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SHORT, LONG, AND BEYOND: Molecular and Embryological Approaches to Insect Segmentation

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■ **Abstract** Over the past dozen years, studies comparing the expression of orthologues of the *Drosophila* segmentation genes among various insects have served to broaden our view of the ways in which insects make segments. The molecular data suggest that, although the overall genetic mechanisms of segmentation during embryogenesis have been conserved, the details of this process vary both within and between various insect orders. Here we summarize comparative gene expression data relevant to segmentation with an emphasis on understanding the extent of molecular patterning prior to gastrulation. These results are discussed in embryological context with an eye toward understanding the evolution of segmentation within insects.

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INTRODUCTION

Molecular approaches have both deepened our understanding of biological phenomena and provided fresh avenues that we have only begun to explore. Amidst this bounty, however, molecular data have not always squared well with the results

of previous morphological studies. This is particularly true for systematics, where decisions about how to either integrate or variously privilege molecular and morphological results have not come easily. A similar dilemma faces the field of comparative insect development. Pioneering work on the genetics of early development in *Drosophila* (53, 68) has led to the identification of genes crucial to patterning the *Drosophila* embryo. As a result, the longstanding tradition of comparative insect embryology has been confronted with a slow but steady stream of data concerning the embryonic expression of candidate developmental genes in various insects. On the one hand, the results have revealed a great deal of conservation in the developmental processes responsible for early pattern formation. Although this impression is surely a product, in part, of our tendency to recognize the familiar, conservation at the molecular level is intriguing in light of dramatic variation in the embryology of different insects. On the other hand, the results also reveal that these developmental processes vary, often in subtle and interesting ways. Understanding the form and extent of this variation is an important first step toward understanding how development evolves.

Here we attempt to take stock of the variation in the process by which insects make segments. In particular, we review comparative molecular data on segmentation among various insect orders, focusing on the expression of homologues of the pair-rule and segment polarity genes identified in *Drosophila*. In the spirit of integrating molecular and morphological approaches, we have tried not to neglect the embryological context of our subject, discussing the results of embryological manipulations where relevant. The discussion is prefaced with both a review of basic concepts in insect embryogenesis and a short description of the genetic hierarchy responsible for segmentation in *Drosophila*, since, for the most part, genes identified for their role in segmentation in fruit flies have served as the basis for molecular studies of segmentation in other insects.

INSECT EMBRYOS: SMALL AND LARGE, SHORT AND LONG

For almost all insects embryogenesis begins with repeated divisions of the zygote nucleus without cell division. The result is a yolky syncytium containing a population of centrally located energids, each consisting of a nucleus surrounded by a yolk-free island of cytoplasm. After several rounds of division, the majority of energids migrate to the periphery to form a blastoderm. In some insects (e.g., *Drosophila*) this early blastoderm is syncytial, consisting of a monolayer of nuclei surrounding yolk. Eventually, this syncytial blastoderm will cellularize, resulting in a cellular blastoderm—essentially a monolayer of cells. In other insects (e.g., grasshoppers) there is no prolonged syncytial blastoderm, and nuclei cellularize immediately or soon after they reach the surface of the egg. In any event, at about this time a portion of the blastoderm is specified as the embryonic rudiment, or germ anlage, while the remaining portion is slated to form extraembryonic tissue. Beyond the blastoderm, embryogenesis among insects also varies quite substantially

with regard to when and how the segmental body plan is patterned. To describe these differences, three modes of embryogenesis are generally recognized: short, intermediate, and long germ embryogenesis.

Whereas the designations short, intermediate, and long germ are now used to describe both the initial size of the germ anlage and differences in embryogenesis, the terminology originally coined by Krause in 1939 was more specialized (47, 97). The terms "kleinkeim" and "grosskeim" (small and large germ) were intended to describe the length of germ anlage relative to the length of entire egg (Figure 1). The terms "kurzkeim," "halblangkeim," and "langkeim" (short, half-long, and long germ) were instead meant to capture a suite of differences in embryogenesis, among them the number of segments specified in the germ anlage prior to gastrulation. In many long germ insects (e.g., *Drosophila*) all segments are specified almost simultaneously within the blastoderm (i.e., prior to gastrulation). In short germ insects (e.g., grasshoppers) only segments of the head (including the procephalon and gnathos) are specified in the blastoderm, whereas the remaining segments of the thorax and abdomen form progressively from a posterior growth zone following gastrulation. The terms were meant to represent opposite poles of a continuum, with intermediate (half-long) germ insects falling somewhere between these two extremes. In Krause's words, "The 'short germ' mainly represents the head region . . . the 'long germ' maintains the natural proportions of the body regions of the larva . . . Therefore short germ, half-long germ and long germ differ by the number of presumptive segments within the segment formation zone" (47, translated in 85). In short germ insects (and to a lesser extent intermediate germ insects) posterior

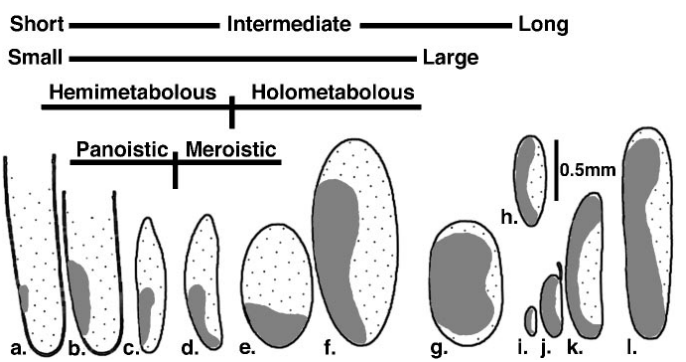

Figure 1 Insect eggs of various germ types with correlated characters (adapted from 63, 82, 84). Insect eggs are drawn to scale and represent lateral views with anterior at top. Area covered by germ anlage is shaded. Orthoptera: *a*, *Oecanthus pellucens*; *b*, *Acheta domesticus*. Odonata: *c*, *Platycnemis pennipes*. Hemiptera: *d*, *Euscelis plebejus*. Coleoptera: *e*, *Atrachya menetriesi*; *f*, *Leptinotarsa decemlineata*; *h*, *Bruchidius obtectus*. Lepidoptera: *g*, *Bombyx mori*. Diptera: *i*, *Smittia* sp.; *j*, *Drosophila melanogaster*; *k*, *Calliphora erythrocephala*. Hymenoptera: *l*, *Apis mellifera*.

segments that have not yet been specified originate from a disproportionately short posterior region of the germ anlage, the segment formation or growth zone, which undergoes substantial growth as these segments are defined. Thus, whereas the entire process of segmentation in a long germ insect is by proportionate subdivision of the pregastrulation anlage, segmentation in a short germ insect requires a phase of secondary growth coupled with the sequential specification of more posterior segments (83).

As Krause was well aware, the small/large and short/long distinctions are not independent. Indeed, the majority of insects exhibiting the short and long germ types of embryogenesis possess small and large germ anlagen, respectively (Figure 1). Thus, workers in the field have come to use the terms short, intermediate, and long germ to indicate not only the number of presumptive segments in the growth zone of the anlage, but also its relative size. Typically this poses no problems, although equating the distinctions can sometimes be misleading, as in the case of the silk moth discussed below.

Differences between short and long germ insects are also reflected in the results of embryological manipulations. To begin with, the typically small size of short germ anlagen, as well as their relative autonomy, is reflected in the ability to withstand ligations and lesions directed at portions of the egg far from the site of anlage formation. When the germ anlage is affected by manipulations, the resulting pattern of defects reflects the delayed and sequential nature of posterior segment specification in short and intermediate germ insects, whereas the more specific defects of long germ insects reflect the early and simultaneous specification of all segments (reviewed in 2, 3, 82). It is also generally the case that long germ insects exhibit a reduced capacity to regulate in response to perturbation, a trait that likely has much to do with the fact that long germ insects develop much more quickly than either short or intermediate insects. A related difference is that in long germ embryos, molecular-patterning events tend to take place earlier relative to embryological events. Indeed, the difference between the short and long germ modes can be characterized in part as a heterochronic shift in the process of segmentation and patterning relative to gastrulation (48; Figure 2). The tendency for greater "prefabrication" in long as opposed to short germ embryos (82) is a theme we encounter again as we compare the molecular data of various insects as well as their modes of oogenesis.

