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Early embryo patterning in the grasshopper, *Schistocerca gregaria*: *wingless*, *decapentaplegic* and *caudal* expression

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SUMMARY

Although the molecular pathways that pattern the early embryo of *Drosophila melanogaster* are well understood, how these pathways differ in other types of insect embryo remains largely unknown. We have examined the expression of three markers of early patterning in the embryo of the African plague locust *Schistocerca gregaria*, an orthopteran insect that displays a mode of embryogenesis very different from that of *Drosophila*. Transcripts of the *caudal* gene are expressed maternally and are present in all cells that aggregate to form the early embryonic rudiment. First signs of a posterior-to-anterior gradient in the levels of *caudal* transcript appear in the early heart-stage embryo, shortly before gastrulation. This gradient rapidly resolves to a defined expression domain marking segment A11. The *decapentaplegic* (*dpp*) gene, which encodes a transforming growth factor β family ligand, is first expressed in a circle of cells that delimit the margins of the embryonic primordium, where embryonic and extra-embryonic tissues abut. Patterned transcription of *wingless* reveals that the first segments are delineated in the *Schistocerca* embryo substantially earlier than previously thought, at least 14-16 hours before the onset of *engrailed* expression. By the late heart-stage, gnathal and thoracic segments are all defined. Thus, with respect to the molecular patterning of segments, the short germ *Schistocerca* embryo differs little from intermediate germ embryos. The expression of these marker genes suggests that embryonic pattern formation in the grasshopper occurs as cells move together to form the blastodisc.

Key words: Locust, Wingless, Decapentaplegic, Caudal, Hunchback, Segmentation, Pattern formation, *Schistocerca gregaria*

INTRODUCTION

The study of *Drosophila* molecular development has established that the syncytial character of the early embryo is vital for early patterning. The syncytium allows free diffusion of transcription factors between adjacent nuclei. By contrast, the Acridid grasshopper *Schistocerca gregaria* displays a very different mode of early development, the most obvious difference being the early formation of closed cells during cleavage, before formation of a defined embryonic primordium or blastodisc (Ho et al., 1997). Concomitant with this difference, the molecular basis of pattern formation in this hemimetabolous insect appears to differ from that of *Drosophila* and other holometabolous insects, at least with respect to the expression of several marker genes involved in segmentation (Patel et al., 1992; Dawes et al., 1994; Dearden et al., 2000; Jockusch et al., 2000). Here, we use additional markers to define early patterning in the *Schistocerca* embryo.

The embryonic rudiment in *Schistocerca* forms through the aggregation of cells at the posterior pole of the egg. The zygote nucleus lies beneath the posterior pole of the egg, where it divides to form the primary cleavage energids (nuclei surrounded by islands of cytoplasm but without cell membranes). Some of these energids reach the surface near the posterior pole. Others travel forwards deep in the yolk before surfacing. Thus, the egg surface becomes populated with energids from posterior to anterior. Cell membranes form around energids shortly after they surface (Ho et al., 1997); then many of these cleavage cells migrate back along the egg cortex, coalescing at the posterior pole where they continue to divide to form a circular blastodisc.

The blastodisc becomes visibly asymmetric at 13-15% of development (35-48 hours after egg laying (AEL)), about the same time that gastrulation begins. It develops expanded head lobes at the anterior and an elongated posterior growth zone. This stage is known as the ‘heart-stage’ embryo. Two extra-embryonic membranes, the serosa and the amnion, migrate over and enfold the embryo at this time (Dearden et al., 2000). The posterior part of the embryo then elongates to form the segmented germband.

It is not known to what extent the energids and cells of the grasshopper egg are patterned before they coalesce to form the blastodisc or whether all patterning of the embryo occurs after the blastodisc has formed, through local interactions. The only molecular markers whose expression has been examined during these early cleavage stages are the homologues of the

zerknüllt (*zen*) (Dearden et al., 2000) and *fushi-tarazu* (*ftz/dax*) (Dawes et al., 1994) genes. Zen protein is initially present in all cleavage energids, derived at least in part from maternal RNA. Levels of Zen protein are downregulated, first in aggregating cells at the posterior pole of the egg, before the formation of the blastodisc, and then more anteriorly, in all cells of the forming blastodisc (Dearden et al., 2000). However, this apparent wave of protein regulation has not been positively linked to subsequent patterning. The *Schistocerca ftz* homologue is first expressed in some cleavage energids at approx. 24 hours after egg laying. By the time a circular blastodisc has formed, its expression defines a crescent of cells in the forming posterior growth zone (Dawes et al., 1994).

We now describe the expression of three marker genes that are expressed during these early stages – *caudal*, *wingless* (*wg*) and *decapentaplegic* (*dpp*). These represent three gene families that play roles in the early patterning of many animal embryos, at least some of which seem to be widely conserved.

caudal has been cloned from many metazoan phyla (for examples see Macdonald and Struhl, 1986; Schulz et al., 1998; Bürglin et al., 1989; Frumkin et al., 1991; Gamer and Wright, 1993; Xu et al., 1994). It is a homeobox-containing transcription factor with a conserved role in regulating posterior development (Joly et al., 1992; Hunter and Kenyon, 1996; Epstein et al., 1997; Isaacs et al., 1998).

wg and *dpp* are representatives of two widely conserved families encoding extracellular signalling molecules. In *Drosophila*, *wg* is involved in a range of patterning events, including the maintenance of parasegment boundaries (reviewed by Cohen and DiNardo, 1993; Di Nardo et al., 1994). As an extracellular signalling molecule with a conserved role in arthropod segmentation (Nagy and Carroll, 1994; Nulsen and Nagy, 1999), Wg protein is a candidate for regulating early segmentation in the cellularised grasshopper embryo.

dpp encodes a *Drosophila* representative of the transforming growth factor/bone morphogenetic protein (TGF/BMP) family of signalling proteins. This family is also involved in many developmental processes in insects and other phyla, at least one of which, dorsoventral (D/V) patterning, appears to be widely conserved (reviewed by Hoffmann, 1992; Holley and Ferguson, 1997).

These markers demonstrate patterning of the *Schistocerca* embryo as energids aggregate at the posterior pole and in the early heart-stage. The expression of *wg*, in particular, shows that segment patterning occurs earlier in the *Schistocerca* embryo than has previously been apparent. They allow us to define a molecular fate map for the late heart-stage embryo in which all gnathal and thoracic segments are already defined.

