Pair-rule expression patterns of *even-skipped* are found in both short- and long-germ beetles

Nipam H. Patel*, Barry G. Condron† & Kai Zinn†

* Department of Embryology, Carnegie Institution of Washington, 115 West University Parkway, Baltimore, Maryland 21210, USA
† Division of Biology, California Institute of Technology, Pasadena, California 91125, USA

Now that the genes controlling embryonic patterning have been identified in several model organisms, long-standing questions concerning the evolution of developmental systems are open to investigation. Examination of the expression of *even-skipped* in a variety of insects reveals that insect germ-type designations apparently do not reflect the variations in the mechanisms of segmentation evident throughout insect phylogeny.

GENETIC and molecular approaches have yielded a wealth of information about the mechanisms of pattern formation in the Dipteran *Drosophila melanogaster*. The anterior–posterior axis of the embryo is first established by gradients of maternal information derived from products transported into the oocyte from nurse cells during oogenesis[1]. These gradients initiate the successive expression of zygotic gap, pair-rule, and segment polarity genes, which rapidly subdivide the embryo into progressively smaller units, leading to the nearly simultaneous establishment of segmental pattern throughout the embryo[2,3].

There are reasons to believe, however, that not all aspects of the *Drosophila* patterning system are shared by all insects. Even though all insect embryos ultimately establish similar body plans, various experimental perturbations suggest that the initial mechanisms of pattern formation vary considerably among different insects[4]. A general classification scheme derived from some of these manipulations places insects into three broad categories: long, intermediate and short germ[4]. Extreme long-germ embroyos, such as those of *Drosophila*, contain a complete representation of the body plan by the end of the blastoderm state. By contrast, extreme short-germ embryos, such as those of the Orthopteran *Schistocerca americana* (grasshopper), generate all body segments during a post-blastoderm growth phase. Intermediate-germ insects occupy a middle range; segments are established as far posterior as the thorax or anterior abdomen at the blastoderm stage, and the remaining, more posterior segments are formed after gastrulation.

A comparison of a phylogenetic tree of insect evolution with the distribution of germ types (Fig. 1) indicates that the different germ types are distributed throughout the insect orders and that

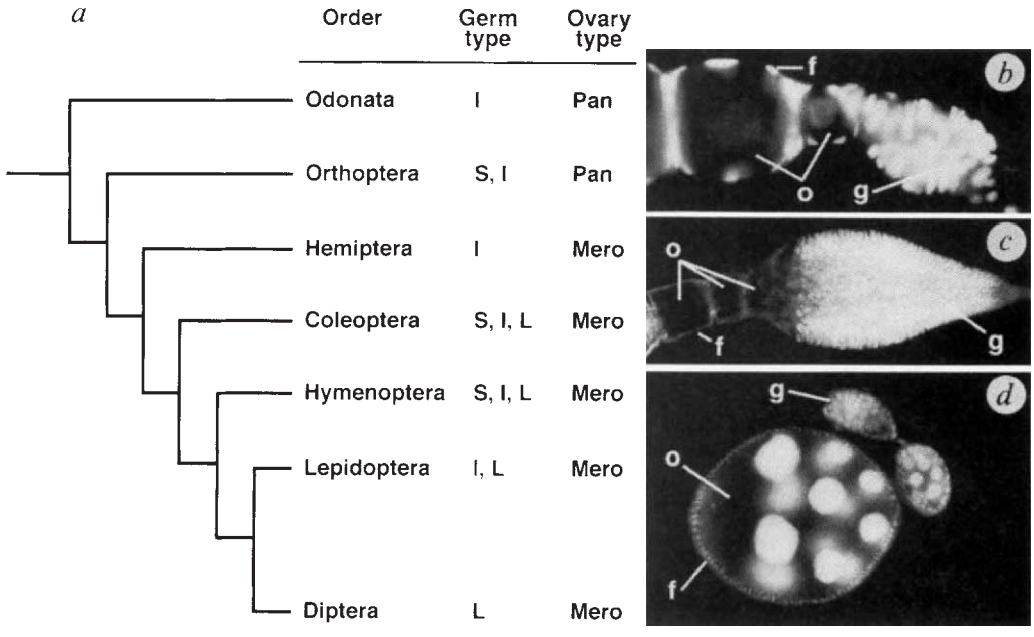

FIG. 1 Relationships of insect orders, germ types, and ovary types. *a*, Phylogenetic tree of a subset of insect orders. The tree is adapted from refs 24 and 25. The germ-type distribution is derived from ref. 4 (S, short; I, intermediate; L long). The ovary type distribution is taken from ref. 26 (Pan, panoistic, no nurse cells; Mer, meroistic, with nurse cells). Germ types do not form separate monophyletic groups, and certain orders include representatives of all three germ types. One general trend is visible: long-germ development appears to be restricted to the more phylogenetically 'advanced' orders, which also all contain meroistic ovaries. *b–d*, Diaminophenylindole (DAPI)-stained ovaries. o, Oocytes; f, follicle cells; g, germarium. *b*, The panoistic ovary of the Orthopteran *Schistocerca americana*. Stem cells in the germarium produce oocytes that are not attached to nurse cells. The germinal vesicle of the oocyte transcribes all the maternal mRNA of the egg[26]. The small, flat cells surrounding the oocyte are the follicle cells, which secrete the chorion of the egg. *c*, The meroistic ovary of the Hemipteran *Oncopeltus fasciatus*. This is referred to as a telotrophic meroistic ovary because the nurse cells, which supply various maternal mRNAs, remain in the germarium and are attached to the anterior end of each developing oocyte by nutritive cords[26]. *Tribolium*, *Dermestes* and *Callosobruchus* all possess this type of ovary. *d*, The meroistic ovary of the Dipteran *Lucillia cuprina*. This is an example of a polytrophic meroistic ovary, in which the nurse cells are attached to the anterior end of the oocyte and supply various maternal mRNAs[1], but, unlike in telotrophic meroistic ovaries, the nurse cells move along with the oocyte down the ovariole.

