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Pax group III genes and the evolution of insect pair-rule patterning

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SUMMARY

Pair-rule genes were identified and named for their role in segmentation in embryos of the long germ insect *Drosophila*. Among short germ insects these genes exhibit variable expression patterns during segmentation and thus are likely to play divergent roles in this process. Understanding the details of this variation should shed light on the evolution of the genetic hierarchy responsible for segmentation in *Drosophila* and other insects. We have investigated the expression of homologs of the *Drosophila* Pax group III genes *paired, gooseberry* and *gooseberry-neuro* in short germ flour beetles and grasshoppers. During *Drosophila* embryogenesis, *paired* acts as one of several pair-rule genes that define the boundaries of future parasegments and segments, via the regulation of segment polarity genes such as *gooseberry,* which in turn regulates *gooseberry-neuro,* a gene expressed later in the developing nervous system. Using a crossreactive antibody, we show that the embryonic expression of Pax group III genes in both the flour beetle *Tribolium* and the grasshopper *Schistocerca* is remarkably similar to the pattern in *Drosophila*. We also show that two Pax group III genes, *pairberry1* and *pairberry2,* are responsible for the observed protein pattern in grasshopper embryos. Both *pairberry1* and *pairberry2* are expressed in coincident stripes of a one-segment periodicity, in a manner reminiscent of *Drosophila gooseberry* and *gooseberry-neuro. pairberry1,* however, is also expressed in stripes of a two-segment periodicity before maturing into its segmental pattern. This early expression of *pairberry1* is reminiscent of *Drosophila paired* and represents the first evidence for pair-rule patterning in short germ grasshoppers or any hemimetabolous insect.

Key words: Pax, *Drosophila*, *Schistocerca*, *Tribolium*, Grasshopper, Paired, Gooseberry, Segmentation, Pair-rule, Pattern formation

INTRODUCTION

We currently know a great deal about how the *Drosophila* embryo becomes progressively subdivided into its future body segments. Gradients of maternal information act at the top of a genetic hierarchy that involves the sequential activation of the zygotic gap, pair-rule, and segment polarity genes. In phylogenetically derived long germ insects such as *Drosophila,* this genetic hierarchy functions to define and pattern all segments almost simultaneously within the blastoderm. In short germ insects, however, only segments of the head are defined in the initial blastoderm, while the remaining segments of the thorax and abdomen form progressively from a posterior growth zone (Sander, 1976). Thus, crucial questions arise as to which components of the *Drosophila* segmentation hierarchy are shared by different short germ insects and how they might function in the short germ context.

To better understand segmentation in short germ insects, we have chosen here to focus on homologs of the pair-rule and segment polarity class of segmentation genes in flour beetles and grasshoppers. Segment polarity genes were originally defined by their loss-of-function phenotypes in *Drosophila,* which reveal patterning defects within each segment of the embryonic cuticle (Nüsslein-Volhard and Wieschaus, 1980). Consistent with their phenotypes, most of these genes are expressed in *Drosophila* just before and throughout the morphologically segmented germ band stage in a segmentally reiterated pattern. The segment polarity genes that have been most widely studied in other insects are *engrailed* (*en*) and *wingless* (*wg*). In *Drosophila*, each is expressed as a single ectodermal stripe within each individual segment, defining the anterior and posterior boundaries, respectively, of each parasegment. These same patterns have thus far been found in all insects examined (reviewed by Patel, 1994a; Dearden and Akam, 2001), suggesting that segment polarity genes constitute part of the ancestral insect segmentation system. Indeed the role of these genes in patterning segments is likely to be ancient, as they are also expressed in segmental stripes in embryos of non-insect arthropods such as crustaceans (reviewed by Patel, 1994b; Nulsen and Nagy, 1999) and spiders (Damen et al., 1998).

In contrast to segment polarity genes, pair-rule homologues tend to exhibit more divergent patterns. Pair-rule genes were also originally defined by their loss-of-function phenotypes in *Drosophila*, in which regions of the embryonic cuticle are deleted with a two-segment periodicity (Nüsslein-Volhard and

Wieschaus, 1980). The three that have been widely studied outside of *Drosophila* are *even-skipped* (*eve*), *hairy* (*h*), and *fushi-tarazu* (*ftz*). In *Drosophila*, all three of these genes are expressed in stripes of a two-segment periodicity before the onset of gastrulation. After gastrulation, *eve* is expressed segmentally with refined late stripes in odd-numbered parasegments and weaker so-called minor stripes in even-numbered parasegments (Frasch et al., 1987). The expression of all three of these genes is conserved in the short germ flour beetle *Tribolium* (reviewed by Patel, 1994a). In the case of *eve*, its pair-rule function appears to be conserved as well, in that chromophore-assisted laser inactivation of Eve protein results in a pair-rule phenotype (Schröder et al., 1999). A deletional mutant of the *Tribolium* Hox complex that includes the *ftz* ortholog, however, does not exhibit any pair-rule defects (Stuart et al., 1991), indicating that despite its two-segment periodicity, this gene appears to be functioning differently from its *Drosophila* ortholog. Additionally, genetic screens in *Tribolium* have produced at least two, possibly three, mutants that display pair-rule phenotypes (Maderspacher et al., 1998; Sulston and Anderson, 1996; Sulston and Anderson, 1998).

In the short germ grasshopper *Schistocerca* – a more distant relative of *Drosophila* – evidence of pair-rule patterning has instead proven elusive. *eve* and *ftz* orthologs are reportedly not expressed in periodic stripes in the early embryo, but in broad posterior domains (Dawes et al., 1994; Patel et al., 1992). This suggests that *eve* and *ftz* might play altogether different roles in grasshoppers, and raises the possibility that these insects might manage to define and pattern their segments without the use of pair-rule patterning. Alternatively, grasshoppers might use a form of pair-rule patterning that uses only some of the *Drosophila* pair-rule genes.

We have investigated the embryonic expression of homologs of the *Drosophila* pair-rule gene *paired* (*prd*), the segment polarity gene *gooseberry* (*gsb*) and *gooseberry-neuro* (*gsbn*), a gene that is expressed in the developing nervous system, but whose function has not yet been defined (reviewed by Noll, 1993). These three fly genes are the products of two duplication events, the first of which gave rise to *prd* and an ancestral *gsb/gsbn* gene, which then subsequently duplicated, giving rise to *gsb* and *gsbn*. Together with their vertebrate homologues *Pax3* and *Pax7*, the three genes belong to Pax group III (PgIII), one of at least four subgroups of the Pax family of transcription factors (Balczarek et al., 1997) whose members all possess both a paired domain (PD) and an extended S_{50} paired-like homeodomain (HD).

In spite of their different roles in *Drosophila*, the Prd, Gsb and Gsbn proteins appear to be interchangeable with regard to patterning the embryonic cuticle and nervous system. The ubiquitous expression of each gene results in a similar cuticular phenotype. Furthermore, when placed under the control of *gsb cis*-regulatory elements, the coding region of *prd* is capable of rescuing *gsb*- defects in both the cuticle and the expression of target genes (Li and Noll, 1994). The coding region of *gsb*, when placed under the control of *prd cis*-regulatory elements, is likewise able to rescue a *prd* deficiency (Xue and Noll, 1996). Thus, differences in the roles played by *prd* and *gsb* in segmentation (as well as *gsbn* in the developing nervous system) appear to derive solely from their different *cis*-regulatory systems and their resulting differential expression.

In *Drosophila*, Prd protein is found in seven primary stripes of a two-segment periodicity at the onset of cellularization,

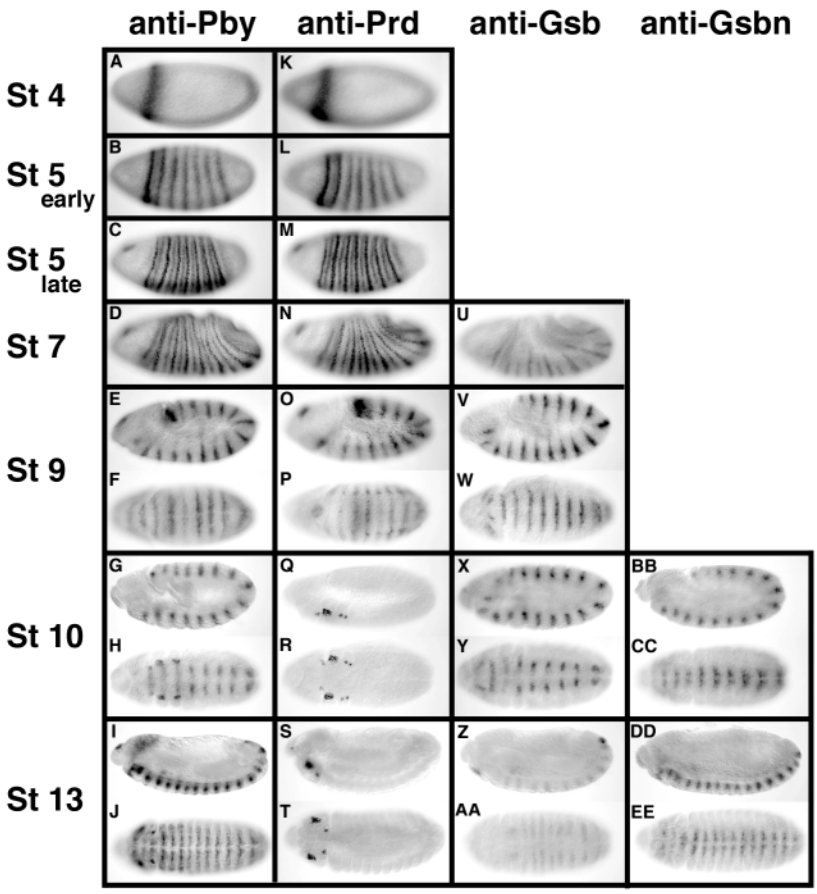

Fig. 1. Anti-Pby crossreacts with Prd, Gsb and Gsbn. *Drosophila* embryos are stained with anti-Pby (A-J) and gene-specific monoclonal antibodies (mAbs) against Prd (K-T), Gsb (U-AA) and Gsbn (BB-EE). (A-J) As anti-Pby reacts to Prd, Gsb and Gsbn protein, the Pby pattern is a fusion of the Prd, Gsb and Gsbn patterns, as well as an additional glial pattern appearing at stage 13. (K-T) At stage 4 Prd is expressed in an anterior broad stripe (K), which itself resolves into stripes 1 and 2 as it is joined by stripes 3-7 (L). During stage 5, the primary stripes resolve into a segmental pattern of 14 secondary stripes of alternating intensity as an anterior dorsal domain appears (M). By stage 10, Prd stripes are mostly absent, but the protein persists in the gnathal protuberances (Q-T). (U-AA) At stage 7, Prd and Gsb are expressed in a coincident pattern (N,U), although Gsb stripes persist through stage 10, at which time they restrict to the neuroectoderm (X-Y). (BB-EE) Gsbn is expressed in a defined neuroectodermal pattern at stage 10, and persists after the neural stripes of Gsb have diminished. The Prd-specific MAb DP201 has not been previously described. Staining in the gnathal protuberances (Q-T) is also present in G,I but is not apparent because focal planes are sagittal. Anterior is towards the left and all views are lateral, except F,H,J,P,R,T,W,Y,AA,CC,EE, which are ventral views.

