Krüppel is a gap gene in the intermediate germband insect *Oncopeltus fasciatus* and is required for development of both blastoderm and germband-derived segments

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Summary

Segmentation in long germband insects such as *Drosophila* occurs essentially simultaneously across the entire body. A cascade of segmentation genes patterns the embryo along its anterior-posterior axis via subdivision of the blastoderm. This is in contrast to short and intermediate germband modes of segmentation where the anterior segments are formed during the blastoderm stage and the remaining posterior segments arise at later stages from a posterior growth zone. The biphasic character of segment generation in short and intermediate germ insects implies that different formative mechanisms may be operating in blastoderm-derived and germband-derived segments. In *Drosophila*, the gap gene *Krüppel* is required for proper formation of the central portion of the embryo. This domain of *Krüppel* activity in *Drosophila* corresponds to a region that in short and intermediate germband insects spans both blastoderm and germband-derived segments. We have cloned the *Krüppel* homolog from the milkweed bug, *Oncopeltus fasciatus* (Hemiptera, Lygaeidae), an intermediate germband insect. We find that *Oncopeltus Krüppel* is expressed in a gap-like domain in the thorax during the blastoderm and germband stages of embryogenesis. In order to investigate the function of *Krüppel* in *Oncopeltus* segmentation, we generated knockdown phenotypes using RNAi. Loss of *Krüppel* activity in *Oncopeltus* results in a large gap phenotype, with loss of the mesothoracic through fourth abdominal segments. Additionally, we find that *Krüppel* is required to suppress both anterior and posterior Hox gene expression in the central portion of the germband. Our results show that *Krüppel* is required for both blastoderm-derived and germband-derived segments and indicate that *Krüppel* function is largely conserved in *Oncopeltus* and *Drosophila* despite their divergent embryogenesis.

Key words: *Krüppel* (*Kr*), Gap gene, Short germband, Segmentation, *Oncopeltus*, Milkweed bug, RNAi

Introduction

During embryogenesis, the *Drosophila melanogaster* blastoderm is patterned along the anterior-posterior axis by action of the segmentation gene cascade (reviewed by Hulskamp and Tautz, 1991; St Johnston and Nüsslein-Volhard, 1992; Pankratz and Jäckle, 1993). Initially, maternal gradients specify the anterior- and posterior-most regions of the animal and set up the primary co-ordinate system that dictates the position of action for the downstream segmentation genes. The downstream zygotic gap genes that are expressed in broad, largely non-overlapping domains in the blastoderm follow the maternally supplied gene products. Reflecting this expression pattern, the gap genes function to specify large swaths of the animal, each of which encompasses several adjoining segments. When mutant for a gap gene, embryos develop abnormally and lack several contiguous segments yielding the canonical 'gap' phenotype. Following the gap genes in turn are the primary pair-rule genes that are responsible for setting up a two-segment periodicity in the developing blastoderm. The segment polarity genes that serve to subdivide each individual segment into anterior and posterior compartments then follow the pair-rule genes. In sum, *Drosophila melanogaster* segmentation occurs by subdivision of the blastoderm into finer and finer regions with the output being the final metameric body plan. In *Drosophila*, specification of all the body regions occurs nearly simultaneously and in fact, the entire future body plan is already proportionally represented on the early blastoderm fate map (Hartenstein et al., 1985; Lohs-Schardin et al., 1979).

Although the developmental genetics regulating *Drosophila* segmentation is well understood, what we have learned from *Drosophila* cannot be universally applied to the other insects. In many ways, embryonic development in *Drosophila* is not representative of most insects. For example, insects can be categorized as being 'short', 'intermediate', or 'long germband' with the classifications based largely on the number of segments specified before gastrulation (Davis and Patel, 2002; Krause, 1939; Sander et al., 1985). Since *Drosophila* specifies its entire body plan essentially simultaneously, *Drosophila* is classified as a long germband insect. In short and intermediate germband segmentation, only the anterior segments are specified at the blastoderm stage. The remainder of the segments arises later

during embryogenesis from disproportionate growth of the posterior, from a region described as the 'posterior growth zone'. This region occupies the posterior-most portion of the elongating germband and growth of this region gives rise to the posterior segments which are specified sequentially in an anterior to posterior progression as the germband elongates. Thus, while long germband insects pattern their entire bodies via successive subdivision of the blastoderm, short and intermediate germ insects allocate their blastoderms into only their anterior-most segments and then produce the remaining segments during a later phase of posterior growth. (Since the short and intermediate forms of segmentation are conceptually so similar, for convenience sake we will henceforth refer to both the short and intermediate forms as 'short'.)