While short and intermediate germ types are widely found among various insect orders, the long germ type is for the most part restricted to those orders utilizing nurse cells during oogenesis (Figure 3). Nurse cells may allow for an increased and spatially polarized maternal contribution to the developing oocyte. This evolutionary innovation was potentially an important precondition for rapid development, if not actually a direct product of its selection. The correlation between germ and ovary types was first made by Bier, who linked the presence of nurse cells (meroistic ovaries) with the long germ type and the lack of nurse cells (panoistic ovaries) with the short germ type (8). As there are plenty of examples of meroistic short germ insects, this correlation is now expressed in the assertion that, with possible exceptions such as fleas, most long germ insects have nurse cells (Figure 3) and

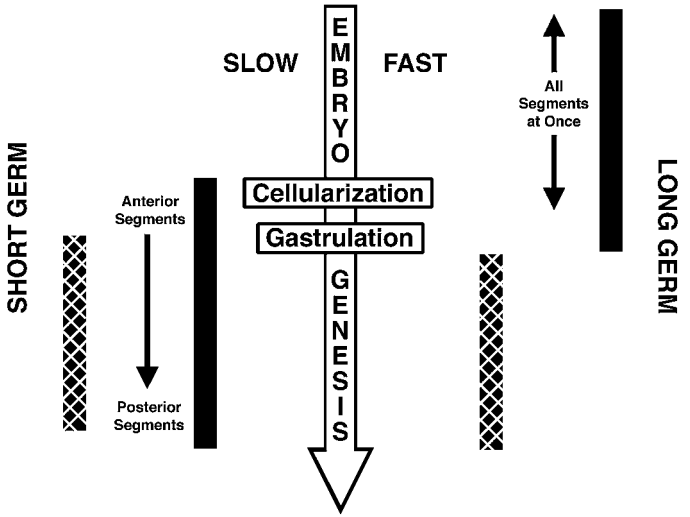

Figure 2 Relative timing of cellularization, gastrulation, segment specification, and elongation in short versus long germ embryogenesis. Molecular process of segmentation is shown as a solid bar; germ band elongation/extension is shown as a hatched bar.

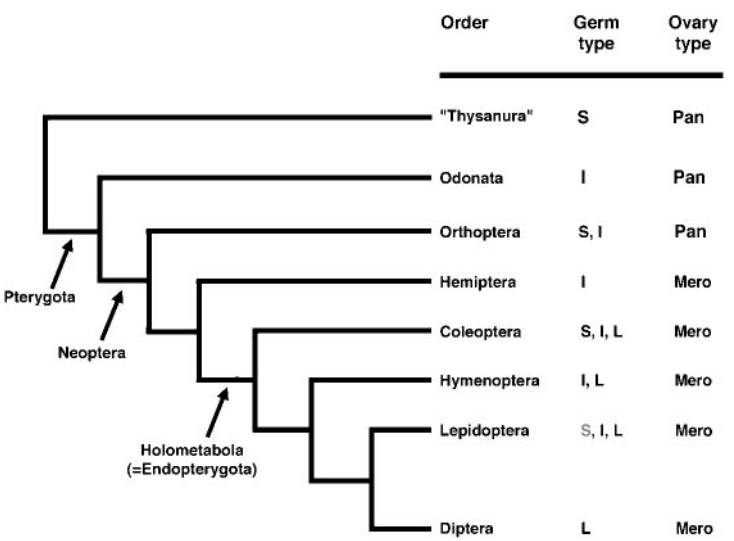

Figure 3 Phylogeny of insect orders discussed in text (plus Hemiptera) showing distribution of germ and ovary types (adapted from 73). Phylogeny and ovary type distribution (based on 43, 50). Germ type distribution (based on 82 and text). The inclusion of the short germ type in lepidopterans (in gray) is tentative.

thus that the provisioning of maternal information by nurse cells might have been a prerequisite for the evolution of long germ embryogenesis.

Closer inspection of the phylogenetic distribution of germ types reveals, however, that the long germ type is more closely correlated to holometaboly, which is characterized by both rapid development and the possession of larvae whose morphology differ substantially from adults (Figure 3). The fact that the long germ type is found in multiple clades within the Holometabola suggests one of two possibilities. Either long germ embryogenesis has been secondarily lost in orders possessing representatives of multiple germ types (e.g., Coleoptera and Lepidoptera), or it has evolved more than once. In essence, we are left uncertain as to the homology of long germ embryogenesis between different groups, a question we return to (see below).

FRUITS OF THE FLY

Because it is not uncommon to treat *Drosophila* as the archetypal example of long germ embryogenesis, it may surprise the reader that, as the sole dipteran discussed in Krause's 1939 paper, *Drosophila* was instead described as "fast langer grosskeim" (almost long large germ). The germ anlage of *Drosophila*, as well as those of other dipterans, indeed occupies the entire length of the egg and is clearly large (Figure 1). With regard to the long germ designation, Krause's hesitation stemmed in part from the observation that this insect, as well as other dipterans and long germ beetles, undergoes a phase of elongation known as germ band extension, in which the germ anlage stretches by cell rearrangement followed by contraction to its original length. Whether germ band extension is related to the elongation observed in short and intermediate germ types, however, is not clear. One important difference is that, in contrast to short and intermediate germ insects, germ band extension in *Drosophila* and other long germ insects is not the result of disproportionate growth in the prospective abdomen. This difference is mirrored by UV fate maps that indicate that, whereas all segments are proportionally represented in the *Drosophila* blastoderm (54), the posterior segments of short and intermediate germ insects originate from a disproportionately small region of the germ anlage. Although fate maps provide some insight into the nature of segmentation, ideally they are supplemented with molecular descriptions of these events.

In this regard, it is fortunate that we know a great deal about the molecular means by which the *Drosophila* embryo is progressively subdivided. mRNAs transcribed from maternal genes and transferred to the oocyte by nurse cells are later translated and act at the top of a genetic hierarchy involving the sequential activation of the zygotic gap, pair-rule, and segment polarity genes. In the first step of this process, the maternal protein products establish the expression patterns of gap genes. Gap genes, which all encode transcription factors, were originally defined by their loss-of-function phenotypes in *Drosophila*, in which regions of the embryonic cuticle spanning several segments are deleted (68).

Consistent with their phenotypes, gap genes are expressed in domains that span several segments, and together with maternal genes are responsible for the earliest patterning events in *Drosophila* embryogenesis. It is important that these initial events take place prior to cellularization. This is because several maternal genes (e.g., *bicoid* and *nanos*) and gap genes (e.g., *hunchback* and *Krüppel*) are thought to act as morphogens via protein gradients that form by diffusion across the syncytial blastoderm. Such a syncytial requirement would seem to present a problem to short and intermediate germ insects, since in these cases patterning and segmentation of the posterior portions of the germ anlage must take place in a cellular environment. Nevertheless, as we discuss below, the expression of gap gene orthologues in several short germ insects suggests that these molecular mechanisms are conserved.

The spatial pattern of maternal coordinate and gap gene expression is responsible for setting up patterns of pair-rule gene expression, which represent the first periodic gene expression in the *Drosophila* embryo. The pair-rule genes, like the gap genes, all encode transcription factors, and again, the genes were originally defined by their loss-of-function phenotypes in *Drosophila*, in this case deletions of the embryonic cuticle occuring with a two-segment periodicity (68). Consistent with this phenotype, pair-rule genes in *Drosophila* are typically expressed in stripes of a two-segment periodicity in both the syncytial and cellular blastoderm. The pair-rule genes that have thus far been examined outside of *Drosophila* are *even-skipped* (*eve*), *hairy*, *runt*, *fushi-tarazu* (*ftz*) and *paired* (*prd*). In *Drosophila*, *hairy* and *runt* are expressed in the early blastoderm in complementary patterns, each consisting of seven stripes of a two-segment periodicity (29, 39; Figure 4). Similarly, *eve* and *ftz* are also found in complementary seven-stripe patterns (27, 34, 36, 56). Unlike *hairy* and *runt*, however, the *eve* and *ftz* stripes loosely obey the boundaries of units known as parasegments (52). These are divisions of the developing embryo defined by the expression of segment polarity genes (see below). Although each parasegment is one segment wide, parasegmental boundaries are slightly out of phase with segmental boundaries so that each parasegment contains approximately the posterior 1/3–1/4 of one segment and the anterior 2/3–3/4 of the adjacent segment (Figure 4). The pair-rule stripes of *e*ve and *ftz*, then, are roughly centered on the odd- and even-numbered parasegments, respectively (Figure 4). Finally, *prd* is also found in seven pair-rule stripes in the early blastoderm, and like *ftz*, these stripes fall primarily within even-numbered parasegments, but in this case extend slightly across the parasegmental boundaries (33, 42; Figure 4).