consistent with its role as a pair-rule gene (Gutjahr et al., 1993a). The stripes, each approximately six cell rows wide, are centered on even-numbered parasegments and extend across both anterior and posterior parasegmental boundaries (Fig. 1L). By mid-cellularization the protein is upregulated in the posterior region of stripes 2-7, resulting in a step pattern within each primary stripe. Beginning at mid-gastrulation repression of *prd* in the middle two cell rows of stripes 2-7 results in the 'splitting' of these stripes. Before germ band extension, this splitting process, together with the narrowing of stripe 1 and the addition of an eighth posterior stripe, results in 14 secondary stripes of alternating intensity, each approximately two cell rows wide (Fig. 1M). These secondary *prd* stripes correspond to parasegmental boundaries: the anterior cell row expresses *wg*, while the posterior cell row expresses *en* (Gutjahr et al., 1993a). Consistent with this pattern of co-expression, *prd* is required for the activation of both *wg* and *en* in the odd-numbered stripes (DiNardo and O'Farrell, 1987; Ingham and Hidalgo, 1993). *prd* is also required for the activation of the odd-numbered stripes of *gsb* (Baumgartner et al., 1987).

gsb was originally identified for its role in patterning the epidermis, as evidenced by the loss of naked cuticle in mutant embryos (Nüsslein-Volhard and Wieschaus, 1980). This cuticle phenotype appears to be mediated almost entirely by *wg*, which requires *gsb* for its maintenance after stage 11 (Li and Noll, 1993). Gsb protein is first detected at the end of cellularization and by the onset of germ band extension is found in 14 segmental stripes at the posterior of each parasegment, consistent with the role of *gsb* as a segment polarity gene (Gutjahr et al., 1993b; Fig. 1U). At the end of germ band extension, stripes 4-14 undergo restriction to the ventral neuroectoderm (Fig. 1, compare W with Y). At this stage, *gsb* expression includes the Wg domain and extends across the parasegmental boundary one to two cell rows into the anterior portion of the En domain. The neuroectodermal stripes of *gsb* are required for the proper patterning of neuroblasts of rows 5 and 6, plus the most medial neuroblast of row 7 (Duman-Scheel et al., 1997). *gsb* is also responsible for the subsequent expression of *gsbn* in a subset of the ganglion mother cell and neuronal progeny of the *gsb*-expressing neuroblasts (Gutjahr et al., 1993b, Fig. 1BB-EE).

To better understand the role PgIII genes play in the segmentation of short germ insects, we have focused on both flour beetles, in which expression of pair-rule and segment polarity homologs have thus far been found to resemble *Drosophila*, and grasshoppers, in which an expression pattern consistent with pair-rule function has not yet been reported. Our approach has been to develop a polyclonal antibody that has allowed us to visualize the products of PgIII genes in embryos of these insects. In both flour beetle and grasshopper embryos, we find that the expression pattern of PgIII genes resembles the pattern in *Drosophila*. Although these data are consistent with previous results for flour beetles, we show for the first time that a grasshopper segmentation gene is expressed in stripes of a two-segment periodicity, suggesting the existence of a pair-rule prepattern in this insect. To identify the PgIII genes responsible for the observed expression in grasshoppers, we isolated cDNAs of two PgIII genes, *pairberry1* (*pby1*) and *pairberry2* (*pby2*). We describe the structure of these genes as well as their expression patterns in grasshopper embryos.

MATERIALS AND METHODS

Antibody production

The *Drosophila*-specific mouse anti-Prd monoclonal antibody (mAb) DP201 and the crossreactive rat anti-Pairberry polyclonal antibody (anti-Pby) were generated by injection of full-length *Drosophila* Prd (derived from the cDNA c7340.1, kindly provided by Marcus Noll) fused to the product of the *TrpE* gene using pATH expression vectors (Koerner et al., 1991). Bacterial expression and purification were carried out as previously described (Patel et al., 1992). Affinity purification of anti-Pby serum was carried out as previously described (Patel et al., 1992), using a column bound with a portion of Gsbn fused to the product of the *TrpE* gene. The region of Gsbn used for purification was the C-terminal 289 amino acids that contain the Gsbn HD, but not the PD or the octapeptide (derived from cDNA bsh4c4, kindly provided by Marcus Noll).

Immunohistochemistry

Embryos of *Drosophila melanogaster* were prepared according to the standard protocol (Patel, 1994c). Embryos of *Tribolium castaneum* were prepared as previously described (Patel et al., 1994). Embryos of *Schistocerca americana* were prepared as previously described (Patel et al., 1989b), except that embryos were fixed in PEM-FA for 15 minutes, and then washed immediately with PT before incubation with PT-NGS, followed by overnight incubation with primary antibody at 4°C.

In general, staining was completed as previously described (Patel et al., 1989b; Patel, 1994c). In addition to anti-Pby serum and MAb DP201, staining was performed with mAbs 4D9 (anti-En; Patel et al., 1989b), 2B8 (anti-Eve; Patel., 1994) and PP7C11 (anti-Hb, Patel et al., 2001), as well as 16F12 (anti-Gsb) and 9A1 (anti-Gsbn), which were kindly provided by Bob Holmgren.

Cloning and sequence analysis

Total RNA was isolated with TRIzol Reagent (Gibco BRL) from three collections of embryos of the grasshopper *Shistocerca americana* at ~20-25%, 25-30% and 30-35% of development (Bentley et al., 1979; Patel et al., 1989a). Three separate pools of cDNA were then generated from these RNA collections with the SuperScript Preamplification System (Gibco BRL) and used for initial PCR screens using nested degenerate primers (first, 5′-GGN GGN GTN TTY ATH AAY GG-3′ and 5′-RTT NSW RAA CCA NAC YTG-3′, then 5′-MAR ATH GTN GAR ATG GC-3′ and 5′-RTA NAC RTC NGG RTA YTG-3′) based on the PD and HD of *Drosophila* and mouse PgIII genes. The three cDNA pools were sampled with seven independent PCR reactions from which 87 clones were sequenced. No PgIII genes other than *pby1* and *pby2* were found.

Additional sequence 3′ of the HD of *pby1* and *pby2* was obtained using 3′ RACE (Gibco BRL). Phylogenetic trees are based on an amino acid alignment of a partial PD (98 amino acids) or both a partial PD plus the HD (63 amino acids; see Fig. 3A). The optimality criterion was maximum parsimony using the ProtPars step matrix (PHYLIP (Phylogeny Inference Package), J. Felsenstein, 1993) and searches were performed in PAUP* v4.0b8 (Swofford, 2001) using the branch and bound algorithm.

In situ hybridization

Whole-mount in situ hybridization using a digoxigenin-labeled RNA probe was performed as previously described (Patel, 1996) with the following changes. Embryos of *Schistocerca americana* were fixed for 15-20 minutes in 3.7% formaldehyde after dissection in 1× phosphate-buffered saline (PBS; pH=7.4) and were then dehydrated stepwise in methanol before storing them at 4°C. Riboprobes for *pby1* (~1500bp) and *pby2* (~700bp) were generated by digesting cDNA plasmids with *Blp*I and *Nde*I, respectively, such that only non-conserved regions 3′ of the HD were transcribed. Fixed embryos were not treated with xylene or proteinase K, and the hybridization was

carried out overnight at 65°C in an sodium dodecyl sulfate (SDS) hybridization solution (SDS-Hyb; 50% formamide, 5× saline sodium citrate (pH 4.5), 0.1% Tween-20, 0.3% SDS, 50 μg/ml heparin, and 100 μg/ml sonicated salmon sperm DNA).

The embryos were then washed at 65°C in the following solutions of SDS-Hyb and 1× PBS containing 0.1% Tween-20 (PTw): 2×20 minutes (80% SDS-Hyb/20% PTw); 2×20 minutes and 2×1 hour (50% SDS-Hyb/50% PTw); 2×20 minutes in (20% SDS-Hyb/80% PTw); then 2×20 minutes (100% PTw). Finally, embryos were washed at room temperature for 2×20 minutes in 1× PBS with 0.1% bovine serum albumin and 0.1% Triton X-100 (PBT) before adding sheep anti-digoxigenin-AP diluted 1:3000 in PBT, and incubating at 4°C overnight. The next day, embryos were washed 4×20 minutes and 2×60 minutes in PBT before performing the color reaction.

RESULTS

Antisera raised to *Drosophila* Paired crossreact to Gooseberry and Gooseberry-Neuro

Antisera raised against *Drosophila* Prd protein have previously shown reactivity not only to Prd, but also to Gsb and Gsbn (Gutjahr et al., 1993a). We thus considered it likely that such antisera would contain antibodies to epitopes shared by all three proteins, as well as the homologous proteins of other insects. We therefore raised antisera to Prd and enriched for crossreactivity by positive adsorption to an affinity column made with the HD of Gsbn. The resulting polyclonal antibody reveals a combined pattern of Prd, Gsb and Gsbn in stained *Drosophila* embryos, confirming that the reagent recognizes the products of all three genes (Fig. 1A-J). The antibody additionally recognizes the products of other genes possessing paired-like homeodomains, such as *aristaless* and *repo* (see below). Because such genes are expressed in non-stripe patterns later in development, this additional crossreactivity did not interfere with our analysis of segmentation.

