A pair-rule gene circuit defines segments sequentially in the short-germ insect *Tribolium castaneum*

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In *Drosophila*, a hierarchy of maternal, gap, pair-rule, and segment polarity gene interactions regulates virtually simultaneous blastoderm segmentation. For the last decade, studies have focused on revealing the extent to which *Drosophila* segmentation mechanisms are conserved in other arthropods where segments are added sequentially from anterior to posterior in a cellular environment. Despite our increased knowledge of individual segmentation gene details of their interactions in non-Drosophilid insects are not well understood. We analyzed the *Tribolium* orthologs of *Drosophila* pair-rule genes, which display pair-rule expression patterns. *Tribolium castaneum* paired (*Tc-prd*) and sloppy-paired (*Tc-slp*) genes produced pair-rule phenotypes when their transcripts were severely reduced by RNA interference. In contrast, similar analysis of *T. castaneum* even-skipped (*Tc-eve*), runt (*Tc-run*), or odd-skipped (*Tc-odd*) genes produced severely truncated, almost completely asegmental phenotypes. Analysis of interactions between pair-rule components revealed that *Tc-eve*, *Tc-run*, and *Tc-odd* form a three-gene circuit to regulate one another as well as their downstream targets, *Tc-prd* and *Tc-slp*. The complement of primary pair-rule genes in *Tribolium* differs from *Drosophila* in that it includes *Tc-odd* but not *Tc-hairy*. This gene circuit defines segments sequentially in double segment periodicity. Furthermore, this single mechanism functions in the early blastoderm stage and subsequently during germ-band elongation. The periodicity of the *Tribolium* pair-rule gene interactions reveals components of the genetic hierarchy that are regulated in a repetitive circuit or clock-like mechanism. This pair-rule gene circuit provides insight into short-germ segmentation in *Tribolium* that may be more generally applicable to segmentation in other arthropods.

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Abbreviations: En, Engrailed; RNAi, RNA interference.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database [accession nos. DQ414246 (*Tc-odd*), DQ414247 (*Tc-prd*), and DQ414248 (*Tc-slp*)].

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including mandibular, labial, thoracic (T2), and four abdominal segments (Fig. 1e). Corresponding germ-band embryos lacked odd-numbered Tc-En stripes (Fig. 3c and d), suggesting that Tc-prd is essential for the expression of Tc-En in odd-numbered parasegments. Complementary to Tc-prdRNAi, Tc-slpRNAi cuticles lacked even-numbered segments (Fig. 1f). Corresponding germ-band embryos lacked even-numbered Tc-En stripes (Fig. 3e and f), indicating that Tc-slp is required for the expression of Tc-En in even-numbered parasegments. Interestingly, hypomorphic slp mutants in Drosophila affect odd-numbered segments (16), whereas Tc-slpRNAi affects even-numbered segments, implying that the requirement for slp function is different in flies and beetles.

The two classes of cuticular phenotypes seen in RNAi embryos suggest that in Tribolium, pair-rule genes may operate at two functional levels, as in Drosophila. In addition, nascent stripes of Tc-run and Tc-odd appear in the posterior growth zone, whereas stripes of Tc-prd and Tc-slp appear later in the anterior growth zone (see Fig. 4a and Supporting Results). Taken together, these data suggest that Tc-eve, Tc-run, and Tc-odd may function as primary pair-rule genes, whereas Tc-prd and Tc-slp function as secondary pair-rule genes.

We also analyzed the functions of the remaining candidate pair-rule genes, Tribolium h, ftz, odd-paired (opa), and Tenascin major (Ten-m). However, no segmentation defects were observed (data not shown), with the exception of Tc-hRNAi, which produced anterior defects (Fig. 6 and Supporting Results, which are published as supporting information on the PNAS web site). The truncated, asegmental phenotypes shown by Tc-eveRNAi, Tc-runRNAi, and Tc-oddRNAi embryos; the modified pair-rule function of Tc-slp; and the fact that not all pair-rule gene orthologs participate in segmentation in Tribolium strongly suggest that segments are prepatterned by different pair-rule gene interactions in Tribolium and Drosophila.

