**even-skipped** is not a pair-rule gene but has segmental and gap-like functions in *Oncopeltus fasciatus*, an intermediate germband insect

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**Summary**

The pair-rule gene *even-skipped* is required for the initiation of metameric pattern in *Drosophila*. But *Drosophila* segmentation is evolutionarily derived and is not representative of most insects. Therefore, in order to shed light on the evolution of insect segmentation, homologs of the pair-rule gene *even-skipped* have been studied in several insect taxa. However, most of these studies have reported the expression *eve* but not its function. We report the isolation, expression and function of the homolog of *Drosophila even-skipped* from the intermediate germband insect *Oncopeltus fasciatus*. We find that in *Oncopeltus*, *even-skipped* striped expression initiates in a segmental and not pair-rule pattern. Weak RNAi suppression of *Oncopeltus even-skipped* shows no apparent pair-rule like phenotype, while stronger RNAi suppression shows deletion of nearly the entire body. These results suggest that in *Oncopeltus*, *even-skipped* is not acting as a pair-rule gene. In almost all insects, prior to its striped expression, *even-skipped* is expressed in a conserved broad gap-like domain but its function has been largely ignored. We find that this early broad domain is required for activation of the gap genes *hunchback* and *Krüppel*. Given the large RNAi deletion phenotype and its regulation of *hunchback* and *Krüppel*, *even-skipped* seems to act as an über-gap gene in *Oncopeltus*, indicating that it may have both upstream and downstream roles in segmentation.

Key words: *even-skipped*, *Oncopeltus*, Milkweed bug, RNAi, Segmentation, Growth zone, Convergent extension, *hunchback*, *Krüppel*, Pair-rule, Evolution, Short germband

**Introduction**

All adult insect bodies are composed of repeated metameric units called segments. The fruit fly *Drosophila melanogaster* undergoes what is termed ‘long germband’ segmentation, where the entire set of body segments are specified almost simultaneously during early embryogenesis. The action of the segmentation gene cascade serves to subdivide the embryo into finer and finer domains. The upstream maternal and gap genes first allocate the early blastoderm into broad regions, each of which will eventually correspond to several body segments (reviewed by Hulskamp and Tautz, 1991; Pankratz and Jackle, 1993; St Johnston and Nusslein-Volhard, 1992). The downstream pair-rule genes then subdivide these initial broad domains into repeated units that will form the segments. Reflecting this role, the pair-rule genes are expressed in a two-segment periodicity and as such, represent the first periodic gene expression in the segmentation cascade. Thus, the *Drosophila* pair-rule genes occupy an important position in this cascade, translating the broad gradients of the upstream genes into the periodic patterns of the segmented insect body plan.

The *Drosophila* mode of segmentation is not representative of all insects and is actually evolutionarily derived. Evolutionarily basal insects undergo what is termed ‘short’ or ‘intermediate’ germband development where only the anterior segments are initially specified with the posterior body regions arising later in a sequential anterior to posterior progression (Davis and Patel, 2002; Krause, 1939). The sequential nature of posterior segmentation in short and intermediate germband insects implies that the underlying mechanisms that govern the production of posterior segments potentially differ from *Drosophila*. Given the importance of pair-rule genes in producing the first periodic gene expression patterns in *Drosophila*, they make a logical choice for understanding posterior segmentation in short and intermediate germ insects. Here, we focus our attention on the pair-rule gene *even-skipped*.

*even-skipped* (*eve*) was originally identified in *Drosophila* as a member of the pair-rule class of segmentation genes because hypomorphic alleles produced embryos that lacked the denticle band and adjacent cuticle from even-numbered segments (odd-numbered parasegments) – a canonical ‘pair-rule’ phenotype (Nusslein-Volhard et al., 1984). The gene encodes a homeodomain-containing transcription factor and acts as a transcriptional repressor (Biggin and Tjian, 1989; Macdonald et al., 1986). *even-skipped* is initially expressed in a broad blastoderm domain that first resolves into a striped primary pair-rule pattern with a two segment periodicity (a pattern that correlates well with the hypomorphic *eve* phenotype). This primary pair-rule pattern then matures into a secondary segmental one (Frasch et al., 1987; Macdonald et al., 1986).

Previous studies of *eve* in other insects have found its expression to be variable, with patterns similar to *Drosophila*, or having only the pair-rule or segmental phases, implying that the role of *even-skipped* may be highly plastic during insect evolution (Binner and Sander, 1997; Grbic et al., 1996; Grbic
and Strand, 1998; Kraft and Jackle, 1994; Miyawaki et al., 2004; Patel et al., 1992; 1994; Rohr et al., 1999; Xu et al., 1994). However, the vast majority of these studies have examined only the expression but not function of the gene. Outside of Drosophila, eve function has only been examined in the beetle Tribolium castaneum where its pair-rule function is consistent with its pair-rule expression pattern (Scheroder et al., 1999). Thus, the evolution of even-skipped function within the insects, especially among more basal groups, is not at all clear.

In order to gain insight into the evolution of even-skipped function in the insects, we have examined the expression and function of even-skipped in the milkweed bug, Oncopeltus fasciatus (Hemiptera:Lygaeidae) an intermediate germband insect. We find that in this insect, even-skipped does not act as a pair-rule gene, but rather is expressed in a segmental pattern and is required for proper growth and patterning of nearly all segments.

Moreover, all previous studies have only examined the role of striped eve expression, ignoring the earlier broad domain seen in almost all insects. The Drosophila eve phenotype does not suggest any function for this early domain, and has therefore been largely ignored. Here, we present the first report that this earlier domain actually has an important function during segmentation. Our results indicate that in Oncopeltus, this early domain is required for proper expression of the gap genes hunchback and Krüppel.