When applied to embryos of other insects the affinity-purified antibody reveals striped patterns similar to those in *Drosophila*, suggesting that it recognizes the products of PgIII genes more generally. In light of this crossreactivity, we refer to this polyclonal antibody as anti-Pairberry (anti-Pby), and to the pattern it reveals as the Pairberry (Pby) pattern.

Expression of Pax group III genes in *Tribolium* mimics the pattern in *Drosophila*

The expression of segment polarity and pair-rule homologs in the flour beetle *Tribolium* have thus far been shown to be similar to *Drosophila* (reviewed by Patel, 1994a; see Brown et al., 1994a; Brown et al., 1994b). *Tribolium* probably also possesses at least three PgIII genes (Wim Damen and Martin Klingler, personal communication). We thus predicted that the Pby pattern in *Tribolium* should closely mimic the combined pattern of Prd, Gsb and Gsbn in *Drosophila*.

As with *Drosophila* Prd, the Pby pattern (the sum product of presumptive PgIII genes) in *Tribolium castaneum* appears in the blastoderm as a broad anterior domain, corresponding in *Tribolium* to the presumptive serosa (Fig. 2A,B). Concurrently, Eve protein is found in a broad posterior domain (Fig. 2C). Before gastrulation, a Pby stripe corresponding to the mandibular segment appears de novo (Fig. 2D,E). The stripe is positioned just anterior to the first of two broad Eve stripes, which form sequentially from the posterior domain before gastrulation (Fig. 2F). Like the mandibular stripe of Prd in *Drosophila*, this Pby stripe does not split.

As the amniotic fold advances (Fig. 2H, asterisk shows initial amniotic fold), a broad Pby stripe appears de novo between the two broad Eve stripes (Fig. 2I,J, arrowhead). The relative positions of these domains mimic expression in *Drosophila*, where primary Prd and Eve stripes are centered on even- and odd-numbered parasegments, respectively. In *Tribolium*, broad Pby stripes continue to appear de novo in sequential fashion between broad Eve stripes at the posterior (Fig. 2L,M,O,P, arrowheads). In that the broad Eve stripes have previously been shown to be stripes of a two-segment periodicity (Patel et al., 1994), the complementary Pby stripes are likewise of a two-segment periodicity.

As the *Tribolium* germband continues to extend posteriorly, the broad primary Pby stripes, like the early Prd stripes of *Drosophila*, split by loss of expression in the center of each stripe (Fig. 2J-N). Of the resulting two secondary stripes, the anterior stripe is transiently narrower than the posterior stripe. This is similar to the splitting of broad primary Eve stripes in this insect, though in the case of Eve it is the posterior secondary stripe that is transiently narrower and the primary stripes originate from a posterior domain, rather than appearing de novo (Patel et al., 1994). As in *Drosophila*, this splitting process is followed by the appearance of stripes of En, which partially overlap the posterior of the now segmental secondary Pby stripes (Fig. 2M-S). After the appearance of En, the secondary Pby stripes undergo restriction to the neuroectoderm in a manner similar to *Drosophila* Gsb (Fig. 2, compare R with Q). Later, during *Tribolium* neurogenesis, the Pby pattern is similar to the neural pattern for *Drosophila* Gsb and Gsbn (not shown). These observations suggest that anti-Pby recognizes the products of PgIII genes generally, as well as demonstrating that the expression pattern of these genes in *Tribolium* closely mimics their pattern in *Drosophila*.

Schistocerca possesses at least two Pax group III genes

Anti-Pby also revealed a striped pattern in embryos of the grasshopper *Schistocerca americana*. Like *Tribolium*, this pattern is similar to the pattern in *Drosophila*, but with some important differences. In order to identify the PgIII genes responsible for the grasshopper Pby pattern, we screened embryonic cDNA pools for PgIII genes by degenerate PCR followed by 3′ RACE. Our screen yielded two unique partial cDNAs, which we named *pairberry1* (*pby1*) and *pairberry2* (*pby2*).

The cDNA sequences of *pby1* and *pby2* predict proteins that each possess a PD plus an extended S_{50} paired-like HD and terminate 39 and 148 amino acids, respectively, after the HD (Fig. 3A,B). Within the PD and HD, the grasshopper genes are highly similar to one another and to *Drosophila prd*, *gsb* and *gsbn*, yet lack significant sequence similarity C-terminal to the HD. Both *pby1* and *pby2* also possess the octapeptide sequence shared by *gsb*, *gsbn*, *pax-3* and *pax-7*, but not by *prd* (Frigerio et al., 1986). Finally, we were able to detect alternatively spliced forms not reported for any of the *Drosophila* genes. The alternate splice forms result in the insertion of five amino acids in the PD of Pby1 and the deletion of three amino acids from the HD of Pby2 (Fig. 3A). The *pby1* splice site is shared by *gsbn* but not by *gsb* or *prd*. The splice site in *pby2* appears to be unique among insect PgIII genes.

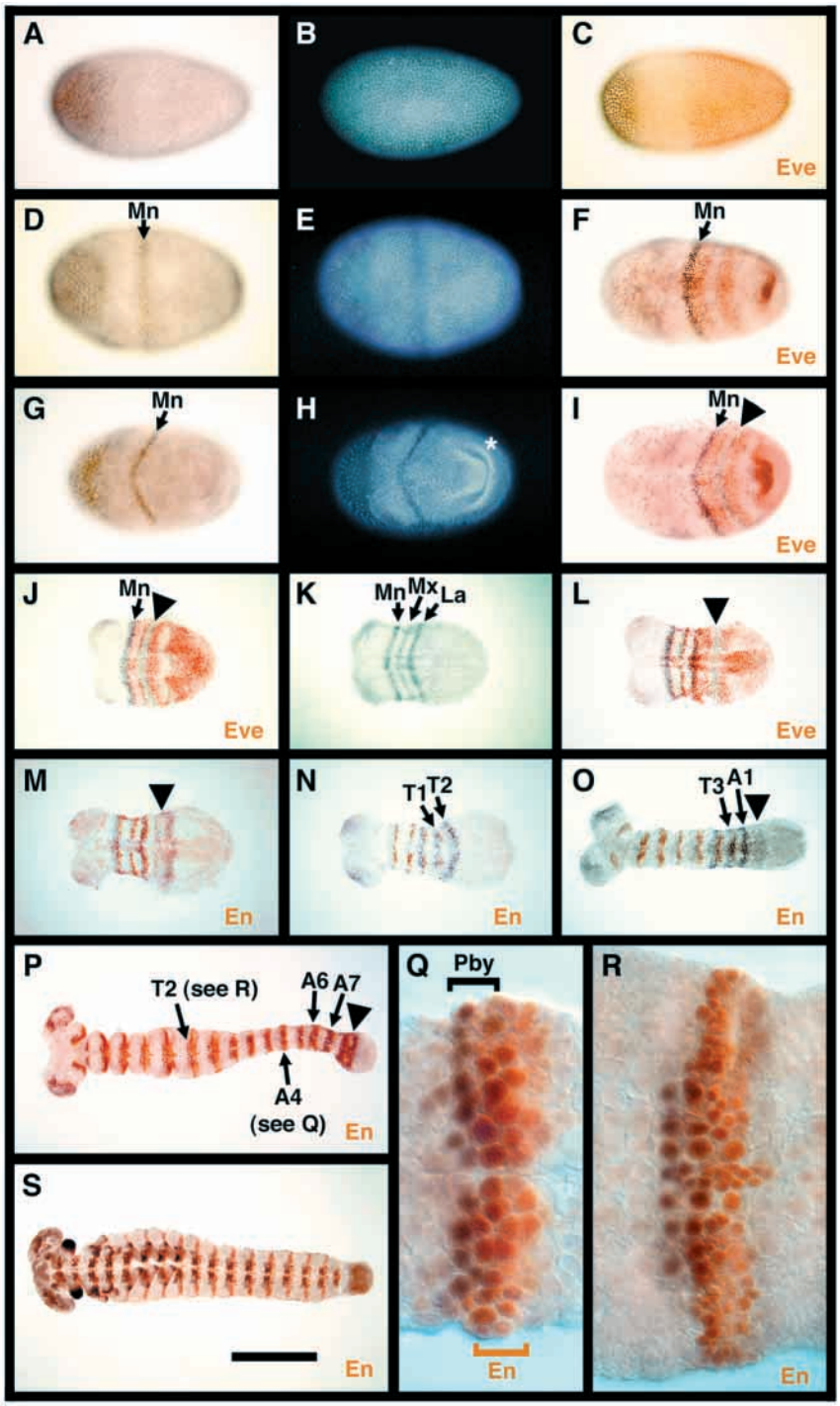

Fig. 2. The Pairberry pattern in *Tribolium* mimics *Drosophila*. *Tribolium* embryos are stained with anti-Pby (in black; A,C,D,F,G,I,J-S) with Dapi counterstains (B,E,H) that correspond to (A,D,G), respectively. In addition, embryos are immunostained with second labels (in brown) of Eve (C,F,I,J,L) or En (M-S). (Q,R) Higher magnification images of A4 and T2 segments of P. Embryos are blastoderm stage (A-C) and increasingly older from D to S. (A-C) At the blastoderm stage, anti-Pby reveals protein in a broad anterior domain, while Eve is expressed in a posterior domain (C). (D-H) The mandibular Pby stripe (Mn arrow) appears anterior to the first of two nascent primary Eve stripes (F). No additional stripes appear before the amniotic fold advances (asterisk, H), concurrent with the onset of gastrulation. (I-K) A weak broad Pby stripe appears between the two primary Eve stripes (arrowhead, I,J) and splits, giving rise to the maxillary and labial stripes (Mx and La arrows, K). (L-N) A second broad stripe appears between two primary Eve stripes (arrowhead, L) and splits (M), giving rise to the first and second thoracic stripes (T1 and T2 arrows, N). Stripes of En appear just posterior to individuated stripes (M,N). (O-S) The third thoracic and first abdominal stripes have appeared, as well as a de novo broad stripe (O). The broad stripe corresponding to the eighth and ninth abdominal stripes splits (P, arrowhead), while more anterior segmental stripes overlap with stripes of En by ~one cell row (P,Q). Still more anterior stripes are restricted to the neuroectoderm (P,R). Later in development, all stripes are present and restricted to the neuroectoderm, with additional Pby staining in preantennal domains, the mandibles, maxillae and labial appendages, as well as the tips of the gnathal palps and limb primordia (S). Anterior is towards the left and all views are ventral except F, which is slightly oblique. J-S have been dissected off of the yolk and L-S have had the amnion removed. Scale bar: 250 μm for A-P,S; 19 μm for Q; and 28 μm for R.

