Expression of *engrailed* during segmentation in grasshopper and crayfish

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Summary

We have used a monoclonal antibody that recognizes *engrailed* proteins to compare the process of segmentation in grasshopper, crayfish, and *Drosophila*. *Drosophila* embryos rapidly generate metameres during an embryonic stage characterized by the absence of cell division. In contrast, many other arthropod embryos, such as those of more primitive insects and crustaceans, generate metameres gradually and sequentially, as cell proliferation causes caudal elongation. In all three organisms, the pattern of *engrailed* expression at the segmented germ band stage is similar, and the parasegments are the first metameres to form. Nevertheless, the way in which the *engrailed* pattern is generated differs and reflects the differences in how these organisms generate their metameres. These differences call into question what role homologues of the *Drosophila* pair-rule segmentation genes might play in other arthropods that generate metameres sequentially.

Key words: *engrailed*, segmentation, homeobox, arthropods.

Introduction

The genetic hierarchy controlling segmentation in *Drosophila* involves both maternal components and the sequential expression of gap, pair-rule, and segment polarity genes in the zygote (reviewed by Ingham, 1988). Under the control of these genes, the cellular blastoderm is progressively subdivided into a series of repeated units of increasingly smaller size and greater number. Although segments are not apparent as morphological subdivisions until gastrulation has occurred, developmental commitments by cells (Simcox and Sang, 1983) and the patterning of gene expression (Hafen *et al.* 1984) point to the generation of metameric organization during the blastoderm stage. This mode of *Drosophila* segmentation, in which the metameric units form rapidly and without growth, does not represent the only form of segmentation in the arthropods, or indeed, even in all insects. Rather, many arthropods, including more primitive insects and crustaceans, undergo a quite different form of segmentation. They generate metameres during a phase in which a subterminal growth zone adds segmental primordia through caudal elongation (Anderson, 1972).

The existence of these two modes of segmentation – addition one at a time in a rostrocaudal gradient vs progressive subdivision of a sheet of cells – raises several questions. First, what mechanisms are conserved between more primitive and more advanced arthropods, and what mechanisms are unique to different subgroups? Second, how did segmentation by subdivision evolve from sequential segmentation? Finally, what can we infer about the evolution of

segmentation and its molecular genetic control by comparing segmentation in different arthropods?

To address these questions, we used a molecular marker to compare the generation of metameres in *Drosophila*, grasshopper, and crayfish. We examined expression of *engrailed* gene in early embryos of these animals because it has been previously shown that, in *Drosophila*, *engrailed* is expressed in the posterior compartment of every segment from the embryo to the adult, and thus can be considered a molecular marker defining *Drosophila* segments (Kornberg *et al.* 1985; Fjose *et al.* 1985). This study was possible because the *engrailed* gene is conserved within the arthropod lineage, and a monoclonal antibody, MAb 4D9, has been characterized that recognizes a conserved epitope in the *engrailed* proteins of these animals (Patel *et al.* 1989). We find that MAb 4D9 also reveals that *engrailed* protein is localized to the posterior region of each developing grasshopper and crayfish segment. As described below, many aspects of *engrailed* expression are conserved among these arthropods, but other aspects of *engrailed* stripe formation are not. These disparities expose fundamental differences in pattern formation.

We note that metamerization of the *Drosophila* embryo involves both the generation of parasegments at the cellular blastoderm stage and the generation of segments later during gastrulation. Both these processes are included in the common usage of the term segmentation, and we retain this usage here.

Materials and methods

The production of the 4D9 monoclonal antibody and the

staining protocol are described in Patel *et al.* (1989). Cells were counted by staining embryos with Hoechst dye after the peroxidase reaction, and counting nuclei using a Zeiss epi-fluorescence microscope. To estimate the number of cells in a grasshopper parasegment at the onset of *engrailed* expression, we used embryos that showed a slight left/right asynchrony. The side that was slightly ahead in development marked the position of the next stripe to form on the opposite side. This position was taken to be the start of the next parasegment on the side that was lagging behind. In crayfish embryos, the *engrailed* stripe widening process lags 2–3 parasegments behind the formation of new stripes and therefore adjacent parasegments exist in which neither has begun the next round of division (see Fig. 8B).

Results

Sequential expression of engrailed *stripes in the grasshopper embryo*

We used MAb 4D9 to examine the expression of *engrailed* in approximately 70 grasshopper embryos.

The embryos ranged in age from early gastrulation (13 % development; each 5 % interval is approximately one day of development (Bentley *et al.* 1979)) to dorsal closure and organogenesis (60 % of development). *engrailed* expression was first observed at around 17 % and the final *engrailed* stripe appears at about 31 % of development. For the most part the *engrailed* stripes appear one at a time (Figs 1, 2) and, in each segment, precede the first morphologically visible of segmentation – the constriction of the mesoderm into an arrowhead-like shape.

The first *engrailed* stripe appears shortly after the onset of grastulation in a region that corresponds to the first thoracic segment (T1) and in some embryos the stripes of the first and second (T2) thoracic segment appear simultaneously. The T3 stripe is the next stripe to appear, at about 18–19 %. At 19 % of development, stripes begin to appear in the subesophageal segments. Usually S1 appears first, followed by S3 and then S2, but in some embryos S1 and S3 appear simultaneously. At about 20 %, the most anterior two pairs of cephalic
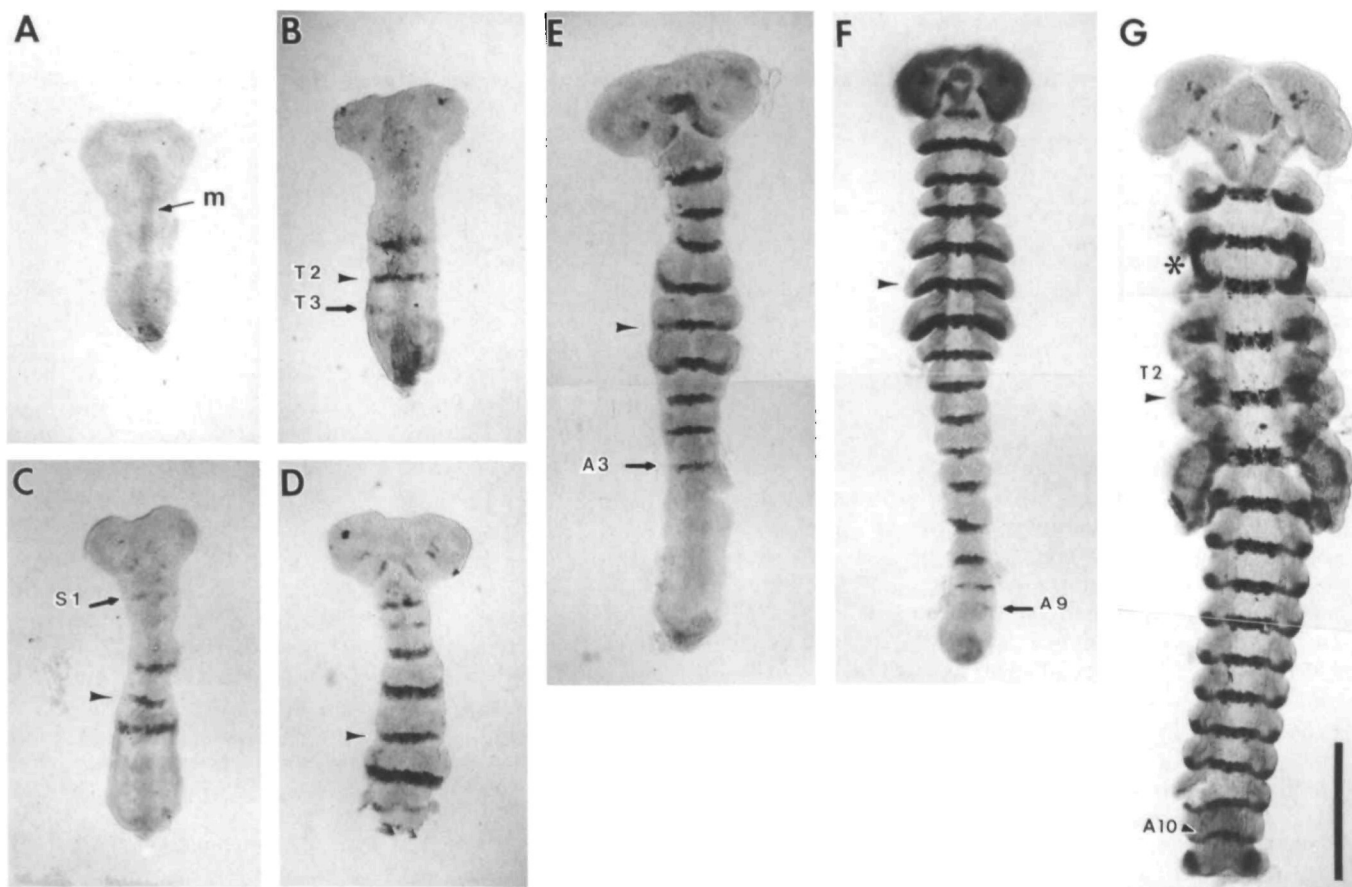