Although the "pair-rule pattern"—stripes of a two-segment periodicity—is intuitively consistent with a pair-rule phenotype, the expression patterns of these genes are actually quite dynamic. For example, several pair-rule genes are additionally expressed in stripes of a one-segment periodicity. In particular, *eve*, *runt*, and *prd* are expressed in a "segmental" pattern following gastrulation. Such secondary patterns are accomplished in at least two ways. In the case of *eve*, each of the original stripes becomes refined to the anterior of odd-numbered parasegments, and weaker so-called minor stripes appear de novo in even-numbered parasegments

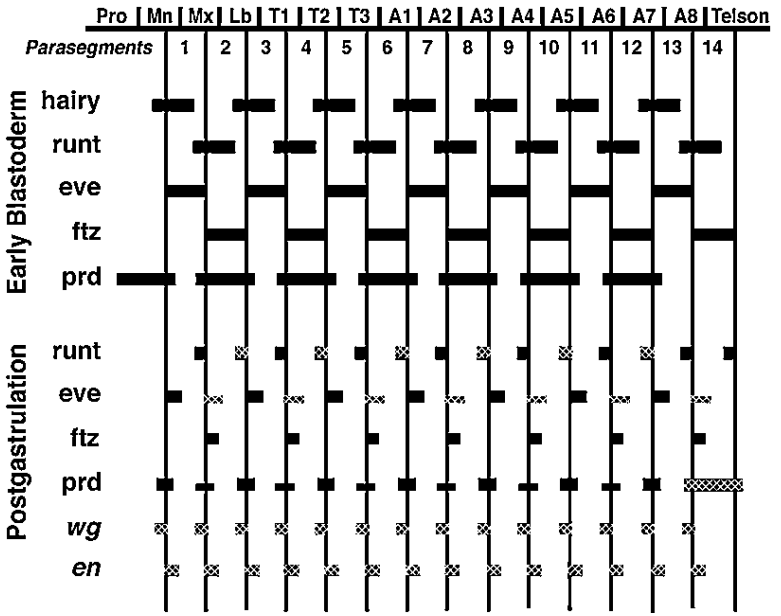

Figure 4 Protein expression of various pair-rule and segment polarity genes in *Drosophila melanogaster* in both blastoderm and postgastrulation stages (adapted from 69, 91). Pair-rule genes are expressed in the early blastoderm, but their expression is joined by the expression of segment polarity genes (in italics) immediately after the onset of gastrulation (ventral furrow formation). Postgastrulation solid domains are temporally and spatially continuous with early blastoderm expression, whereas hatched domains appear de novo.

(27, 56; Figure 4). Likewise, primary *runt* stripes refine to the posterior of each odd-numbered parasegment as de novo stripes appear in the even-numbered parasegments (29; Figure 4). The segmental secondary stripes of *prd*, however, instead arise by a process of "splitting." This occurs when, just prior to gastrulation, the centrally located cell rows of primary pair-rule stripes 2–7 gradually lose expression (33, 42; Figure 4). With the addition of a broad eighth stripe, the result is a segmental pattern of 14 stripes of alternating intensity, each spanning a parasegmental boundary. The point at which *eve*, *runt*, and *prd* are expressed in segmental patterns coincides with the segmental expression of genes of the next level of the hierarchy, the segment polarity genes.

Pair-rule genes regulate the expression of segment polarity genes, most of which are expressed in a segmentally reiterated pattern immediately following the onset of gastrulation and throughout the morphologically segmented germ band stage. Like the gap and pair-rule genes, the 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 (68). Unlike the gap and pair-rule genes, however, not all segment polarity genes are transcription factors, the other members of the class being components of signaling pathways. For example, the segment polarity genes most widely studied in other insects, *engrailed* (*en*) and *wingless* (*wg*), encode a transcription factor and a secreted signaling molecule, respectively. In *Drosophila*, both *en* and *wg* are expressed as a single ectodermal stripe within each individual segment, defining the anterior and posterior boundaries, respectively, of each parasegment (6, 101; Figure 4).

The expression of pair-rule and segment polarity genes in stripes represents the earliest periodic organization of the *Drosophila* embryo. The number of these stripes present in the blastoderm and immediately following the onset of gastrulation is thus a reliable gauge of the extent to which segments along the anteroposterior axis have been specified. In *Drosophila*, the full set of pair-rule and segment polarity stripes appear prior to, and shortly after, the onset of gastrulation. For example, in the *Drosophila* blastoderm we find seven *eve* stripes and 14 *en* stripes shortly thereafter, corresponding to the 14 morphological segments of the larvae. The stripes of each gene appear along the anteroposterior axis almost simultaneously, with equal spacing, reflecting the long germ mode of segmentation by subdivision. This somewhat simplified description is made more complex by the observation that, in the case of *en*, even-numbered stripes appear slightly before odd-numbered stripes, and stripes appear in a slight anterior-to-posterior progression (23, 24, 41, 101). Details notwithstanding, it is fair to say that the *Drosophila* segmentation hierarchy specifies all segments nearly simultaneously within the blastoderm, consistent with the classification of *Drosophila* as a long germ insect on morphological grounds (47).

The ability to detect a molecular prepattern in the form of stripes has afforded us with an opportunity to ask whether molecular data generally support germ type assignments based on morphology and experimental embryology. The answer is generally yes, although as we examine pair-rule and segment polarity data below we encounter examples where this is not the case. Before we begin, it is worth noting that the use of pair-rule and segment polarity orthologues to assess the number of segments specified at the blastoderm stage requires that expression and function be minimally conserved in other insects. If this were not the case, meaningful comparisons would elude us. So, is it?

With regard to segment polarity genes, similar patterns of both *en* and *wg* have thus far been found in all insects examined (reviewed in 70, 71). Genetic studies in *Drosophila*, together with functional studies in the flour beetle (68a), allow us to reasonably infer conservation of function as well, suggesting that segment polarity genes, and parasegments, constitute aspects of the ancestral insect segmentation system. Indeed the role of these genes in patterning segments is likely to be ancient since they are also expressed in segmental stripes in embryos of noninsect arthropods such as crustaceans (58, 67, 71, 75, 76, 87, 88) and spiders (17). In contrast, the expression of pair-rule orthologues has also been investigated in a number of insects, and the results vary from highly conserved to subtly different to radically

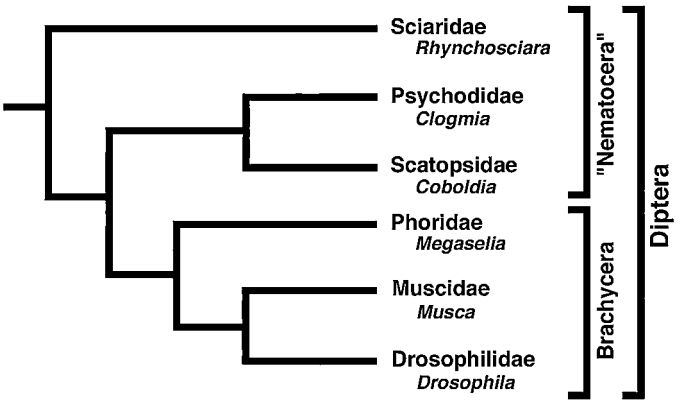

Figure 5 Phylogeny of dipteran species discussed (based on 105).

divergent (reviewed in 70). We discuss these results below and conclude by asking how they inform our current picture of the evolution of insect segmentation.

DIPTERA

In general, the available molecular and experimental data (reviewed in 82) support the long germ designation for members of both major dipteran suborders, the Brachycera (true flies) and the "Nematocera" (a paraphyletic group that includes mosquitoes, gnats and midges) (Figure 5). Despite the single germ type designation, however, there are interesting differences between these groups with regard to segmentation. In particular, brachycerans exhibit a greater degree of molecular prepattern in the blastoderm, whereas nematocerans have retained the tendency to delay segmentation and patterning of the most posterior segments (79).