Phylogenetic analysis supports the inclusion of *pby1* and *pby2* within PgIII, but fails to resolve their relationship to the fly genes (Fig. 3C). The PD and HD amino acid and nucleotide sequence for *pby1* and *pby2* show >80% identity to each other and >60% identity to each of the three fly genes. Thus, *pby1* and *pby2* may be more closely related to each other than either is to *prd*, *gsb*, or *gsbn*. Finally, the combined distribution of *pby1* and *pby2* mRNA fully accounts for the *striped* Pby pattern in grasshopper embryos (Fig. 4). Thus, while we cannot rule-out the existence of additional homologs, we believe that we have found all of the PgIII genes in *Schistocerca*.

The combined expression of *pairberry1* and *pairberry2* fully accounts for the striped Pairberry pattern in *Schistocerca*

While possessing important differences, the overall Pby pattern in *Schistocerca*, like *Tribolium*, is similar to the combined pattern of Prd, Gsb and Gsbn in *Drosophila*. At ~35% of development, the grasshopper Pby pattern, as well as the pattern of *pby1* and *pby2* mRNA, includes 19 segmentally reiterated stripes corresponding to the antennal (An), intercalary (Ic), mandibular (Mn), maxillary (Mx), labial (La), thoracic (T1-3) and abdominal (A1-11) segments (Fig. 4K,L,N). The combined expression of *pby1* and *pby2*, then, fully accounts for the 19 stripes of protein observed with anti-Pby. That anti-Pby indeed reacts to the protein products of both *pby1* and *pby2* was confirmed by western blots of recombinant protein (not shown).

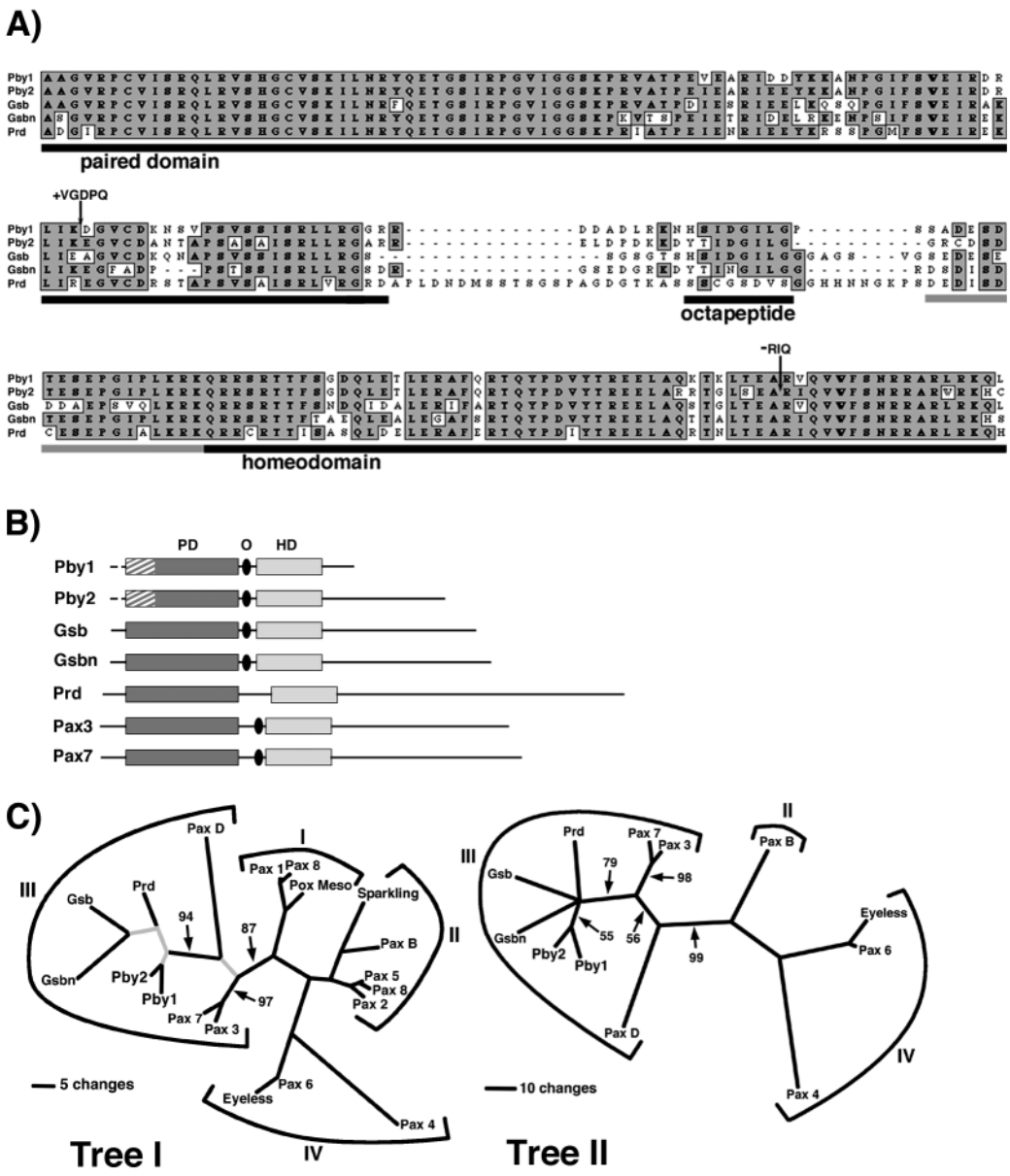

Fig. 3. *Schistocerca* possesses at least two Pax group III genes. (A) Amino acid sequence alignment of Pby1 and Pby2 (*Schistocerca americana*) with Gsb, Gsbn and Prd (*Drosophila melanogaster*) reveals conservation in the paired domain, homeodomain and octapeptide (black underline). The extended region of the homeodomain is also shown (gray underline). The addition and deletion of amino acids that result from the alternative splice forms of *pby1* and *pby2*, respectively, are shown above the alignment. Available cDNA sequence for both *pby1* and *pby2* have been deposited in GenBank (Accession Numbers, AY040535 and AY040536, respectively). (B) Schematic of *Schistocerca*, *Drosophila* and vertebrate PgIII gene products. The relative lengths and conserved regions of each gene product are shown. PD, paired domain; HD, extended homeodomain; O, octapeptide. Hatching indicates presumed regions not yet sequenced. Pax3 and Pax7 are from mouse. (C) Phylogenetic analysis of Pby1 and Pby2 amino acid sequences using maximum parsimony. Based on the alignments shown in A, Tree I (partial paired domain alone) is the most parsimonious tree and Tree II (partial paired domain + homeodomain proper) is a strict consensus of the three most parsimonious trees. Both trees support the placement of Pby1 and Pby2 within PgIII. Pax genes that lack full homeodomains were not included in Tree II. Pax groups I, II, III and IV are those originally defined (Balczarek et al., 1997). Pax 1-9 are from mouse; Gsb, Gsbn, Prd, Eyeless, Sparkling and Pox Meso are from *Drosophila*; and Pax B and Pax D are from the cnidarian *Acropora millepora* (Miller et al., 2000). Numbers shown reflect the percentage of 1000 bootstrap replicates supporting the indicated node (only values >50% for PgIII nodes are shown, other PgIII nodes are shown in gray).

Before ~20%, *pby1* is transcribed in a complex pattern that culminates in seven segmental stripes of the head and thorax (An, Mn, Mx, La, T1-3; Fig. 4A). Transcripts of *pby2*, however, are not detectable at these stages (Fig. 4B). As the pattern of Pby stripes mimics the pattern of *pby1* stripes at these stages (Fig. 4A,D), we presume that these Pby stripes are the product of *pby1* and not *pby2*. In 20-27% embryos, abdominal *pby1* stripes appear at least four segmental stripes more posterior of the most posterior *pby2* and En stripes (Fig. 4F-H). Thus, in the abdomen, as in the head and thorax, *pby1* is transcribed before *pby2*. As the pattern of posterior abdominal Pby stripes is identical to the pattern of posterior abdominal *pby1* stripes at these stages (Fig. 4F,I), we also presume that the Pby stripes in the posterior pre-En region of the embryo are the product of *pby1* and not *pby2*, while Pby stripes in the anterior post-En region reflect the protein products of both genes.

In addition to these 19 stripes, the Pby pattern also includes repeating patterns of neural expression and various non-stripe domains in the pre-antennal region of the head, in appendage primordia, in lateral ectoderm of the abdomen and in the telson (Figs 4K-O, 5H). Most of these non-stripe domains are found in the mRNA pattern of either *pby1* or *pby2*, or both, and the majority do not appear until after ~30%, well after segmentation is complete. The portions of the Pby pattern that are not due to either *pby1* or *pby2* are probably due to crossreactivity to proteins possessing paired-like homeodomains, such as *aristaless* and *repo*.