In *Drosophila*, the gap genes are expressed in broad domains in the blastoderm, each of which encompasses several contiguous body segments. Reflecting this expression pattern, *Drosophila* embryos mutant for gap genes show segmental deletions spanning several contiguous segments. Thus the gap genes are early patterning genes involved in the initial subdivision of the blastoderm. Since one of the essential differences between short and long germ segmentation lies in how the early blastoderm is allocated into broad body regions, comparing the action of the gap genes between long and short germ insects should serve as a good starting point for better understanding the differences between these two modes of insect segmentation.

The *Drosophila* gap gene *Krüppel* (*Kr*) is required for proper formation of the central portion of the fly embryo. *Krüppel* encodes a transcription factor that contains four zinc-finger motifs that are important for its DNA-binding function (Gaul et al., 1989; Rosenberg et al., 1986). Null alleles of *Krüppel* result in embryos that have a canonical 'gap phenotype' and lack the first thoracic through fourth abdominal segments with the fifth abdominal segment partially deleted. Additionally, the posterior boundary of this deleted region is frequently marked by the presence of a mirror image duplication of the sixth abdominal segment (Gloor, 1950; Wieschaus et al., 1984).

The region deleted in *Drosophila Krüppel* mutants spans segments that in short germband insects are specified both during the blastoderm stage and also later during germband growth. For example, in the intermediate germband insect *Oncopeltus fasciatus* (Hemiptera, Lygaeidae), only the mandibular through the third thoracic segments are specified during the blastoderm stage. It is later, during germband elongation, that the abdominal segments become specified (Butt, 1947; Liu and Kaufman, 2004). Thus, using *Drosophila* as an analogy, the putative region of *Kr* function in *Oncopeltus* would span segments specified during the blastoderm stage (thoracic) as well as segments specified during the germband stage (abdominal). Since nothing is known about *Krüppel* activity at the functional level in the context of short germ segmentation and given the striking differences in segment formation between the blastoderm-derived and germband-derived body regions, it is difficult to imagine how *Krüppel* would act in both of these regions of the *Oncopeltus* embryo.

Would *Kr* function as a canonical gap gene in only the anterior segments, leaving the posterior segments untouched or would *Krüppel* action span both body regions? In order to shed light on this question and also to better understand segmentation in short germband insects in general, we investigated the developmental role of *Krüppel* in the milkweed bug, *Oncopeltus fasciatus*. We isolated the milkweed bug homolog of the *Drosophila* gap gene *Krüppel* and report its expression pattern during milkweed bug embryogenesis. Then using RNA-mediated interference (RNAi), we depleted *Krüppel* activity, which allowed us to examine its function in *Oncopeltus* segmentation.

Materials and methods

Cloning

We prepared total RNA from mixed stage *Oncopeltus* embryos using the TriZol reagent (Life Technologies). Total RNA was poly(A) selected using the Qiagen RNA mini kit and this RNA was used to synthesize cDNA for both 5′ and 3′ RACE using the First Choice RLM-RACE kit (Ambion). Primer sequences for all PCRs are listed in Table 1. In order to clone the short initial fragment of *Of'Kr*, we performed two rounds of PCR on *Oncopeltus* embryonic cDNA using nested degenerate primers designed to conserved regions of *Krüppel*. This initial clone provided sequence to which we designed exact primers for use in 5′ and 3′ RACE. We found that two rounds of amplification using nested exact primers and anchor primers supplied in the RLM RACE kit were also required for the RACE reactions. Several independent RACE reactions were performed and several clones from each were sequenced in order to minimize PCR and sequencing artifacts.