Fig. 1. Appearance of *engrailed* stripes in the grasshopper embryo. Photographs of *engrailed* staining in grasshopper embryos at (A) 15 %, (B) 17 %, (C) 19 %, (D) 22 %, (E) 23 %, (F) 29 %, and (G) 33 % of development. Anterior is up in all panels and the position of T2 is indicated by an arrowhead. All photos are at the same magnification. At 15 % (A), no *engrailed* stripes are visible and gastrulation has begun to generate mesoderm (m). At 17 % (B), the third thoracic stripe (T3) is just beginning to form. At 19 % (C), the S1 stripe is appearing and at 19 % (D) the cephalic stripes are forming. The third abdominal stripe (A3) is forming at 23 % (E), and the ninth abdominal stripe (A9) appears at 29 % (F). Note that the embryos in E and F are almost the same length, but the *engrailed* stripes are still progressively appearing down the length of the abdomen. By 33 % (G) the final *engrailed* stripe (A10) has appeared. Asterisk indicates lateral connection between S2 and S3 stripes. The embryo has increased in size due to growth by further cell divisions. Scale bar: 500 μm.
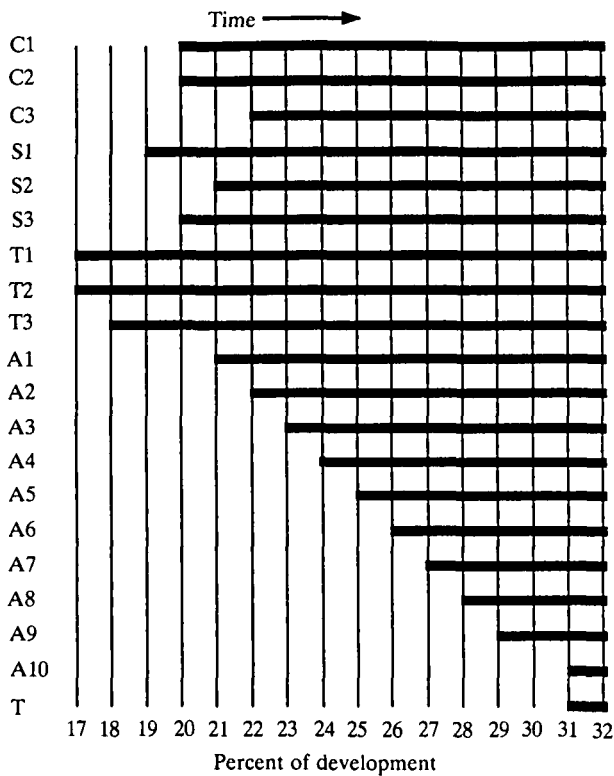
Fig. 2. Appearance and timing of the *engrailed* stripes in the grasshopper embryo. The horizontal axis represents time in terms of per cent development and the vertical axis denotes the different body segments. The spatiotemporal expression pattern matches Seidel's concept of the anterior thoracic region as the morphological differentiation center (reviewed in Sander, 1976). The abdominal *engrailed* stripes appear in a sequential pattern with one stripe appearing about every one percent of development. See text for details.

patches (C1 and C2) appear simultaneously and do not take on the appearance of stripes until much later in development (see below). At about 22%, the final cephalic stripe (C3) begins to form.

Starting at about 18% of development, the abdominal anlagen undergoes a major phase of growth and the embryo rapidly elongates caudally. Interestingly, the appearance of *engrailed* stripes does not keep pace with the rate of elongation. The abdominal stripes appear sequentially (Fig. 2) with the first becoming visible at approximately 20–21% of development, and the next eight appearing at approximately 1% intervals. At 24%, the abdomen stops elongating, even though only the first three to four abdominal stripes have formed. This leaves a large gap between the most recent stripe and the end of the abdomen. The ninth abdominal stripe forms at 29% of development, and the tenth 2–3% later. A ring of *engrailed*-positive cells also appears around the telson at this time.

Maturation of engrailed *stripes*

The *engrailed* stripes are initially 2–3 cells wide (anterior–posterior dimension) with about 7–8 unstained cells separating the stripes (Figs 3, 4). Thus in

both *Drosophila* and grasshopper, initial *engrailed* expression is characterized by a ratio of approximately one *engrailed*-positive cell among four total cells (Fig. 4). In the grasshopper, staining first appears about half way between the midline and the lateral edge of the embryo, and then rapidly extends both medially and laterally.

Embryos are often bilaterally asymmetric with respect to onset of *engrailed* stripes. Stripes may appear on one side of the embryo that lack a matching stripe on the other side. In these same embryos, the asymmetry is also reflected in the more anterior stripes that are more mature (i.e. Fig. 3B,C). The appearance of such embryos suggests that the two halves of the embryo do not develop synchronously. This asynchrony indicates some level of independence of the two halves of the embryo during this period of pattern formation. Asymmetry of this nature has been observed previously in an analysis of axon outgrowth. For instance, it is not uncommon to find grasshopper embryos in which the neurons on one side of the segment are about 1% of development ahead of the other side (Raper *et al.* 1983*a,b*). Similar temporal asymmetries are also occasionally visible during *Drosophila* development.

About 1 to 2% in development after its formation, each *engrailed* stripe begins to widen. A single row of cells with lightly stained nuclei appear immediately posterior to the original 2–3 rows of cells with darkly stained nuclei. Following cell division, the stripe widens to about 5–6 cells. The number of rows of unstained cells also increases to 12–13. Thus, stripes appear to widen both by recruitment of new cells to their posterior border, and as a result of cell division (Fig. 4). Although cell mitoses occur during widening, the absolute length of the segment changes very little. Rearrangements cause the cells to become more staggered in the medial/lateral plane. In addition, the cells are smaller after dividing.