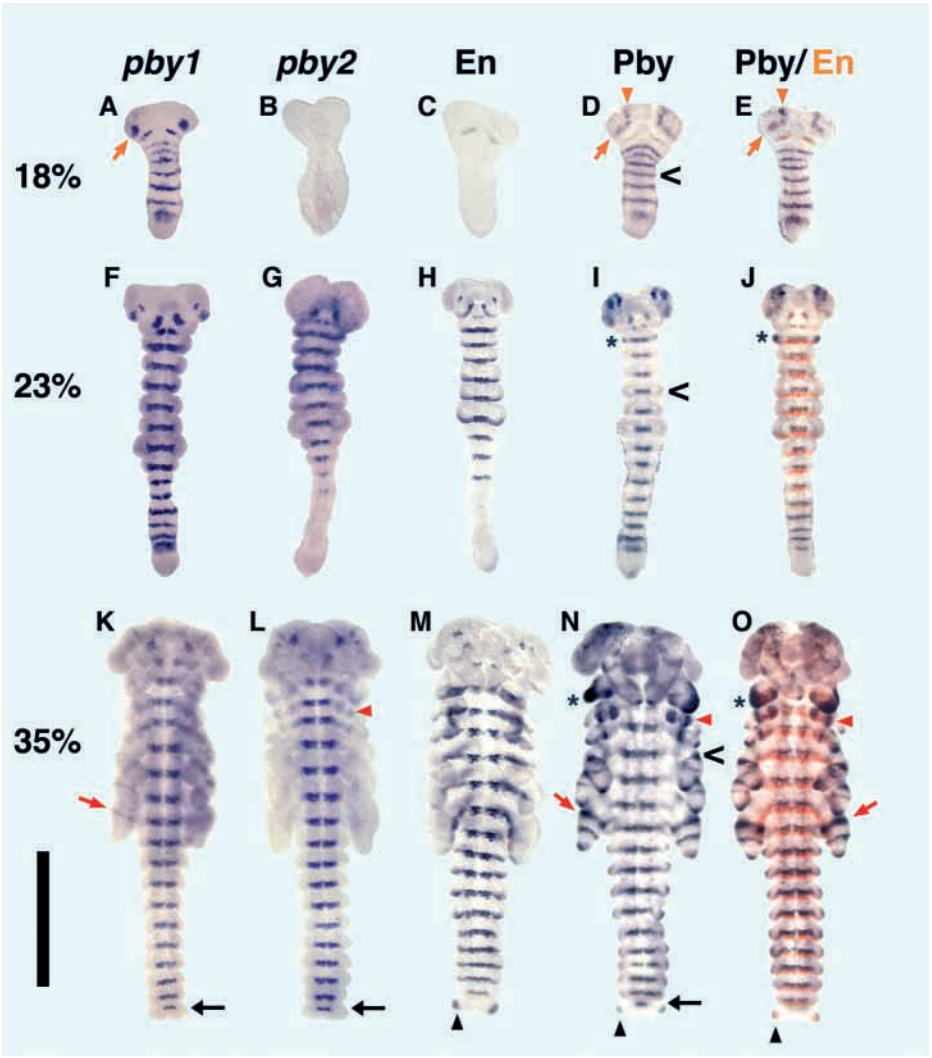

Fig. 4. The Pairberry pattern comprises both *pby1* and *pby2* in *Schistocerca*. Grasshopper embryos stained for *pby1* mRNA (A,F,K) and *pby2* mRNA (B,G,L), En protein (C,H,M), Pby protein (D,I,N), or both Pby (black) and En (brown) protein (E,J,O). Approximate stages are as indicated. Open arrowheads (<) mark T1 in Pby embryos. (A-E) ~18%: *pby1* mRNA is found in seven stripes that correspond to the antennal, gnathal and thoracic segments, as well as a broad posterior domain and pre-antennal eyespots (brown arrows, A,D,E). (B,C) *pby2*, by contrast, is not expressed at this stage (B) while only the antennal stripes of En (C) have appeared. Anti-Pby staining reveals a pattern similar to *pby1* mRNA as well as several, more anterior, pre-antennal domains of the head lobes (brown arrowheads, D,E). (F-J) ~23%: additional *pby1* stripes have appeared in the intercalary segment and A1-A6, along with a broad posterior domain (A7/A8). Note that the gnathal, thoracic, and A1 *pby1* stripes are now restricted to the neuroectoderm (F). *pby2* mRNA is found in stripes, coincident with *pby1*, that are entirely restricted to the neuroectoderm and are, in this embryo, only just appearing in A4 (G). *pby2* mRNA appears about the same time as En, which in this embryo is found only as far posterior as A3 (H). The *pby1* eyespots have refined to two lateral domains, which are still not present in the *pby2* pattern (compare F with G). The two Pby embryos are slightly older, showing laterally restricted stripes in A3 and A4 as well individuated A7 and A8 stripes (I,J). The Pby/En embryo reveals that En stripes appear before the restriction of Pby1 protein (J). (K-O) ~35%: ventrally restricted stripes of *pby1*, as well as *pby2*, are found in the antennal, intercalary as well as all gnathal, thoracic and abdominal segments (K,L). *pby1* and *pby2* stripes are also found in A11 (black arrows, K,L), which does not express En (the A11 stripe is present, but not visible in O). At this stage, *pby1* is also expressed in at least one ring in the limb primordia, of which three are visible in the Pby embryos (red arrows, K,N,O). For the first time, *pby2* is also expressed in pre-antennal domains coincident with *pby1*, as well as the gnathal appendages (red arrowheads, L,N,O). Anti-Pby staining recapitulates the combined *pby1* and *pby2* patterns, while also revealing non-PgIII domains found in the pre-antennal regions of the head lobes, the mandibles (in which staining appears before the appearance of *pby2* mRNA in the mandible) (asterisk, I,J,N,O), as well as tips of gnathal palps, limbs, abdominal buds and the telson (where En is also expressed) (black arrowheads, M-O). Scale bar: 1.0 mm.

Striped expression of *pairberry1*

Head and thoracic stripes

pby1 is first expressed in the grasshopper embryo shortly after gastrulation at ~40 hours after oviposition. At this point, anti-Pby reveals low levels of Pby1 protein in a posterior domain spanning six to ten cells along the anteroposterior axis (Figs 5B, 6B, 9%). As the embryo begins to extend posteriorly, a second, more anterior, domain appears medially and spreads laterally to form an arc of expression spanning ~ten cells along the anteroposterior axis (Fig. 5C, arrowhead; Fig. 6B, 10-11%). This early arc corresponds to the region of the future gnathal segments (Mn, Mx and La) and is thus referred to as the ‘gnathal arc’. As the embryo continues to extend (~12–13%), the posterior domain disappears while the gnathal arc persists. At ~15% of development, a de novo stripe spanning ~four cell rows arises ~ten cells posterior to the gnathal arc and expands posteriorly, becoming a broad domain spanning ~15 cell rows, which comprises the future stripes of both T2 and T3 (Figs 5D, 6B, 15-16%).

After the T2/T3 broad domain appears, the gnathal arc begins to split. The split results from loss of Pby1 protein in the middle six cell rows of the arc, leaving two stripes corresponding to regions of the future Mn and La segments (Fig. 5E, black staining; Fig. 6B, 15-16%). As the gnathal arc splits, expression in the more posterior portion of the T2/T3 broad domain diminishes, while expression levels increase in the most anterior two cell rows, forming the T2 stripe (Fig. 5E, black staining). Concurrently, the T3 stripe, which spans ~four cell rows, appears

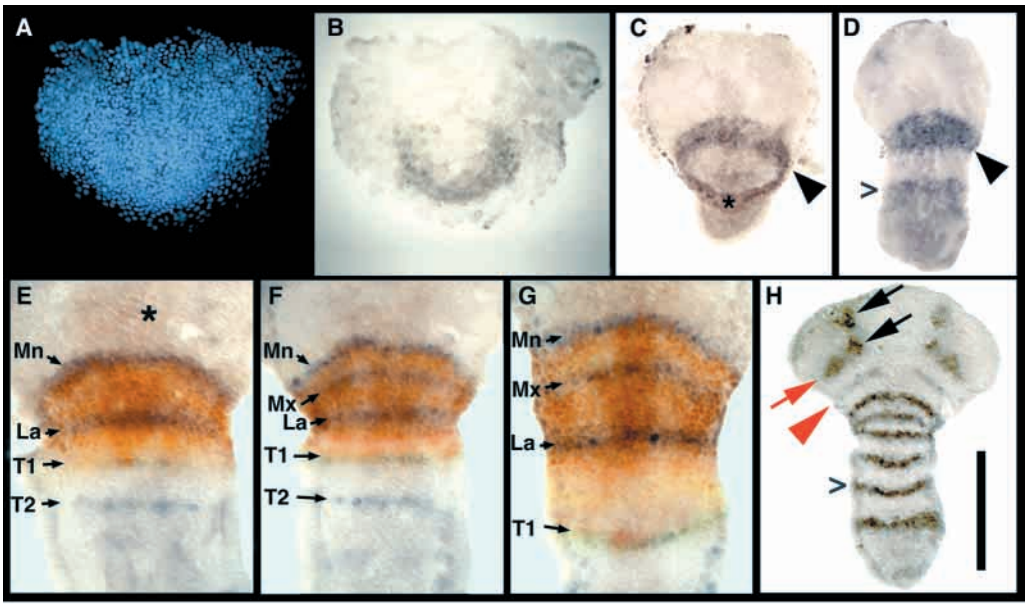

Fig. 5. Early Pairberry pattern in *Shistocerca*. (A-D) Anti-Pby immunostaining of early grasshopper embryos. (A) Dapi stain of embryo, ~9% of development. (B) Same embryo, showing weak posterior Pby domain also observed with *pby1* mRNA (not shown). (C) ~11% embryo showing gnathal arc (black arrowhead). The amniotic fold has been left intact (asterisk). (D) ~15% embryo showing gnathal arc (black arrowhead) and T2/T3 broad domain with higher levels at the anterior edge where the T2 Pby stripe will form (open arrowhead). (E-G) Anti-Pby (black) and anti-Hb (brown) immunostaining of ~16-17% embryos. (E) At ~16% the Mn, La and T2 Pby stripes have formed, while T1 is just beginning to appear. The strong subdomain of Hb protein extends from the Mn Pby stripe to just posterior of the La Pby stripe, where the weak subdomain continues through the T1 Pby stripe. Low levels of Hb are also found throughout the more anterior head lobes (asterisk). (F) Slightly later, the Mx Pby stripe has formed while the Hb domain remains static. (G) At ~18%, extension results in the concomitant separation of Pby stripes and increased length of the Hb domain, particularly in T1. (H) ~17% embryo after the split of the gnathal arc and intercalation of the Mx and T1 Pby stripes, plus addition of antennal (red arrowhead) and T3 Pby stripes. Open arrowhead indicates position of T2 Pby stripe. Embryo also shows several pre-antennal domains, which include the eyespot present in the *pby1* mRNA pattern (red arrow) and domains not seen in either the *pby1* or *pby2* pattern (black arrows). Scale bar: 300 μm for A-D,H; and 170 μm for E-G.

near the posterior edge of the previous broad domain (not shown). After the formation of the Mn, La, T2 and T3 stripes, stripes of expression corresponding to the Mx and T1 segments intercalate de novo (Fig. 5E-G, black staining; Fig. 7B, 16-17%).