MATERIALS AND METHODS

Cloning

Degenerate primers specific for *caudal* were designed using the methods of Rose et al. (Rose et al., 1998) and used in a RT-PCR reaction (Dearden and Akam, 2000) on mixed stage embryonic poly(A)$^+$ RNA (15-45%). A 167 bp product was amplified, isolated and cloned. Fifteen separate clones were sequenced and found to have identical sequence. Primers were ACCCGCACCAAGGATAAGTA-CMGNGTNGTNTA and CGGCGGTTCTGGAACCADATYTT.

A cDNA library was produced from mixed embryo (20-50%) poly(A)$^+$ RNA in Lambda Zap II (Stratagene) following the manufacturers instructions. 400,000 clones from this library were screened using the methods of Mason and Vulliamy (Mason and Vulliamy, 1995) with the initial *caudal* fragment as a probe. A single positive plaque was isolated and characterised. This clone, SgCAD4114a, was sequenced and found to contain a partial *Sgcaudal* cDNA. The 3′ end of the cDNA is truncated in the centre of the homeodomain, and the start codon is missing. The sequence of this cDNA has been deposited with GenBank accession number, AF374724.

A clone of *wg* from *Schistocerca americana* was kindly provided by M. Friedrich (Friedrich and Benzer, 2000). *Schistocerca americana* is a close sister species to *Schistocerca gregaria*. Most antibodies and in situ hybridisation probes crossreact between these two species.

A clone of *dpp* from *Schistocerca americana* was kindly provided by S. J. Newfeld and W. M. Gelbart (Newfeld and Gelbart, 1995). Nested primers were designed based on this sequence. Primers were CGACGTGCCGCCGATGAG and CCTGCGAATGTGGGGAATG-AAATG for the initial PCR, and CGCTTACACCGACGACAA and CCAGAGATTAAAACGGAGATACG for the nested reaction. *S. gregaria dpp* sequences (GenBank accession number, AF374725) were PCR amplified from plasmid DNA made from mass-excised Lambda Zap II phage from the zygotic library. Mass excision and plasmid DNA preparation were performed according to the manufacturers instructions (Stratagene).

RT-PCR

RT-PCR was performed as per Dearden and Akam (Dearden and Akam, 2000) using *caudal* specific primers (ACCCGCACCAAGGA-CAAGTACCGGGTGGTGTA and CGGCGGTTCTGGAACCAGA-TCTT) or *engrailed* degenerate primers (GGAATTCGARAAYCGI-TAYCTIACIGA and GCTCTAGACGYTTRTTTTGRAACCA). RT-PCR was carried out on poly(A)$^+$ RNA extracted from whole eggs using the polyA pure mRNA isolation kit (Ambion).

In situ hybridisation

In situ hybridisation was performed using the methods of Broadus and Doe (Broadus and Doe, 1995) modified in the following ways: early eggs and embryos (up to 20%) were fixed by pricking whole eggs 20-30 times in 1× phosphate-buffered saline (PBS), 50 mM EGTA, 9.25% formaldehyde with a fine needle and incubating at room temperature for 4 hours. The chorion was then peeled off using fine forceps and the eggs washed in PTw (PBS + 0.1% Tween 20). Heart-stage and extended heart-stage embryos (13-20%) were dissected from the fixed yolk and washed in PTw. Older embryos (20% onwards) were dissected from eggs and fixed in 1× PBS, 50 mM EGTA, 9.25% formaldehyde for 50 minutes, and then washed in PTw. Ovaries were fixed and dissected as previously described (Dearden and Akam, 2000).

Digoxigenin (DIG)-labelled probes were produced by run-off transcription (Dearden and Akam, 2000). Probes (excepting that for *dpp*) were digested to aid penetration by incubation in an equal volume of 120 mM Na_2CO_3, 80 mM $NaHCO_3$, pH 10.2, at 60°C for 40 minutes, and neutralised with 30 volumes of hybridisation buffer.

Tissue was hybridised for 24 hours at 55°C in hybridisation buffer (50% formamide, 4× standard saline citrate (SSC), 5% dextran sulphate, 1× Denhardt's solution, 250 μg/ml tRNA, 0.1% Tween 20, 500 μg/ml ssDNA), and washed six to eight times over 16 hours in 50% formamide, 4× SSC, 0.1% Tween 20, at 55°C.

Detection of bound probe using anti-DIG-AP antibody was performed as described by Broadus and Doe (Broadus and Doe, 1995). Stained preparations were dehydrated and washed in methanol to remove pink coloured staining (Patel, 1994), rehydrated, cleared in 50% glycerol and finally mounted in 70% glycerol.

Images were captured on a Zeiss Microscope using a Coolsnap digital camera and Openlab software (Improvision).

Antibody staining

Antibody staining was carried out with anti engrailed 4D9 (1:1) (Patel et al., 1989), and anti grasshopper Hunchback 7c11 (1:3) (Patel et al., 2001). Antibody staining was performed as described previously (Dearden et al., 2000) and visualised with DAB (Sigma).

Grasshopper husbandry

Grasshopper embryos and ovaries were collected from a culture of *Schistocerca gregaria* maintained at the Zoology Department, University of Cambridge.

Early grasshopper embryos (up to approximately 55 hours AEL) were staged using timed collections of eggs. Eggs in these collections developed at 30°C. Later embryos (15% and onwards) were staged according to the methods of Bentley et al. (Bentley et al., 1979) with reference to Patel et al. (Patel et al., 1989). Embryos at 48-52 hours correspond to the 15% stage (Bentley et al., 1979).

RESULTS

caudal

Fifteen identical 167 bp *caudal*-like sequences were amplified from mixed embryonic stage RNA (15-45%) using RT-PCR. This sequence was used to isolate a 761 bp partial cDNA from an embryonic cDNA library (Fig. 1). This cDNA contained a sequence identical to that of the initial PCR fragments, suggesting that a single *caudal* gene is expressed during early embryogenesis in *Schistocerca*. We designate the gene from which this clone derives *Sgcaudal* (*Sgcad*).

caudal expression

RT PCR reveals that *Sgcad* RNA (hereafter just termed *caudal*) is expressed in the ovary, and in all stages of egg development examined (Fig. 2A). This implies that there is both maternal and zygotic expression of the gene. We do not know when maternally derived transcript is replaced by zygotic.