All stripes widen except those in A10 and in the head. Head stripes extend laterally along the posterior margins of C1 and C2 by 27–28% of development. The stripe in A10 never widens, and remains narrower than other abdominal ones. The tenth abdominal stripe of the grasshopper is similar to the ninth abdominal stripe of *Drosophila*: both lag behind the others in development, both remain narrower than other abdominal stripes, and both are the most posterior.

When the *engrailed* stripes appear, their posterior and anterior margins are uneven. Numerous *engrailed*-containing nuclei are out of alignment. Some are well outside the main stripe (Fig. 3E,F). Shortly after stripe formation, however, the anterior border of the stripe forms a sharp boundary. Given the sharpness of the border, this event might reflect some degree of cell movement or realignment. The fate of ectopic cells is not clear. They persist for a few per cent of development, but are never seen in older embryos, and thus either cease to express *engrailed*, or alternatively migrate into the *engrailed* stripe. The posterior border of the stripe (the segmental boundary) eventually sharpens, but never to the extent of the anterior one
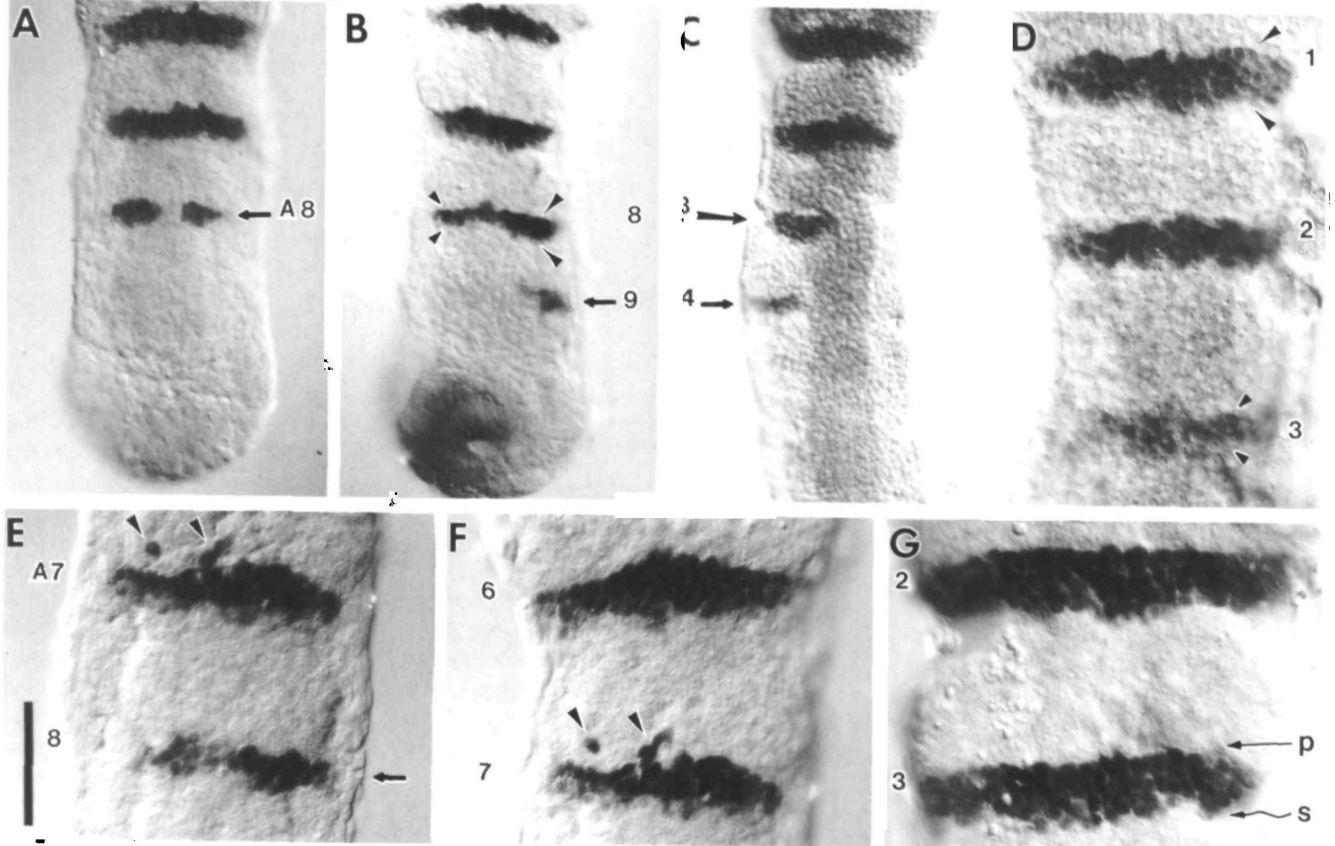
Fig. 3. Maturation of *engrailed* stripes in the grasshopper embryo. Photographs of *engrailed* staining in 28 % (A,E,F, and G), 29 % (B), 23 % (C), and 24 % (D) grasshopper embryos. Anterior is up in all photos and numbers at the edge of each embryo refer to the level of each abdominal segment. A shows an eighth abdominal stripe beginning to form symmetrically on each side of the embryo. B shows an embryo where the ninth abdominal stripe has only begun to form on one side of the embryo. In the eighth abdominal segment, the stripe on the left side has just formed and is only about 2–3 nuclei wide (small arrowheads). The stripe on the right side, however, has already begun the widening process (large arrowheads). Thus, the right side of this embryo is about one percent of development ahead of the left side. C shows an extreme case where the third and fourth abdominal stripes have formed on only one side of the embryo. The widening process is visible in D where the third abdominal stripe is only 2–3 nuclei wide (small arrowheads), but the first abdominal stripe has widened to 5–6 nuclei (large arrowhead). Panels E, F, and G are photographs from a single embryo and show the progressive straightening of the parasegmental boundary. In E the seventh and eighth abdominal stripes have irregular boundaries and there are even some ectopic cells (arrowheads). In the sixth abdominal stripe (F), the parasegmental boundary has sharpened somewhat. In G, the third abdominal stripe illustrates that the parasegmental boundary (straight arrow) is quite sharp, but the segmental boundary (wavy arrow) is still somewhat irregular. The cells at the segmental boundary edge also stain somewhat less intensely than cells within the stripe or at the parasegmental boundary. Scale bar: (A,B, and C) 100 μm; (D) 50 μm; (E,F, and G) 70 μm.

(Fig. 3G). By comparison with *Drosophila*, we suggest that the anterior border of the *engrailed* stripe designates the parasegmental boundary (Lawrence, 1988).

engrailed *stripes in head, limbs and body*

Several other features of the *engrailed* staining pattern are noteworthy. Grasshopper embryos, unlike their *Drosophila* counterparts, possess limb buds that grow as lateral evaginations from the thoracic segments. These limb buds possess the same pattern of *engrailed* expression as do the segments from which they arise; the posterior margin of each bud is composed of *engrailed*-positive cells (Fig. 5B). Cross-sections through legs reveal that between 1/3 and 1/4 of the circumference of the leg is composed of *engrailed*-positive cells. This same pattern is also seen in the developing antennae, which form from the C2 segment,

but the level of expression is quite low. An interesting modulation occurs in the legs just before they subdivide in the distal–proximal axis (Fig. 5C). Cells along the posterior margin that sit in the proximal portion of each limb subdivision stain darker than those at the distal portion, and the pattern does not appear to be due to regional changes in cell shape or density. This pattern probably has nothing to do with limb bud subdivision itself, since the *engrailed* staining is limited to the posterior part of the limb while the constrictions encircle the entire circumference of the leg. Rather, *engrailed* could be responding to some proximal–distal cues that are actively involved in limb bud subdivision.