For the Hox genes, gene fragments of *Oncopeltus Deformed* (*Of'Dfd*) and *Sex combs reduced* (*Of'Scr*) were isolated previously

Table 1. PCR primers used for cloning

Gene, primer	Primer sequence (5′→3′)
Of'Kr, degenerate primary forward	TAYAARCAYGTGYTRCARAAYCA
Of'Kr, degenerate nested forward	TTYGARTGYWCNGARTGYCA
Of'Kr, degenerate primary reverse	TANGGNCKYTCNCCNGTRTGNAC
Of'Kr, degenerate nested reverse	GCNACYTGNACRAAYTGNCKRTC
Of'Kr, 5′RACE primary	ATGACAGTGGTAAGGTTTCTCGCC
Of'Kr, 5′RACE nested	GATGATGGTCCCTTCGGAATC
Of'Kr, 3′RACE primary	GGAGATTCCGAAGGGACCATCATC
Of'Kr, 3′RACE nested	CGGACTCACACGGGCGAGAAA
Of'pb, 3′RACE primary	TGGATGAAGGAGAAGAAGACAGC
Of'pb, 3′RACE nested	AGCAACCAGCAAGAGAATGGAC
Of'Ubx, 5′ degenerate forward	ATGAAYTCNTAYTTYGARCARGGNTTYTAYGG
Of'Ubx, 5′ degenerate reverse	CCRTTNGCNCCNGCDATNGCCATCCANGGRTARAA
Of'Ubx, 3′ degenerate forward	TTYCAYACNAAYCAYTAYYTN
Of'abd-A, degenerate forward	TAYCCNTGGATGTCNATHACNGAYTGGATG
Of'abd-A, degenerate reverse	GGNACYTTNGANACNGCYTTNAGNAGRTC
Of'abd-A, 3′RACE primary	TTGGATGAGCCCGTTCGACAGAGTC

(Hughes and Kaufman, 2000) and we used these fragments to synthesize in situ probes. The previously published *Oncopeltus proboscipedia* (*Of'pb*) sequence (Rogers and Kaufman, 1997) was used to design primers for 3′ RACE which allowed us to isolate a large cDNA (approximately 3 kb) corresponding to the 3′ end of the transcript. The *Oncopeltus Ultrabithorax* (*Of'Ubx*) and initial *abdominal-A* (*Of'abd-A*) had not been isolated so we cloned fragments of these genes using degenerate primers designed to the conserved homeodomain, and isolated larger fragments using exact primers.

GenBank accession numbers for submitted sequences are: *Of'Kr*: AY627357, *Of'pb*: AY627358, *Of'Ubx*: AY627359, AY627360, *Of'abd-A*: AY627361.

Embryo fixation, in situ hybridization, and RNAi

Embryo fixation and in situ hybridizations were performed as previously reported (Liu and Kaufman, 2004). We found that it was much easier to dissect the germband stage embryos out of the yolk before carrying out the in situ hybridization procedure. In order to do this, embryos removed from the eggshell but undissected from the yolk balls were first rocked in a SYTOX solution (a fluorescent DNA dye; Molecular Probes) for 2 hours. These embryos were then dissected in PBT under a fluorescence stereomicroscope. Both embryonic (eRNAi) and parental RNAi (pRNAi) RNA-mediated interference were carried out as previously reported (Hughes and Kaufman, 2000; Liu and Kaufman, 2004).

Results

Although descriptions of *Oncopeltus fasciatus* embryogenesis and segmentation have been reported previously (Butt, 1947; Liu and Kaufman, 2004), we will briefly describe segmentation in this insect in order to orient the reader who is unfamiliar with milkweed bug embryogenesis. Segmentation in *Oncopeltus* occurs in two phases – during the blastoderm and germband stages of embryogenesis. During the blastoderm stage, anterior segmentation progresses at least to the level of the segment polarity genes, as *Oncopeltus engrailed* (*Of'en*) transcript is detected in six vertical stripes, corresponding to the mandibular through third thoracic segments (Fig. 1C) (Liu and Kaufman, 2004). Thus at the end of the blastoderm stage, the *Oncopeltus* blastoderm has been subdivided into six anterior segments, leaving the remainder to develop during the germband stage.

