (PS) and its function as a fundamental unit of segmentation and pattern has been debated and largely supported (Martinez-Arias, 1993; Lawrence, 1988; Sander, 1988). Most of the evidence presented for the existence and function of the parasegment has been taken from *Drosophila*, although some has also been obtained from other insects (Patel, 1994b). The accumulation patterns of EN documented here, together with some additional information gathered on the expression pattern of homeotic genes, adds new fuel to this discussion.

The *Drosophila* embryo produces a transient set of constrictions corresponding to the formation of the parasegment boundary (Ingham and Martinez-Arias, 1992). However, parasegmental grooves have not been reported for other insects. It has been documented that the anterior border of EN accumulation sharpens prior to that of the posterior border, which is thought to reflect the formation of parasegment borders prior to segments and that this event is a general feature of insect development (Patel, 1994b). Although this sequential sharpening also appears to be true for milkweed bugs, grooves do not initiate in the head or thorax until the EN stripe is at least four cells wide. This grooving occurs on either side of the EN accumulation giving a compartmental periodicity, rather than segmental or parasegmental one. In contrast, the grooves in the abdomen of the milkweed bug initiate in a parasegmental register. These grooves initiate just anterior to the EN stripe when the stripe is only one cell wide. Only after the stripe has widened does a groove form at the posterior edge of the EN stripe. These compartmental and parasegmental groove patterns eventually give way to a segmental groove pattern. We interpret the formation of grooves as indicative of an 'event' in the process of segmentation, and the timing of segment and parasegmental grooves, as flexible. Whether the formation of grooves represents a critical stage in the process of segmentation is unknown; however, this 'event' might signal the formation of compartment boundaries. In this case, the formation of compartment boundaries, which are clonal boundaries, is not identical to the definition of the EN expression patterns, an association that is often assumed (Patel, 1994b).

Some authors have pointed to the initiation of homeotic gene expression as an example of parasegmental patterning, and indeed the expression of *abd-A* orthologs in *Drosophila*, *Tribolium* and *Schistocerca* (Stuart et al., 1993; Tear et al, 1990; Karch et al. 1990) appears to initiate and maintain parasegmental borders. However, it has also been stated that the products of the Antennapedia-complex (ANT-C) homeotic genes accumulate first in a parasegmental pattern which resolves into a segmental one (Martinez-Arias, 1993; Finkelstein and Perrimon, 1991). A careful examination of the initiation of homeotic genes from the ANT-C, however, has revealed that these genes do not initiate in a simple paraseg-

mental register. The protein product of *Scr* (SCR) accumulates in a jagged stripe that is neither parasegmental nor segmental. This stripe then resolves into a pattern that is segmental in the dorsal and lateral regions and parasegmental only in the ventral region (Gorman and Kaufman, 1995). An examination of the *Dfd* (DFD) product reveals that the DFD pattern develops similarly to that of SCR (Rogers and Kaufman, unpublished). Furthermore, the expression of ANT-C orthologs in the milkweed bug, cricket, firebrat (Rogers, Peterson and Kaufman, unpublished data) and grasshopper (Hayward et al., 1995) reveals that they also do not initiate expression in parasegmental domains.

Others have pointed to the organogenesis of the salivary gland as a process dependent on parasegmental cues (Martinez-Arias, 1993). The *Drosophila* salivary gland forms within PS2 (Panzer et al., 1992), composed of compartments of the Lb and Mx segments suggesting that parasegmental cues may be important for organogenesis as well as segmentation. Our observation that the salivary gland of the milkweed bug extends into and invaginates in the posterior compartment of the Lb segment suggests that its salivary gland may be patterned by non-parasegmental cues. Alternatively, these EN-expressing cells may represent those not recognized as part of the *Drosophila* gland.

Although there is certainly enough evidence to support the idea that the parasegmental border plays a key role in insect development, there is considerably less evidence that the segment-wide parasegment is a fundamental unit of pattern. The best evidence for the parasegment as a fundamental unit, comes from observations of posterior embryonic development. The embryos of Malacostraca (Crustaceans) elongate by adding one parasegment at a time (Scholtz et al., 1994) and homeotic expression in the posterior of insect embryos largely obey parasegmental boundaries. Observations of anterior development have provided significantly less evidence for the parasegment as a fundamental unit. In the insect head, the parasegment is just one possible unit and clearly not the primary unit of homeotic gene expression. The observation that the salivary gland may be defined in a parasegmental (*Drosophila*) register or not (milkweed bug) is evidence that the parasegmental cues are not fundamentally required for its organogenesis. Finally, the formation of compartmental grooves in the milkweed bug is at least suggestive enough to revive a compartmental model of segmentation. In this model, the two compartment borders might be defined independently without a requirement for the order of their formation; however, the requirement for EN expression at both borders remains. The order of groove formation in the abdomen reflects the timing of expression of EN stripes, while the grooves in the head and thorax do not. The formation of parasegmental grooves and compartmental grooves in the milkweed bug may reflect the difference in relative time of formation of the posterior and anterior compartmental boundaries in the growing abdomen compared to those of the blastoderm.

### The process of segmentation in the insect head may be conserved

The process of segmentation of the *Drosophila* head is considerably different from that of the trunk. The segments of the head anterior to the gnathos do not use the familiar hierarchy of gap and pair-rule genes to define the segment borders but instead use overlapping patterns of head gap gene expression to define the segments in a still mysterious process (Finklestein and Perrimon, 1991; Cohen and Jurgens, 1991). Even when the same genes are utilized for segmentation of both head and trunk, the relationships among these genes are not the same in both locations. For example, *wg* is required for the maintenance of *en* expression in the trunk (Heemskerk et al., 1991) but not in the gnathos or anterior head (van den Heuvel, 1993; unpublished observation). Also, in contrast to the trunk, where segmentation occurs at different times in short and long germband insects (Patel, 1995b), the pattern and order of segment development in the head is highly conserved.

We have observed that the order of expression of EN in the cephalic segment primordia is highly conserved. The only variation is in *Drosophila* where the An expression is delayed slightly. Otherwise, EN accumulation in the An segment is initiated in the blastoderm along with gnathal, thoracic and abdominal segments depending on germ type. EN then accumulates in the Oc segment and finally in the Ic segment only after the stomodeum is formed. This accumulation literally 'intercalates' between the pre-existing An and Mn stripes. The high conservation of order and pattern of EN accumulation suggests that the mechanism of segment formation is also highly conserved. However, as the mechanism of head segmentation is still not well understood in *Drosophila* and, since no head-specific segmentation gene homologs have been studied in any other insects, the extent of conservation of these mechanisms is unknown.

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