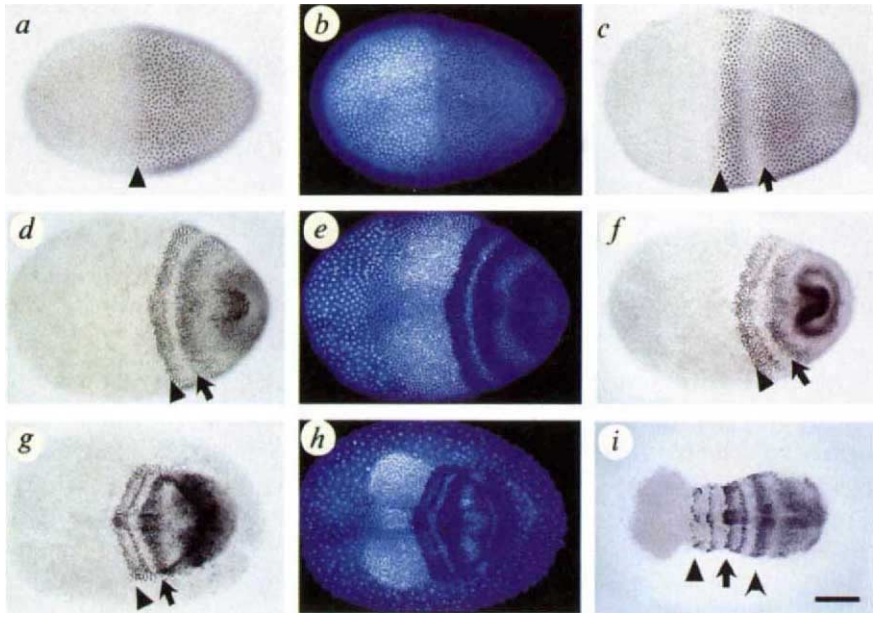

FIG. 2 *Tribolium even-skipped* protein patterns. Nomarski (*a*, *c*, *d*, *f*, *g*, and *i*) and fluorescence (*b*, *e* and *h*) images of embryos stained with mAb 2B8 and counterstained with DAPI (DNA stain). Triangles and arrows mark the anterior boundaries of *even-skipped* primary stripes 1 and 2, respectively, from the time that the boundary is first visible to the time its associated primary stripe resolves into secondary stripes. Arrowhead indicates the position of *even-skipped* primary stripe 3. *a* and *b*, Initial *even-skipped* expression in the posterior half of the embryo. *c*, The first *even-skipped* primary stripe forms as *even-skipped* protein disappears from a circumferential interstripe zone. An irregular 'wave' of mitosis then begins and *even-skipped* protein temporarily diffuses from the nucleus to the cytoplasm of mitotic cells (not shown). *d* and *e*, The primitive pit begins to form and cells continue to aggregate and divide to form the embryonic germ anlage. Gastrulation now begins at the ventral midline. The second *even-skipped* primary stripe forms as an interstripe appears anterior to the primitive pit. *f*, *even-skipped* primary stripe 1 begins to resolve into two secondary stripes. *g* and *h*, The posterior region of the embryo continues to dive into yolk. At this time, *even-skipped* staining disappears from the extraembryonic serosa, and *even-skipped* primary stripe 1 has resolved into two secondary stripes (1a and 1b). As caudal extension continues, *even-skipped* stripes form in the amnion that are somewhat irregular but are aligned to the stripes in the embryo. *i*, Embryo dissected free from the egg to expose the more posterior regions. *even-skipped* primary stripes 1 and 2 are both split into secondary stripes (1a, 1b and 2a, 2b) and the interstripe region between *even-skipped* primary stripes 3 and 4 is forming. Anterior is to the left and ventral side is up in all panels. Scale bar, 100 μm for all panels.