Materials and methods

Cloning

Embryonic total RNA was isolated from mixed stage Oncopeltus fasciatus embryos using the Trizol reagent (GibcoBRL/Life Technologies). Poly(A+) RNA was isolated using the Oligotex mRNA minikit (Qiagen). cDNA was synthesized using the FirstChoice RLM-RACE kit (Ambion). We first performed PCRs with degenerate primers designed to conserved even-skipped sequences from other arthropod species. This was followed by 5′ and 3′ RACE for isolation of the remainder of the transcript. For the degenerate PCR, the primer pairs were as previously reported (Patel et al., 1992). 5′ RACE PCR was performed using the gene-specific primer TTCATGACGCT- GAAAGGCCTTCTC, while 3′ RACE required nested PCRs with gene-specific primers ACTACGTTTACGACCAAGGGTTGCGG- GAGC and TGCGCAGCTCAACTGGGCTTCT, along with the anchor primers supplied in the FirstChoice RLM-RACE kit. All PCRs were performed using the Advantage2 polymerase mix (BD Biosciences). After separation on an agarose gel, candidate PCR products were gel-extracted if necessary (Qiagen), and cloned using either the PCR-Script Amp Cloning kit (Stratagene) or the TOPO-TA for Sequencing kit (Invitrogen). At least three independent PCRs were performed and several clones sequenced in order to minimize PCR and sequencing artifacts. The Of′eve cDNA sequence has been submitted to GenBank with Accession Number AY870400.

Embryo fixation, in situ hybridization and antibody staining

Embryo fixation, probe synthesis and in situ hybridization were carried out as previously reported (Liu and Kaufman, 2004a). The final color development step was carried out essentially as described by Liu and Kaufman (Liu and Kaufman, 2004a), except for two-color in situus using BCP/NBT and BCIP/INT where the first AP antibody was inactivated by heating to 70°C for 30 minutes in Tris-EDTA followed by additional fixation for 2 hours before continuing with the second AP antibody.

Results

Isolation of Oncopeltus even-skipped

We took an RT-PCR approach in order to clone the Oncopeltus fasciatus homolog of even-skipped. First, degenerate primers designed to the conserved even-skipped homeodomain from other insect species was used to PCR a short initial fragment. Sequence of this fragment allowed us to design exact primers for subsequent 5′ and 3′ RACE reactions. We sequenced several clones from independent PCRs and found no evidence for additional copies of Of′eve.

Using this strategy, we were able to isolate a 1038 bp fragment of the Of′eve transcript encoding a polypeptide of at least 236 amino acids. As there is no in frame stop codon in the 5′ sequence prior to the first methionine codon, it is possible that this fragment does not include the entire open reading frame. However, alignments of the predicted Oncopeltus polypeptide with other insect eve sequences show very strong conservation at the N terminus, suggesting that our clones represent most of, if not the entire, open reading frame. Alignments with other insect eve sequences show sequences similar to the homeodomain, the Groucho co-repressor interaction domain, and an additional region of similarity at the N terminus (Fig. 1).

Oncopeltus even-skipped expression in the blastoderm

In order to gain insight to the potential function of Of′eve in milkweed bug segmentation, we used in situ hybridization to determine its pattern of expression during embryonic development. Probes for in situ hybridization were synthesized to two non-overlapping regions of the Of′eve cDNA, an ~600 bp 3′ fragment that contained a region of the homeodomains and RNAi

Double-stranded RNA used in parental and embryonic RNAi was in vitro transcribed from template prepared one of two ways. Plasmid containing the insert of interest was linearized by restriction digest, or template was prepared from a PCR where T3 and T7 phage promoter sequences were added to the primers. Sense and antisense RNA was synthesized in two separate reactions using the MEGAscript kit (Ambion). Following in vitro transcription and DNase treatment, the transcription reactions were immediately mixed in a single tube and annealed. The RNA was annealed in a PCR machine set to incubate at 94°C for 3 minutes, then set to quickly reach 85°C followed by a slow cooling to 25°C over the course of 1 hour. We removed unannealed single-stranded RNA by digestion with RNase A (Ambion) for 15 minutes. A small amount of annealed RNA was analyzed on an agarose gel to confirm successful annealing and digestion and we found that the RNase A treatment resulted in much less smearing of the dsRNA on the gel when compared with previous methods. Injections for embryonic and parental RNAi was performed as previously reported (Liu and Kaufman, 2004a).

Image capture and processing

Images of blastoderms and RNAi embryos were captured using a Nikon SMZ1500 stereomicroscope with attached Nikon DXM1200 digital camera. As these samples were relatively large, a single focal plane was not sufficient to capture all the detail of the entire embryo. Therefore several focal planes were taken for each sample and were combined into a single composite image in Photoshop (Adobe). Images of germband-stage embryos were captured on a Zeiss Axioshot microscope with attached Nikon DXM1200 digital camera.
an approximately 260 bp 5’ fragment that did not include homeodomain sequences (Fig. 1A). Both probes gave identical results.