In the ovaries, in situ hybridisation reveals *caudal* transcript in only the germline (Fig. 3A). Transcript levels are highest in oocytes in the germarium and the first oocytes in the vitellarium (Fig. 3B). Expression is lower in older oocytes. In the oldest oocytes, *caudal* transcripts become cortically located (reflecting the exclusion of cytoplasm from the yolk-packed centre of the egg (Dearden et al., 2000). *caudal* transcripts are never obviously localised along the A/P axis of oocytes.

We have examined the localisation of *caudal* transcripts in whole eggs at 4,18, 30 and 40 hours after egg laying (AEL). In 18 hour eggs (Fig. 3C) and 30 hour eggs (data not shown), *caudal* mRNA is readily detected in all superficial energids. No differential expression is seen. Before energids reach the surface (e.g. at 4 hours AEL) no *caudal* transcript can be detected at the surface of the egg.

By very early heart-stage (38-40 hours AEL), *caudal* transcripts are present in all cells of the blastodisc, but are present at higher levels in the posterior regions of the forming embryo (Fig. 3D). Transcript levels within the blastodisc initially appear to be smoothly graded, but slightly later, as gastrulation occurs, a discontinuity in the levels of *caudal* transcript is apparent near the centre of the embryo (arrowheads in Fig. 3E). We surmise that this discontinuity may be coincident with the posterior boundary of Hunchback expression (Patel et al., 2001), but because *caudal* transcript levels are low at this stage, we have been unable to detect them in double staining with Hunchback.

By late heart-stage (48 hours/15% of development), *caudal* transcripts are undetectable throughout the embryo, except in the most posterior terminal regions of the germ band, where they persist as the germ band extends (Fig. 3F-I). By the time visible segmentation reaches the posterior abdomen, *caudal* expression extends from the parasegment 16 boundary in A10 to the terminus of the embryo in A11 (Fig. 3J,K). Transcripts are initially present in all cells of A11, but as the proctodeum forms, *caudal* expression is reduced in the invaginating cells (Fig. 3L). As the invagination deepens, *caudal* becomes restricted to the anal cerci and a ring of cells at the anterior end of the hindgut, possibly the Malpighian tubule primordia

Fig. 1. Analysis of the *Schistocerca caudal* encoded protein. Alignment of Sgcaudal against metazoan caudal proteins. Shading denotes similarity; boxes indicate identity. A.Ga, *Anopheles gambiae*; B.mori, *Bombyx mori*; D.mel, *Drosophila melanogaster*; G.do, *Gallus domesticus* caudal 1; M.mu, *Mus musculus* caudal 1; S.greg, *Schistocerca gregaria* (*Sgcad*); T.cast1 and T.cast2, *Tribolium castaneum* caudal proteins 1 and 2; X.Lae2, *Xenopus laevis* caudal 2.

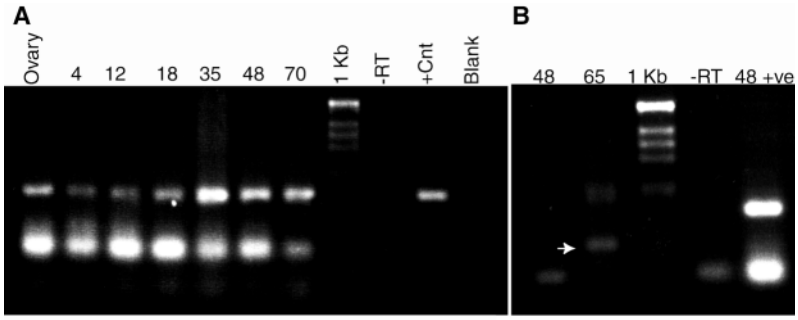

Fig. 2. RT-PCR analysis of *Schistocerca caudal* and *engrailed* expression. (A) Expression of *Sgcaudal* RNA in ovary and early development. *Sgcaudal* is expressed in the ovary, and all stages of embryogenesis shown. Timing is in hours. 1 Kb, calibration ladder; –RT, representative control reaction run without reverse transcriptase enzyme; +Cnt, positive control PCR from plasmid clone; Blank, reaction run with no template. Amounts of PCR product are not representative of amounts of *Sgcaudal* RNA in each stage. The lower band in the experimental lanes is a spurious product produced by using these primers on cDNA. Comparison with the positive control lane identifies the upper band as *Sgcaudal* transcript. (B) Expression of *engrailed* RNA at two stages of embryogenesis, 48 hours AEL (late heart-stage, 15% development) and 65 hours AEL. *engrailed* RNA is detected at 65 hours (arrow) but not 48 hours. –RT, control reaction run with no RT enzyme (on 65 hour RNA); 48 +ve, RT-PCR reaction performed on 48 hour RNA with caudal primers.

(arrow in Fig. 3L). This pattern is almost complementary to that of *wg* in A11 (Fig. 3M).

dpp

We have used a probe made from a *Schistocerca americana* clone of *dpp* to detect *dpp* expression in *S. gregaria* (Fig. 4). Hybridisations using this probe were found to give the same pattern as those performed using a small region of the *Sgdpp* gene isolated from our embryonic cDNA library (data not shown).

At heart-stage (15%) *dpp* is expressed at high levels in a ring of cells with large nuclei that surround the forming embryo (Fig. 4A-C). The position and size of the nuclei in these cells identifies them as the necklace cells, which also express *Sgzen* (Fig. 4B; Dearden et al., 2000). These are the marginal cells of the serosa that migrate over the embryo between 15 and 17% of development. Expression persists in these cells until 20% of development.

Between 15% and 16%, *dpp* is also transiently expressed in a u-shaped patch in the posterior of the germband (Fig. 4C), and diffusely in the head lobes (arrow). Later, from about 20% of development, *dpp* is re-expressed in the thoracic segments and in a one-cell wide strip around the margins of the gnathal and thoracic regions of the embryo, in cells that will form the dorsal regions of the embryo (Fig. 4D; Jockusch et al., 2000).

wg expression during segmentation

We had expected that segmental stripes of *wg* would first appear around the same time as engrailed protein is expressed, in 58-60 hour (17%) embryos. However, well-resolved stripes of *wg* RNA appear much earlier than engrailed protein, in early heart-stage embryos (42-44 hours). Perhaps more surprisingly, the first stripe of *wg* does not appear in the prothorax, which is where engrailed protein is first expressed, but in the mandibular segment (see below).

wg transcripts can first be localised in early heart-stage embryos (42-44 hours). They define bilateral patches of cells in the head lobes, a patch of cells at the posterior of the germ band, and a single stripe of cells across the embryo that lies 5-10 cell diameters posterior to the stomodeum (star in Fig. 5A). The characteristics of this initial stripe differ in several respects from those that appear later. It runs across almost the entire width of the blastodisc and it is distinctly curved (Figs 5A,B).