Epistasis Analysis of Tc-eve, Tc-run, and Tc-odd. To understand how genes expressed in pair-rule stripes produce truncated and asegmental RNAi embryonic cuticles, we examined the RNAi effects of each gene on the expression of the others. In severely affected Tc-eveRNAi embryos, expression of Tc-run and Tc-odd was lost or greatly reduced, indicating that Tc-eve is required for the activation of Tc-run and Tc-odd (Fig. 2h–j). The expression patterns of Tc-eve and Tc-odd are almost completely complementary and show only slight overlap (Fig. 5b). Therefore, Tc-eve probably indirectly activates Tc-odd. In severely affected Tc-oddRNAi embryos, the broad initial expression domains of Tc-eve and Tc-run failed to resolve into stripes (Fig. 2t–v). Thus, Tc-odd is required for repression of Tc-eve and Tc-run to produce pair-rule stripes. However, it is unlikely that Tc-odd directly represses Tc-run, because their expression patterns overlap (Fig. 4a and Supporting Results). Instead, Tc-odd might repress Tc-run through repression of Tc-eve. In Drosophila, the initial expression of the primary pair-rule genes eve and run is not altered by mutations in odd (17), a secondary pair-rule gene. The ectopic expression of Tc-eve and Tc-run in Tc-oddRNAi indicates

![Fig. 1](image1.png)  
Fig. 1. Cuticle preparations of severely affected T. castaneum pair-rule gene RNAi embryos. (a) This WT first-instar larval cuticle contains a head, three thoracic segments (T1–T3), eight abdominal segments (A1–A8), and terminal structures. Lr, labrum; Ant, antennae; Md, mandibles; Mx, maxillae; Lb, labium. (b) This spherical, asegmental Tc-eveRNAi cuticle contains labrum and antennae but no trunk segments. (c) In this severely affected Tc-runRNAi cuticle, the preoral and mandibular segments developed normally, but all other segments are missing, resulting in a spherical body similar to that of the Tc-eveRNAi embryo in b. (d) Preoral, mandibular, and maxillary segments developed normally in this severely affected Tc-oddRNAi cuticle, but the absence of posterior segments produced a spherical body similar to the Tc-eveRNAi and Tc-runRNAi embryos. (e) This severely affected Tc-prdRNAi cuticle contains maxillary, T1, T3, and four abdominal segments. (f) In this severely affected Tc-slpRNAi cuticle, T2 and four abdominal segments formed, whereas all gnathal and even-numbered trunk segments are missing.

![Fig. 2](image2.png)  
Fig. 2. Expression of Tribolium pair-rule genes in primary pair-rule gene RNAi embryos. Expression of Tc-En and pair-rule genes in WT (a–f), Tc-eveRNAi (g–j), Tc-runRNAi (m–t), and Tc-oddRNAi (s–x) embryos is shown. (g) Antennal and intercalary Tc-En stripes formed in this severely affected Tc-eveRNAi embryo. (h–l) In severely affected Tc-eveRNAi embryos, expression of Tc-eve (h), Tc-run (i), and Tc-odd (j) were severely reduced or abolished, and Tc-prd (k) and Tc-slp (l) failed to resolve into stripes. (m) In this severely affected Tc-runRNAi embryo, only antenna and mandibular Tc-En stripes formed. (n–r) In severely affected Tc-runRNAi embryos, Tc-eve (n), Tc-prd (o), and Tc-slp (p) were ectopically expressed, but Tc-run (q) and Tc-odd (p) expression was strongly reduced. (j) Tc-deformed (purple) and Tc-En are expressed normally in the mandibular and maxillary segments of this severely affected Tc-oddRNAi embryo. (t–x) In severely affected Tc-oddRNAi embryos, Tc-eve (t) and Tc-run (u) were expressed in broad continuous domains, but Tc-odd (v), Tc-prd (w), and Tc-slp (x) expression was abolished.
that different genetic interactions between these genes evolved in the lineages, leading to beetles and flies. Strongly affected \( Tc-run^{RNAi} \) caused broad expression of \( Tc-eve \) as well as severe reduction of \( Tc-odd \) expression in the growth zone, implying that \( Tc-run \) is required for activation of \( Tc-odd \) and repression of \( Tc-eve \) (Fig. 2 n–p). However, the overlap between \( Tc-eve \) and \( Tc-run \) expression (Fig. 7, which is published as supporting information on the PNAS web site) suggests that the repression of \( Tc-eve \) by \( Tc-run \) is an indirect effect mediated by \( Tc-odd \). These interactions indicate that these three genes provide primary pair-rule functions in \( T. castaneum \).