The germband forms via a process termed 'germband invagination'. Germband invagination follows the blastoderm stage of embryogenesis and involves migration of the blastoderm cells towards the posterior pole of the egg where they then plunge into the yolk mass to contribute to the forming germband (Fig. 1C-D2). This process of germband invagination results in the germband ending up upside-down and backwards in the egg – the germband lies on the ventral surface of the egg with its head towards the posterior pole and its ventral surface facing dorsal, towards the yolk mass. (The embryo does eventually right itself to its proper final position before hatching with its head at the anterior pole of the egg during katatrepsis via further embryonic movements.) Since these embryonic movements can potentially lead to confusion, when discussing blastoderm-staged embryos, we will refer to 'dorsal' and 'ventral' in terms of the final position of the embryo in the egg at hatching. Note that this convention is the reverse of the convention that we followed previously (Liu and Kaufman, 2004), but seems more intuitive for many readers.

During the germband stage, the remaining posterior body segments that were not specified during blastoderm stage are now produced through elongation of the posterior portion of the germband, the 'growth zone'. Progressive abdominal segment specification can be tracked by observing the appearance of abdominal *en* stripes developing in an anterior to posterior direction (Fig. 1E-H). This biphasic mode of segmentation, with anterior segment specification during the blastoderm stage and subsequent posterior patterning during

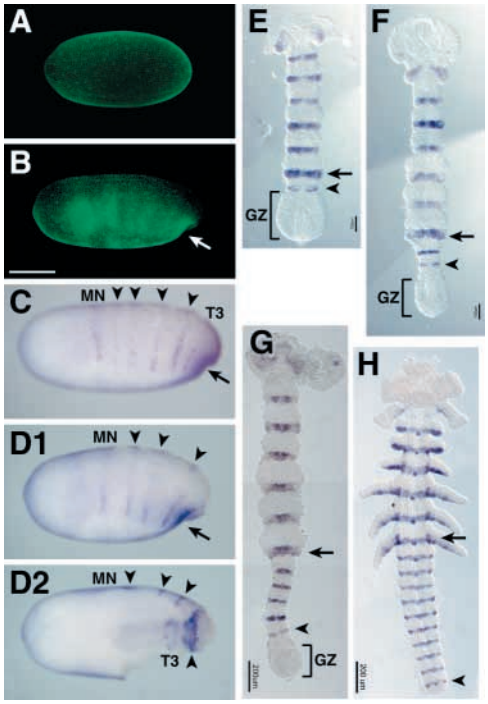

Fig. 1. *Oncopeltus* embryogenesis. (A) Blastoderm embryo shortly after cellularization stained with SYTOX, a fluorescent DNA dye. (B-D1) Since germband invagination and other embryonic movements can be potentially confusing, blastoderm images are oriented so that anterior is to the left, and the future dorsal region of the embryo at hatching is up. Note that dorsal/ventral fates of blastoderm cells may not correspond to final position of the embryo at hatching. See text for details. (B) Late blastoderm embryo undergoing early germband invagination, stained with SYTOX. Arrow marks site of invagination. 'Lateral plates' can be seen as areas of higher cell density. Also note that segmentation is now apparent. (C) Embryo at approximately the same stage as in B, hybridized with probe made to *Oncopeltus engrailed* (*en*) to mark segmental boundaries. At this stage, six *en* stripes corresponding to the mandibular through third thoracic segment can be seen (MN, mandibular; T3, third thoracic *en* stripes). Arrow marks site of invagination. (D1) Embryo undergoing germband invagination and at a later stage than in C stained for *en*. Note that only four *en* stripes can be seen. (D2) Same individual embryo as in D1, but rotated to view ventral aspect with yolk partially removed to reveal underlying early germband. Note that the two most posterior *en* stripes now appear on the germband. (E-H) Germband stage embryos stained for *engrailed*. The arrow marks the third thoracic segment and the arrowhead marks the most newly formed abdominal *en* stripe. GZ, growth zone. Notice that the size of the growth zone decreases as more abdominal segments are added. Embryos oriented such that anterior is up. Scale bars: 200 μm (A,B and E-H).

germband growth, marks *Oncopeltus* as an intermediate germband insect.