Three additional stripes rapidly appear posterior to the initial stripe (Fig. 5B) to define the thoracic segments (see below). These stripes at first contain only single rows of *wg*-expressing cells and are almost straight. As the posterior region of the embryo grows out to form the extended heart-stage, these three stripes widen to encompass three to four cell rows, and two more thin stripes (two cells wide) appear between them and the initial stripe of *wg* (Fig. 5C). The pattern remains with just these six stripes until at 17% an additional bilateral pair of stripes appears in the head lobes (Fig. 5D). At 18%, *wg* stripes begin to appear in the abdomen in anteroposterior sequence (Fig. 5E).

The first six stripes of *wg* are continuous across the midline, but quickly separate into domains on either side of the midline (Fig. 5B,C). Abdominal stripes form as two domains originating near the midline and extending laterally (Fig. 5E). We have observed no pair-rule modulation in the appearance of these stripes.

Engrailed protein (as determined by the 4D9 cross-reacting antibody) first appears in the prothoracic segment at 17% of development (Patel et al., 1989). Double staining for Engrailed protein and *wg* RNA demonstrates that expression of *engrailed* starts just posterior to the fourth stripe of *wg*, identifying this as the prothoracic stripe (parasegment 3; Fig. 5F). This enables us to assign segment identity to the other stripes of *wg*. The initial stripe of *wg* becomes the posterior of parasegment 0, in the mandibular segment. The next three stripes to appear are in the thoracic segments (parasegments 3-5). The maxillary and labial stripes then form anterior to the prothoracic segment. Finally, antennal and abdominal stripes form (Fig. 5E). In the abdomen, stripes of *wg* RNA form before stripes of Engrailed protein, running two to three segments ahead of Engrailed (Fig. 5G).

Because the precocious expression of *wg*, so long before *engrailed*, was unexpected, we tested the possibility that another *engrailed* gene might be expressed in the early heart-stage *Schistocerca* embryo, producing a protein that is not detected by the 4D9 cross-reacting antibody. However, RT-PCR using degenerate primers for *engrailed* genes failed to detect the expression of any *engrailed* gene at 48 hours AEL (15% of development; Fig. 2B). When the same experiment was repeated at 65 hours AEL, a band of the correct size for *engrailed* was amplified. This band was cloned, and found to contain a sequence identical to that of the *engrailed* gene already cloned from *S. americana*.