**Oncopeltus even-skipped** transcript first appears during the blastoderm stage ~20-24 hours after egg lay (AEL). At this stage, **Of’ eve** transcript accumulates as a broad band covering the posterior two thirds of the blastoderm (Fig. 2A) and is reminiscent of early eve expression in Drosophila, where it also first accumulates in all nuclei before the appearance of its later striped expression (Frasch et al., 1987; Macdonald et al., 1986). Shortly thereafter, at 24-28 hours AEL, the **Oncopeltus** pattern becomes weaker on the ventral surface (not shown). This is not likely to reflect a role in determining the dorsal/ventral axis, but probably reflects the distribution of embryonic and extra-embryonic cells. Indeed expression of **hunchback**, **Krüppel**, and **Deformed** are also weaker on the ventral blastoderm surface (Liu and Kaufman, 2004a; Liu and Kaufman, 2004b) (P.Z.L., unpublished). Given the extreme embryonic movements during development, the embryo actually rotates twice during embryogenesis relative to the eggshell. In the interest of consistency, we orient all blastoderm images as if the egg is held constant and the embryo moves within it. A consequence of this is that blastoderm cells that are near the dorsal surface of the egg are actually fated to become ventral in the embryo.

The anterior boundary of this initial broad domain then sharpens so that by 32-36 hours AEL, a strong stripe circumscribing the early blastoderm can be seen superimposed on the early broad domain (Fig. 2B). In embryos 36-40 hours AEL, the expression pattern then changes in two ways. The broad diffuse domain fades from most of the blastoderm, but remain in a small patch in the very posterior. Interestingly, this maintenance **Of’ eve** expression corresponds well with the concomitant clearing of **Oncopeltus** Krüppel expression in late blastoderm (Fig. 2F), I). Additionally, as the initial broad domain fades, **Of’ eve** stripes appear in its place and seem to arise in a slight anterior to posterior progression (Fig. 2A-F). Thus, by 40 hours AEL, this expression dynamic results in a total of six vertical even-skipped stripes spaced on the blastoderm surface (Fig. 2F). As **Oncopeltus** is a short-germ insect, the number and positions of these stripes do not correspond to the same segments as they would on a long-germ insect, such as Drosophila. In milkweed bugs, only the mandibular through third thoracic segments are specified during the blastoderm stage as can be seen by **Oncopeltus** engrailed (**Of’ en**) expression (Fig. 2G) (Butt, 1947; Liu and Kaufman, 2004a). The six **Of’ eve** stripes appear very similar to the **Of’ en** expression and thus probably also correspond to these same segments.

In Drosophila, gap genes such as **hunchback** and **Krüppel** regulate the position and spacing of the primary even-skipped stripes (Clyde et al., 2003; Frasch and Levine, 1987; Small et al., 1992). We wished to know if expression of these same genes correlated with the even-skipped stripes on the Oncopeltus blastoderm. To this end, we performed double in situ hybridizations for **Oncopeltus even-skipped** with **hunchback** (**Of’ hb**) and **Krüppel** (**Of’ Kr**). **Of’ hb** is expressed in two broad domains in the blastoderm, a weaker anterior band which is anterior to the mandibular segment and corresponds to the anterior head, and a stronger central one corresponding to the posterior of the mandibular through labial segments (Liu and Kaufman, 2004a). **Of’ Kr** is expressed in a broad posterior domain in the blastoderm, corresponding to the thoracic segments (Liu and Kaufman, 2004b). **Of’ eve** stripes 1-3 (mandibular through labial) coincide with the strong central domain of **hb**, while stripes 4-6 (thoracic) underlie the **Kr** domain (Fig. 2H, I). This is in contrast with Drosophila, where **hb** and **Kr** only span two stripes each.

**Oncopeltus even-skipped expression in the germband**

**Oncopeltus** embryos undergo ‘germband invagination’ during which cells of the late blastoderm migrate to the posterior pole of the egg and dive into the interior of the yolk mass to contribute to the formation of the germband (Butt, 1947; Liu and Kaufman, 2004a). This process results in cells that originally occupied the posterior tip of the blastoderm ending up as part of the posterior growth zone of the early germband.

During germband invagination and throughout the remainder of germband growth and segmentation, **Oncopeltus even-skipped** is continuously expressed in both the mesoderm and ectoderm of the posterior growth zone (Fig. 2J-K; Fig. 3), reminiscent of even-skipped expression in the grasshopper Schistocerca (Patel et al., 1992). Additionally, there are a few stripes of expression directly anterior to this growth zone domain. As **Oncopeltus** is an intermediate-germ insect and posterior segments are specified sequentially in an anterior-to-posterior progression, these even-skipped stripes do not correspond to any particular segments but rather are always expressed in the chronologically youngest (most posterior) ones.

We next wished to determine the segmental register of the

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**Fig. 1.** Predicted structure and sequence analysis of Oncopeltus even-skipped. (A) Cartoon of predicted Of’ eve protein structure with conserved N-terminal domain (N), homeodomain (HD) and C-terminal domain (C). Underlined are the regions used for synthesizing dsRNA and in situ probes. The 3’ fragment is approximately 600 bp long and spans a small region of the homeodomain. The 5’ fragment is approximately 260 bp long and does not include any of the homeodomain. (B) Conserved N-terminal domain of even-skipped from Drosophila melanogaster, Tribolium castaneum, Oncopeltus fasciatus and Schistocerca americana. (C) Conserved C-terminal domain from the same insect species. The Groucho co-repressor interaction domain is underlined. (D) Conserved homeodomain region from the four insect species.
striped *Of’ eve* expression. As the stripes fade before segmental grooves form, we used expression of *Oncopeltus engrailed* as a segmental marker. *Of’ en* is eventually expressed in all body segments but during germband growth, *Of’ en* stripes initiate anterior to the growth zone, just as the stripes of *Of’ eve* expression are fading. As abdominal segmentation proceeds in an anterior to posterior progression, expression of these *Of’ eve* stripes slightly precedes initiation of the *Of’ en* stripes. However, there is some overlap of both genes. For example, Fig. 3C2 shows three young segments (labeled as A3-A5), each of which shows some expression of both *Of’ eve* and *Of’ en*. This co-expression in the germband shows that *even-skipped* and *engrailed* stripes have a one-to-one correspondence. For at least the stripes that are far outside the growth zone then, it seems that *Of’ eve* is expressed in a segmental rather than pair-rule pattern.