Along with the genus *Drosophila*, the house fly *Musca domestica* and the more basal phorid fly *Megaselia abdita* are some of the brachycerans that have been the subject of molecular study. In the case of house flies, the overall expression of gap genes such as *hunchback*, *Krüppel*, and *knirps*, is well conserved with respect to *Drosophila* (92). Expression of the pair-rule gene *hairy* and the segment polarity gene *en* reveals that the number of segments specified at gastrulation is similar to that of *Drosophila*; seven and 14 stripes, respectively, are present in the late blastoderm and early gastrula (92; Table 1). *Megaselia* also appears to exhibit a similar blastoderm prepattern, as seven primary *eve* stripes are reported to appear prior to gastrulation and are soon followed by the de novo appearance of seven weak secondary *eve* stripes (79; Table 1).

More distantly related to *Drosophila*, nematoceran flies exhibit some interesting differences. In the psychodid midge *Clogmia albipunctata*, the gap genes *Krüppel*

and *hunchback* are expressed in mostly conserved patterns. The likely equivalent of the abdominal hunchback stripe found in the *Drosophila* blastoderm, however, appears during germ band extension in *Clogmia*, suggesting that posterior development is delayed in this insect (78, 79). Consistent with this notion, only six primary *eve* stripes, centered on the odd-numbered parasegments, appear simultaneously in the syncytial blastoderm. These stripes start out broad (2–3 cell rows), but later narrow (1 cell row) in an anterior to posterior progression. The seventh primary stripe, which overlaps the delayed *hunchback* domain, likewise appears later during germ band extension (78, 79; Table 1). What appears as a delay specific to the posterior, however, may in actuality result from an overall delay in patterning, rendering what in *Drosophila* appears as a subtle anteroposterior progression of pattern into the more obvious posterior delay observed in *Clogmia*. Consistent with this notion, all of the *en* stripes in *Clogmia* are delayed, arising later during germ band extension (78, 79; Table 1).

It is perhaps surprising that no secondary stripes of *eve*, located in even-numbered parasegments in *Drosophila*, were observed in *Clogmia* (79; Table 1). This may be a problem of detection, however, as the same anti-Eve monoclonal antibody used in this study (MAb 2B8) only rarely detects these minor stripes in *Drosophila* (N. Patel, unpublished data). On the other hand, it is interesting to note that in *Drosophila* the developmental role of these minor stripes is still not clear. Although in *Drosophila* complete loss of *eve* function results in the absence of both odd- and even-numbered *en* stripes (36, 56), the activation and maintenance of even-numbered *en* stripes is likely to be mediated solely by the posterior trailing edge of the early primary stripes centered on odd-numbered parasegments (28). Thus even if the results obtained with MAb 2B8 do reliably indicate a lack of secondary *eve* stripes in *Clogmia*, a more general role for *eve* in segmentation is likely to be conserved.

Delayed patterning observed in other nematocerans suggests a trend in this group. In the scatopsid midge, *Coboldia fuscipes*, *eve* is expressed in at most five primary stripes prior to gastrulation. Furthermore, although secondary stripes are observed, they appear relatively late (at full extension) and only in the second and fourth parasegments (79; Table 1). In the sciarid gnat *Rhynchosciara americana*, gastrulation begins long before germ band extension, and yet stripes detected with the monoclonal antibody 4D9 do not appear until after germ band extension (99). If the stripes are in fact due to *en*, then this represents an even greater delay in patterning associated with a relatively slow rate of development. In this case, however, it is possible that only products of the *en*-paralogue *invected* are being detected, as this gene in *Drosophila* is expressed in a pattern coincident, but delayed, with respect to *en* (16, 99). MAb 4D9 was raised against *Drosophila* Invected protein, and although the antibody reacts to both Engrailed and Invected in *Drosophila*, this cross-reactivity does not necessarily extend to other insects.

In sum, molecular data reveal variation among dipterans in the temporal dynamics of segment specification, specifically in the extent to which posterior segmentation lags. The anteroposterior gradient of pattern formation found in lower

TABLE 1 Numbers of stripes of pair-rule orthologues and *en* in various insects

			Diptera					Lepidoptera		Hymenoptera		Coleoptera		
			Brachycera			"Nematocera"								
			Drosophila	Musca	Megaselia	Clogmia	Coboldia	Manduca	Bombyx	Apis	Copidosoma	Tribolium	Dermestes	Callosobruchus
En		# of stripes immediately after onset of gastrulation	14	14		*0*			1	0	0-3	1		
		Total # of stripes	15	15		15			16	16	15	16	16	16
Even-skipped	1° (Early) Stripes	# of stripes at onset of gastrulation	7		7	6	5		2	6	3 (not PR)	2	4	6
		Total # of stripes	7		7	7	7		8	8	15 (not PR)	8	8	8
		Anterior border (parasegment)	odd		*odd*	odd	*odd*		odd	odd	na	odd	odd	odd
		Initial width	broad			broad				broad	narrow	broad	broad	broad
	2° (Late) Stripes	Total # of stripes	7		7	-	2 (2,4)		-	8	na	16	8	8
		Anterior border (parasegment)	even		*even*	-	*even*		-	even	na	even	even	even
		Origin of stripes	DN		DN	-	DN		-	Split 1°	na	Split 1°	Split 1°	Split 1°
		Relative intensity of stripes	weak		weak	-	weak		-	strong (in ant.)	equal	narrow (trans.)		

Hairy	# of stripes at onset of gastrulation		7	7								2		
	Total number of stripes		7	7								8		
Runt	# of stripes at onset of gastrulation		7					8						
	Total number of stripes		7					8						
	Secondary stripes?		Yes											
Ftz	# of stripes at onset of gastrulation		7									1		
	Total number of stripes		7									8		
Paired-like	1° (Early) Stripes	# of stripes at onset of gastrulation	8									2		
		Total # of stripes	8									9		
		Anterior border (parasegment)	even									even		
		Initial width	broad									broad		
	2° (Late) Stripes	Total # of stripes	6											
		Anterior border (parasegment)	odd											
		Origin of stripes	Split 1°											
		Relative intensity of stripes	weak											

Only gnathal, thoracic, and abdominal stripes are considered. Grey areas are unknown or unpublished; italics indicate inference based on published data. The 15th *en* stripe of dipterans corresponds to the rudimentary segment A9. Secondary *eve* stripes are observed only for parasegments 2 and 4 in *Colboldia. eve* stripes in *Apis* alternate in intensity only in the anterior and in *Copidosoma* do not exhibit a two-segment periodicity. The *Tribolium hairy* stripe 3/4 is not included as a pair-rule stripe appearing prior to gastrulation. -, not present; PR, pair-rule; na, not applicable; trans, transient; DN, de novo; split 1°, splitting of primary stripe. Data derived from the following sources. Dipterans: *Musca* (92), *Megaselia*, *Clogmia*, and *Coboldia* (79); Lepidopterans: *Manduca* (46), *Bombyx* (103); Hymenopterans: *Apis* (9, 25), *Copidosoma* (30); Coleopterans: *Tribolium* (11–14, 73, 93), *Dermestes* and *Callosobruchus* (73).

dipterans, however, is also a feature of short and intermediate germ insects. Why, then, should nematoceran flies not be reclassified as such? The answer lies in noting that germ type designations are not simply a matter of the slope of this patterning gradient. Dipterans that develop with a relatively steep gradient, as well as the honey bee discussed below, retain the long germ designation because they lack secondary differential growth of the posterior, an essential feature of short and intermediate germ insects. And yet a clear delineation of what we mean by "differential posterior growth" is a tricky business, especially when it comes to lepidopterans.