**Fig. 3.** \( Tc-En \) staining reveals pair-rule defects in severely affected secondary \( T. castaneum \) pair-rule gene RNAi embryos. (a) Sixteen \( Tc-En \) stripes are visible in this fully elongated WT germ band. (b) \( Tc-run \) is transiently expressed in even-numbered parasegments in this elongating WT germ band. (c) There are only seven \( Tc-En \) stripes in this fully elongated \( Tc-prd^{RNAi} \) germ band. (d) The \( Tc-En \) stripes overlap \( Tc-run \) stripes, indicating that the odd-numbered \( Tc-En \) stripes are missing. (e and f) In this \( Tc-slp^{RNAi} \) embryo, all gnathal \( Tc-En \) stripes and every even-numbered \( Tc-En \) stripe in the trunk are missing.

**Fig. 4.** Pair-rule patterning in \( T. castaneum \). (a) The dynamic expression of the primary and secondary pair-rule genes and their regulatory interactions are summarized. The bar at the top indicates that anterior is to the left. Newer segments forming in the growth zone are to the right. In this model of pair-rule patterning in \( T. castaneum \), two-segment units are prepatterned in the posterior region of the growth zone through one cycle of the regulatory circuit (\( Tc-eve \), \( Tc-run \), and \( Tc-odd \)). As the expression of \( Tc-run \) retracts anteriorly in even-numbered parasegments, the expression of \( Tc-prd \) is derepressed. Primary \( Tc-prd \) stripes resolve into two secondary stripes, showing alternatively weak and strong segmental expression. The strong secondary stripes in odd-numbered parasegments regulate \( Tc-En \) expression. \( Tc-run \) also retracts posteriorly in odd-numbered parasegments, resulting in derepression of the primary \( Tc-slp \) stripes. As \( Tc-run \) expression fades, expression of the primary \( Tc-slp \) stripe extends to the posterior border of the odd-numbered parasegment, which is required for the activation of \( Tc-En \). (b) The more complex pair-rule network in \( Drosophila \) (19).
classic pair-rule phenotypes. Based on these discoveries, we propose a model of pair-rule patterning in *Tribolium* that might explain the RNAi phenotypes and discuss major differences in the interactions of pair-rule genes in *Drosophila* and *Tribolium*. Finally, we discuss the implications of these findings on segmentation in short-germ insects and other arthropods.

A Model of Pair-Rule Gene Interaction in *Tribolium*. We describe a pair-rule gene circuit in Fig. 4a in which *Tc-eve* expression is required to activate *Tc-run*, which, in turn, is required to activate *Tc-odd*. *Tc-odd* expression in even-numbered parasegments is required to repress *Tc-eve* there, separating a primary *Tc-eve* stripe from the broad expression domain. As *Tc-eve* expression is repressed in even-numbered parasegments, the posterior edges of *Tc-run* and then *Tc-odd* expression fade. *Tc-eve* expression is also repressed in odd-numbered parasegments (regulated by an as yet unknown gene) to produce segmental *Tc-eve* secondary stripes that are coincident with *Tc-En* stripes (8, 18). Loss of *Tc-eve* expression in odd-numbered parasegments causes *Tc-run* stripes to fade from their anterior edge, resulting in narrow *Tc-run* stripes that are coincident with every even-numbered *Tc-En* stripe. For reasons yet unknown, all three genes remain coexpressed with *Tc-En* in even-numbered parasegments. Consequently, a two-segment unit is prepatterened through one cycle of this primary pair-rule gene circuit. Restriction of *Tc-run* expression leads to the derepression of *Tc-prd* and *Tc-slp*, which are responsible for the activation of *Tc-En* in odd- and even-numbered parasegments, respectively.