*Of’ eve* stripes also seem to be generated from the growth zone in a segmental fashion. In *Drosophila* the secondary segmental stripes arise de novo after the primary stripes are refined, while in other insects with both primary pair-rule and secondary segmental patterns, the secondary stripes are generated from ‘splitting’ of the broader primary stripes (Binner and Sander, 1997; Macdonald et al., 1986; Patel et al., 1994). Thus, the pair-rule nature of expression is revealed by the dynamics of stripe formation – a broad primary stripe of expression followed by narrower segmental secondary ones. In *Oncopeltus*, early stripes close to the growth zone often have a characteristic ‘V’ shape at the midline where they remain contiguous with the growth zone (Fig. 3B,E). These stripes seem to ‘peel’ off of the growth zone in a segmental register as they maintain their width as they mature (compare chronologically younger and older stripes in Fig. 3B,C2) and do not ‘split’ to form secondary stripes.

Moreover, early growth zone expression often shows three or four stripes of *Of’ eve* within the unelongated growth zone (Fig. 3A2). These stripes may correspond to anterior abdominal stripes that migrate into the rest of the germband as the germband elongates. If this is the case, then the growth zone may become patterned before actual elongation. At any rate, these stripes also do not appear to be any broader than the abdominal stripes to which they then give rise. Thus, the dynamics of *Of’ eve* stripe formation reveal no obvious pair-rule phase of expression.

**Oncopeltus even-skipped RNAi**

With *Of’ eve* expression suggesting roles in segmentation and growth zone function, we wished to functionally test its developmental role. We therefore used RNAi to specifically knockdown *even-skipped* function in *Oncopeltus* in order to gain insight into its role in milkweed bug...
Development

female did produce moderate and weak phenotypes, we found (Liu and Kaufman, 2004a). Moreover, when an individual clutches – later clutches show gradually weaker phenotypes suppression effect eventually fades over the course of several reported previously, for a given injected female, the pRNAi milder and moderate phenotypic classes were produced. As embryos and it was only at this low concentration that the typically use for pRNAi) still yielded the most severe class I 0.002

Table 1. Of’ eve RNAi results

<table>
<thead>
<tr>
<th>dsRNA</th>
<th>[dsRNA] (µg/ul)</th>
<th>Nonspecific [%]</th>
<th>Wild type [%]</th>
<th>Class III [%]</th>
<th>Class II [%]</th>
<th>Class I [%]</th>
<th>Totals</th>
</tr>
</thead>
<tbody>
<tr>
<td>3’ eRNAi</td>
<td>0.002</td>
<td>2 (0.5)</td>
<td>169 (44.8)</td>
<td>21 (5.6)</td>
<td>26 (6.9)</td>
<td>159 (42.2)</td>
<td>377</td>
</tr>
<tr>
<td>3’ pRNAi</td>
<td>0.002</td>
<td>2 (0.5)</td>
<td>169 (11.8)</td>
<td>21 (1.5)</td>
<td>26 (1.8)</td>
<td>120 (84.1)</td>
<td>1429</td>
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<tr>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>150 (100)</td>
<td>150</td>
</tr>
<tr>
<td>0.2</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>540 (0)</td>
<td>548</td>
</tr>
<tr>
<td>2.0</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>171 (99.4)</td>
<td>172</td>
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<td>0 (0)</td>
<td>0 (0)</td>
<td>0 (0)</td>
<td>64 (24.8)</td>
<td>258</td>
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<td>0 (0)</td>
<td>64 (24.8)</td>
<td>258</td>
<td></td>
</tr>
</tbody>
</table>

The nonspecific class included embryos which underwent at least some development, but whose final morphology was uninterpretable. Percentages may not add up to 100 due to rounding.

Table 2. Of’ eve RNAi suppression fades over subsequent egg clutches

<table>
<thead>
<tr>
<th>Clutch number</th>
<th>Nonspecific [%]</th>
<th>Wild type [%]</th>
<th>Class III [%]</th>
<th>Class II [%]</th>
<th>Class I [%]</th>
<th>Clutch totals*</th>
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</thead>
<tbody>
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<td>0 (0)</td>
<td>6 (100)</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>0 (0)</td>
<td>6 (17.6)</td>
<td>7 (20.6)</td>
<td>6 (17.6)</td>
<td>15 (44.1)</td>
<td>34</td>
</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>12</td>
</tr>
</tbody>
</table>

All egg clutches from a single female injected with 0.002 µg/ul of Of’ eve dsRNA were collected and scored. Percentages may not add up to 100 due to rounding.

embryogenesis. We directly injected double-stranded RNA into early Oncopeltus embryos (termed embryonic RNAi, eRNAi) (Hughes and Kaufman, 2000), and also injected double-stranded RNA into the abdomens of adult females (termed parental RNAi, pRNAi) (Liu and Kaufman, 2004a), and both yielded equivalent knockdown phenotypes. We found that occasionally, the first clutch from a given injected female would contain wild-type embryos, while later clutches would then show the even-skipped phenotype. This is probably because in the developing oocytes that give rise to these early broods, the egg chorions were likely already deposited, preventing the entry of the dsRNA. We therefore excluded these clutches from our analysis.