An interesting parallel in the staining pattern of grasshopper and *Drosophila* occurs in the connection of the stripes of the second and third (S2, S3) subesophageal segments. In germ-band-extended *Drosophila* embryos, the S2 and S3 stripes are initially uncon-
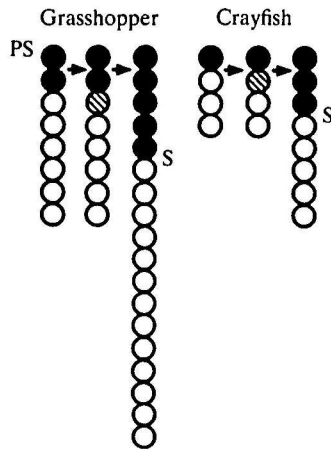
Fig. 4. Widening of the *engrailed* stripe. Grasshopper *engrailed* stripes begin approximately 2–3 nuclei wide. A short while later, a row of nuclei begin to stain lightly at the posterior margin of the stripe. Then a round of mitosis occurs and the combination of recruitment and cell division yields about 5–6 rows of *engrailed*-positive nuclei. The parasegment does not actually double in absolute length due to cell rearrangements and the reduction in the size of the daughter cells. A very similar process occurs in the crayfish. Expression begins in a single row of cells (the *a* row) at a time when there are four rows of cells per parasegment. Then staining is seen in some of the *b* row cells just before divisions begin. The combination of divisions and recruitment yield a final pattern in which slightly more than one-fourth of the cells are *engrailed*-positive. The parasegment does not actually double in absolute length because the daughter cells are smaller than the original cells and some divisions are oriented obliquely. PS, parasegmental boundary; S, segmental boundary (and groove).

nected, but then cells between the dorsal ends of the two stripes begin to express *engrailed*. The expression extends anterior and posterior resulting in the bridging of the ends of the S2 and S3 stripes (Fig. 6A). This same connection occurs in grasshopper embryos (Fig. 6B,C). At about 28 % of development, nuclei begin staining between the lateral ends of the S2 and S3 stripes (Fig. 6B). The initial nuclei are located somewhat closer to S2 than S3, and then the expression spreads to connect the ends of the two stripes together. By 32 % of development, the S2 and S3 stripes are connected together by a lateral bridge of *engrailed*-positive cells (Figs 6C and 1G).

In grasshopper, the head segments are more clearly visible than they are in *Drosophila*, since in grasshopper they do not undergo involution and are less fused. Studies of *Drosophila* development indicate that in the three rostral head segments (C1, C2, C3), *engrailed* is expressed in four patches that result from distortions of the original three stripes (DiNardo *et al.* 1985). In grasshopper, three distinct patches appear in these head segments (Fig. 5A) and eventually elongate laterally into faint stripes along their posterior margins (28 %). The C2 stripe also extends along the posterior portion of the antennae as it develops. The first two of these head segments show an obvious segmental structure,
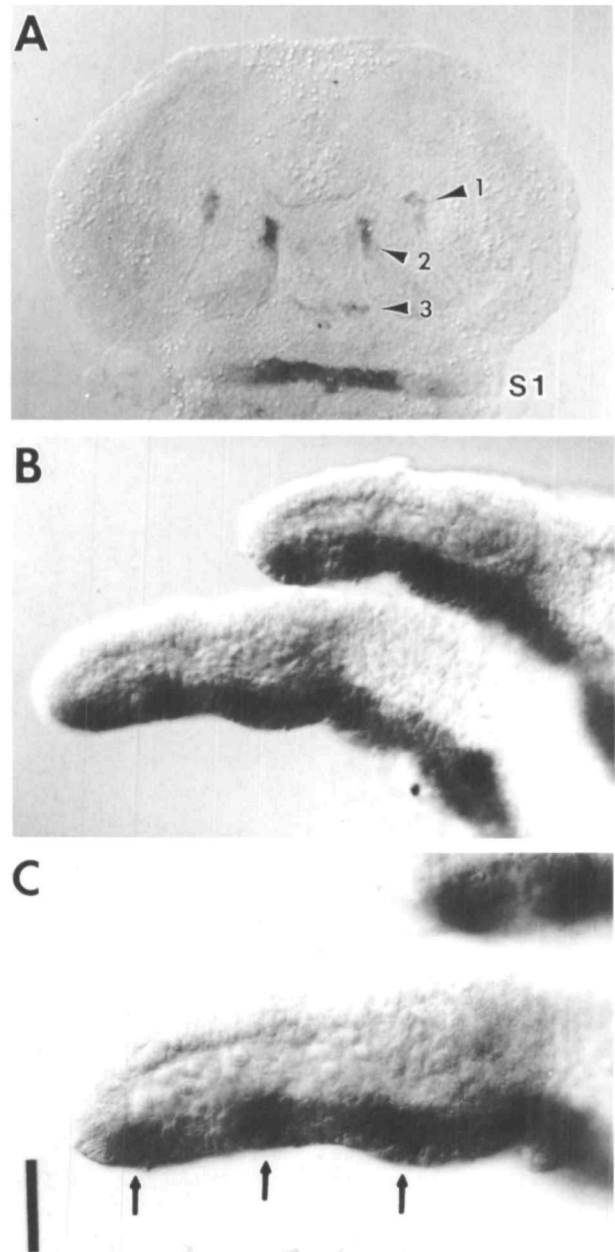

Fig. 5. Head and limb expression of *engrailed* in the grasshopper embryo. Photographs of the head of a 22 % embryo (A) and limbs of a 32 % embryo (B and C) stained with the 4D9 MAb. The head of the embryo (A) at 22 % shows three pairs of patches (arrowheads) that are part of the three head segments (C1, C2, C3; S1 is the stripe of the first subesophageal segment). The grooves delineating C1 and C2 are clearly visible, but the *engrailed* expression will not cover the entire posterior margin of these segments until 28–29 % of development. The limbs (B) show *engrailed* expression along their entire posterior margin and in C a slight undulation in the pattern is visible that seems to correlated with the distal to proximal pattern of subdivision. Scale bar: (A and B) 150 μm; (C) 95 μm.