This early segmental Pby1 pattern allowed us to determine more precisely the position and modulation pattern of the early anterior gap domain of *hunchback* (*hb*) (Patel et al., 2001). At 10% (~50 hours AEL), this domain of Hb protein comprises both weak expression in the head lobes and a strong band of expression lying just posterior. The strong band then modulates into a step-pattern that consists of a strong anterior subdomain and weaker posterior subdomain. While we have mapped the position of the these subdomains by double labeling for En (Patel et al., 2001), double labeling with anti-Pby confirms that the formation of the step-pattern occurs via reduction of expression in the most posterior portion of the original band. Embryos stained for both Pby1 and Hb protein reveal that from the outset this modulated Hb stripe (in brown) extends from the posterior edge of the Mn Pby1 stripe through to the Pby1 stripe of T1 (Fig. 5E). Within this larger stripe, the boundary between the strong and weak subdomains lies just posterior to the La Pby1 stripe (Figs 5F-G, 6C). Importantly, this reveals that the apparent 'expansion' of the weak subdomain is due to differential growth within T1 (compare Fig. 5F with 5G), while domain boundaries are maintained.

After the appearance of the gnathal and thoracic Pby1 stripes, anti-Pby reveals a bilateral pair of stripes in the An segment (Fig. 5H, red arrowhead). A bilateral pair of En stripes arises just posterior to the An Pby1 stripes (Figs 4C-E, 6B, 17%). En stripes then appear in the Mn, T1 and T2 segments. Each En stripe lies posterior to a Pby1 stripe, the two stripes overlapping by one to two cell rows (Fig. 4F-J). This ordering of the appearance of En stripes (An at ~17%, followed by Mn, T1 and T2 at ~18%, Fig. 6A) differs from the order previously reported for this species (Patel et al., 1989a), suggesting that the timing of En stripes may be polymorphic. The only remaining anterior Pby1 stripe, that of the Ic segment, does not appear until ~20-21%, just before the Ic En stripe (Figs 4F, 6B). After the appearance of adjacent En stripes, both the gnathal and thoracic Pby1 stripes undergo restriction to the ventral neuroectoderm (Figs 4F, 6B, 20-22%), in a manner similar to *Drosophila gsb*.

Abdominal stripes

The origin of the T2 and T3 stripes from within a single broad domain of expression suggests a transient two-segment periodicity for the stripes of *pby1* in the thorax. In the abdomen as well, this process – two adjacent stripes arising from within an initially broad domain – is repeated five times, reminiscent of the process by which *prd* acquires its segmental pattern in *Drosophila*. The process in grasshoppers consists of the following steps: (1) appearance of a broad domain (10–15 cells wide) near the extending posterior end; (2) increased expression in a stripe (~four cells wide) at the anterior edge of the broad domain with a concomitant decrease in expression levels in the posterior portion; and (3) appearance of a second posterior stripe (~four cells wide) arising from within the fading posterior portion of the broad domain (Fig. 7). An essential observation in this regard is that broad domains are only observed posterior to stripes of even-numbered abdominal segments. Hence, abdominal Pby1 stripes emerge from the following broad domains: A1/A2, A3 /A4, A5/A6, A7/A8 and A9/A10. At least at the protein level, broad domains typically appear less refined at their posterior edge, so that low levels of

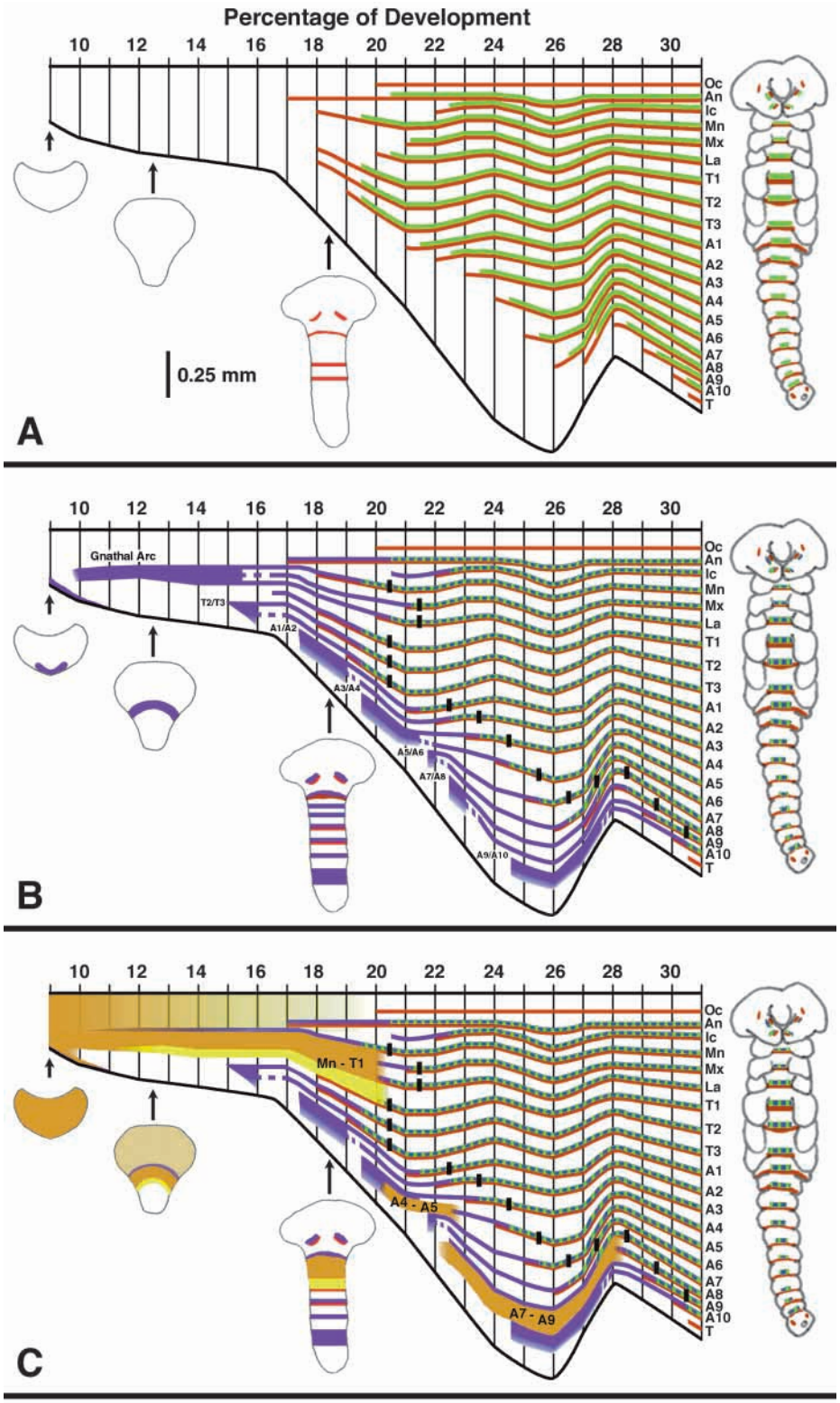

Fig. 6. Striped patterns of Pby1, Pby2, En and Hunchback protein during *Schistocerca* embryogenesis. (A-C) The horizontal axis represents time, as measured in percentage development, while the vertical axis represents the length of the embryo, as measured from the tip of the head lobes to the end of the abdomen. 9-26%: extension occurs throughout the embryo but is concentrated in the abdomen. 26-28%: overall length decreases, owing to morphological segmentation, which contracts the embryo in accordian-like fashion, despite the persistence of extension in the abdomen. >28%: overall length increases as the result of nondifferential growth throughout embryo. (A) The schematic shows the timing of appearance and position along the anteroposterior axis of En protein (red) and Pby2 protein (green) during ~9-31% of development. Estimates of the timing of appearance of Pby2 protein are based on *pby2* mRNA. (B) Same schematic as A, but showing Pby1 protein (purple), coincident Pby1 and Pby2 protein (purple and green hatched), and En protein (red) stripes. Broken purple lines indicate relatively weak expression as detected with anti-Pby. Vertical black bars indicate when Pby1 becomes restricted to the neuroectoderm (see text). Note that stripes of En join Pby1 stripes prior to their restriction. Also note that T2, T3 and the abdominal Pby1 stripes originate from within broader stripes of a two-segment periodicity (see text and Fig. 6). The A11 Pby1 and Pby2 stripe, which does not appear until ~35%, is not shown. Non-stripe domains of Pby1 and Pby2 have also been omitted. A,B are based on 23 embryos stained with anti-Pby and anti-En, which were used to measure embryo length and the position of stripes; tracings of four representative embryos are shown (the intercalary stripe, Ic, is shown through the tissue of the antennae in the 31% embryo). The distribution of Pby1 and Pby2 protein was estimated by comparing the Pby and En pattern with the distribution of *pby1* and *pby2* mRNA (Fig. 4). (C) Same schematic as B, with the estimated position of three ectodermal Hb expression domains (Patel et al., 2001). The early anterior band occupies parasegments 1-3 and thus extends from the Mn to T1 segments, while concurrent weaker expression extends throughout the head (brown, strong subdomain; yellow, weak subdomain). It is not known when the domain extending from A4 to A5 disappears, as the mesodermal expression obscures the domain at ~22%. The posterior extent of the domain extending from A7 to A9 should also be regarded as provisional. Staging estimates are ±1%. Oc, ocular; T, telson.

protein often extend to the posterior tip of the embryo. In general, low levels of protein are also observed between nascent stripes, but these interstripe regions are quickly cleared as the stripes mature, as can be seen in more anterior segments (Fig. 7B). Finally, the last stripe to appear – that of A11 – appears in restricted form at ~35% and is not associated with an En stripe (Fig. 4K-O), as is the case for the A11 Gsb stripe in *Drosophila* (Gutjahr et al., 1993b).