We also injected two different dsRNAs corresponding to two non-overlapping regions of the Of’ eve transcript and both regions produced identical knockdown phenotypes (Table 1). RNAi of other genes in Oncopeltus results in phenotypes that range in severity and the resulting hypomorphic series often aids in the interpretation of the phenotype (Angelini and Kaufman, 2004; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b). We took advantage of this and injected dsRNA to Of’ eve in a range of concentrations (Table 1). Based on their phenotypic severity, the RNAi embryos were categorized into three classes, ranging from the strongest (class I) to the mildest (class III).

Milkweed bug embryogenesis seems to be very sensitive to even-skipped RNAi as injections with concentrations as low as 0.002 µg/ul (one thousandth of the concentration that we typically use for pRNAi) still yielded the most severe class I embryos and it was only at this low concentration that the milder and moderate phenotypic classes were produced. As reported previously, for a given injected female, the pRNAi suppression effect eventually fades over the course of several clutches – later clutches show gradually weaker phenotypes (Liu and Kaufman, 2004a). Moreover, when an individual female did produce moderate and weak phenotypes, we found that there was a very rapid transition from severely affected progeny, to weakly affected, and finally to wild type, often within the span of a single clutch. For example, we tracked a single injected female and scored each of her successive clutches of progeny (Table 2). For this individual, the first and second clutches were composed only of strongly affected progeny. The third clutch contained a mixture of class I, II and III progeny, and in the next clutch, all the progeny were wild type. This rapid transition between clutches of severely affected animals to clutches with only wild-type embryos along with the extremely low Of’ eve dsRNA concentrations needed to produce the phenotypes suggest that Oncopeltus development is very sensitive to even-skipped function.

even-skipped RNAi phenotype

Suppression of Oncopeltus even-skipped results in defects in germband growth and segmentation of almost the entire body, including both growth-zone and blastoderm-derived segments. The more weakly affected RNAi embryos were rare, but nonetheless informative in understanding the phenotype. The hypomorphic series shows that the abdomen is most sensitive to RNAi depletion and as the depletion increases in severity, the thorax and eventually the gnathal segments also become affected.

The mild class III embryos constituted only 1.7% of the total affected pRNAi embryos and as noted were produced only from injection of low concentrations of Of’ eve dsRNA (Table 1). Several abdominal segments were formed, but appeared much smaller than normal (Fig. 4B1-B3). The mandibular and maxillary styles were present, although not extended (not unexpected because the uncoiling of the internal stylets usually occurs at hatching). Segmental grooves of the thorax were occasionally less prominent giving the thorax a smoother appearance. The thoracic legs appeared slightly deformed, although this may be due to steric deformation within the confines of the eggshell rather than reflecting defects in

patterning. Importantly, any weak disruptions in the thorax affected all segments. Any bias in sensitivity seemed graded, increasing towards the posterior, with no evidence of any skipping of segments. In order to more clearly examine the phenotype, we used Oncopeltus engrailed expression as a convenient segmental marker in germband stage RNAi embryos. Fig. 4D1,D2 shows late-stage class III germband stained for engrailed and shows that the abdomen is reduced with highly disorganized segmentation (compare Fig. 4D1 and D2 with Fig. 3D1) while the head and thoracic segments appear normal. Thus, class III embryos have defective abdomens but relatively normal heads and thoracic segments.

We should note that in Drosophila, even-skipped is required for expression of engrailed, raising the issue of why engrailed expression is detected at all in the RNAi germbands. In Drosophila, after the segment polarity genes wingless and engrailed are initiated, they maintain each other’s expression (reviewed by Perrimon, 1994) and a similar process of mutual reinforcement may be occurring here.

The moderate class II embryos were also rare, making up only 2.1% of the RNAi embryos and were also only produced at lower dsRNA concentrations (Table 1). When compared with the milder class III embryos, these embryos show stronger abdominal defects as well as defects in the thorax – the abdomen is severely reduced and thoracic segmentation is defective (Fig. 4E1,E2). In these embryos, posterior thoracic legs are either reduced or missing but anterior structures are left relatively unaffected. Germband stage embryos corresponding to this phenotypic class stained for engrailed reveal strong disruption of posterior growth and patterning but relatively normal anterior segmentation (Fig. 4F). As with class III, these embryos also seem to show a gradient of defect, stronger in the posterior and weaker in the anterior, without any pair-rule like defects.

Class I constituted 81.8% of the RNAi embryos (Table 1). These embryos are characterized by a very large deletion of almost the entire body (Fig. 4G-H2). Given this severity, it is difficult to capture all aspects of the phenotype photographically, so we will describe their morphology based on observations of several class I embryos. These embryos lack most of the body, with no apparent gnathal, thoracic or abdominal segments (as the intercalary segment is so small, we cannot determine its presence or absence). Antenna, eyes and a labrum can still be found and seem morphologically normal, albeit smaller in size (Fig. 4G). Mandibular and maxillary styles are missing, suggesting that the deleted region spans these segments as well. Late-stage RNAi germ bands stained with engrailed probe show that although anterior head elements do form, the mandibular through abdominal regions are entirely missing (Fig. 4H1,H2), consistent with what is seen in hatching-staged embryos. Thus, strong suppression of Of’eve results in loss of almost the entire body, from the mandibular segment to the posterior of the animal.

**even-skipped RNAi results in gap gene misexpression**

The severe Of’eve phenotype, complete loss of the mandibular through abdominal segments, was much stronger than we had expected. Although loss of abdominal segments may be explained by disruption of patterning in the growth zone, we noticed that loss of the gnathal and thoracic segments correlated well with the early broad blastoderm domain of expression (mandibular to the posterior of the blastoderm). We reasoned that as the head and thorax are normally specified
during the blastoderm stage, blastoderms that are depleted for Of’eve function might become repatterned to reflect loss of the deleted regions.