METHODS. Mice were immunized with the previously described *Schistocerca* eve/trpE fusion protein[8]. Of the several hundred hybridoma lines that recognized *Schistocerca even-skipped*, three lines, 3B9, 7H5 and 2B8, were expanded for further study. The staining patterns of mAb 2B8 in *Drosophila* and *Schistocerca* are identical at all stages to published descriptions of *even-skipped* protein expression in these two insects[8,12]. Based on the mid-embryonic staining of the characteristic pattern of cells in the dorsal mesoderm, anal pad and nervous system (RP2, aCC, pCC, U, CQ and EL neurons, a medial neuron in the first suboesophageal ganglia, and a pair of bilaterally symmetric neurons in the brain)[27,28], mAb 2B8 seems to recognize *even-skipped* in the following insects: Diptera, *Drosophila melanogaster*, *Anopheles gambiae*, *Lucillia cuprina*; Hymenoptera, *Camponotus laevigatus*; Coleoptera, *Tribolium castaneum*, *Dermestes frischi*, *Callosobruchus maculatus*; Dermaptera, *Anisolabis annulipes*; Orthoptera, *Schistocerca americana*, *Locusta migratoria* and *Acheta domesticus*. Furthermore, mAb 2B8 staining patterns in *Tribolium* match the distribution of *Tribolium even-skipped* mRNA (J. Parrish, S. Brown and R. Denell, personal communication). Immunohistochemistry has been described[18]. Detailed protocols for optimizing egg collection, fixation, and devitellinization for the various insect species are available on request.

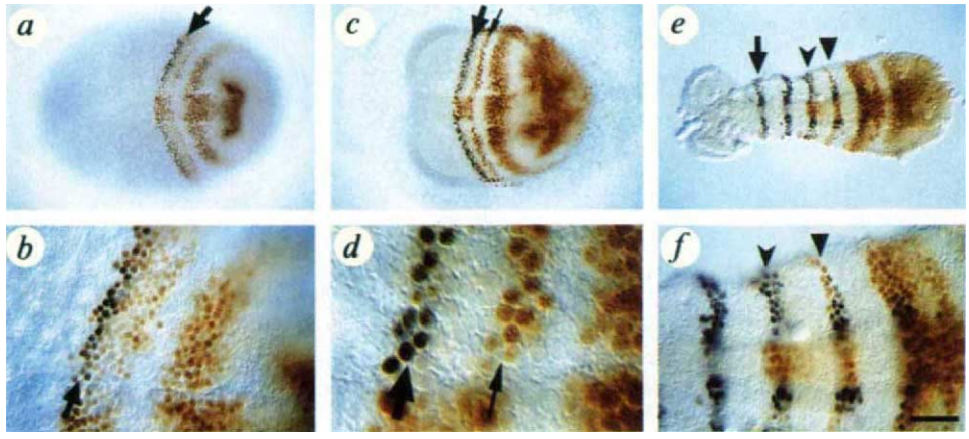

FIG. 3 Relationship of *even-skipped* and *engrailed* expression in *Tribolium*. Embryos are stained for *engrailed* in black and *even-skipped* in brown. *b*, *d* and *f*, High-magnification views of the embryos shown in *a*, *c* and *e*, respectively. *a*, *b*, At the onset of gastulation, *engrailed* stripe 1 (wide arrow) appears at the anterior edge of *even-skipped* primary stripe 1. Note that *even-skipped* primary stripe 1 is just beginning to resolve into secondary stripes. *c*, *d*, As caudal elongation begins, *engrailed* stripe 1 (wide arrow) and *even-skipped* stripe 1a are coincident. *engrailed* expression has not yet started in *even-skipped* stripe 1b (thin arrow). *e*, *f*, In this embryo, *engrailed* stripe 1 is indicated with a wide arrow; *even-skipped* stripes 1a and 1b have faded away; *even-skipped* stripe 2a coincides with *engrailed* stripe 3 (arrowhead) and *even-skipped* stripe 2b coincides with *engrailed* stripe 4 (triangle; *even-skipped* stripes 2a and 2b are most obvious at the midline because *even-skipped* stripes fade from the ectoderm before the mesoderm). *engrailed* stripe 5 is forming at the anterior margin of *even-skipped* primary stripe 3. Anterior is to the left and ventral side is up in all panels. Scale bar: *a*, 100 μm; *b*, 25 μm; *c* and *e*, 75 μm; *d*, 10 μm; *f*, 30 μm.

METHODS. *engrailed* expression was detected using mAb 4D9, which recognizes *engrailed* in a wide variety of organisms[18]. The inclusion of nickel chloride in the staining reaction was used to generate the black reaction product. Embryos were then washed, stained with mAb 2B8, and reacted without metal ions to reveal the expression of *even-skipped* in brown.

insects with shared germ types do not sort into individual monophyletic groups. Thus although the germ-type classification scheme is an important reminder of the diversity of embryonic development, the simple categorization of long, short and intermediate germ types is insufficient to describe the evolutionary origins of insect pattern formation[5]. Examination of homologues of *Drosophila* segmentation genes in other insects may help establish a more comprehensive framework for describing the evolution of insect pattern formation.

A number of molecular studies have compared *Drosophila* and *Schistocerca* development, in part because *Schistocerca* represents the opposite extreme of several characteristics found in *Drosophila*[6–9]. Not only is *Schistocerca* a short-germ insect but, in contrast to *Drosophila*, *Schistocerca* is considered to be relatively phylogenetically primitive, it is hemimetabolous, and its eggs are formed without the benefit of maternal input from nurse cells (Fig. 1*b–d*). Previous experiments compared the *Drosophila* and *Schistocerca* expression patterns of *even-skipped*[8], a homeobox-containing gene required for proper segmentation and neurogenesis in *Drosophila*[10,11]. In *Drosophila*, *even-skipped* is expressed during segmentation in a pair-rule pattern and later is a segmentally reiterated subset of neurons[12,13]. *Schistocerca* shows a similar pattern of *even-skipped* expression during neurogenesis, but no pair-rule pattern was observed during segmentation[8]. This result suggested that short-germ *Schistocerca* embryos, rather than using a pair-rule prepattern, might generate segmental patterns by a different mechanism, such as a system of cell–cell interactions during the growth of the embryo.