namely deep segmental grooves, well before the *engrailed* expression has begun to spread laterally. By 40 % of development, these head stripes are barely visible, but in the regions where the patches began, a
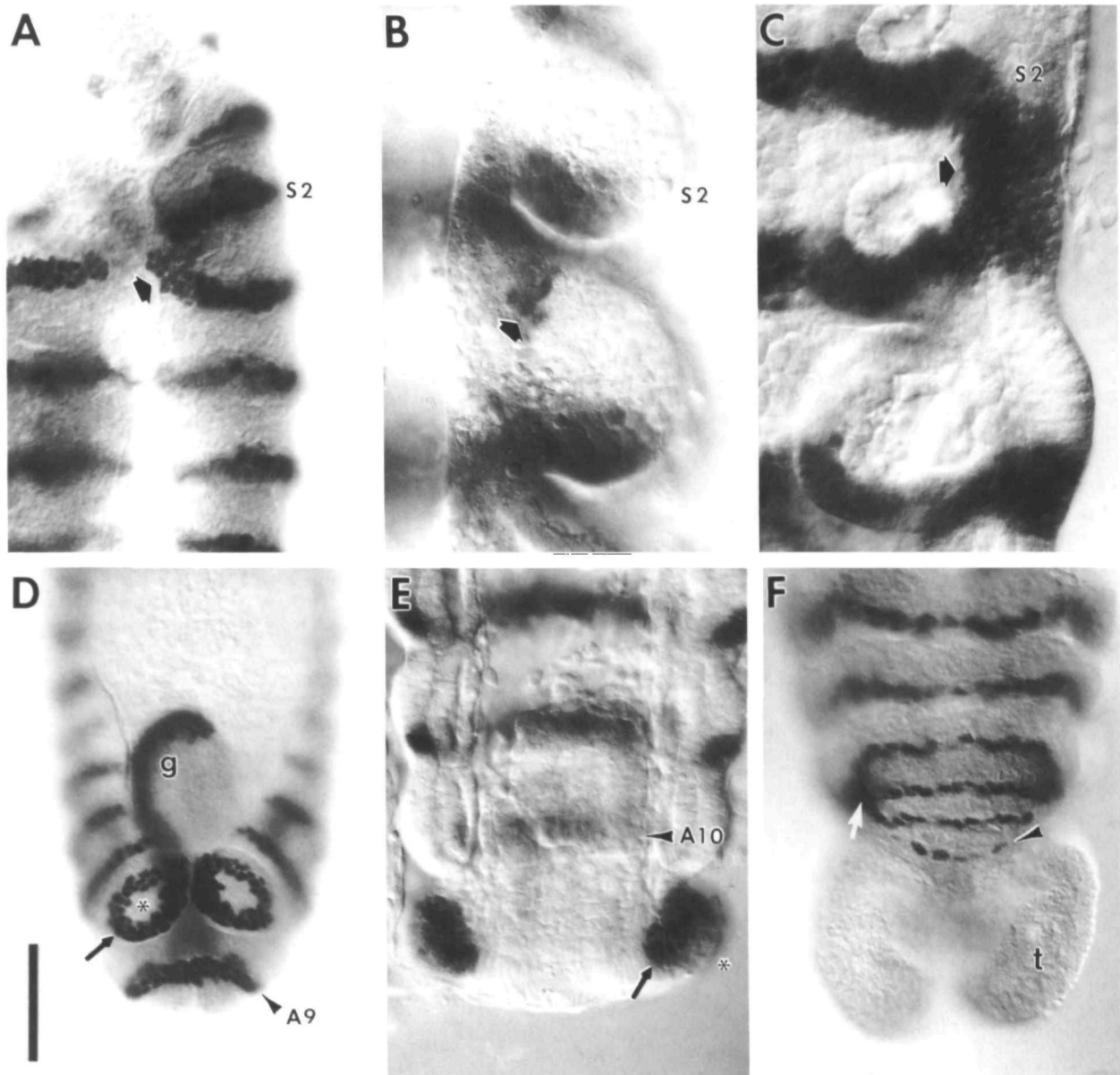
Fig. 6. Connections of *engrailed* stripes and stripes in terminal structures. *Engrailed* staining of *Drosophila* embryos at 7 h (A) and 10 h (D) of development, grasshopper embryos at 29 % (B), 33 % (E), and 34 % (C), and a crayfish embryo (F) at approximately 30 % of development. Anterior is up; A is a lateral view, B,C,E and F are ventral views, and D is a dorsal view. In *Drosophila*, cells between S2 and S3 begin to express *engrailed* and result in the connection of the S2 and S3 stripes (arrowhead in A). This same process occurs in the grasshopper. B shows a small number of nuclei (arrowhead) beginning to stain between S2 and S3 and eventually a continuous band of staining (arrowhead in C) connects the S2 and S3 stripes. The terminal end of *Drosophila* (D) reveals *engrailed* expression along a portion of the hindgut (g), the stripe of the ninth abdominal segment (A9), and ring of staining around the spiracle (arrow; asterisk indicates unstained cells within the spiracle). The staining around the spiracle is from the dorsal part of the eighth abdominal stripe. The posterior region of grasshopper (E) shows no staining of the developing hindgut, the presence of a stripe in the tenth abdominal segment (A10), and a ring of staining (arrow; asterisk marks unstained cells within the right) on each side of the telson. The terminal segments of crayfish (F) reveal four *engrailed* stripes (arrowhead points to final stripe) that are fused together at the lateral edge (white arrow), and a telson (t) which shows no *engrailed* expression. Scale bar: (A) 55 μm; (B) 70 μm; (C and E) 110 μm; (D and F) 65 μm.

few darkly stained nuclei remain, and these appear to be small groups of neurons within the developing brain.

Grasshoppers also have a simpler stripe pattern in their terminalia, but unfortunately this does not help our understanding of the pattern in the fly. In grass-

hopper the last clear stripe is part of the tenth abdominal segment (Fig. 6E), while in fly it is part of the ninth abdominal segment. As discussed above, there are several striking parallels between these two stripes. Thus, changes in the total number of visible segments

could reflect changes in the number of segments anterior to this terminal stripe. In *Drosophila*, the ring of *engrailed* staining around each spiracle (Fig. 6D) is thought to be a component of the dorsal part of the eighth abdominal *engrailed* stripe (DiNardo *et al.* 1985). In the grasshopper embryo we also observe rings of staining at the posterior end of the embryo, but they seem to represent a group of cells independent of any other stripe (Fig. 6E), and we do not know if they give rise to the grasshopper spiracles. Additionally, one surface of the *Drosophila* hindgut is composed of *engrailed*-positive cells, but no expression is detected in the grasshopper hindgut.

Segmentation in crayfish as revealed by engrailed *expression*

We also examined the expression pattern of *engrailed* during germ band formation in the crayfish, *Procambarus clarki*. In crustacean embryos, the germ band extends and develops through an orderly process in which the pattern of cell divisions is quite stereotyped. This mode of development has been studied extensively in the crustacean, *Diastylis rathkei* (Dohle, 1970, 1976; Dohle and Scholtz, 1988), and our observations of caudal elongation in the crayfish embryo correspond closely with Dohle's analysis of *Diastylis rathkei*. A brief summary of Dohle's observations on the caudal extension of the germ band in *Diastylis rathkei* will help clarify our interpretation of *engrailed* expression in the crayfish.

Development of the caudal germ band begins with approximately eight ectoteloblasts on each side of the midline (Fig. 7). The ectoteloblasts divide asymmetrically in relative synchrony, each round of division giving rise to a row of smaller ectoteloblast progeny. With each round of division, the ectoteloblasts are displaced caudally, leaving behind rows of progeny. These progeny cells are arranged in precise longitudinal and transverse rows so as to create a grid-like pattern. Each row of eight ectoteloblastic progeny is designated by a Roman numeral (I, II, III, IV, etc; see Fig. 7).

Cells in each Roman numeral row then undergo two series of symmetrical divisions to yield four rows, designated *a*, *b*, *c*, and *d*. Thus, from an initial single Roman numeral row of ectoteloblast progeny (e.g. III) arise four adjacent rows of cells (e.g. III*a*, III*b*, III*c*, and III*d*; see Fig. 7). At this point a series of differential cleavages begins and each cell divides in a characteristic and recognizable manner. Several of these cleavages are oriented laterally, thereby distorting the perfect rows.