We first noticed that RNAi embryos showed defects in germband invagination. In normal 48-52 hour embryos, germband invagination is nearly complete with the site of invagination at the posterior pole of the blastoderm (arrow in Fig. 5A). Of’eve RNAi embryos at a similar stage show a failure of germband invagination with a mislocalization of the invagination site to a more ventral position on the blastoderm (arrow in Fig. 5B). When dissected, these embryos do not have an elongating germband in the yolk (not shown), which is consistent with loss of the entire body in the class I animals. These defects suggest that depletion of Of’eve function may repattern the blastoderm.

We further reasoned that blastoderm repatterning might be reflected in abnormal gap gene expression. As noted earlier, Of’hb is normally expressed in two broad blastoderm domains, a weaker anterior domain corresponding to the head and a stronger central domain corresponding to the maxillary through first thoracic segments (Fig. 5C) (Liu and Kaufman, 2004a). We found that when Of’eve function is reduced, we detect only a single Of’hb domain spanning the posterior half of the blastoderm (Fig. 5D1,D2). In uninjected animals, the two Of’hb bands are clearly distinct on the blastoderm but in the RNAi embryos, this remaining band of expression appears uniform throughout its domain. We interpret the remaining single band as probably representing a loss of the stronger central gnathal Of’hb domain, with a concomitant expansion and posterior displacement of the remaining head domain for two reasons. First, the severe Of’eve RNAi phenotype is a loss of all segments except the anterior head, a region that spans the central, but not anterior head, domain of Of’hb expression. Second, the broad domain of Of’eve expression spans the central gnathal, but not anterior head, domains of Of’hb expression (compare Fig. 2B with Fig. 5C), indicating that Of’eve is spatially positioned to activate the central gnathal Of’hb domain. These results suggest that Of’eve is required for activation of Of’hb in the mandibular to first thoracic segments; when Of’eve function is reduced, the remaining head domain of Of’hb expands to fill the posterior of the blastoderm.

In uninjected animals, Of’Kr is expressed in the posterior third of the blastoderm, corresponding to the thoracic segments (Fig. 5E) (Liu and Kaufman, 2004b). Of’Kr expression in
Fig. 5. hunchback and Krüppel expression in Of eve RNAi blastoderms. (A,C,E) Uninjected animals. (B,D1-2,F1-2) Of eve RNAi animals. (A) Lateral view of uninjected 44-48 hour embryo stained for DNA. Germband has fully invaginated, leaving behind head lobes on outside posterior of yolk ball (densely staining region). Arrow indicates site of invagination. (B) Lateral view of 44-48 hour Of eve RNAi embryo stained for DNA. Odd wrinkles and putatively mislocalized invagination site (arrow) can be seen. (C) Dorsal aspect of uninjected 36-40 hour blastoderm stained for Oncopeltus hunchback showing that Of’hb normally accumulates in two broad bands. Weaker anterior band corresponds to anterior head, while central band corresponds to posterior mandible through anterior first thoracic segments. (D1) Dorsal view of 36-40 hour Of eve RNAi blastoderm, showing that Of’hb is now expressed in a single uniform domain in the posterior half of the blastoderm. (D2) Same embryo as in (D1), but rotated to show lateral view. Of’hb expression is restricted to the dorsal part of the blastoderm and is typical in late stage blastoderms. (E) Dorsal view of uninjected 36-40 hour embryo stained for Oncopeltus Krüppel. Of’Kr normally accumulates in posterior one-third of blastoderm. (F1) Of eve RNAi blastoderm at 36-40 hour. Of’Kr expression is largely absent, with only a very small posterior patch remaining. (F2) Same embryo as in F1, end-on view of posterior tip of blastoderm, showing remaining Of’Kr patch. Scale bars: 200 µm.

Of eve RNAi blastoderms is highly reduced, so that only a tiny patch of expression remains at the posterior tip of the blastoderm (Fig. 5F1,F2). This suggests that Of eve is also required for proper expression of Of’Kr in the blastoderm.

Of eve is expressed in a dynamic pattern during the blastoderm stage, with an early broad domain covering the posterior two thirds of the blastoderm and a later striped phase of expression. Temporally and spatially, it seems most likely that it is this early broad blastoderm domain (rather than the later striped expression) that is responsible for these gap gene-regulating functions. Thus, in the absence of Of eve function, the early blastoderm becomes re-allocated to represent only the anterior head.

Discussion

We have isolated the Oncopeltus fasciatus homolog of the Drosophila pair-rule gene, even-skipped. In order to determine the role of even-skipped during Oncopeltus development and its evolution within the insects, we examined Of eve expression using in situ hybridization and its developmental function using RNAi. There are two major findings of our work: first, that even-skipped in Oncopeltus does not act as a pair-rule gene; and second, that even-skipped acts as a gap gene. We discuss these two findings and their implications in turn.

even-skipped does not act as a pair-rule gene in Oncopeltus

One of the characteristics of Drosophila even-skipped and most of the other pair-rule genes is that they are expressed in the blastoderm in a series of seven transverse stripes with a two-segment periodicity (Carroll et al., 1986; Frasch et al., 1987; Sander, 1997; Patel et al., 1994). However, in Oncopeltus, even-skipped expression has been examined in a number of insects, and in many species, a primary pair-rule pattern is followed by a later segmental one. For example, in Drosophila, even-skipped primary stripe expression is in odd numbered parasegments, and then later minor stripes arise de novo in the even numbered parasegments (Nusslein-Volhard et al., 1984; Frasch et al., 1987; Macdonald et al., 1986). In both the long germ honeybee Apis mellifera and short germ beetle Tribolium, secondary stripes appear through ‘splitting’ of the primary pair-rule stripes (Binner and Sander, 1997; Brown et al., 1997; Patel et al., 1994). The Oncopeltus eve expression dynamic shows none of these pair-rule patterns, instead initiating in a segmental manner.