The conclusions from comparisons between *Schistocerca* and *Drosophila* may not, however, explain why germ-type differences are also observed within individual insect orders. For example, within the order Coleoptera (beetles), which is considered to be phylogenetically intermediate between Orthoptera and Diptera, species are found that span the entire range of germ types[4]. To study the events that underlie germ-type transitions, we examined *even-skipped* expression in the short-germ beetle *Tribolium*[14], the intermediate-germ beetle *Dermestes*[4], and the long-germ beetle *Callosobruchus*[4]. In all three beetles, *even-skipped* is expressed in a pair-rule manner, with the only difference being the relative number of stripes formed before, versus after, the onset of gastrulation. Thus, germ-type designations do not necessarily predict the mechanistic details of development: short-germ *Tribolium* is probably more closely related to long-germ *Drosophila* than to short-germ *Schistocerca* with regard to the mechanisms that generate the segmental pattern.

Tribolium even-skipped stripe formation

To examine *even-skipped* expression in the Coleoptera, we used a monoclonal antibody (mAb 2B8) that was generated against the *Schistocerca even-skipped* protein but that also recognizes *even-skipped* in a variety of other insects (see Fig. 2 legend). Before cellularization in short-germ *Tribolium* embryos, *even-skipped* is clearly detectable posterior to 45–50% egg length (100% egg length is the posterior pole of the embryo), and only very low levels are seen in the remaining anterior portion of the embryo (Fig. 2*a*, *b*). As development proceeds, *even-skipped* protein is no longer detectable in the anterior half of the blastoderm. During the subsequent course of embryogenesis, eight primary *even-skipped* stripes form from the posterior domain of *even-skipped* expression. The first primary stripe forms as embryonic cells begin to condense towards the ventral surface of the egg (Fig. 2*c*), and a second primary stripe forms during the onset of gastrulation (Fig. 2*e*, *f*). The remaining six primary stripes form sequentially as the embryo undergoes caudal elongation (Fig. 2*g*, *h*, *i*; see also Figs 3 and 6*a*, *c*). All the primary stripes arise from a combination of the elimination of *even-skipped* protein from interstripe regions and an increase in *even-skipped* protein levels within each stripe. The interstripe region between two primary stripes is roughly two-thirds the width of a primary stripe. After each primary stripe forms, it resolves into two thin secondary stripes (a and b) as *even-skipped* protein disappears from a region within the original primary stripe (Fig. 2*f–i*; see also Fig. 5). These secondary stripes narrow and then fade completely before the morphological appearance of nearby segments. The formation of *even-skipped* primary stripes, the resolution of each primary stripe into secondary stripes, and the eventual disappearance of the secondary stripes, occur sequentially in an anterior–posterior progression along the length of the embryo.

To determine the precise relationship of *even-skipped* stripes with the formation of segmental and parasegmental boundaries, we compared the *even-skipped* and *engrailed* expression patterns. In *Drosophila*, *engrailed* is a member of the segment polarity class of genes and is expressed in the posterior portion of every segment[15–17]. Using a monoclonal antibody (mAb 4D9) that recognizes *engrailed* homologues in a wide variety of organisms, previous studies show that this segmental expression of *engrailed* is highly conserved in insects and crustaceans[18], and we used mAb 4D9 to detect beetle *engrailed*.

In *Tribolium*, *engrailed* protein stripe 1 (anterior portion of parasegment 1) appears just at the onset of gastrulation; with the exception of the cephalic stripes, the remaining *engrailed* stripes appear sequentially along the embryo as development proceeds. Double labelling for *even-skipped* and *engrailed* proteins reveals that ectodermal cells at the anterior margin of *even-skipped* primary stripe 1 begin to express *engrailed* just as this primary *even-skipped* stripe begins to resolve into its two secondary stripes (Fig. 3*a*, *b*). After the first *even-skipped* primary stripe resolves completely into secondary stripes 1a and 1b, *engrailed* stripe 1 and *even-skipped* stripe 1a are coincident (Fig. 3*c*, *d*). *engrailed* stripe 2 then appears when cells of *even-skipped* stripe 1b begin to express *engrailed*. As embryogenesis continues, these overlapping patterns of *even-skipped* and *engrailed* are repeated, so that the anterior margin of each *even-skipped* primary stripe prefigures the anterior boundary of each odd-numbered *engrailed* stripe or, in other terms, the parasegmental boundary of each odd-numbered parasegment. The odd-numbered *engrailed* stripes thus correspond to the *even-skipped* 'a' secondary stripes, and the even-numbered *engrailed* stripes correspond to the *even-skipped* 'b' secondary stripes (Fig. 3*e*, *f*; Fig. 5). The comparison of *even-skipped* and *engrailed* patterns thus shows that the primary *even-skipped* stripes are pair-rule (because the pattern has a two-segment periodicity) and the secondary *even-skipped* stripes are segmental.