The descendants of each Roman numeral row are a genealogical group; each group does not, however, constitute a segment (Dohle, 1976; Dohle and Scholtz, 1988). This is evident in two ways: (1) the segmental groove, which develops in a predictable and reproducible manner, passes transversely and slightly obliquely, running anterior to or between or behind the descendants of the *b* row (the exact location depends on the medial lateral position), and (2) a limb is composed of *c* and *d* cell descendants from one Roman numeral row
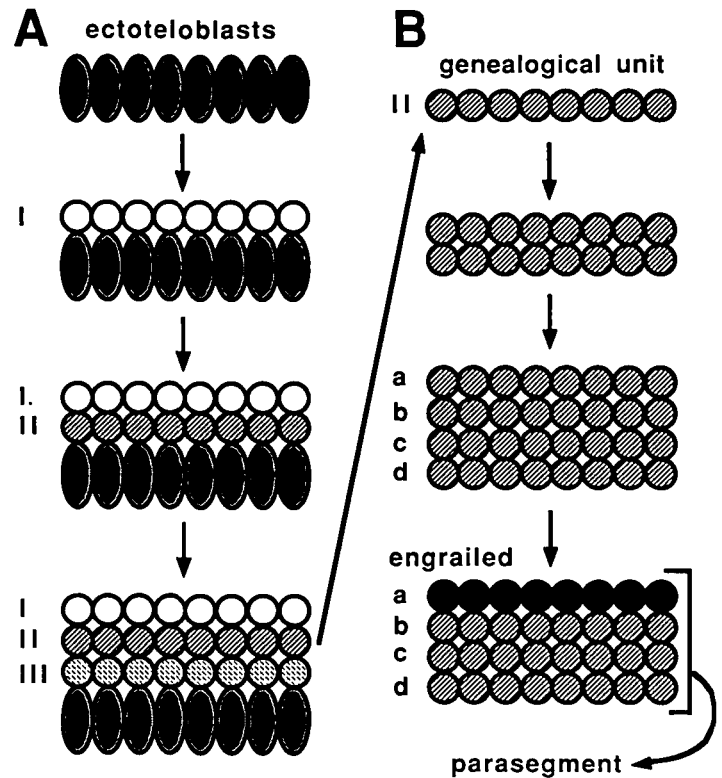

Fig. 7. Ectoteloblastic growth of crustacean embryos. Schematic illustration of divisions of ectoteloblasts (A) and divisions of Roman numeral rows (B) adapted from descriptions of Dohle (1976). In A, the rows of ectoteloblasts undergo synchronous rounds of asymmetric divisions to progressively generate rows of progeny (Roman numeral rows). Here we show the result of the first three rounds of divisions that generate Roman numeral rows I, II, and III. The ectoteloblasts continue their pattern of divisions and are displaced caudally down the embryo. B illustrates the divisions that each of the Roman numeral rows undergoes (in the case we follow row II). Each Roman numeral row goes through two rounds of synchronous divisions to yield four rows (*a*,*b*,*c*, and *d*). It is the entire *a* row that then begins to express *engrailed* (filled in cells). Some *b* row cells also express *engrailed* just before the start of the next divisions. Each Roman numeral row is a parasegment, in that each row gives rise to a geneological unit of cells *a*, *b*, *c*, and *d* which form a parasegment. The parasegmental boundary forms first at the anterior border of the *engrailed* stripe: the anterior border of the *a* row. The segmental boundary forms later after further cell division; the segment groove forms in a reproducible position between or at either side of the descendants of the *b* row at the same time as the *engrailed* stripe expands posteriorly. See text for discussion.

and *a* cell descendants from the next Roman numeral row. Martinez-Arias and Lawrence (1985) and Dohle and Scholtz (1988) speculated that this genealogical unit may be equivalent to a *Drosophila* parasegment.

Our data reveal a number of parallels between the *Drosophila* parasegment and this crustacean genealogical unit. We first observe *engrailed* expression at the

stage when a Roman numeral row has divided twice to yield its four *a, b, c,* and *d* rows. The initial expression is in the *a* row, in one out of four rows of cells (Figs 4, 7, 8). The pattern and precision of this ratio is clear among the grid-like arrangement of cells in crustaceans, and this ratio correlates well with similar observations in *Drosophila* and grasshopper. In *Drosophila,* the anterior margin of the *engrailed* stripe coincides with the parasegmental border. Using this designation in crayfish, the anterior border of the *engrailed* stripe (the anterior border of row *a*) is the parasegmental boundary, and thus the clonal descendants (*a, b, c, d* rows) of a single Roman numeral row, populate a single parasegment.

Just as in *Drosophila* and grasshopper, so in crayfish the *engrailed* stripe widens. Widening begins before the next round of cell divisions, and includes new *engrailed* expression by some of the cells of the *b* row (Fig. 8B). It is also at this time that the segmental groove begins to form. Once the differential cleavages begin, it appears that all progeny of the *a* row continue to express *engrailed*. The situation for the *b* row is more complex with both *engrailed*-negative and *engrailed*-positive progeny being generated (Fig. 8C). This pattern of expression must occur for *engrailed* expression to reach the segmental border as it does, for the segmental

groove will pass posterior to or between the descendants of the *b* row for part of its length. This expression pattern is also consistent with the staining pattern in the limbs; the most posterior part of each limb is *engrailed*-positive since the posterior margin of each limb is composed of descendants of an *a* row. Thus *engrailed* is initially expressed in one-fourth of the cells, but due to the new expression in some *b* progeny, the final number of *engrailed*-positive cells is greater (Fig. 3). The divisions that occur in the *a, b, c,* and *d* rows do not double the absolute length of the segment, since some cells divide obliquely and the daughter cells are somewhat smaller than the initial cells.

Finally, the last four abdominal stripes of *engrailed* expression are fused together at their lateral margins and there is no staining in either the hindgut or the telson structure of the crayfish (Fig. 5). In addition, there is a large arc of *engrailed*-positive nuclei in the extraembryonic membrane that extends from the level of the head to the abdominal segments.

Discussion

By virtue of its ability to recognize a conserved region in *engrailed* proteins from a wide array of organisms, MAb 4D9 can be used for comparative analyses (Patel
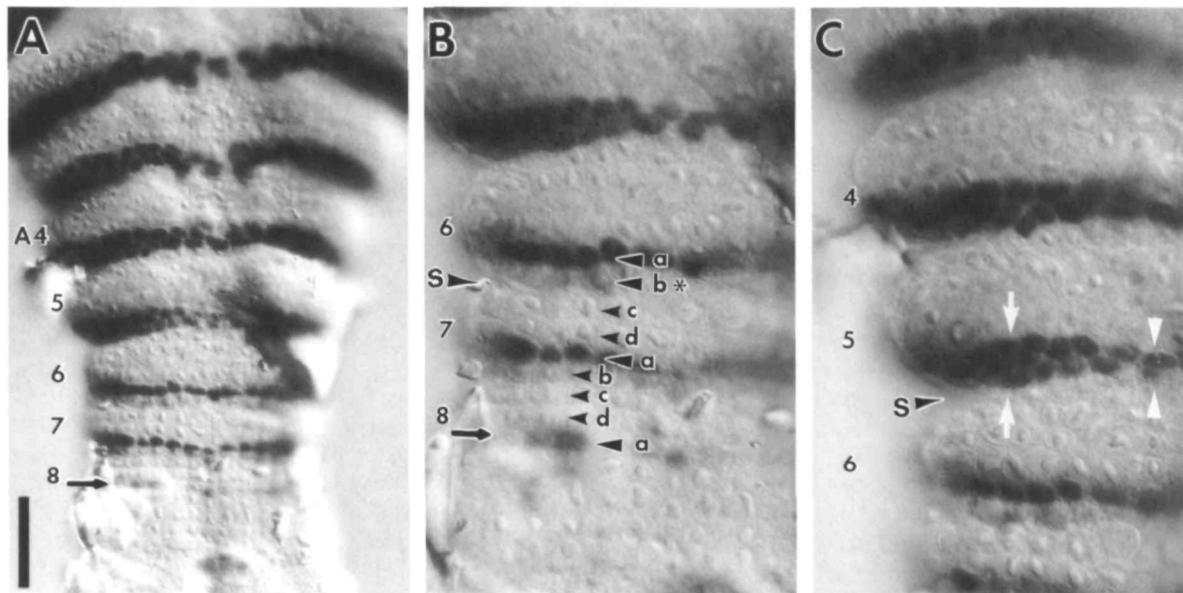