The asegmental phenotypes produced by RNAi analysis of *Tc-eve*, *Tc-run*, and *Tc-odd* are readily explained by this model. The knock-down of *Tc-eve* abolishes *Tc-run* expression, which induces ectopic expression of both *Tc-prd* and *Tc-slp*. *Tc-En* expression is not properly regulated to define the parasegmental borders, which results in an asegmental phenotype. Similarly for *Tc-run* RNAi, in the absence of *Tc-run*, *Tc-prd* and *Tc-slp* are expressed ectopically, *Tc-En* is not activated, and segmental grooves are not formed. However, the mechanism that generates the asegmental phenotype in *Tc-odd* RNAi embryos is different from that in *Tc-eve* or *Tc-run* embryos; the knock-down of *Tc-odd* leads to ectopic expression of *Tc-eve*, which induces ectopic expression of *Tc-run*. As a result, *Tc-prd* and *Tc-slp* are fully repressed, which leads to misregulation of *Tc-En* expression and produces the asegmental *Tc-odd* phenotype. Thus, either loss or ectopic expression of *Tc-prd* or *Tc-slp* leads to misregulation of *Tc-En*, ultimately resulting in asegmental phenotypes.

Major Differences of Pair-Rule Interactions Between *Drosophila* and *Tribolium*. Our model of pair-rule interactions in *Tribolium* is not predicted by simple application of the *Drosophila* pair-rule gene paradigm (19) (Fig. 4b). In *Drosophila*, the three primary pair-rule genes (*h*, *eve*, and *run*) are key players in initiating pair-rule patterning. However, *Tc-h* seems not to function as a pair-rule gene at all. Although *odd* is a secondary pair-rule gene in *Drosophila* that is repressed by * eve*, *Tc-odd* functions as a primary pair-rule gene in *Tribolium* that represses *Tc-eve*. Repression of *slp* and * odd* by * eve* is critical to activate *prd*-dependent odd-numbered and *fitz*-dependent even-numbered *en* stripes, respectively, in *Drosophila* (19, 20) (Fig. 4b). In contrast, *Tc-eve* is required for the activation of *Tc-fitz* which in turn represses *Tc-eve* to prepattern a two-segment unit. Furthermore, *Tc-run*, which is induced by *Tc-eve*, is important for the formation of *Tc-prd*-dependent odd-numbered and *Tc-slp*-dependent even-numbered *Tc-en* stripes. *Drosophila fitz* is a secondary pair-rule gene that activates even-numbered *en* stripes, but *Tc-fitz* does not function in segmentation (11). Differences in the primary pair-rule genes result in different genetic interactions between primary and secondary genes and likely affect the regulatory interactions between pair-rule and segment polarity genes. For example, loss of *slp* affects odd-numbered parasegments, whereas loss of *Tc-slp* affects even-numbered parasegments.

Our model provides a core mechanism for pair-rule patterning in *Tribolium* segmentation. However, additional components remain to be discovered. *Tc-eve*, *Tc-run*, and *Tc-odd* have different anterior boundaries of expression that correspond to the number of gnathal segments remaining in RNAi embryos. These boundaries are likely regulated by gap genes, as in *Drosophila*.

By using the candidate gene approach, we determined that orthologs of genes previously identified as pair-rule genes in *Drosophila* function in *Tribolium* segmentation. However, the gene(s) responsible for resolution of primary pair-rule *Tc-eve* stripes into secondary segmental stripes as well as genes that limit the expression of *Tc-run* within the *Tc-eve* domain and *Tc-odd* within the *Tc-run* domain have yet to be determined. Furthermore, we do not yet know which genes function to activate *Tc-prd* and *Tc-slp*. It still must be determined how the pair-rule gene circuit is initiated in blastoderm embryos and stopped after elongation. If this pair-rule gene circuit is regulated by genes involved in anterior–posterior patterning, *Te-caudal* is a likely candidate. It is strongly expressed in the growth zone throughout germ-band elongation (21, 22) and produces a severely affected RNAi phenotype (23) that is identical to that described for *Tc-eve*. Gap genes such as *Tchunchback*, which is expressed in the posteriormost regions of the elongating germ band (24), may be involved in regulating the pair-rule gene circuit there. However, because pair-rule patterning occurs in a cellular environment in *Tribolium*, it is possible that intercellular signaling pathways are involved in regulating the pair-rule gene circuit as components or targets of a segmentation clock. Indeed, the sequential function of the pair-rule gene circuit during *Tribolium* segmentation provides evidence for regulation by some type of periodic mechanism in insects. In vertebrates, somitogenesis is regulated by a segmentation clock (25). Homologs of vertebrate segmentation clock components, such as Notch and Delta, are required for proper segmentation in basally branching arthropods such as the spider *Cupiennius* and have led to the speculation that this mode of segmentation might be very ancient (26). Although a Notch homolog has not been implicated in insect segmentation (27), other signaling molecules may provide the regulatory link between pair-rule genes and a segmentation clock.