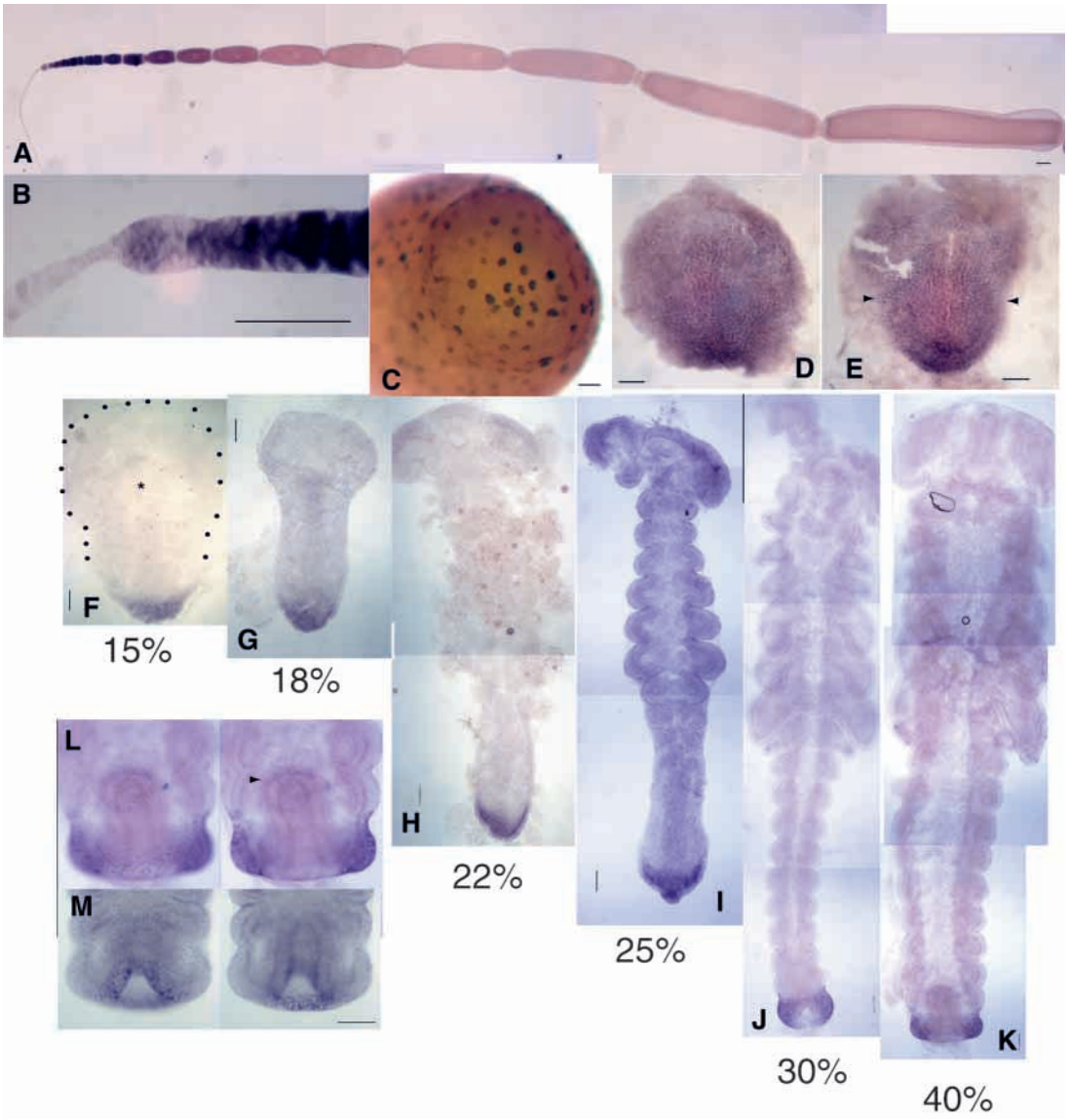

Fig. 3. Expression of *Sgcaudal* during embryogenesis. (A) Ovariole hybridised for *caudal* RNA. *caudal* RNA is expressed strongly in the germarium and early vitellarium (left), but staining becomes weaker as oocytes mature. In the most mature eggs, *caudal* RNA is cortically located. (B) Close up of ovariole seen in A, *caudal* RNA can be seen in oocytes as they form next to the terminal filament. (C) Egg at 18 hours. *caudal* RNA is expressed in energids as they move to the posterior pole. Counter staining for DNA shows that all superficial energids express *caudal* at this stage (data not shown). (D) Expression of *caudal* RNA in late blastodisc embryo (36-38 hours APF; slightly damaged at posterior (bottom)); *caudal* expression is graded: low at the anterior, high at the posterior. (This embryo has not been treated with methanol after staining to ensure faint expression is detected, hence the pink colour.) (E) Expression of *caudal* RNA in early heart-stage embryo (38-40 hours APF). The gradient has a discontinuity forming a curved boundary between higher levels (posterior) and lower levels (anterior; arrowheads). Expression is stronger in the posterior around the terminus of the embryo. (This embryo has also not been treated with methanol.) (F) Expression of *caudal* at 15% development (48 hours). *caudal* RNA is restricted to a posterior domain at the end of the germ band. Dots demarcate the anterior boundary of the embryo. Star marks the stomodeum. (G-I) Expression of *caudal* at 18% (G), 22% (H) and 25% (I) of development. *caudal* RNA is present in a small posterior domain and absent from the rest of the embryo. (J,K) Expression of *caudal* at 30% development (J) and 40% (K). As segmentation finishes, *caudal* is expressed in the posterior of A10 and in A11. (L) Two focal planes of an enlargement of K showing A11 and the invaginating proctodeum. *caudal* RNA is absent from the invaginating regions of the proctodeum, and present only in the margins of the segment. Faint expression is also seen in a ring of cells at the anterior end of the invaginating hindgut (arrowhead), possibly the Malpighian tubule primordium. (M) Expression of *wg* RNA in A11 and the proctodeum at 40% development (two focal planes). *wg* is expressed in a ring around the proctodeal invagination, a pattern almost complementary to that of *caudal*. Scale bars: 100 μm.

We also defined the position of the *wg* stripes in relation to the early expression of Hunchback protein in *Schistocerca*. In heart-stage embryos, high levels of Hunchback protein are expressed in a broad crescent of cells crossing the embryo, and in the presumptive serosa (described for *Schistocerca americana* by Patel et al., 2001). Lower levels are also present in the head, and just posterior to the crescent of high expression (Patel et al., 2001). The lower level domains are not detectable

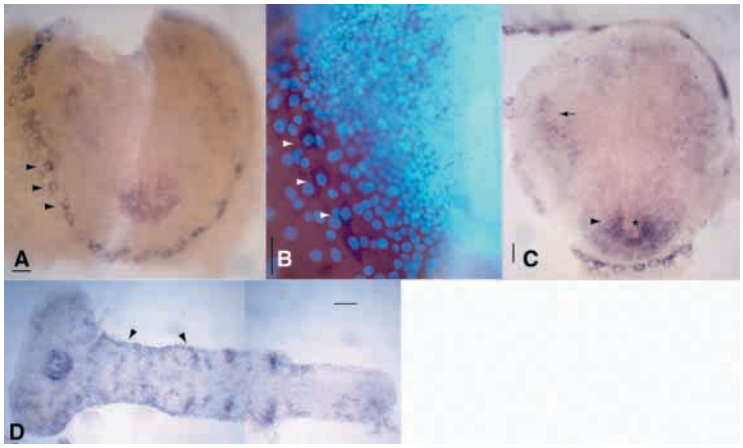

Fig. 4. Expression of *dpp* during embryogenesis. (A) Expression of *dpp* RNA in heart-stage (48 hours/15%). *dpp* RNA is present in a ring of cells that run around the embryo. Arrowheads mark three such cells. (B) Close up view of the specimen in A, stained with Hoechst 33342, to show the *dpp* staining cells. These are likely to be the necklace cells (Dearden et al., 2000). Arrowheads mark the same cells shown in A. (C) A similar stage to A, but with clearer expression in the U-shaped patch (arrowhead) around the posterior end of the gastrulation furrow (star). Diffuse expression is also seen in the head lobes (arrow). (D) Expression of *dpp* RNA in a 20% embryo. *dpp* RNA is located in two pairs of stripes located laterally in each appendage bearing segment, and in a single longitudinal row of cells at the lateral margin of the germ band in the gnathal and thoracic region (arrowheads). Scale bars: 100 μm.

in our specimens, probably because of reduced sensitivity caused by performing antibody staining after in situ hybridisation. The initial *wg* stripe forms at the anterior edge of the high expression Hunchback domain from Hunchback-expressing cells (Fig. 5H,I). Hunchback expression is quickly lost, however, from the *wg*-expressing cells. As the *wg* stripe broadens it remains abutting the anterior edge of the Hunchback stripe.

The first thoracic stripe of *wg* appears posterior to the crescent of Hunchback and is not directly apposed to it (Fig. 5H,J). The two gnathal stripes appear entirely within the crescent of high Hunchback expression (Fig. 5J,K). Thus, the high Hunchback expression domain stretches from the boundary of parasegment 0 to beyond the posterior of parasegment 2. Its posterior limit lies at, or close to, the boundary between the labial segment and the first thoracic segment. High levels of Hunchback are thus expressed in gnathal, rather than thoracic regions. Hunchback is also expressed in gnathal regions in *Tribolium* (Wolff et al., 1995).

Other domains of *wg* expression

In the heart-stage embryo (15%), *wg* is also expressed in paired regions in the headlobes, in the posterior of the gastrulation furrow, and faintly in the newly formed mesoderm (Fig. 5A). The paired domains of *wg* expression in the head lobes of the heart-stage embryo go through a number of shape changes during early development. Comparison with the expression of *wg* in the *S. americana* head (Friedrich and Benzer 2000) suggests that these patches mark the developing eyes.