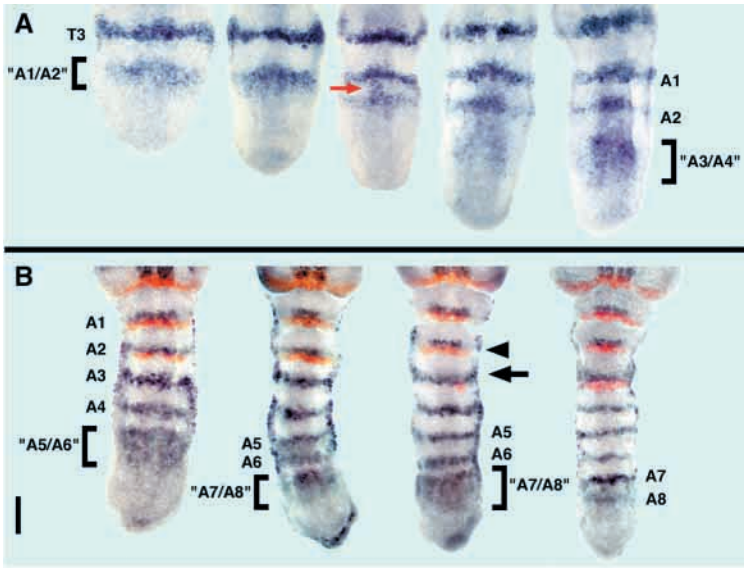

Fig. 7. Early *pby1* stripes are of a two-segment periodicity. (A) The posterior portion of ~18-20% grasshopper embryos stained for *pby1* mRNA are shown. A1 and A2 *pby1* stripes arise from within an initial A1/A2 broad domain, comprising the future *pby1* stripes of A1 and A2. Note residual expression connecting A1 and A2 stripes during the individuation process (red arrow), as well as the low level of expression posterior to the A1 stripe through to the posterior tip. The individuation of stripes A1 and A2 is accompanied by the appearance of the A3/A4 broad domain, which will later refine into stripes A3 and A4. (B) The extending abdomens of ~21-24% embryos immunostained for Pby (black) and En (brown) are shown. A5 and A6 Pby stripes arise from within the broad 'A5/A6' domain and A7 and A8 Pby stripes from within the broad 'A7/A8' domain. As in A, note the low level of expression throughout the posterior beginning from the most recently individuated anterior stripe. Also note that En stripes appear before the restriction of Pby stripes to the ventral neuroectoderm: the third embryo from the left shows both a restricted stripe (arrowhead, A2) with En and an unrestricted stripe (arrow, A3) posterior to which an En stripe is just beginning to appear. Although some broad domains appear after the previous (more anterior) broad domain has split (A), other broad domains appear before the anterior nascent stripes have fully individuated (B), though this may reflect a difference in the turnover rates of transcript versus protein. Scale bar: 100 μm. Embryos in A have swelled somewhat as a result of the in situ protocol.

After forming, each of the segmental abdominal stripes narrows along the anterior-posterior axis to span only 2-3 cell rows. This narrowing is followed by the appearance of an En stripe posterior to the Pby1 stripe, such that the two stripes overlap by ~one cell row (Fig. 4, 23%; Fig. 7B). In part owing to their early appearance, stripes of Pby1 protein, unlike En, appear to keep pace with the extending posterior tip (Fig. 6B). After the appearance of an adjacent En stripe, each abdominal Pby1 stripe, like the gnathal and thoracic stripes, undergoes restriction to the ventral neuroectoderm and then expands to span four to five cell rows (Fig. 4, 23%). Unlike the segmental secondary stripes of *Drosophila* Prd, in which the posterior *prd*-expressing cell also expresses *en* (Gutjahr et al., 1993a), nascent En stripes are not situated entirely within Pby1 stripes. Pby1 stripes instead behave more like Gsb in this respect, overlapping En stripes by just one cell row.

Striped expression of *pairberry2*

The distribution of *pby2* mRNA reveals that this gene is expressed in a pattern spatially coincident with, but temporally delayed with respect to, *pby1*. In particular, *pby2* is expressed exclusively in stripes coincident with mature (i.e., ventrally restricted) *pby1* stripes and these stripes appear at approximately the same time as stripes of En protein and slightly before stripes of Pby1 protein become ventrally restricted (Fig. 4G). The earliest *pby2* stripes appear at ~19-21% in the An, thoracic and gnathal segments, followed by the Ic and abdominal stripes, the latter appearing in strict anterior to posterior progression (Fig. 6A,B). Based on its spatial and temporal dynamics, *pby2* expression is reminiscent of *Drosophila gsbn* and late *gsb* expression.

The expression of *pairberry1* and *pairberry2* partially account for the non-striped Pairberry pattern in *Schistocerca*

As *pby1* and *pby2* were the only grasshopper PgIII genes found in our screen, we reasoned that these genes together should be responsible for most, if not all, of the Pby pattern in this insect. Surprisingly, *pby1* expression alone recapitulates the entire striped Pby pattern (Fig. 4). *pby1* also recapitulates some (but not all) of the Pby domains in the pre-antennal region of the head, the earliest of which (Fig. 4A,D,E, brown arrows; Fig. 5E, red arrow) is found in the eye lobe and is likely to be coincident with a similar domain of *wg* (Dearden and Akam, 2001; Friedrich and Benzer, 2000). At ~23%, *pby1* mRNA is found in two separate domains in the eye lobe (Fig. 4F), while at 35% the pre-antennal domains of *pby1* are located more anteriorly (Fig. 4K). *pby1* is also expressed in at least one of three circumferential rings in the limb primordia (Fig. 4K,N,O, red arrows) and these rings are also part of the *Tribolium* Pby pattern (Fig. 2S).

pby2 expression also recapitulates some non-stripe features of the Pby pattern. After ~30% *pby2* joins *pby1* in apparently coincident pre-antennal domains (Fig. 4L) and by ~40% in at least one limb ring (not shown). *pby2* is also expressed in the mandibles and in the maxilla and labium primordia (corresponding to the emerging galea, lacinia and lingula) (Fig. 4L,N,O, red arrowheads) and at later stages (>40%) *pby1* mRNA is found in these domains as well (not shown). This late gnathal expression is also part of the *Tribolium* Pby pattern (Fig. 2S) and is reminiscent of the late expression of *Drosophila prd*, which is also found at the base of the embryonic gnathal protuberances (Fig. 1Q-T).

Although *pby1* and *pby2* account for most of the Pby pattern in *Schistocerca*, some non-stripe features of the Pby pattern are apparently not due to either of these genes. These Pby domains are instead probably due to crossreactivity to additional proteins possessing the putative epitope shared by the products of PgIII genes. These non-PgIII Pby patterns include: (1) staining in the amnion (not shown); (2) pre-antennal domains that initially lie more anterior than *pby1* (Fig. 4D,E, brown arrowheads; Fig. 5H, black arrows); (3) additional staining in the mandibles (Fig. 4I,J,N,O, asterisk) plus the distal tips of the maxillary and labial palps, limb primordia, lateral

abdominal ectoderm (Fig. 4N,O) and the telson (Fig. 4N,O, black arrowheads); and (4) glia of the developing central and peripheral nervous system (not shown). All four of these patterns are also observed in *Tribolium* and the latter three in *Drosophila* (not shown). The source of Pby staining in the amnion of *Schistocerca* and *Tribolium* is unknown, but staining in the pre-antennal region of all three insects is probably due to one or several of the paired-like homeodomain proteins known to be expressed in the developing brain and eye of *Drosophila*. Pby domains in the mandibles and the tips of the maxillary and labial palps, limb primordia, lateral abdominal ectoderm and the telson are probably due to *aristaless*, as this gene is expressed in analogous domains in *Drosophila* and possesses a paired-like homeodomain. Furthermore, anti-Pby staining of *Drosophila* wing and leg imaginal discs matches the published description of *aristaless* (Schneitz et al., 1993, not shown). The Pby staining in glia is almost certainly due to *repo*, as the product of this gene also possesses a paired-like homeodomain and is found in the glia of *Drosophila* and *Schistocerca* embryos (Halter et al., 1995). Consistent with this, the Pby glial pattern is observed in wild-type *Drosophila* embryos, but lost in $repo^{4e25}$ embryos (Xiong et al., 1994, not shown). The non-PgIII crossreactivity of anti-Pby should be useful in comparing the expression of these additional genes between various arthropods.

DISCUSSION

Using a crossreactive antibody (anti-Pby), we have observed that the pattern of presumptive PgIII gene products (the Pby pattern) in flour beetle and grasshopper embryos closely resembles the combined pattern of *prd*, *gsb* and *gsbn* in *Drosophila* (Figs 1, 2, 4). Importantly, Pby stripes in grasshopper embryos appear before the segment polarity gene *en* and exhibit a 2-segment periodicity, indicating that pair-rule patterning is a facet of segmentation in this insect. In order to identify the genes responsible for the Pby pattern in grasshoppers, we conducted a screen for *Schistocerca* PgIII genes. The screen resulted in two genes whose expression accounts for the entire striped Pby pattern. We discuss the evolution, expression and possible regulatory interactions of these two genes. We then reflect on how these data shape our picture of the ancestral insect segmentation system.

pairberry1 and *pairberry2* may be the result of duplication of the ancestral PgIII gene

With the exception of possible nematode homologs (reviewed by Hobert and Ruvkun, 1999), protostome PgIII genes have thus far not been reported outside *Drosophila*. We have isolated two PgIII genes from *Schistocerca*, which we have named *pairberry1* (*pby1*) and *pairberry2* (*pby2*). Each gene possesses both a paired box and an extended S_{50} paired-like homeobox (Fig. 3A,B). Phylogenetic analysis and high sequence similarity to *Drosophila prd*, *gsb* and *gsbn* supports the inclusion of *pby1* and *pby2* within PgIII (Fig. 3C). Additionally, *pby1* and *pby2* appear to be more closely related to each other than either is to *prd*, *gsb* or *gsbn*, suggesting they may be the result of an independent duplication (Fig. 3C). This conclusion is tempered, however, by the possibility of homogenization of *pby1* and *pby2* via gene conversion.

Although the two grasshopper genes may be closely related, we were unable to unequivocally resolve their relationship to the fly genes (Fig. 3C). Although it is possible that *pby1* and *pby2* result from the duplication of the ancestral *gsb/gsbn* gene along the lineage leading to *Schistocerca* after its split with *Drosophila*, this scenario implies that a grasshopper *prd* ortholog either exists and has not been found, or was subsequently lost. As the expression patterns of both *pby1* and *pby2* include elements similar to the expression of each of the three *Drosophila* genes, we think it more likely that *pby1* and *pby2* result from an independent duplication of a single ancestral insect PgIII *(prd/gsb/gsbn)* gene.

The early expression of *pairberry1* constitutes evidence for pair-rule patterning in *Schistocerca* and is reminiscent of *Drosophila paired*

The early transcript and protein expression patterns of *pby1* provide, for the first time, evidence of pair-rule patterning in the grasshopper *Schistocerca*. Indirect evidence is provided by the order of appearance of the gnathal and thoracic Pby1 stripes. In particular, the onset of the Mx and T1 stripes is delayed relative to their adjacent stripes (Figs 5F-H, 6B, 16-17%). Thus, like many segment polarity genes in *Drosophila*, the order of appearance of these segmental stripes follows a two-segment periodicity. This may reflect, as it does in *Drosophila*, regulation by an underlying pair-rule patterning mechanism.