Primary Pair-Rule Genes in Germ-Band Elongation. In *Tribolium*, a short, wide germ rudiment elongates into a long, narrow germ band during segmentation (28). In the absence of concerted cell division, this morphological change may be due to cell movement and intercalation, similar to convergent extension in *Drosophila* (29). Germ-band elongation is not disrupted in *Tc-prd* and *Tc-slp* RNAi embryos; the classic pair-rule phenotypes result from loss of patterning in alternating segments. In contrast, defective elongation in *Tc-eve*, *Tc-run*, and *Tc-odd* RNAi embryos produces short, amorphous germ bands in which posterior segments are not initiated. These results, taken together with their WT expression patterns, implicate primary (but not secondary) pair-rule genes in elongation as well as segmentation. Interestingly, *eve* and *run* have been implicated in convergent extension of the *Drosophila* germ band (29).

One Segmentation Mechanism Functions in the Blastoderm and During Elongation. In *Tribolium*, up to three pair-rule stripes form in the cellular blastoderm, prepatterned the three gnathal and three thorax segments; abdominal segments are subsequently added from the growth zone during germ-band elongation. Gap gene RNAi and mutant phenotypes display specific homeotic pheno-
types in the gnathum and thorax with severely disrupted segmentation in the abdomen (30, 31). These results have led to the hypothesis that segmentation mechanisms differ between the blastoderm and elongation phases of short-germ development. The pair-rule gene circuit that we describe prepatterns segments in both elongation and segmentation in short-germ arthropods, providing continuity between the blastoderm and germ-band elongation phases. Thus, it appears that the biggest difference between these phases occurs at the level of the gap genes.

**Several Insights into Segmentation in Other Short-Germ Arthropods.**

Our results provide several insights into segmentation in *Tribolium* that may apply to other short-germ arthropods in general. First, a smaller complement of genes may comprise the core pair-rule mechanism. Second, primary and secondary genes may be different from those in *Drosophila*. Indeed, the dynamics of pair-rule gene homolog expression in the spider *Cupiennius* (32) suggest pair-rule gene functions that differ from those of their *Drosophila* counterparts. Third, if primary pair-rule genes function in both elongation and segmentation in short-germ arthropods, they may produce dramatically stronger RNAi phenotypes than secondary pair-rule genes. RNAi analysis in non-model arthropods is required to test these insights and provide a better understanding of the logic of the ancestral pair-rule patterning mechanism.

**Materials and Methods**

**Molecular Analysis.** *Tc-odd*, *Tc-prd*, and *Tc-slp* sequences were computationally identified in the *Tribolium* genome sequence by TBLASTN analysis of *Drosophila* protein sequences. PCR amplicons from total embryonic RNA were cloned to use as templates for *in situ* probes or dsRNA.

**Parental RNAi.** Parental RNAi was performed as described in ref. 13. Injection of 900 ng/μl (*Tc-eve*), 500 ng/μl (*Tc-run*, *Tc-prd*, and *Tc-slp*), or 350 ng/μl (*Tc-odd*) into pupae produced strong RNAi effects. Injection buffer (1×) or 1 μg/μl *Tc-fz* dsRNA was injected and produced no mutant effects.

**Whole-Mount *in Situ* Hybridization and Immunocytochemistry.** Whole-mount *in situ* hybridization was carried out as in refs. 8 and 9 with digoxigenin-labeled RNA probes. The anti-digoxigenin antibody conjugated with alkaline phosphatase (Roche Diagnostics) was preadsorbed and used at 1/2,000 dilution. Immunocytochemistry was performed as described in ref. 8 with the anti-Eve antibody diluted to 1/20 or the anti-En antibody diluted to 1/5. Germ bands were dissected from the yolks of embryos, mounted in 80% glycerol, and photographed using Nomarski optics on a BX50 compound microscope (Olympus, Melville, NY).

**Phenotype Analysis.** Cuticle preparations of RNAi embryos were performed as described in ref. 11. First-instar larvae were observed and photographed under dark-field optics.

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