LEPIDOPTERA

Although historically lepidopterans have been classified as intermediate germ (e.g., 3), they have generally proven problematic for Krause's classification scheme (for discussion see 63). Indeed it is possible that multiple germ types exist within this single order, as is the case for Coleoptera and perhaps Hymenoptera (Figure 6). Members of the group Ditrysia, which encompasses the vast majority of lepidopterans (e.g., *Tineola*, *Bombyx*, and *Manduca*), tend to possess large anlagen and develop relatively quickly, features correlated with long germ embryogenesis. In contrast, the superfamilies Micropterigoidea (*Neomicropteryx*) (3a, 44) and Hepialoidea (*Endoclyta*) (4, 5) appear to possess small anlage and develop slowly,

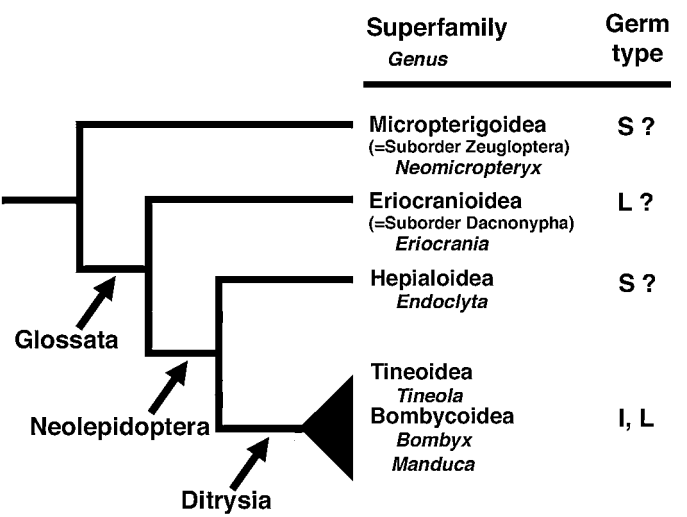

Figure 6 Phylogeny of lepidopteran groups (based on 50a), showing species discussed and hypothetical distribution of germ types (based on discussion in 63).

features correlated with short germ embryogenesis. At least one member of the superfamily Eriocranioidea (*Eriocrania*) appears instead to possess large germ anlagen and develop at a rate comparable to ditrysians (45, 63).

Although ligation experiments have proven difficult in lepidopterans owing to the rigidity of the chorion (63), lesions made with UV light have been informative. In *Tineola biselliella*, UV-irradiating the egg surface in transverse strips at cleavage stages produces segment-specific defects, and the resulting fate map includes all of the abdominal segments (55). In the silk moth, *Bombyx mori*, UV-irradiating small spots on the egg surface prior to the first nuclear division can also produce single-segment defects for all segments (62). Although the results of these experiments do not indicate that cells have either received instructive signals or have been determined, the ability to construct such a fate map prior to blastoderm stage does constitute evidence that segments are specified relatively early in lepidopterans. Indeed, lepidopterans would seem to represent an extreme case, as typically it is not possible to construct such a fate map until the energids have arrived at the periphery, as in *Drosophila* (54). Regardless, the results of perturbation experiments seem to favor the long germ designation for ditrysians, consistent with their large germ anlagen.

A closer look at the embryology of *Bombyx*, however, complicates the issue. Following cleavage, energids arrive at the periphery and cellularize in an anterior to posterior wave to form the cellular blastoderm (65). Soon after, it is possible to distinquish the ventrally located germ anlage from the more dorsal serosa. Strikingly, the silk moth anlage is large, occupying most of the diameter and almost the entire length of the egg (49, 65; Figure 1). This anlage, however, soon contracts to occupy only half the length of the egg (see Figure 7 in 65). By the onset of gastrulation the germ band has taken on a short germ appearance and subsequently undergoes a phase of elongation during which segmental grooves are formed, first in the gnathal and thoracic regions and then sequentially in the abdomen. While this description perhaps favors a short germ designation, closer inspection reveals that the observed elongation is likely to result from cell rearrangements such as convergent extension rather than cell division, as no discrete concentration of mitotic figures is observed in the posterior (49, 65). As this embryology does not fall easily into any germ type, we are fortunate that a fair amount of gene expression data are available for this insect.

While the gene *caudal* does ultimately form an mRNA and protein gradient emanating from the posterior, this gradient forms during gastrulation instead of in the blastoderm as in *Drosophila* (104). At the onset of gastrulation, *hunchback* mRNA is found in an anterior-thoracic domain, but like the midge *Clogmia*, the abdominal *hunchback* domain does not appear until after gastrulation (103). As far as pair-rule and segment polarity orthologues are concerned, at the onset of gastrulation only the most anterior *eve* stripes 1–2 and the most anterior *en* stripe are present (Table 1). During gastrulation and elongation, six additional *eve* stripes appear in rough anterior to posterior progression, the more anterior stripes fading by the time the eighth *eve* stripe appears. Similarly, the remaining 15 stripes of

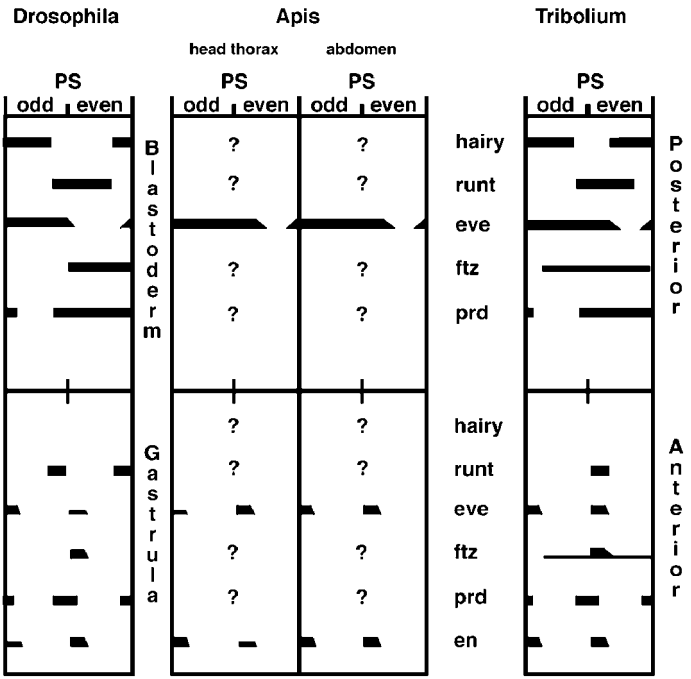

Figure 7 Schematic showing *hairy*, *runt*, *eve*, *ftz*, *prd*, and *en* expression for *Drosophila*, *Apis*, and *Tribolium* (adapted from 9, 11, 12, 73). Upper panel represents the blastoderm prior to gastrulation for *Drosophila* and *Apis* but for *Tribolium* represents the more posterior ("younger") segments. Lower panel likewise represents the postgastrulation germ band for *Drosophila* and *Apis* but for *Tribolium* represents the more anterior ("older") segments. For simplicity the developmental lag between the head/thorax and abdomen in *Apis* is not represented. Expression of *Tribolium runt* based on unpublished observation (described in 11). Domains of *Tribolium hairy* encompass the expression domains of both *Drosophila hairy* and *deadpan* (unpublished observation, described in 11).

en appear sequentially, both during and following gastrulation and elongation. Distances between adjacent *eve* stripes during elongation support the suggestion made above that no discrete posterior growth zone exists in this insect (103). As expected, the anterior borders of these primary *eve* stripes coincide with the anterior borders of odd-numbered *en* stripes, but as reported for *Clogmia*, no secondary stripes are observed (103). Although data were obtained by in situ hybridization, the failure to observe secondary *eve* stripes may still be a problem of detection. In any case, the available molecular data suggest that in *Bombyx* the majority of segments are not patterned prior to the onset of gastrulation. Thus in spite of the initially large germ anlage, the lack of a discrete region of cell

division during elongation, and the fate maps, this insect appears to be of the short germ type.

How are we to explain the fate map results? One possibility is that the fate maps do indeed reflect cell fate but that at early cleavage stages the processes responsible for defining segments have not yet been deployed. Thus cells at this stage might have a specified fate without actually having yet received instructive signals. Alternatively, it is conceivable that a fate map constructed by perturbing embryogenesis does not actually reflect cell fate per se. It is possible, for example, that UV-irradiation disrupts extracellular cues in the cortex that subsequently manifest themselves as pattern defects (for discussion see 82 and 63). In principle this would explain how such a fate map could be constructed for a point prior to the actual molecular subdivision of the embryo into segments. What, then, about the lack of a discrete region of cell division during elongation? Past literature on the process of germ band elongation in short and intermediate germ insects has admittedly not always been clear as to the basis for this morphological change. The terms often used to describe this process—elongation, extension, and growth—are examples of convenient ambiguity. In fact, several clear cases of short germ embryogenesis, such as the flour beetle *Tribolium* (discussed below), may not employ cell division as the principal means of germ band elongation, thus casting doubt on the usefulness of this feature as an indicator of germ type.