Fig. 8. *Engrailed* expression during crayfish segmentation. Photographs of *engrailed* staining during the formation of the eighth abdominal stripe in a crayfish embryo. Anterior is up, ventral view. (A) Low magnification photograph shows that stripes form in a rostral–caudal progression and the eighth abdominal stripe is just beginning to form in this embryo. Higher magnification photograph (B) of the posterior region shows that the *engrailed* staining begins in the *a* row of each set of *a,b,c,d* rows (each *a,b,c,d* row is marked by an arrowhead). The most caudal staining is in an *a* row (arrow) that will contribute to the posterior part of the eighth abdominal segment. The next four more anterior rows will produce the anterior portion of A8 and the posterior portion of A7 and contain an *engrailed* stained *a* row (large arrowhead), and *engrailed*-negative *b,c* and *d* rows (small arrowheads). The next set of four rows will produce anterior A7 and posterior A6 and contains *engrailed* staining (pair of large arrowheads) in the entire *a* row and weaker staining in a subset of the *b* row cells (asterisk). The segmental groove (S) is beginning to form just posterior to this *b* row. There is no *engrailed* staining in the *c* and *d* rows (pair of small arrowheads). The final panel (C) shows some of the segments further anterior. A clear segmental groove (S) can be seen forming between the fifth and sixth abdominal segments. The entire posterior portion of A5 contains *engrailed*-positive cells as all descendants of the *a* row continue to express *engrailed*, and those descendants of the *b* row that are anterior of the groove also express *engrailed*. Scale bar: (A) 100 μm; (B,C) 50 μm.

et al. 1989). Here we have used MAb 4D9 to compare *engrailed* expression in a relatively advanced insect (*Drosophila*), in a relatively primitive insect (grasshopper), and in a crustacean (crayfish). Our motivation was to better understand the mechanisms and evolution of arthropod segmentation. These animals were chosen for study because, unlike *Drosophila* embryos, where all segments are generated by progressive subdivision during the blastoderm stage, the segments of grasshopper and crayfish embryos appear to be generated sequentially as embryos elongate caudally by cell proliferation. *engrailed*, a molecular marker for segments in *Drosophila*, provides a means to directly compare mechanisms of segmentation.

The metameric pattern of the *Drosophila* germ band is thought to be a consequence of the sequential action of a number of different types of genes: of zygotic gap and pair-rule genes which establish the repeating patterns of segment polarity genes, and of homeotic genes which endow the individual segments with an identity. Sander has observed that the segmented germ band (i.e. embryos that have completed gastrulation and are overtly segmented) is a highly conserved stage in insect development, but that earlier stages of embryonic development are not (Sander, 1976, 1988). Even though the strategies for early arthropod development are remarkably diverse, all arthropod embryos pass through the segmented germ band stage. Sander (1988) has concluded '...that gene interactions guiding development up till the germ band stage might differ more between various insects forms than the genes active in the germ band'. Thus, while the segmented germ band is highly conserved and those genes active at this stage might also be conserved, the mechanisms for generating the segmented germ band may not be, and those genes active before this stage might vary considerably. In this view, it is not surprising that the segmentally repeated expression pattern of *engrailed* is well conserved throughout many arthropods. It might also be expected that patterns of homeotic gene expression are also conserved (Akam *et al.* 1988). However, the segmentation functions of other segmentation genes (e.g. pair-rule genes) which are active before the germ band becomes segmented may not be conserved in all arthropods (see below).

Similarities in the pattern of engrailed *stripes*

The basic pattern of *engrailed* expression within a segment (or parasegment) is the same in *Drosophila*, grasshopper, and crayfish. There is an initial ratio of 1:4 of *engrailed*-positive cells to total cells. The initial *engrailed*-positive cells form the anterior border of the final *engrailed* stripe. By comparison with *Drosophila*, the anterior border of the *engrailed* stripe is the parasegmental boundary (Lawrence, 1988), and thus the early appearance of the parsegmental boundary (and the subsequent appearance of the segment border) in all three arthropods is a common feature of segmentation. The *engrailed* stripes in grasshopper and crayfish then widen, apparently due to both cell divisions of *engrailed*-positive cells as well as the recruitment of new

cells to the posterior portion of each stripe. In *Drosophila*, the *engrailed* stripes also widen, with the original single row of *engrailed*-positive cells becoming two to three cells wide (Kornberg *et al.* 1985; Fjose *et al.* 1985; DiNardo *et al.* 1985). Unfortunately, the rapid cell movements and rearrangements of germ band extension and the absence of the developmental gradients characteristic of grasshopper and crayfish embryos obscure the mechanism of widening. On the basis of our studies, we suggest that expansion of the stripes in *Drosophila* also involves recruitment. This has interesting implications since *engrailed* had been thought to define the cells of the prospective posterior compartment at the onset of *engrailed* expression during the cellular blastoderm stage. If recruitment does contribute cells to the widening stripes, then lineage compartments do not form until after the stripes have fully widened during or after germ band extension.

Several other similarities also exist in the final pattern, including the lateral connection of the S2 and S3 stripes during development in *Drosophila* and grasshopper and the formation of three head stripes in all three arthropods. The expression of *engrailed* in neuroblasts and neuronal progeny is also highly conserved throughout arthropod evolution (Patel and Goodman, unpublished). Differences do exist in the total number of *engrailed* stripes, but this probably reflects the differences in the terminal structures of these different organisms.

Differences in the generation of engrailed *stripes*

The order of appearance of *engrailed* stripes differs between the long germ band *Drosophila* embryo and the shorter germ band grasshopper and crayfish embryos. This variation in the sequence of *engrailed* expression is related to differences in embryogenesis and suggests that the mechanisms of metameric pattern generation may differ fundamentally between these different arthropods.

In *Drosophila*, the segmentation genes interact in the early syncytial blastoderm embryo to generate the entire body plan. However, in the blastoderm of shorter germ band insects, only the most anterior portions of the embryo are specified. The more posterior regions, including the entire abdomen, arise from a subterminal growth zone. Therefore, the steps of segmentation and determination occur sequentially as the embryo elongates by cell divisions.

These differences in development are clearly manifest in the generation of the *engrailed* pattern. Most notably, the *engrailed* stripes in grasshopper and crayfish are generated sequentially as the embryo elongates. The posterior growth zone does not show a compressed pattern of *engrailed* stripes that expands with growth. That the segments are generated sequentially is further supported by the experiments of Mee and French (1986). By heat-shocking grasshopper embryos at progressively later stages during caudal extension, they altered increasingly more posterior segments (Mee and French, 1986). The progressive appearance of *engrailed* stripes in the abdominal segments is striking (Fig. 1),

and there is no evidence of a transient two segment periodicity as seen in *Drosophila* (DiNardo *et al.* 1985; Weir and Kornberg, 1985). Using *engrailed* as an early molecular marker of segmentation, we infer that segments are added caudally one at a time as the germ band elongates via cell proliferation. It appears that at the molecular level, just as at the morphological level, the mechanisms of pattern formation appear to be different in grasshopper and crayfish as compared to *Drosophila*.