Function of even-skipped had previously only been examined in Drosophila and Tribolium, and was found to have a pair-rule requirement, reflecting the pair-rule expression pattern in both of these insects (Nusslein-Volhard et al., 1984; Schroeder et al., 1999). However, in Oncopeltus, there is no apparent pair-rule phenotype. Instead, there seems to be a gradient of sensitivity, with posterior segments being more sensitive to RNAi depletion. We should note that as Of eve is expressed in a broad blastoderm domain, in the growth zone and in segmental stripes, teasing apart the functions of each of these individual domains is difficult without more sophisticated genetic techniques. Because we can assay for a pair-rule phenotype only in the thorax, we cannot rule out a hidden pair-rule function in the abdominal segments. Nevertheless, as neither the expression nor functional analyses reveal an apparent pair-rule role, even-skipped is probably not acting as a pair-rule gene in this insect.

Divergent regulation of the segmentation genes in Oncopeltus

The non-pair-rule role of Oncopeltus even-skipped suggests
that in this insect, the genetic paradigm regulating the segmentation gene cascade must differ from *Drosophila* in several respects.

First, regulation of striped expression of *Of’eve* by the upstream gap genes must be divergent. In *Drosophila*, *even-skipped* is directly regulated by gradients of gap gene proteins that bind to stripe-specific enhancers in the *eve* promoter (Clyde et al., 2003; Frasch and Levine, 1987; Small et al., 1992). The anterior *Drosophila hunchback* domain covers *eve* stripes 1 and 2, while the *Krüppel* domain covers stripes 3 and 4. This is in contrast to *Oncopeltus*, where during the blastoderm stage, *Of’hb* spans the first three *Of’eve* stripes and *Of’Kr* spans stripes 4, 5 and 6. Moreover, the stripes in *Drosophila* are pair-rule but in *Oncopeltus*, are segmental in register. Although *Of’eve* stripes are expressed in a manner consistent with their potential regulation by the gap genes, the precise mechanism governing this regulation is likely to be fundamentally different from that in *Drosophila*. Moreover, during germband elongation, *Of’eve* stripes are generated sequentially out of the growth zone, a dynamic very different from *Drosophila*. Given these differences, the cis-regulatory elements that govern *Of’eve* regulation should prove to be very interesting.

Second, the overall pair-rule mechanism is likely to show fundamental differences in *Oncopeltus*. In *Drosophila*, the primary *eve* stripes act within the context of a pair-rule network (Carroll and Vavra, 1989). But given the segmental register of *Of’eve*, this network of cross-regulation is likely to be significantly different. Moreover in *Drosophila*, striped *eve* expression initiates in the pre-cellular blastoderm, where these early primary stripes each act as morphogenetic gradients to regulate the other pair-rule genes (Fujioka et al., 1995). In *Oncopeltus*, cellularization of the blastoderm nuclei occurs at around 17 hours AEL (Butt, 1947), well before the initiation of *Of’eve* striped expression at around 32 hours. The lack of a prolonged syncytial blastoderm stage in *Oncopeltus* suggest that morphogenetic gradients are not involved in the same fashion in the regulation of *Of’eve* or in its regulation of other pair-rule genes.

The third aspect which may differ between *Drosophila* and *Oncopeltus* is the regulation of *engrailed* by *even-skipped*. The expression dynamics of these two genes strongly suggest that *Of’eve* regulates *Of’en*. *Of’eve* expression slightly precedes and becomes coincident with expression of *Of’en* during both the blastoderm and germband stages. Thus, *Oncopeltus even-skipped* is temporally and spatially poised to regulate *engrailed*. However, the details are again likely to differ between *Oncopeltus* and *Drosophila*. In *Drosophila*, both the odd- and even-numbered *engrailed* stripes are initiated solely through action of the primary *eve* stripes, while the role of the minor stripes is unclear (Fujioka et al., 1995; Jaynes and Fujioka, 2004). It may be that *engrailed* activation in *Oncopeltus* is more similar to the activation of either the odd- or even-numbered *engrailed* stripes in *Drosophila* and that all *engrailed* stripes are generated using the same mechanism. It is interesting that the *Of’eve* pattern has more affinity to the late *Drosophila* (14 stripe) pattern, and may indicate that the minor stripes in *Drosophila* are an evolutionary vestige of a previous function.

*eve* expression has been found to be surprisingly variable in several insects, with some insects showing pair-rule only, segmental only, both, or neither patterns (Fig. 6A) (Binner and Sander, 1997; Gribi et al., 1996; Gribic and Strand, 1998; Kraft and Jackle, 1994; Miyawaki et al., 2004; Patel et al., 1992; Patel et al., 1994; Rohr et al., 1999; Xu et al., 1994) (S. Noji, personal communication). Additionally, *eve* expression has been examined in a crustacean and found to have no obvious pair-rule pattern (Davis and Patel, 2003). Thus, it is not clear what the ancestral state was in insects. Perhaps what this variability in striped expression is telling us is that we should be focusing on what inherent architectural features in the pair-rule network are allowing such easy change. This will require in-depth functional analysis of multiple pair-rule genes in an insect such as *Oncopeltus*, as well as other arthropods.