Expression in *Dermestes* and *Callosobruchus*

At the cellular blastoderm stage of intermediate germ *Dermestes*, *even-skipped* protein is detected in all cells posterior to 35–40% egg length. In long-germ *Callosobruchus*, this expression boundary is located at 25–30% egg length. In both of these beetles, as in *Tribolium*, eight primary *even-skipped* stripes form sequentially in an anterior–posterior progression from this posterior domain of *even-skipped* expression, and each of these primary stripes subsequently resolves into two secondary stripes that then disappear (Fig. 4). As in *Tribolium*, the formation of primary stripes involves the elimination of *even-skipped* protein from interstripe regions, and the secondary stripes resolve as *even-skipped* protein disappears from a zone within each primary stripe. Double labelling for *even-skipped* and *engrailed* reveals that the relationship between *engrailed* and *even-skipped* expression in *Dermestes* and *Callosobruchus* is identical to that already described for *Tribolium:* the eight primary *even-skipped* stripes have a pair-rule periodicity and demarcate the anterior margins of odd-numbered *engrailed* stripes, and the secondary stripes are coincident with each *engrailed* stripe (Fig. 5).

A conspicuous difference between these three beetles is, however, seen in the temporal relationship between morphological development and the molecular progression of segmentation. In *Tribolium*, the first primary *even-skipped* stripe appears as cell condensation begins, and a second primary stripe resolves at the

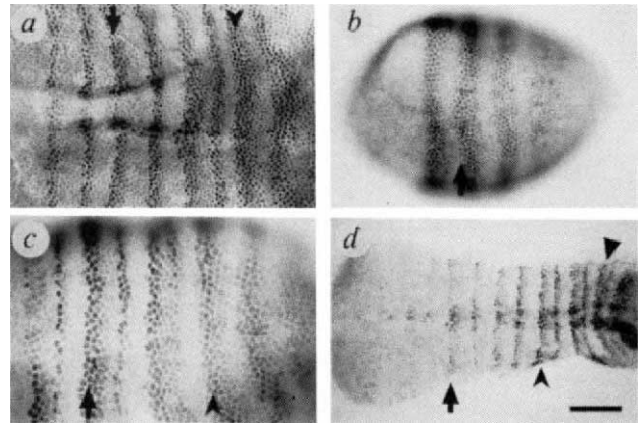

FIG. 4 Expression of *even-skipped* in *Dermestes* (*a*) and *Callosobruchus* (*b*, *c*, *d*). *a*, At the onset of gastrulation in *Dermestes*, primary stripes 1, 2 and 3 are in various phases of resolving into secondary stripes (arrow marks stripe 2a). Primary stripe 4 (arrowhead) has just separated from the posterior domain of expression. The folds in the middle of the embryo are the edges of the forming gastral furrow. *b*, Early in the cellular blastoderm stage, three *even-skipped* primary stripes have already formed in *Callosobruchus* embryos. Low levels of *even-skipped* expression are found in all cells posterior to the third *even-skipped* stripe (the entire posterior region is not visible in this focal plane). The anterior margin of primary stripe 2 is indicated with an arrow. *c*, Shortly before the start of gastrulation in *Callosobruchus*, *even-skipped* primary stripes 1, 2 and 3 are in various phases of resolving into secondary stripes, whereas primary stripes 4 and 5 are still in their wide, pair-rule pattern. Stripe 2a is indicated with an arrow, primary stripe 4 is indicated with an arrowhead. As primary stripe 2 resolves into secondary stripes, it does so asymmetrically: the 'a' secondary stripe is initially wider than the 'b' secondary stripe. This is the case for the initial formation of all *even-skipped* secondary stripes in all three beetles (see primary stripe 3 in Fig. 3*e* and *f* for example). *d*, At the onset of gastrulation and caudal extension in *Callosobruchus*, *even-skipped* stripes 1a and 1b have faded entirely; primary stripes 2, 3, 4, 5 and 6 are at various points in the process of resolving into secondary stripes; and stripes 7 and 8 have not yet separated from each other. Stripe 2a is indicated by an arrow, stripe 4a by an arrowhead, and stripe 6a by a triangle. Anterior is to the left and ventral side is up all panels. Scale bar: *a*, 130 μm; *b*, *d*, 100 μm; *c*, 50 μm.

onset of gastrulation. In *Dermestes*, two primary *even-skipped* stripes have formed by the time cells condense toward the ventral side, two more primary stripes have resolved by the onset of gastrulation (Fig. 4*a*), and the remaining four primary stripes appear during caudal extension. In *Callosobruchus*, the first three primary *even-skipped* stripes are formed by the time cells begin to condense on the ventral side (Fig. 4*b*), and three additional primary stripes form by the time gastrulation begins (Fig. 4*c*, *d*). The seventh and eighth stripes finally separate from one another during caudal elongation.