Expression of *wg* in the posterior of the gastrulation furrow continues throughout early development, and continues as this region forms the proctodeum. *wg* is initially expressed in a domain overlapping that of *caudal* in the proctodeum. After 30%, *caudal* is downregulated in the regions expressing *wg* (Fig. 3M).

DISCUSSION

The molecular markers that we have cloned provide no evidence that cleavage energids are patterned at the time when they emerge at the egg cortex. The two maternally expressed genes that we have examined, *zen* (Dearden et al., 2000) and *caudal*, are uniformly expressed in all superficial energids. However, pattern emerges as cells aggregate to form the blastodisc. By the late heart-stage (48 hours/15%), the patterns of expression of *wg*, *caudal*, *dpp* and *hunchback* indicate that major divisions of the grasshopper germ band have been established and segments of the gnathum and thorax are already defined. In this respect, the molecular markers reveal a pattern more akin to that expected for an ‘intermediate germ embryo’, than that predicted for a ‘short germ’ embryo (see below).

These markers allow us to propose fate maps for both the early and late heart-stage embryos (Fig. 6). The embryonic primordium is bounded by a ring of cells that express *dpp*, and probably Zen protein (the necklace cells, Dearden et al., 2000). The entire pattern of the embryo and its associated amnion is generated within this boundary, while the necklace cells themselves and those that lie outside it will detach from the embryo to form the serosal membrane (Dearden et al., 2000). Within the necklace, a crescent containing high levels of Hunchback protein demarcates the future gnathal territory. At early heart-stages, *caudal* expression extends throughout the presumptive thorax and abdomen, but by late heart-stages, *caudal* has already retracted to a small posterior region. Segmental stripes of *wg* show that, at this stage, most cells of the growth zone are fated to form thorax. The abdomen is represented only by the *caudal*-expressing region and a small territory anterior to it.

Patterning the A/P axis of the embryo

The first sign of A/P patterning that we see in the grasshopper embryo is the graded expression of *caudal* transcript in the very early heart-stage embryo.

The appearance of this gradient is no surprise – RNA concentration gradients of *caudal* have been described in all insect species examined (Xu et al., 1994; Schulz et al., 1998). However, the relatively late appearance, compared with, for example, the gradient established during cleavage stages in *Drosophila* (Macdonald and Struhl, 1986), suggests that A/P polarity of the embryo may be specified during aggregation, and not maternally or during cleavage. A/P polarity of the egg must be specified earlier, during oogenesis, but at early stages, the A/P axis of the embryo lies perpendicular to this axis.

At 38-40 hours AEL, a discontinuity in the *caudal* gradient becomes visible, separating the head lobes, with low levels, from a more posterior region, with higher levels. This is the