A look at another well-studied lepidopteran, the tobacco hawkmoth (*Manduca sexta*), provides an interesting counterpoint. Like *Bombyx*, the initial germ anlage is quite large; but also like *Bombyx*, only the head lobes plus a nonsegmented terminus are apparent at the point invagination is first detected in the gnathal region (see Figure 1*A–B* in 10). At this stage there are no obvious signs of morphological segmentation; segmental grooves first appear in the thorax when the embryo has elongated enough to have formed most of the prospective abdomen (see Figure 1*D–E* of 10). As in *Bombyx*, elongation would seem to result mostly from cell rearrangement rather than cell division. Indeed, elongation can occur in the presence of mitotic inhibitors (personal communication from A. Dorn, cited in 63), and mitotic figures are equally present in anterior and posterior regions (63). Despite these similarities to *Bombyx*, the molecular data for *Manduca* paint a different picture.

Historically, *Manduca* is noteworthy as it provided the first evidence of pair-rule patterning outside of *Drosophila* in the form of an unidentified cell surface antigen that is expressed in a mesodermal pair-rule pattern during gastrulation (15). More direct evidence for pair-rule patterning was later obtained by visualizing the expression of the pair-rule gene *runt* in eight stripes of a two-segment periodicity. In spite of the overall embryological similarity to *Bombyx*, all eight of these *runt* stripes are present and equally spaced prior to gastrulation (46; Table 1). Consistent with this early specification, the abdominal stripe of *hunchback* is also present at this stage, and all 16 stripes of the segment polarity gene *wg* appear by the end of gastrulation (46). The process of elongation in this insect is thus more akin to germ band extension in *Drosophila* where stretching of the germ band occurs only

after its molecular subdivision. As a molecular prepattern exists for all segments prior to gastrulation, it is difficult not to conclude that *Manduca* is best described as a long germ insect (46). Nevertheless, in light of their relatively close kinship and embryological similarity, one can only wonder if these two insects are truly as different as the molecular data would have us believe. By allowing for more direct comparisons, patterns of *eve* and *runt* expression in *Manduca* and *Bombyx*, respectively, should shed considerable light in this regard.

HYMENOPTERA

In 1939 it was the honey bee, *Apis*, rather than *Drosophila*, that received Krause's title for the most extreme long germ type. Not only does the honey bee germ anlage occupy almost the entire length of the egg (Figure 1), it also undergoes almost no germ band elongation (47). In light of the past tendency to associate germ band elongation with the secondary formation of segments, this latter point indeed suggests that this insect is developmentally well ahead of the game. However, like many short germ insects, the honey bee exhibits a pronounced anteroposterior gradient of development. This progression is evident during gastrulation, a process that begins in the gnathal region but moves posteriorly as development proceeds. Instead of invagination at the ventral midline, gastrulation in the honey bee begins with the separation of prospective mesoderm from ectoderm along two longitudinal furrows. The resulting ectodermal-free edges then migrate over the mesoderm to join at the ventral midline (26, 66).

The anteroposterior developmental progression is also evident during segmentation. Before the two gastrulation furrows reach the posterior, transverse segmental grooves form in the gnathal and thoracic regions. These are followed by the sequential appearance of grooves in the abdomen (26). Concomitantly, stripes detected with the monoclonal antibody 4D9 appear within these grooves in gnathal, thoracic, and abdominal regions (25). Like the gnat *Rhynchosciara*, this appearance of stripes at the point of morphological segmentation may mean that the antibody is only detecting products of the *en*-related gene *invected*. The two *en*-related genes from honey bee, E30 and E60, do exhibit minor differences in the 4D9 epitope, but it is not clear if these are sufficient to disrupt binding (76, 100). Upstream of the segment polarity genes, expression of *eve* has been reported, and six of eight primary stripes appear in the blastoderm prior to the onset gastrulation; all of these stripes appear sequentially and share an anterior boundary with odd-numbered *en* stripes (9; Table 1). Thus at least at the pair-rule level, the extent of molecular prepattern in the honey bee blastoderm is comparable to that of the lower dipteran *Colboldia*, consistent with its long germ designation (Table 1).

With regard to pair-rule patterning in *Apis*, a particular observation bears mention. Regardless of whether they represent Engrailed or Invected protein, the *en*-related stripes in the gnathal and thoracic regions alternate in intensity, a feature shared by *Drosophila en* and *invected*, reflecting control by upstream pair-rule

genes. It is intriguing, however, that the strong-weak phasing in honey bee (MN-mx-LA-t1-T2-t3) is opposite to that of *Drosophila* (mn-MX-la-T1-t2-T3) (25). Secondary *eve* stripes in the even-numbered parasegments are also detected in this insect, but like the pair-rule gene *prd* in *Drosophila*, these stripes form by "splitting" broad primary stripes rather than appearing de novo (Table 1). Consistent with the *en* result, these segmental *eve* stripes also possess an alternating intensity in the gnathal and thoracic regions (mn-MX-la-T1-t2-T3), but again, opposite to that of *Drosophila* (MN-mx-LA-t1-T2-t3), preserving the relationship between weak *eve* and strong *en* stripes in the head and thorax (9; Figure 7). This perhaps suggests that, whereas regulatory interactions of the genetic hierarchy in honey bee are conserved, the specifics of its deployment along the anteroposterior axis vary. A similar phenomenon is discussed below regarding the phasing of pair-rule stripes of a *prd*-like gene in grasshopper.

It would be remiss to leave Hymenoptera without mentioning some of its most peculiar members. From the point of view of early patterning, the parasitic wasps are intriguing because some are polyembryonic. *Copidosoma floridanum*, the species of wasp that exhibits the most extreme form of polyembryony known, is capable of generating up to 2000 larvae from a single egg deposited into the egg of its moth host (31). This occurs by repeated rounds of cell division beginning with complete cleavage of the original zygote. Several days later, in the thoracic hemocoel of the host's fourth instar larva, groups of $\sim$20 wasp cells (morulae) undergo morphogenesis to form embryonic primordia that are completely cellular and lack a yolk-filled cavity. These appear as typical-looking germ bands only after transverse dorsal furrows separate the anterior and posterior ends of each coiled anlage. Gastrulation is by ingression, and by the time the ventral gastrulation furrow reaches the posterior the first segmentation groove can be observed in the labial segment. As the embryo uncoils, grooves appear sequentially in the gnathal, thoracic, and abdominal segments (A1–A9).

Molecular data supplement this description. For example, *eve* is first expressed in the embryonic primordium in a broad posterior domain but then resolves directly into 15 segmental stripes that appear in rapid anteroposterior progression. At least three of these stripes are present at the onset of gastrulation (see Figure 5*B* in 30; Table 1). No two-segment periodicity of *eve* stripes is reported, and consistent with this, *en* stripes appear with equal intensity in rough anteroposterior sequence following the onset of gastrulation (see Figure 6*A*, *B*, and *E* in 30; Table 1). Like the honey bee, these stripes form in the absence of any obvious germ band elongation. If the germ band does elongate, then this process is likely to be proportional along the anteroposterior axis and not concentrated at the posterior. Thus although their extremely derived early development may have persuaded previous workers to regard polyembryonic wasps as short germ, both the later embryology and gene expression data would seem to put *Copidosoma* squarely in the long germ camp (30).

With the caveat that pair-rule patterns are often transient and difficult to detect, the purported absence of pair-rule stripes of *eve* in *Copidosoma* invites

speculation regarding the evolution of this gene's expression. In that both *Apis* and the flour beetle *Tribolium* exhibit both pair-rule and segmental patterns of *eve*, the former has likely been secondarily lost in *Copidosoma* (30). This loss is perhaps better correlated with early cellularization rather than with polyembryony, as pair-rule stripes of *eve* are also absent in other monoembryonic wasps that cellularize early (31, 32). However, early cellularization also occurs in insects that do exhibit evidence of pair-rule patterning such as grasshoppers and the silk moth (19, 38, 65, 103). On the other hand, grasshoppers and silk moths do not exhibit the extreme version of cellularization (i.e., holoblastic cleavage) found in parasitic wasps. Thus it should prove interesting to see if a similar loss of pair-rule patterning is observed for springtails, the only other hexapod group reported to undergo holoblastic cleavage.