Comparisons with *Drosophila*

The expression of *even-skipped* and *engrailed*, and the relationship between the two patterns, have been extensively investigated in *Drosophila*[13,19], and many aspects of *even-skipped* and *engrailed* expression are strikingly similar in *Drosophila* and beetles. In both groups of insects, *even-skipped* is first expressed in a broad domain, pair-rule stripes form by a combination of interstripe repression and stripe activation, and the anterior margin of each *even-skipped* pair-rule stripe prefigures the anterior margin of each odd-numbered *engrailed* stripe[19]. The *Drosophila even-skipped* stripes start out approximately six cells wide, with the highest expression within a region of four cells (about one segment width)[13]. Instead of resolving into two secondary stripes as in the beetles, however, the *Drosophila even-skipped* pair-rule stripes narrow to coincide ultimately with the odd-numbered *engrailed* stripes, and weak stripes appear *de novo* that are coincident with even-numbered *engrailed* stripes[12,13]. The segmental stripes of *even-skipped* alternate in intensity in *Drosophila* but are of equal intensity in the beetles. The resolution of *even-*

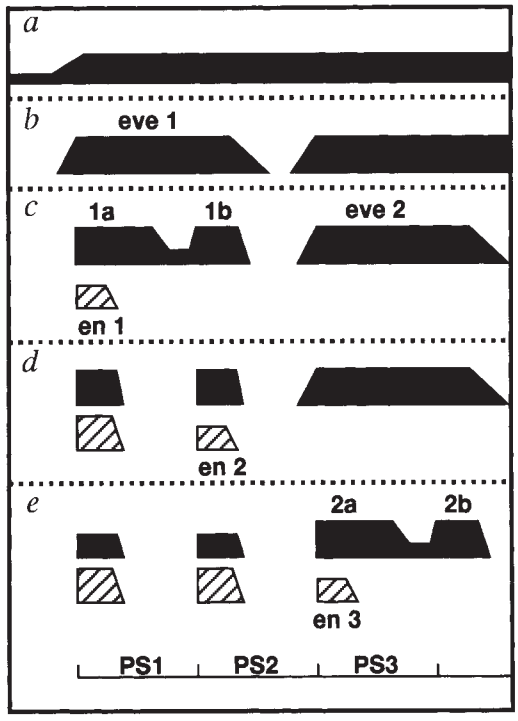

FIG. 5 Diagram of the relationship between *even-skipped* and *engrailed* expression in all three Coleoptera. Solid blocks represent *even-skipped* expression, and hatched boxes represent *engrailed* expression. *a*–*e*, Successively later stages during the establishment of *even-skipped* and *engrailed* patterns in the anterior regions of the embryo. Parasegment boundaries (PS) are indicated at the bottom of the diagram. The heights of the boxes reflect the approximate levels of protein expression. The pattern shown here continues down the length of all three beetle embryos as development proceeds.

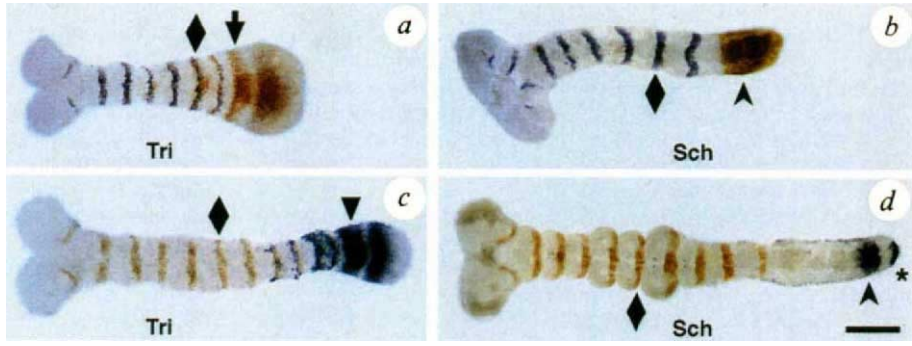

FIG. 6 Comparison of *even-skipped* and *engrailed* patterns in *Schistocerca* and *Tribolium* during abdominal segmentation. *Tribolium* (Tri; *a* and *c*) and *Schistocerca* (Sch; *b* and *d*) embryos stained for *engrailed* in black and *even-skipped* in brown (*a* and *b*) or *even-skipped* in black and *engrailed* in brown (*c* and *d*). In all four panels, a diamond marks *engrailed* stripe 5 (second thoracic segment). The *Tribolium* and *Scistocerca* embryos in *a* and *b* are at similar stages of generating segments because both have six *engrailed* stripes. Likewise, those in *c* and *d* are at a similar stage because both have just started to form the ninth *engrailed* stripe (obscured by *even-skipped* stripe 5a in the *Tribolium* embryo). In the *Tribolium* embryos, pair-rule (primary) stripes of *even-skipped* are clearly visible. The arrow in *a* indicates the position of primary *even-skipped* stripe 4. In *c*, a triangle marks primary *even-skipped* stripe 6. Primary stripes 7 and 8 are still fused, and stripes 4a and 4b have almost completely faded away (the irregularly positioned black cells in 4a and 4b are in the amnion overlying the embryo). In the *Schistocerca* embryos, *even-skipped* is expressed in a posterior domain (arrowhead in *b* and *d*), but no pair rule pattern of stripes is seen. The staining at the posterior tip in *d* is the start of expression in the anal pad (asterisk), a structure that expresses *even-skipped* in all insects examined, and the isolated *even-skipped* expressing cells in the region of the thorax are the ganglion mother cells that produce RP2 neurons. These photographs show that both *Schistocerca* and *Tribolium* embryos increase in length as they develop. As the *Schistocerca* abdomen changes in length, it maintains a constant width, but as the *Tribolium* abdomen increases in length, it decreases in width. *Tribolium* embryos appear to 'pinch down' as sequential *engrailed* stripes form. Preliminary studies suggest that *Tribolium* embryos undergo phases of regional proliferation followed by convergent extension and that *Schistocerca* embryos also exhibit phases of regional proliferation, but without convergent extension (N.H.P., unpublished observations). It is possible that at least part of the shape changes seen in *Tribolium* are due to the same type of cell intercalations that occur during *Drosophilia* germband extension. More detailed cell lineage analysis will be required to accurately establish the relationship between 'growth' and patterning in these insects. Anterior is to the left and ventral side is up in all panels. Scale bars: *a*, *c*, 100 μm; *b*, *d*, 225 μm.