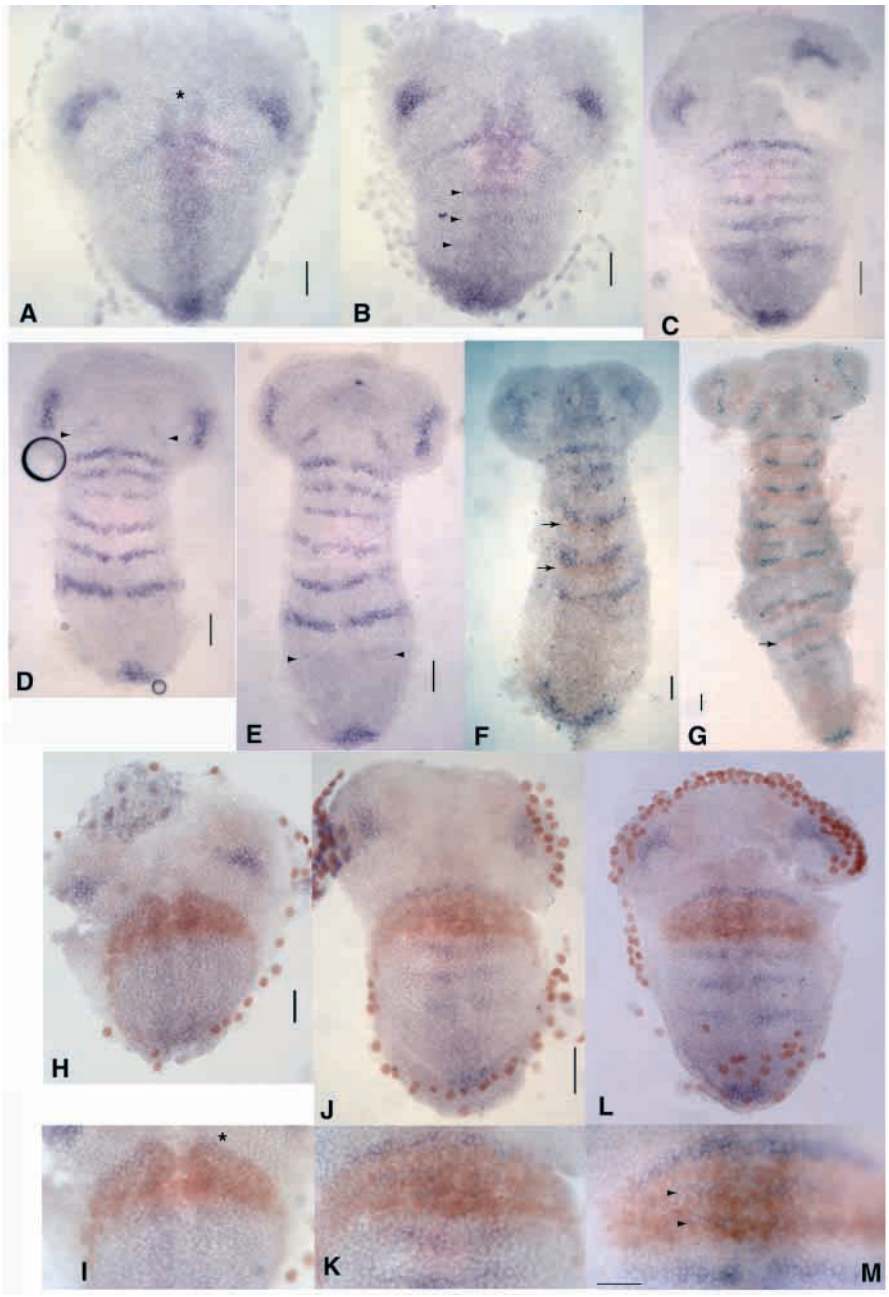

Fig. 5. Expression of *wg* during embryogenesis and comparison with other markers. (A-C) Expression of *wg* RNA at 44 hours (A), 48 hours (15%) (B), 50 hours (C). *wg* first appears in paired domains in the protocephalon, in a single band across the embryo (marking the parasegment 0/1 boundary), faintly in the mesoderm, and in a posterior domain (A). Three thoracic stripes, posterior to the initial one, appear soon afterwards (arrowheads in B). These stripes thicken, and two more stripes (gnathal) appear between them and the initial stripe (C). (D) Expression of *wg* at 17%. *wg* RNA is present in the head lobes and proctodeum, and in six stripes across the germ band, representing the gnathal and thoracic parasegment boundaries. Expression is just starting to appear in the antennal segment (arrowheads). (E) Expression of *wg* at 18%. *wg* RNA is just appearing in the A1 primordia (arrowheads). (F) Expression of *wg* (blue) and engrailed protein (brown) at 17% (same stage as D). Engrailed protein is detectable only in the pro- and mesothoracic regions (arrows). (G) *wg* RNA (blue) and engrailed protein (brown) in a 25% embryo. *wg* RNA is present in A1, A2 and A3. Engrailed expression has only reached A1 (arrow). (H) Expression of *wg* RNA (blue) and Hunchback protein (brown) in an early heart-stage embryo (44 hours). The cells expressing *wg* (marking parasegment 0/1) are the most anterior Hunchback-expressing cells. Hunchback protein is also expressed in the large nuclei of the serosa. (I) Close up of H. Note Hunchback and *wg* co-expressing cells (star). (J) *wg* RNA (blue) and Hunchback protein (brown) in a 48-hour embryo. The thoracic stripes of *wg* lie posterior to the Hunchback domain. (K) Close up of J. Note cells at the parasegment 0/1 boundary express *wg* and not Hunchback. (L) *wg* RNA (blue) and Hunchback protein (brown) in a 50 hour embryo. *wg* RNA is now present in two gnathal stripes inside the Hunchback domain. (M) Close up of L. Note gnathal stripes of *wg* inside the Hunchback domain (arrowheads). Scale bars: 100 μm.

first specific A/P subdivision of the germband that our markers reveal. We surmise that this boundary may abut the posterior boundary of a domain that expresses high levels of Hunchback protein (Patel et al., 2001). In *Schistocerca americana*, Hunchback protein is initially widely expressed in the embryonic primordium, but is cleared from the posterior of the embryonic primordium at the same time as it accumulates to high levels in the gnathal crescent (Patel et al., 2001). It is possible that Hunchback protein is regulating the accumulation of *caudal* transcript at the late heart-stage (and/or vice versa), and perhaps also the earlier graded expression.

D/V patterning and extra-embryonic membranes

In all insects studied, *dpp* is involved in D/V patterning of the embryo. *dpp* is expressed at the dorsolateral edges of the germband, where in *Drosophila* it is required to establish the normal pattern of cell types along the D/V axis of each segment. A very similar pattern of expression is seen in *Schistocerca* embryos, suggesting that this role may be conserved in hemimetabolous insects.

This dorsal expression first appears at 20% development, but we know that the primary D/V axis of the embryo must be specified long before this. In the embryonic primordium of *Schistocerca*, the most ventral structure (the mesoderm) forms along the midline of the disc, and dorsal structures form at its lateral edges. We do not know when mesoderm is first specified, but it must be before the onset of gastrulation in mid heart-stage embryos.

During and shortly after gastrulation, *dpp* is expressed at high levels in the necklace cells that surround the embryo, and

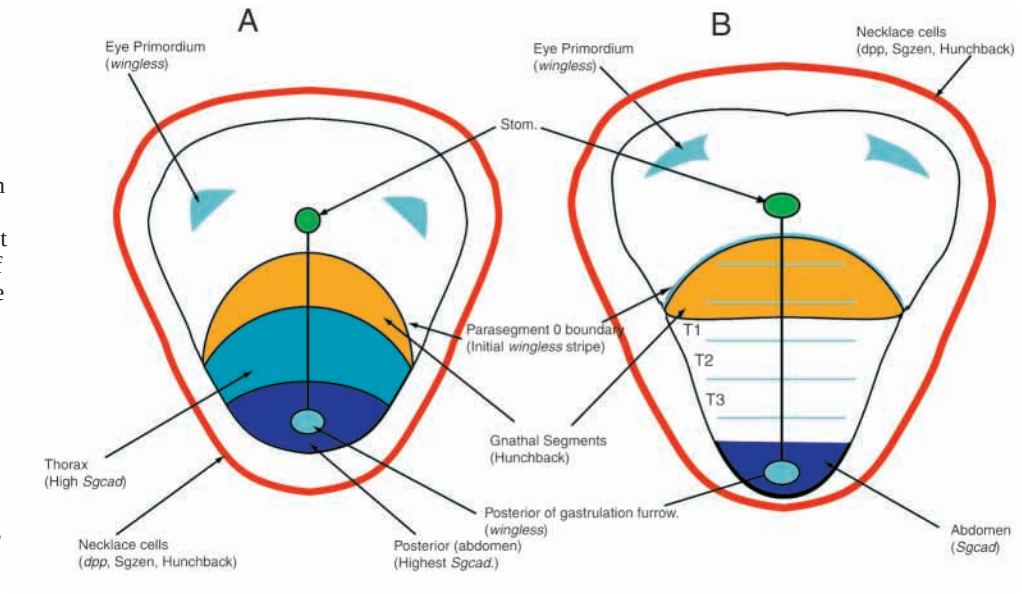

Fig. 6. Fate map of heart-stage embryos derived from the examination of *Sgcaudal*, *wg* and *dpp* expression patterns. The embryo is shown as a flat projection viewed from the position of the pole of the egg with the dorsal side of the egg (anterior of the embryo) uppermost. Stom., Stomodeum. (A) Early heart-stage embryo (38-40 hours APF). The position of the anterior edge of the high *caudal*-expressing domain is not precisely defined. It has been drawn as abutting the Hunchback domain, but may not. (B) Late heart-stage embryo (48 hours APF/15% development).

at lower levels in the lateral parts of the head lobes and around the proctodeum. *dpp* RNA is not detectable in mid-ventral regions. This is consistent with the possibility that this earlier *dpp* expression also mediates some aspects of D/V patterning in the heart-stage embryo, but it seems unlikely that *dpp* expression in the necklace cells is responsible for the initial distinction between embryonic and extra-embryonic tissue. Both *dpp* RNA and Zen protein (Dearden et al., 2000) accumulate specifically in the necklace cells, suggesting that the distinction between embryonic and extra-embryonic tissue is specified by some earlier patterning interaction, and that *dpp* expression at the boundary is a consequence of this patterning event. Patel et al. (Patel et al., 2001) have suggested that maternally synthesised Hunchback protein may mediate this distinction.