**Oncopeltus even-skipped acts like a gap gene**

One of the unexpected findings of this work is that *Oncopeltus even-skipped* RNAi embryos lack such a large region of the body. This complete deletion of the mandibular through abdominal segments is much more severe than the null phenotype in *Drosophila*. On one hand, a complete loss of the abdomen may be explained as an interruption of progressive segmentation in the posterior, as abdominal segments are specified during germband elongation. By preventing proper segmentation of the first abdominal segment, posterior metameres may never be specified. However, this does not explain why the mandibular through thoracic segments are also lacking, as these segments are specified during the blastoderm stage. We found that the early broad blastoderm domain of *Of’eve*, which spans the mandibular through thoracic segments, matches well with the deletion phenotype. Knockdown of *Oncopeltus even-skipped* in blastoderm shows a loss of the central *hunchback* domain with a concomitant expansion of the anterior head domain coupled with a near-complete loss of *Krüppel* expression, indicating that *Of’eve* is required for proper activation and positioning of *Of’hb* and *Of’Kr*. As *Of’eve* regulates the gap genes and also gives a deletion phenotype spanning several contiguous segments (two characteristics usually associated with gap genes), *Of’eve* in some sense also acts as a gap gene.

That a supposedly downstream pair-rule gene regulates supposedly upstream gap genes is not entirely without precedent. The *Drosophila* pair-rule gene *runt* is also required for proper expression of some of the gap genes (Tsai and Gergen, 1994). *Drosophila runt* is initially expressed in an early broad blastoderm domain, before the appearance of the characteristic pair-rule stripes and it is this broad initial domain that is responsible for proper gap gene expression. In *Oncopeltus*, the initial broad blastoderm expression of *Of’eve* may serve a similar function. It may therefore be the case that the early broad blastoderm domain regulates the gap genes, while the later striped expression is in turn regulated by them. This would mean that *Of’eve* occupies both upstream and downstream positions in the segmentation gene hierarchy (Fig. 6B).

**Speculations on non-striped even-skipped function**

Given that the gap-like function of *Of’eve* is novel and has not been reported for other insects, we have much less context in which to discuss its implications. We therefore offer some speculation that we feel is important to discuss explicitly.

First, *eve* in several other insects is also expressed in a
**Fig. 6.** Summary of *Oncopeltus* _even-skipped_ evolution, expression and function. (A) Summary of _even-skipped_ expression within the insects. Species where functional data for _eve_ is known are listed in red. Some species with derived modes of embryogenesis such as polyembryony or complete early cleavage, have altered _eve_ expression and may therefore represent secondarily derived conditions. The early broad domain of _eve_ expression has been reported in several insects, but no function has ever been attributed to it. +, expression present in this species; −, expression not present in this species; ?, neither presence nor absence of expression was reported. Coleopteran, hymenopteran and dipteran phylogenies based on previous work (Lawrence, 1982; Dowton, 1994; Yeates, 1999). (B) *Oncopeltus* _eve_ may occupy both upstream and downstream positions in the segmentation hierarchy. The early broad blastoderm expression (red) is required for activation of the blastoderm expression of the central _hunchback_ and _Krüppel_ domains. These gap genes may then in turn regulate striped _Of’eve_ expression. (C) Summary of putative roles for _Of’eve_. The early broad blastoderm domain (red) is most likely responsible for regulation of _hunchback_ and _Krüppel_. The _eve_ stripes (blue) seen on blastoderms and germbands may regulate _engrailed_. The growth zone expression (red) may be required for proper growth of the posterior. The deleted region in _Of’eve_ RNAi embryos is shown in purple.

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**Fig. 6A** shows the expression domains and putative roles for _Of’eve_. The _eve_ broad domain (red) initiates the _engrailed_ regulatory network. The deleted region in _Of’eve_ RNAi embryos is shown in purple. The _hunchback_ and _Krüppel_ domains are noted by blue and purple, respectively.
Development

It turns out that several segmentation genes, including even-skipped and hunchback, play important roles in convergent extension (Irvine and Wieschaus, 1994). Thus, it is intriguing that both even-skipped and hunchback are so strongly expressed in the Oncopeltus growth zone and that RNAs of these genes result in a failure of posterior growth (Liu and Kaufman, 2004a). This raises the possibility that in addition to posterior patterning, Of eve and Of′ hb may also be required for a process similar to convergent extension in Oncopeltus. However, it is also possible that elongation occurs through increased cell proliferation. Although no convincing increase of mitotic activity has been found in the growth zones of several insects, including Oncopeltus, we cannot rule out cell proliferation as a source of germ band elongation (Brown et al., 1994) (P.Z.L., unpublished; N. Patel, unpublished). But the growth zone shape changes during abdominal growth suggest that cellular rearrangements may at least be one component to posterior elongation.

Interestingly, it has been recently shown that RNAs of the posterior gene caudal in the short germ band beetle Tribolium castaneum, the intermediate germ band cricket Gryllus bimaculatus, as well as in the crustacean Artemia franciscana results in a loss of posterior growth and segmentation (Copf et al., 2004; Shinmyo et al., 2005). In strongly affected Tribolium embryos, only the pregerminal head was formed, a phenotype very similar to the Oncopeltus even-skipped phenotype. In all three organisms, caudal RNAs lead to weakened, abnormal expression of even-skipped. Additionally, caudal RNA in the cricket also leads to loss of hunchback and Krüppel expression, again similar to the situation to Oncopeltus even-skipped. Taken together, this suggests that both caudal and even-skipped are involved in similar functions in posterior growth and patterning, and that possibly, some functions of caudal may be mediated via even-skipped.

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