skipped primary pair-rule stripes into secondary segmental stripes in the beetles is reminiscent of a similar transformation described for the stripes of the pair-rule gene *paired* in *Drosophila*[20].

All the beetles generate eight pair-rule stripes of *even-skipped*, but only seven are seen in *Drosophila*. The seventh *Drosophila even-skipped* stripe is wider than the rest[12,13], however, which is consistent with the idea that the more posterior segments of *Drosophila* have become fused together. Interestingly, *even-skipped* disappears from the posterior end of all three beetle embryos at the same time that the anterior margin of *even-skipped* primary stripe seven becomes visible (Fig. 6*c*). Thus, beetle *even-skipped* primary stripes 7 and 8 start out fused and separate only later in development.

Short-germ *Tribolium* and *Schistocerca* compared

We have examined younger (8–10% of development) *Schistocerca* embryos than previously[8] and find that *even-skipped* is expressed by all cells in the gastral groove and all cells in the posterior two-thirds of the embryo at this stage. As development proceeds, expression rapidly disappears from the proliferating thoracic region and then from the gnathal region, leaving expression in a more posterior domain, as described previously[8]. This posterior expression is highest in the mesoderm but the monoclonal antibodies reveal that *even-skipped* protein is also present in the overlying ectoderm (Fig. 6*b*, *d*). No pattern of pair-rule stripes is seen and no overlap is detected with the developing *engrailed* stripes.

Figure 6 shows the striking differences in the relationship of *even-skipped* and *engrailed* expression in *Schistocerca* and *Tribolium* embryos. As already described, *even-skipped* is expressed in pair-rule and segmental patterns in *Tribolium* before the establishment of *engrailed* stripes (Fig. 6*a*, *c*). In *Schistocerca*, *even-skipped* is expressed in a posterior domain, but no pattern of pair-rule stripes resolves from this domain (Fig. 6*b*, *d*). In addition, both *engrailed* expression and the morphological formation of segments begin in the thorax (parasegments 4 and 5) and spread both anteriorly and posteriorly[6]. By contrast, in *Tribolium*, *engrailed* stripes and the morphological development of segments begin in parasegment 1 and spread posteriorly.

Discussion

To analyse the evolution of insect pattern formation, we examined *even-skipped* expression in three beetle species that span the range of germ types, from short-germ *Tribolium* to intermediate-germ *Dermestes* to long-germ *Callosobruchus*, and compared these patterns with *even-skipped* expression in long-germ *Drosophila* and short-germ *Schistocerca*. We infer two major conclusions from our results: (1) germ-type designations do not necessarily correlate with a particular mechanism of pattern formation, but (2) germ-type designations, at least within the Coleoptera, do accurately predict the temporal aspects of segmentation.

In all three beetles, *even-skipped* is expressed in identical spatial patterns. Eight *even-skipped* pair-rule stripes are established from a posterior domain of *even-skipped* expression by the elimination of *even-skipped* in interstripe regions; these pair-rule stripes then resolve into segmental stripes. In addition, the relationship between *even-skipped* and *engrailed* patterns is identical in all three beetles. This suggests that although each beetle belongs to a different germ type, *Tribolium*, *Dermestes* and *Callosobruchus* seem to use identical mechanisms of pair-rule patterning, at least as determined by the analysis of *even-skipped* and engrailed expression.

The expression of *even-skipped* and its relationship to *engrailed* expression are similar in *Drosophila* and beetles: in both insects, the anterior margins of *even-skipped* pair-rule stripes demarcate the anterior boundary of odd-numbered parasegments, and the segmental secondary stripes align with each *engrailed* stripe. Because the function of beetle *even-skipped* has not been examined genetically, however, it is not yet known whether *even-skipped* is required for the expression of all *engrailed* stripes in beetles as it is in *Drosophila*[13]. Despite the striking similarities in *even-skipped* expression patterns, some aspects of pair-rule patterning are likely to be different between *Drosophila* and Coleoptera. For example, the differences in establishment of

secondary (segmental) *even-skipped* stripes in *Drosophila* and Coleoptera may indicate there are slight differences in the pair-rule gene interactions in the two groups of insects. Thus, our analysis of *even-skipped* expression suggests that long-germ *Callosobruchus* is more closely related to short-germ *Tribolium* than to long-germ *Drosophila* in regard to the mechanisms of pair-rule pattern formation, consistent with the phylogenetic relationships of these three insects.