Stronger evidence for pair-rule patterning lies with the early domains of *pby1* expression from T2 to A10. Stripes of these segments originate as broad domains of a two-segment periodicity at the extending posterior tip, each of which subsequently splits into a pair of adjacent segmental stripes. Thus, adjacent stripes arise by subtly different means. The segmental stripes of T2, A1, A3, A5, A7 and A9 resolve from the anterior edge of sequentially appearing broad domains. By contrast, the segmental stripes of T3, A2, A4, A6, A8 and A10 resolve from within the posterior portions of the same respective broad domains (Figs 6B, 7). This resolution of broad domains into adjacent pairs of segmental stripes is analogous to the process by which *Drosophila prd* acquires its segmental pattern from initial stripes of a two-segment periodicity (Fig. 1B,C).

Although similar to *Drosophila* and flour beetles, the broad domains in grasshopper exhibit at least one notable difference. When compared with either *Drosophila* or flour beetles, the pairing of stripes in grasshoppers is shifted by one segment. For example, in grasshoppers, the Pby1 stripes of A1 and A2 derive from a single A1/A2 broad domain, while in flies and flour beetles the segmental Pby stripes of A1 and A2 derive from the T3/A1 and A2/A3 broad domains (primary Prd stripes 4 and 5 in *Drosophila*). The shift in phasing of stripe pairs in *Schistocerca* when compared with *Tribolium* and *Drosophila* is reflected in the fact that the grasshopper A11 Pby1 stripe (Fig. 4K), which appears relatively late, arises without a sister stripe. Such variation in phasing is likely to reflect a spatial shift in the expression of upstream components of the segmentation hierarchy.

An additional similarity of early *pby1* expression to *Drosophila prd* is its timing relative to segment polarity genes. In *Drosophila*, *prd* is expressed before *en* and *wg*. In *Schistocerca*, *pby1* is expressed before En protein by

approximately four to five stripes from ~20-27% (Figs 4A,C,F,H, 7B). *pby1* is, hence, also likely to be expressed ahead of *wg*, as in *Schistocerca gregaria*, *wg* transcript appears only two to three stripes ahead of En protein (Dearden and Akam, 2001; C. J. and N. P., unpublished). Another feature shared by the early *pby1* pattern and that of *Drosophila prd* is the gnathal arc. This early domain comprises the future Pby1 stripes of the Mn, Mx and La segments (Figs 5C,D,E, 7B, 10-16%). In *Drosophila*, *prd* is also expressed as a single broad stripe before splitting into primary Prd stripes 1 and 2 at the onset of cellularization, just as stripes 3-7 begin to appear (Gutjahr et al., 1993a; Fig. 1A,B,K,L). Primary stripes 1 and 2 in turn give rise to the future Mn, Mx and La secondary stripes of Prd. This early Prd domain in flies is thus remarkably similar to the Pby1 gnathal arc in grasshoppers. We were not able to detect a similar pattern in flour beetle embryos, as the Mn Pby stripe appears de novo (Fig. 2D-F).

The late expression of pairberry1 and pairberry2 is reminiscent of Drosophila gooseberry and gooseberry-neuro

The position of Pby1 stripes just anterior to En with an overlap of ~one cell row, along with their subsequent restriction to the neuroectoderm (Fig. 5B), is reminiscent of *gsb* expression in *Drosophila* (Fig. 1U-Y). Similarly, the delayed appearance of Pby2 stripes, their restricted form, and their coincident expression with Pby1 anterior to En (Figs 4F-J, 6) is reminiscent of late *gsb* expression (Fig. 1X,Y). Additionally, the striped neural expression of both *pby1* and *pby2* as late as 40% (not shown) is reminiscent of *gsbn* expression (Fig. 1BB-EE). Thus, only one of two PgIII genes identified in *Schistocerca*, *pby1*, is potentially functioning in the capacity of all three PgIII genes in *Drosophila* (*prd*, *gsb* and *gsbn*), while *pby2* is potentially functioning in the capacity of one, or perhaps two, of the *Drosophila* genes (*gsb* and *gsb-n*). Finally, although the behavior *pby2* is most similar to *Drosophila gsb* and *gsbn*, the late expression of both *pby1* and *pby2* at the base of the developing gnathal appendages is reminiscent of the late expression of *prd* at the base of the gnathal protuberances in *Drosophila* embryos (Figs 1Q-T, 4K-O).

pairberry1 may regulate the expression of *pairberry2* and segment polarity genes

During *Drosophila* embryogenesis, the pair-rule gene *prd* activates the segment polarity gene *gsb*, which, in turn, activates *gsbn*. Additionally, the products of these three genes are for the most part functionally interchangeable (Li and Noll, 1994; Xue and Noll, 1996). Given both their similarity to the three fly genes and their coincident expression, *pby1* may be required for the activation of *pby2*.

In *Drosophila*, *prd* is also required for the activation of odd-numbered *wg* stripes (Ingham and Hidalgo, 1993). Thus, Pby1 may be required for activation of *wg* in *Schistocerca americana*. The temporal dynamics of *wg* mRNA in the closely related grasshopper *Schistocerca gregaria* are consistent with this suggestion (Dearden and Akam, 2001). In *Drosophila*, *prd* is also responsible for activating and defining the posterior border of odd-numbered En stripes. This is suggested by the absence of odd-numbered En stripes in *prd*-negative embryos (DiNardo and O'Farrell, 1987), as well as their posterior expansion in heat shocked *prd* embryos (Morrissey et al., 1991). Consistent with this role, the posterior borders of secondary Prd stripes in *Drosophila* are coincident with the posterior borders of En stripes (Gutjahr et al., 1993a). In *Schistocerca*, however, Pby1 does not simultaneously share a posterior border with En. Instead, nascent segmental stripes spanning four cell rows narrow to two cell rows just before the appearance of an adjacent En stripe, which overlaps by only a single row of cells. This lack of temporally coincident expression does not, however, rule out a possible role in activating *en*, for it is conceivable that the four-cell row domain of Pby1 may activate *en* before narrowing, with the result that En appears specifically in cells that were previously expressing *pby1*. A similar situation may hold true for *Drosophila*, as it has been proposed that, despite the coincident expression of secondary Prd stripes and En, it is instead the earlier primary stripes of Prd that are responsible for the activation of *en* (Fujioka et al., 1995). Finally, it is important to note that a fully functioning pair-rule mechanism in grasshoppers may well require genes in addition to *pby1* that exhibit pair-rule like expression patterns.

The evolution of insect pair-rule patterning

Based on widespread conservation of expression patterns, it seems likely that the *Drosophila* segment polarity genes functioned as such in the context of the ancestral insect segmentation system. The picture is less clear for pair-rule genes. In light of the more basal phylogenetic position of *Schistocerca*, it is tempting to view the posterior expression domains of *eve* and *ftz* as ancestral for insects, existing before the evolutionary recruitment of these genes to play a role in segmentation (Dawes et al., 1994; Patel et al., 1992). In support of this conjecture, vertebrate orthologs of *eve* are linked to the Hox clusters and expressed in broad Hox-like domains (Bastian and Gruss, 1990; D'Esposito et al., 1991; Dolle et al., 1994; Ruiz i Altaba and Melton, 1989), while the *C. elegans eve* ortholog, *vab-7*, is both expressed in a broad posterior domain and required for posterior cell fates (Ahringer, 1996). *ftz*, a gene closely related to the *Antp*-class Hox genes, is likewise expressed in a broad Hox-like domain in mites (Telford, 2000).

However, grasshoppers in some respects are likely to represent a derived state for insects. This is probably the case for *eve*, as this gene is expressed in stripes in spiders (Damen et al., 2000). Thus, it is possible that *eve* was primitively expressed in both stripes and a posterior domain, but somewhere along the lineage leading to *Schistocerca*, the gene lost its striped expression. Our observation that a PgIII gene is expressed in stripes of a two-segment periodicity in grasshoppers suggests that pair-rule patterning is part of the ancestral insect segmentation system. However, confirmation of this claim will require closer examination of the striped expression of pair-rule orthologs in primitive insects and non-insect arthropods.

An additional consequence of the molecular data presented here is that *Tribolium* and *Schistocerca* appear more similar in their embryology than previously appreciated. Before this study, the non-striped expression of *eve* and *ftz* did not allow comparison with the striped expression of pair-rule genes in other insects. The Pby pattern, however, allows such a comparison. In the case of *Tribolium*, only one Pby stripe, that of the mandibular segment, has formed before the onset of

gastrulation (Fig. 2D); *eve* and *ftz* stripes at this stage have likewise not formed posterior to the gnathal region (Brown et al., 1994a; Patel et al., 1994). In *Schistocerca*, we have been unable to detect any *pby1* expression before the onset of gastrulation (~36 hours AEL), and the first stripe associated with segmentation (the gnathal arc) does not appear until 10% (~50 hours AEL), well after gastrulation has begun (Fig. 5C). Thus, neither *Tribolium* nor *Schistocerca* has specified segmental or parasegmental boundaries posterior to the head at the start of gastrulation, conforming to the classical idea of short (as opposed to intermediate) germ embryogenesis (Sander, 1976).

Drosophila prd is at the bottom of the genetic hierarchy of pair-rule genes and this fact, coupled with its later segmental expression, have led some to suggest that in flies *prd* acts as a bridge between the pair-rule and segment polarity levels of the segmentation hierarchy (Baumgartner and Noll, 1991). If pair-rule patterning is an evolutionarily recent specialization of *prd*, then the segmental secondary Prd stripes of *Drosophila* are best seen as the remnants of an ancestral dual function as a pair-rule and segment polarity gene. It is perhaps not surprising then, that *pby1* – a PgIII gene from a more phylogenetically primitive insect – is expressed in both a pair-rule and segment polarity fashion. As one of only two PgIII genes in *Schistocerca*, *pby1* is expressed in a manner reminiscent of the combined pattern of all three PgIII genes in *Drosophila*. In lacking the specialized expression of the *Drosophila* genes, *pby1* may be our closest approximation of the ancestral insect PgIII gene.

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