COLEOPTERA

It has long been recognized that beetles run the gamut from short to long germ type. In general an interesting correlation among beetles exists between germ type and the extent to which anterior portions of the egg are required for complete patterning of the germ anlage (82). In short germ types, such as *Atrachya*, complete germ bands can develop without a large portion of the egg's anterior. This particular insect also exhibits the rather unusual result of producing two complete germ bands if ligation is performed at the blastoderm stage within a particular region (60). In contrast, intermediate germ types, such as *Necrobia*, appear to require anterior portions of the egg in order to form a complete set of pattern elements. Consistent with this idea, middle segments are often entirely missing when ligature yields partial embryos in both the anterior and posterior egg fragments. This is known as the "gap phenomenon." It is explained by postulating a required interaction between the anterior and posterior regions, possibly the sources of early gradients, as these and the downstream gap genes form the basis of the phenomenon in *Drosophila*. Finally, long germ types, such as *Bruchidius*, appear equally dependent on anterior and posterior egg regions and, not surprisingly, exhibit a pronounced gap phenomenon (82).

Are the germ type designations of beetles also reflected by gene expression data? The answer is yes. In this regard, the short germ flour beetle *Tribolium castaneum* is instructive, as its early development has been studied extensively. In this insect, the germ anlage can be distinguished from serosa by late blastoderm. Soon after, head lobes can be distinguished and germ band elongation begins as the amniotic fold begins to form. During the entire process of elongation the germ band narrows substantially, which suggests that convergent extension plays a significant role in this process. Cell division clearly plays some role as well; but because mitotic figures are found throughout the extending embryo and even within the posterior "growth zone" figures are oriented randomly (14), the extent of its contribution is unclear.

Gastrulation, which occurs by invagination of mesoderm along the ventral midline, begins soon after the amniotic fold passes over the posterior pole (35). At this point the germ anlage possesses defined head lobes plus a nonsegmented posterior region. Not until well after the onset of gastrulation are segmental grooves obvious, forming first in the gnathal region (35). Thus it is difficult, based on morphology alone, to determine the extent of patterning prior to gastrulation. For this task the molecular data are of great help.

Tribolium orthologues of the gap genes *hunchback*, *Krüppel*, and *caudal* are all expressed in the blastoderm in patterns roughly comparable to their patterns in *Drosophila*. Differences stem from the fact that *Tribolium* is not a long germ insect: The *caudal* gradient extends over anterior segments in the blastoderm, and the abdominal *hunchback* domain does not appear until much later during germ band elongation (90, 102). The expression of pair-rule orthologues suggests that the expression of these genes is also largely conserved. *hairy* and *runt* are each expressed in a total of eight stripes of a two-segment periodicity. The stripes of each gene appear in an anteroposterior progression during elongation and, like *Drosophila*, are largely complementary to each other except that *Tribolium hairy* encompasses the domains of both *Drosophila hairy* and a *Drosophila* paralogue of *hairy* known as *deadpan* (93, described in 11; Table 1; Figure 7).

Tribolium eve and *ftz* are also each expressed in eight stripes of a two-segment periodicity (12, 13, 73; Table 1; Figure 7). In the case of *eve*, the broad primary stripes are centered on even-numbered parasegments, but extend more posteriorly than in *Drosophila*. Like *Apis*, these stripes split in an anteroposterior sequence during elongation to yield 16 segmental stripes that correspond to stripes of *en*. In *Tribolium* these secondary *eve* stripes (even-numbered parasegments) are transiently narrower than their odd-numbered sister-stripes but exhibit equal levels of expression (13, 73). In the case of *ftz*, the eight pair-rule stripes are most intense at the anterior of each even-numbered parasegment, as in *Drosophila*. The stripes, however, start out much broader than the fly stripes and maintain a broad domain of low-level expression that overlaps with *eve* (12; Figure 7). Finally, an antibody that cross-reacts between products of the *Drosophila* genes *prd*, *gooseberry*, and *gooseberry-neuro* ("anti-Pairberry") reveals stripes that appear sequentially at the posterior and subsequently split into segmental stripes that lie anterior to *en*, with a slight overlap. These stripes are likely to be the product of a *Tribolium prd* orthologue, as they are roughly complementary to stripes of *eve* and of a two-segment periodicity (19; Figure 7).

While it is common to infer function from expression data, it behooves us to exercise caution in this regard. A deletional mutant of the *Tribolium* Hox complex that includes *ftz*, for example, does not exhibit any pair-rule defects (94), indicating that at least this gene is functioning differently than its *Drosophila* orthologue. In spite of this result, pair-rule patterning is likely to be a critical aspect of segmentation in *Tribolium*. Genetic screens using cuticle preparations have yielded at least one and perhaps two pair-rule mutants (57, 95, 96), and in the case of *Tribolium eve*, chromophore-assisted laser inactivation of *eve* protein results in a

pair-rule phenotype, indicating that the pair-rule function of this gene is conserved (89).

With an impressive array of segmentation genes investigated in *Tribolium*, we are in a good position to use these data to gauge the extent of the molecular prepattern at the onset of gastrulation. If we take the point at which the amniotic fold passes the posterior pole as a proxy for the onset of gastrulation (35), the published descriptions indicate that at this point the extending germ band possesses two stripes of *hairy*, two primary stripes of *eve*, one stripe of *ftz*, and two primary stripes of a *prd*-like gene (12, 13, 19, 73, 93; Table 1). This suggests that the germ band is patterned as far posterior as the third parasegment or the anterior compartment of the first thoracic segment (see Figure 4). As expected, the expression of segment polarity genes at this stage lags somewhat behind pair-rule expression: Only a single stripe each of *en* and *wg* are found prior to gastrulation (14, 64; Table 1). Thus the molecular evidence indicates that at most only the gnathal segments have been specified prior to gastrulation, consistent with a short germ classification.

What about gene expression data in intermediate and long germ beetles? In the intermediate germ beetle *Dermestes*, *eve* is expressed in an overall conserved pattern with *Tribolium*, but instead of two primary *eve* stripes at the onset of gastrulation, this beetle has four. Specifically, *eve* primary stripes 1, 2, and 3 are in various stages of splitting by the time primary stripe 4 originates from the posterior domain (see Figure 4*a* in 73). Thus for this beetle the molecular prepattern at the blastoderm stage appears to extend as far posterior as the anterior compartment of the first abdominal segment (Figure 4). In contrast, the long germ beetle *Callosobruchus maculatus* possesses six of its eight primary *eve* stripes at gastrulation, also in various staging of splitting as one moves along the anteroposterior axis (see Figure 4*b–d* in 73). Thus for this beetle all but the most posterior abdominal segments are represented prior to gastrulation. Within Coleoptera, then, it appears that germ type designations are accurately reflected in the temporal dynamics of segmentation as assayed by molecular markers (73).

ORTHOPTERA

Orthopterans, represented by both short and intermediate types, are interesting in light of their more basal phylogenetic position and the fact that they lack nurse cells (Figure 3). The intermediate germ cricket *Acheta domesticus* has been the subject of more experimental investigations than any other insect (reviewed in 82). As with other intermediate germ insects, local UV-irradiation of the germ anlage produces segment-specific defects only in the gnathocephalon and thorax, whereas abdominal defects map to a disproportionately small posterior region of the anlage (40). Irradiating whole eggs with X-rays after formation of the germ anlage also yields localized defects in the gnathal and thoracic region (37). Application of X-rays later in development yields defects in the abdomen, but this sensitivity disappears progressively from anterior to posterior as the irradiation is applied at later and

later time points. Consistent with the intermediate germ designation, up to six *en* stripes are reportedly found in embryos shortly after the onset of gastrulation (77).

Descriptive and experimental studies have also been performed on short germ orthopterans. The camel cricket *Tachycines asynamorus* received the 1939 title for most extreme short germ type (kopfkeim) (47) and was later investigated by isolating portions of the germ anlage and culturing them (reviewed in 82). More recently, the short germ grasshopper *Schistocerca* has emerged as a popular subject for studies of neurogenesis as well as early development. In this insect, cellularization occurs as soon as energids reach the periphery (38) and the germ anlage forms at the posterior pole of the egg, where localization of maternally provided Hunchback protein may provide the cue for condensation or the distinction betweeen embryonic versus extraembryonic tissue (74). Consistent with this notion, cauterizing the posterior tip during cleavage results in a failure to form a germ anlage (61). Reflecting the progressive determination of segments, heat shock applied at the germ band stage results in a localized deletion that moves posteriorly as the heat shock is applied at later and later time points (59). Although the elongating germ band of *Schistocerca* does not narrow as dramatically as the elongating germ bands of either lepidopterans or flour beetles, it is still difficult to find convincing evidence for posterior proliferation, as no discrete region of mitotic figures is observed (N. Patel, unpublished data). The relative spacing between adjacent stripes of gene expression also suggests that contributions to elongation are distributed throughout the embryo (G. Davis & N. Patel, unpublished data). Although not widely recognized as a feature of short germ development, elongation in *Schistocerca* is followed by contraction, a process not unlike the germ band retraction of dipterans and long germ beetles (see Figure 7 in 19, and Figures 13–15 in 80).