In the blastoderm of *Drosophila*, high levels of *dpp* activity are required to sustain the expression of *zen* in the amnioserosa (Ray et al., 1991); lower levels of *dpp* activity are sufficient to specify dorsal versus neurogenic epidermis. The observed distribution of *dpp* RNA in *Schistocerca* would be consistent with the conservation of these roles, and with a conserved regulatory link between Dpp protein and the *zen* gene in extra-embryonic membranes.

Coincident early expression of both *dpp* and *zen* in the serosa has also been observed in the beetle *Tribolium* (Sanchez Salazar et al., 1996; Falciani et al., 1996), but both here and in *Drosophila* the two genes are initially broadly expressed throughout a dorsal or anterodorsal cap of the egg; there is nothing resembling the local activation seen in the necklace cells of *Schistocerca*. In the case of *Drosophila*, we know that *zen* and *dpp* are regulated by broad maternal gradients; the same may be true in *Tribolium*. In the case of *Schistocerca*, it seems more likely that local cell interactions trigger initial expression of both genes in the serosa.

Segmentation

The pattern of *wg* expression in *Schistocerca* provides evidence that all gnathal and thoracic segments are patterned in the heart-stage embryo, considerably earlier than previously thought. Rows of *wg*-expressing cells appear 14-16 hours (3-4% of development) before the first detectable expression of Engrailed protein, and at a time when no *engrailed* RNA can be detected by RT-PCR. These *wg* stripes persist to stages when *engrailed* is activated, allowing us to be sure that this early pattern is segmental, not pair-rule, and confirming that *wg* is expressed in cells that come to lie immediately anterior to the parasegment boundary, as in *Drosophila* and other insects.

Insect developmental regimes are traditionally divided into three groups, short germ (like *Schistocerca*), intermediate germ (like *Tribolium*) and long germ (like *Drosophila*). Current phylogenies imply that intermediate germ band insects represent the ancestral state for insect development (Tautz et al., 1994). In intermediate germ insects, the head, gnathal, and thoracic regions are defined in the blastoderm, with only the abdominal segments forming from a posterior growth zone. This description applies equally to *Schistocerca* based on the expression of the *wg* gene. The heart-stage embryo, despite being morphologically short germ band, has the whole complement of segments that constitute an intermediate germ band embryo.

With the exception of the first stripe, the patterning of *wg* expression in the trunk proceeds in the same sequence as that of engrailed, stripes of both appearing first in the prothorax, and then spreading to more anterior and posterior regions. This progression is a molecular reflection of the observation that, in Orthoptera, the prothorax constitutes a differentiation centre, from which pattern spreads both anteriorly and posteriorly (reviewed by Anderson, 1973; Sander, 1976). In *Tribolium*, *wg* and *engrailed* stripes form in strict anterior to posterior sequence, from the mandibular (PS 0) segment backwards (Nagy and Carroll, 1994).

In *Schistocerca*, the most anterior *wg* stripe (parasegment 0) is exceptional. It forms before any other, even though the accompanying stripe of engrailed is delayed until after patterning of the thorax. It forms in register with the very early expression of Hunchback protein and, like Hunchback, spans the whole width of the embryonic primordium, not just the medial zone within which segmentation will emerge. For all these reasons, we suspect that this initial expression of *wg* is not simply associated with segment formation, but may reflect

a unique early process – possibly the subdivision of the embryo into the procephalon and the regions of overtly segmented germband ('trunk').

We propose that there may be two distinct segmentation mechanisms in the *Schistocerca* trunk. In the gnathal/thoracic region, segments form within a pre-existing field of cells, almost simultaneously and not in linear sequence. This suggests that the underlying mechanism is analogous to the gap segmentation mechanism in *Drosophila*, where a prepattern of aperiodic signals instructs the formation of each parasegment. Hunchback may provide one component of these signals. In the abdomen, segmentation proceeds more slowly, within a growing field of cells, and in strict A/P sequence; the patterning of each segment follows that of its anterior neighbour. This suggests that pattern may be generated by a process of cell-cell interaction that is reiterated for each segment (or segment pair, see below), much as happens during vertebrate somitogenesis. However, no candidate mechanism has yet been identified for such a process; transcriptional regulation of *Notch* and *fringe* does not appear to be involved (Dearden and Akam, 2000).

Until recently, there was no evidence that a pattern of double segment periodicity preceded definitive segment formation in *Schistocerca*. Homologues of two *Drosophila* pair-rule genes, *fushi-tarazu* and *even-skipped*, have been cloned from *Schistocerca*; neither is expressed in pair-rule stripes (Dawes et al., 1994; Patel et al., 1992). However, Davis et al. (Davis et al., 2001) have now shown that a *Schistocerca paired* homologue is an early marker for segmentation, both in the thorax and the abdomen. Moreover, like its *Drosophila* counterpart, it is expressed transiently in stripes with a double segment periodicity, before each of these resolves into two segmental stripes (Davis et al., 2001). Thus, the initial step in pattern generation, whether instructed by a 'gap' type mechanism, or a reiterated oscillator, must presumably generate double segments.

A posterior patterning focus?

In late heart-stage *Schistocerca* embryos, the patterns of *caudal*, *wg* and Hunchback expression imply that the major divisions of the germ band have been defined. For Hunchback and *wg* it is particularly striking that their initial expression is bounded by an arc apparently centred near the posterior of the blastodisc. These patterns provide some support for the idea that a posterior patterning focus instructs early A/P pattern in the *Schistocerca* embryo, perhaps by inductive signalling.

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