Function of segmentation genes in short vs. long germ band arthropods

In *Drosophila*, the pair-rule genes are intimately involved in the generation of the metameric pattern (Ingham, 1988); pair-rule mutants show alterations in the number and position of *engrailed* stripes (DiNardo and O'Farrell, 1987). After their initial function during segmentation, many of the pair-rule genes also have a second function during neurogenesis. The later segmentally repeated expression pattern of the pair-rule genes *fushi tarazu* and *even-skipped* is essential for the proper determination of certain neurons (Doe *et al.* 1988*a,b*). The constraint of the segmented germ band stage suggests conservation of the genes expressed at that time (see above), but the gene interactions utilized before this stage may not be as well conserved.

On the one hand, a similar hierarchy of genes might function during segmentation in short germ band embryos, but with pair-rule stripes appearing one at a time in alternating segments ahead of the *engrailed* stripes. In this scenario, short germ band insects would differ from long germ band insects in that the expression of zygotic segmentation genes is temporally and spatially dynamic in keeping with the appearance of segments one at a time. On the other hand, given the sequential development of segments in the shorter germ band grasshopper embryo, we might predict that there would be no equivalent function for pair-rule genes during segmentation in the grasshopper. However, those neurons in *Drosophila* that require *fushi tarazu* and *even-skipped* function are present and are generated in the same manner in the grasshopper embryo (Thomas *et al.* 1984). Thus, this second scenario would lead us to predict that homologues of these genes will be present in grasshopper, that their only conserved function will be during the period of neurogenesis, and that during the period of segmentation, they will either be utilized in a very different way, or alternatively not at all. This is consistent with the view that these genes initially evolved for their function during neurogenesis (Doe *et al.* 1988*a*). We previously postulated that an ancestral function of the *engrailed* gene was for neurogenesis, and that early in arthropod evolution it was co-opted for its present function during segmentation (Patel *et al.* 1989). Thus, the pair-rule genes may have also had an ancestral function in neurogenesis, but they may have been recruited into their present pair-rule function in segmentation later in arthropod evolution, perhaps during the transition from shorter germ band to longer germ band embryos.

If in shorter germ band embryos, the pair-rule genes do not regulate the expression of *engrailed* (as they do in *Drosophila*), then which genes do? We consider two possibilities. One alternative is that during the sequential formation of segments, some of the segment polarity genes may interact with one another to generate their pattern of expression one segment at a time. Some of these interactions may still be at work in *Drosophila* where, for example, the maintenance of *engrailed* expression occurs through cellular interactions mediated by the *wingless* gene; this maintenance can be thought of as a re-induction of *engrailed* expression in the germ band (DiNardo *et al.* 1988). This type of cell interaction may be at work in shorter germ band insects to specify the *engrailed* pattern. A second possibility comes from the observation that *engrailed* stripes in the head of *Drosophila* embryos form and are maintained by a mechanism that appears to be independent of at least certain pair-rule genes [for example, *fushi tarazu* is not even expressed in the head (Carroll and Scott, 1985)] and of the *wingless* gene (DiNardo *et al.* 1988). The mechanisms generating *engrailed* stripes in the *Drosophila* head may represent a primitive mode of pattern formation that is utilized in short germ band arthropods.

Whatever molecular mechanism generates *engrailed* stripes in more primitive arthropods, it is reasonable to hypothesize that the evolution of segmentation in more advanced insects involved the co-option of old genes for additional new roles (e.g. the pair-rule genes adding a role during segmentation to their neurogenic function), and thus that the evolution of segmentation in arthropods almost certainly involved changes in the regulation of pre-existing genes in addition to gene duplications.

Crustaceans exhibit a novel manifestation of the parasegment

Dohle (1976), in his study of the crustacean *Diastylis rathkei*, concluded that its segments do not correspond to the genealogical units generated by each ectoteloblast division. We have observed that his descriptions closely match and help explain the patterns of cell arrangements in another crustacean, the crayfish. Interestingly, our analysis of *engrailed* expression suggests that the lineage unit that does exist in the crayfish corresponds to the *Drosophila* parasegment. The progeny of the cells from a single Roman numeral row initially includes one row of cells (*a* cells) that express *engrailed* and three more posterior rows of cells (*b, c,* and *d* cells) that do not. We infer from the manner of cell divisions that follow and from the succeeding patterns of *engrailed* expression, that the descendants of adjacent *a* and *d* rows do not mix. It is the progeny of a single Roman numeral row of cells (the Roman numeral rows in Fig. 7) that constitutes a genealogical unit. Because of the alignment of *engrailed* expression in *Drosophila* and crayfish, we suggest that this unit be designated a parasegment and that the anterior border of *engrailed* expression (the border of *a* and *d* cells) marks the parasegmental boundary. There are several interesting aspects to the crustacean parasegment: (i) it

is a genealogical unit, (ii) the anterior (parasegmental) border of the *engrailed* stripe forms before the posterior (segmental) border; and (iii) thus the parasegment, and the boundaries that define it, appears before the segment and its boundaries. This designation would be consistent with the suggestion of Martinez-Arias and Lawrence (Martinez-Arias and Lawrence, 1985; Lawrence, 1988) that the initial lineage unit in the blastoderm is equivalent to a parasegment, and that the parasegment represents the first sign of metamerization in the embryo.

Several differences exist between the crayfish and *Drosophila* parasegment. In both, parasegments, as defined by the anterior border of *engrailed* expression, represent lineage units. The crayfish lineage unit, however, exists well before *engrailed* expression begins. The progeny of a single Roman numeral row of cells will give rise to a lineage unit (the genealogical units of Dohle). This unit is not visualized by *engrailed* staining until *engrailed* expression begins two cell divisions later, at which time it becomes clear that the lineage units are quivalent to parasegments. No such lineage restriction, however, seems to exist in *Drosophila* prior to the 14th nuclear division which is also the nuclear cycle when *engrailed* stripes form (Karr *et al.* 1989). Thus, the crayfish germ band is made one parasegment at a time as the descendants of each ectoteloblast division populate the parasegments subsequently defined by *engrailed* expression. The invariant lineage pattern in the crayfish might suggest a potential role for lineage in controlling the process of metamerization and the expression of *engrailed*. Alternatively, the lineage pattern might simply be an efficient way to generate a field of cells, with metamerization and gene expression nevertheless being determined by positional information. Determining the relative importance of lineage vs. positional information in the generation of metameric pattern in crustacea will require the use of the *engrailed* MAb and probes for other crayfish segmentation and homeotic genes in conjunction with cell ablation experiments to examine the extent to which regulation can occur.

Thus, in our comparative study of grasshopper, crayfish, and *Drosophila*, we have found two common features – the segmentally repeated pattern of *engrailed* stripes and the early appearance of the parasegmental boundary – which appear to be maintained throughout arthropod evolution. The conservation of these two features despite the dramatic changes in the earlier events leading up to them, suggests to us that the pattern of *engrailed* expression and the establishment of the parasegmental boundary are fundamental events which play a key role in arthropod segmentation.

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