In short-germ *Schistocerca*, *even-skipped* is expressed in a posterior domain, but no pattern of pair-rule stripes resolves from this domain and no overlap is detected between *even-skipped* and *engrailed* expression[8]. In addition, pair-rule patterns for *fushi tarazu* are seen in *Drosophila* and *Tribolium* (S. Brown and R. Denell, personal communication) but not in *Schistocerca* (R. Dawes and M. Akam, personal communication). Thus, the establishment of segments in *Schistocerca* may not involve pair-rule prepatterning. The available data indicate that short-germ *Tribolium* is more closely related to long-germ *Drosophila* than to short-germ *Schistocerca* in regard to the use of pair-rule patterning mechanisms. Taken together, our results indicate that germ-type designations do not correspond to specific mechanisms of pattern formation. Closely related short-, intermediate- and long-germ insects (*Tribolium*, *Dermestes* and *Callosobruchus*) can use similar segmentation mechanisms, whereas two distantly related short-germ insects (*Tribolium* and *Schistocerca*) may use different segmentation mechanisms.

Our results do show, however, that germ-type designations accurately predict the overall temporal aspects of beetle segmentation, as assayed with molecular markers. The expression of pair-rule and segment polarity genes in *Drosophila* shows that the entire segmental pattern is established by the onset of gastrulation[2,3]. Based on *even-skipped* and *engrailed* expression, segmental patterning in *Tribolium* has only proceeded as far as the gnathal segments of the head by the start of gastrulation (Fig. 2*d*). At a similar stage in *Dermestes* development, segments have formed as far posterior as the anterior abdominal region. In *Callosobruchus*, all but the posterior-most abdominal segments are established before the onset of gastrulation. The analysis of *engrailed* expression in *Schistocerca* indicates that all the segments of the gnathal, thoracic and abdominal regions are established after gastrulation[6]. It should be noted that there has been some controversy on the assignment of *Tribolium* as a short- or intermediate-germ insect[14], but our results on *engrailed* expression clearly support the classification of *Tribolium* as a short-germ beetle. It will be interesting to examine *even-skipped* and *engrailed* expression in certain Lepidoptera, whose morphological development is reminiscent of intermediate germ types but for which the results of experimental perturbations are consistent with a long-germ designation[4].

We postulate that the transitions between short-, intermediate- and long-germ development within the Coleoptera is simply the result of heterochrony. That is, the same pair-rule patterning mechanism generates the segmental pattern in all Coleoptera, but changes in the relative timing of morphological development and the molecular process of segmentation have given rise to the continuum of germ types within the Coleoptera. The germ-type differences between *Drosophila* and *Schistocerca*, on the other hand, are more likely to have resulted from changes in the mechanisms that generate anterior–posterior patterns.

Previous results showed that pair-rule patterns of *hairy* expression are seen in *Drosophila* and *Tribolium*[14]. From this it was suggested that pair-rule patterning is used in *Tribolium* and that pair-rule patterning is a fundamental component of segmentation in all insects[14]. Our results support the first conclusion but not the second. Although we cannot absolutely rule out pair-rule patterning mechanisms in *Schistocerca*, our results argue that comparisons between long-germ *Drosophila* and short-germ *Tribolium* cannot be used to predict properties possessed by all insects. Similarly, caution should be applied in interpreting the results from *Schistocerca*. Although *Schistocerca* development has been put forward as a model for the 'primitive' mode of insect pattern formation[6,21], several other species from phylogenetically primitive orders have been described as intermediate germ types, and, more importantly, the results of embryonic perturbations in some of these insects suggest that they develop somewhat differently than *Schistocerca*[4]. We are currently expanding our studies on *even-skipped* to some of these phylogenetically primitive intermediate-germ insects, as well as to crustaceans, to compare their development to *Schistocerca* and to gain a better understanding of the ancestral mode of insect pattern formation. Several unusual species of phylogenetically advanced orders also deserve further investigation, particularly members of the Hymenoptera in which multiple embryos develop from a single egg.

Although germ types do not correlate with potential mechanisms that establish segments, the limited data suggest a correlation between segmentation mechanisms and ovary types. In addition to Diptera and Coleoptera, evidence for pair-rule patterning has been seen in Hymenoptera[22] and Lepidoptera[23], all insects with meroistic ovaries (Fig. 1*c*, *d*). Pair-rule patterns have not been observed in *Schistocerca*, an insect with panoistic ovaries (Fig. 1*b*). Further studies will be required to determine whether ovary types truly limit the systems of pattern formation, and an analysis of phylogentically advanced groups, such as fleas, that have reverted back to panoistic oogenesis would be particularly interesting. It is worth stressing that even though the general classification scheme of germ types may not correlate with segmentation mechanisms, the data collected from embryonic perturbations studies are invaluable, especially in systems where genetic analysis is not yet possible. Ultimately, discerning how insect segmentation has evolved will require genetic, molecular and embryonic perturbation data from species spanning the range of insect phylogeny. □

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