Gene expression data from *Schistocerca* indicate that whereas the gap and segment polarity levels of the segmentation hierarchy may be shared with *Drosophila*, dramatic differences exist at the pair-rule level. The gap gene *hunchback* is expressed in an anterior gap domain, but consistent with being a short germ insect, the abdominal domain does not appear until much later during germ band elongation (74). The segment polarity genes *en* and *wg* are also expressed in conserved patterns, and consistent with a short germ designation, at most a single gnathal stripe of *wg* mRNA and no stripes of En protein are apparent prior to gastrulation (21, 75). In contrast, evidence of a conserved role for pair-rule orthologues has proven elusive. *eve* and *ftz* orthologues are not expressed in periodic stripes in the early embryo but in broad posterior domains, suggesting that *eve* and *ftz* play altogether different roles in grasshoppers (20, 72). Recent evidence, however, does suggest that pair-rule patterning is part of the grasshopper segmentation mechanism.

The grasshopper gene *pairberry1* (*pby1*) is closely related to *Drosophila paired* and the related *Drosophila* genes, *gooseberry* and *gooseberry-neuro*. Unlike *eve* and *ftz*, this gene is expressed in broad stripes of a two-segment periodicity at the posterior portion of the elongating abdomen (19). These broad stripes are transient and subsequently split into segmental stripes in a manner reminiscent of *Drosophila*

paired. Thus grasshoppers may utilize a form of pair-rule patterning that apparently employs only a subset of the *Drosophila* pair-rule genes. Oddly, the phasing of these pair-rule stripes is shifted by one segment, suggesting that, as in the honey bee, the spatial deployment of segmentation genes along the anteroposterior axis may be flexible. Additionally, for the first time we can make meaningful comparisons regarding the expression of a pair-rule homologue in grasshoppers. Consistent with the *wg* result and *Schistocerca*'s short germ status, only gnathal *pby1* stripes are found prior to and shortly after the onset of gastrulation, suggesting that thoracic segments have not yet been patterned at this stage (19).

CONCLUDING REMARKS

The day will eventually come when the utility of the candidate gene approach has been exhausted. In the meantime, it is fair to say that the past dozen years has both broadened and refined our picture of insect segmentation. In particular, the approach has allowed us to revisit a classification scheme based on morphology and embryological experimentation. As predicted, germ type designations do not generally reflect the molecular mechanisms of pattern formation (86). Indeed, comparative expression data suggest that these mechanisms are much more conserved than the diversity of germ types might suggest. Beetles are an excellent example in this regard. Despite spanning all three germ types, the molecular mechanisms of this group so far appear to be conserved. Nevertheless, beetles also reveal that there is a strong correlation between classical germ type and the temporal dynamics of gene expression. In this sense, comparative gene expression has allowed us to confirm and extend previous conclusions based on morphology.

In certain cases, however, molecular data have uncovered aspects of development that were not otherwise obvious. For example, there is currently no compelling reason to reclassify any dipteran as anything but long germ. Still, the molecular data discussed above do reveal a good deal of variation in the extent of early patterning in this group, specifically a steep gradient of segmentation along the anteroposterior axis in nematoceran flies, as well as certain hymenopterans such as *Apis*. In failing to undergo differential elongation of the posterior, however, these insects perform the essential long germ feature of segmentation by subdivision. Among lepidopterans, which do undergo a form of posterior elongation, the available molecular data suggest that, whereas *Manduca* patterns all of its segments prior to gastrulation, posterior patterning in *Bombyx* is delayed and presumably dependent on posterior elongation in the manner of a short germ insect. Although firm conclusions should await more directly comparable data between *Manduca* and *Bombyx*, it is worth entertaining the possibility that *Manduca* is in fact a long germ insect. In light of their similar embryology and close kinship, this difference may indicate a remarkable evolutionary flexibility in the timing of the molecular segmentation hierarchy relative to embryological events. Indeed, a cursory look at the distribution of germ types suggests that the long germ type is likely to have evolved multiple times. This is reflected in the different means

by which insects warrant the long germ description; one has only to consider the different embryological contexts of *Drosophila*, *Manduca*, and *Apis* to suspect that these insects have earned the same germ type designation by different trajectories.

Regardless of whether the long germ type has evolved multiple times, we are left with the question of whether the ancestral insect was of the short or intermediate germ type. Relevant here is the observation that odonates, including dragonflies and damselflies, are of the intermediate germ type (Figure 3). Also relevant is the observation that members of the paraphyletic "Thysanura" possess extremely small germ anlagen, which at gastrulation appear to represent only the most anterior segments of the head and gnathal region (reviewed in 51). A final consideration is that crustaceans, likely the closest relatives of hexapods, develop in a manner reminiscent of short germ embryogenesis. Thus at present the evidence favors an ancestral short germ type, although additional data from primitive hexapods should clarify the situation.

A separate but related question concerns the molecular mechanisms by which the ancestral insect specified and patterned segments. Based on the widespread conservation of expression patterns, the *Drosophila* segment polarity genes probably functioned as such in the context of the ancestral insect segmentation system. The picture is less clear for pair-rule genes. As mentioned above, where the pair-rule expression of *eve* is concerned, parasitic wasps such as *Copidosoma* likely represent cases of secondary loss. The more basal phylogenetic position of the grasshopper *Schistocerca*, however, tempts us to view the posterior expression domains of *eve* and *ftz* as ancestral for insects, existing prior to the evolutionary recruitment of these genes to play a role in segmentation (20, 72). In support of this conjecture, orthologues of *eve* and *ftz* (in vertebrates and mites, respectively) are linked to Hox clusters and expressed in broad Hox-like domains (7, 22, 81, 98). The *C. elegans eve* orthologue, *vab-7*, is also expressed in a broad posterior domain and is required for posterior cell fates (1).

On the other hand, in some respects grasshoppers may represent a secondarily derived state for insects. This is likely the case for *eve*, as this gene is expressed in stripes in earwigs, crickets, and spiders (18; P. Moore, R. Dawes & N. Patel, unpublished data). In this scenario, *eve* was primitively expressed in stripes, but somewhere along the lineage leading to *Schistocerca*, *eve* lost its stripes but retained its posterior domain. The observation that a *paired*-like gene is expressed in stripes of a two-segment periodicity in grasshoppers lends support to the notion that pair-rule patterning is indeed ancestral for insects (19). However, confirmation of this claim will require a closer examination of the striped expression of pair-rule orthologues such as *eve* in primitive insects as well as noninsect arthropods.

Finally, although it is common to speak of a "proliferation" or "growth" zone as being responsible for germ band elongation in short and intermediate germ insects, at least two observations suggest that this notion should be reexamined. Both *Bombyx* and *Tribolium* undergo dramatic reductions in width during the process of elongation, and this narrowing suggests a prominent role for cell rearrangement. Furthermore, direct evidence for a discrete zone of cell division at the posterior is so far lacking in these and other short germ insects such as *Schistocerca*. What we need

are better descriptions of how these embryos elongate, ones that take into account the relative contributions of cell rearrangement and division. Such descriptions should provide important context for the molecular patterns revealed by gene expression studies. However, such descriptions are unlikely to be generalizable across germ type. It is a safe bet, for example, that flour beetles and grasshoppers generate pattern very differently, even as they turn out to utilize similar molecular mechanisms. Although germband designations provide a useful framework, they are ultimately insufficient to describe the actual diversity of insect segmentation. Nevertheless, the posterior region of all elongating short and intermediate germ bands is surely a special place, as this is where periodic pattern first emerges. Understanding exactly how periodic pattern is progressively generated in a variety of insects will surely be an exciting challenge.

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