Isolation of *Oncopeltus Krüppel*

We took a RT-PCR-based approach to isolate the *Oncopeltus* homolog of *Krüppel*. First, degenerate primers were designed to conserved regions of previously isolated *Krüppel* homologs. PCR on milkweed bug embryonic cDNA allowed us to recover a short fragment corresponding to the zinc-finger region of *Oncopeltus Krüppel* (*Of'Kr*). This short initial fragment allowed us to design exact primers for 5′ and 3′ RACE and subsequently isolate both 5′ and 3′ fragments of the gene. Together, our 5′ and 3′ clones include the entire *Of'Kr* open reading frame. *Of'Kr* is predicted to encode a 33.5 kDa protein with a total of four zinc-binding fingers. Additionally, the Of'Kr protein contains sequences similar to the A- and B-boxes found in *Drosophila Krüppel* (Fig. 2).

Of'Kr expression in the blastoderm

Of'Kr is expressed in a spatially and temporally dynamic pattern during embryogenesis with expression in the blastoderm, the germband ectoderm, as well as in mesodermal and neural domains. Since the anterior segments are specified during the blastoderm stage, we wished to examine the expression pattern of *Of'Kr* transcript in *Oncopeltus* blastoderms. In order to do this, we collected and fixed embryos every four hours from 0 to 40 hours after egg lay (AEL; the blastoderm stage begins with cellularization of the cleavage energids between 15 and 17 hours AEL and ends with the beginning of germband invagination between 36 and 40 hours). We then performed in situ hybridization on these fixed embryos with a 1.7 kb probe made to the 3′ end of *Of'Kr*. We were unable to detect any *Kr* expression prior to 28 hours AEL with the earliest detectable transcript appearing in 28- to 32-hour old embryos. *Of'Kr* transcript accumulates in the posterior third of the developing blastoderm (Fig. 3A1). Since *Oncopeltus* is an intermediate germ insect, this portion of the blastoderm corresponds to different segments than the same position on a blastoderm of a long germ insect such as *Drosophila*. *engrailed* (*en*) is expressed very weakly in the milkweed bug blastoderm, and we were unable to perform simultaneous in situ hybridization for both *en* and *Kr*. Therefore in order to determine the approximate segmental register of *Of'Kr* expression on the blastoderm, images of milkweed bug embryos separately stained for *Kr* and *en* were juxtaposed (Fig. 3F). This allowed us to determine that in *Oncopeltus*, *Kr* is expressed in a region of the blastoderm corresponding approximately to the posterior half of the first thoracic segment through the third thoracic segment. In slightly older blastoderms, just prior to germband invagination, *Kr* transcript retreats from the posterior pole (Fig. 3C). Simultaneous staining for both *Oncopeltus hunchback* (*hb*) and *Kr* on early blastoderms shows that the blastoderm *Kr* domain lies immediately posterior to that of *Oncopeltus hb* (Fig. 3B). It is difficult to determine the extent of expression overlap using in situ hybridization with chromogenic precipitates. However, it seems that the expression domains of *hb* and *Kr* transcript overlap little, if at all – a feature that is shared with *Drosophila* (Jäckle et al., 1986; Schröder et al., 1988; Tautz et al., 1987).

Additionally, dorsal-ventral differences in *Kr* expression can be seen. Although earlier blastoderms express *Kr* transcript in a radially symmetrical pattern, by 28-32 hours, cells on the ventral aspect of the blastoderm cease to express *Kr*, while cells on the dorsal and lateral aspects maintain their expression (Fig. 3A1,A2). At approximately the same stage of development, this ventral clearing seen with *Krüppel* is also seen with several other segmentation genes such as *hb*, *even-skipped*, and *en*, as well as the homeotic gene *Deformed* (*Dfd*) (Liu and Kaufman, 2004) (unpublished data). This ventral clearing is coincident with and probably reflects the formation of the blastoderm 'lateral plates' (Butt, 1947).

Of'Kr expression in the germband

The *Oncopeltus Kr* germband expression pattern is partially a continuation of expression initiated during the blastoderm stage but also includes novel domains in the mesoderm and developing nervous system. During the initiation of germband invagination, *Kr* expression reflects the movement of blastoderm cells across the outer surface of the yolk mass. Recall that during germband invagination, blastoderm cells migrate towards the posterior pole of the egg and upon reaching it, dive into the interior of the yolk mass to form the growing germband. These cellular movements can be seen by tracking the movement of *Kr*-expressing cells across the blastoderm. For example, a patch of *Kr* arises in the anterior

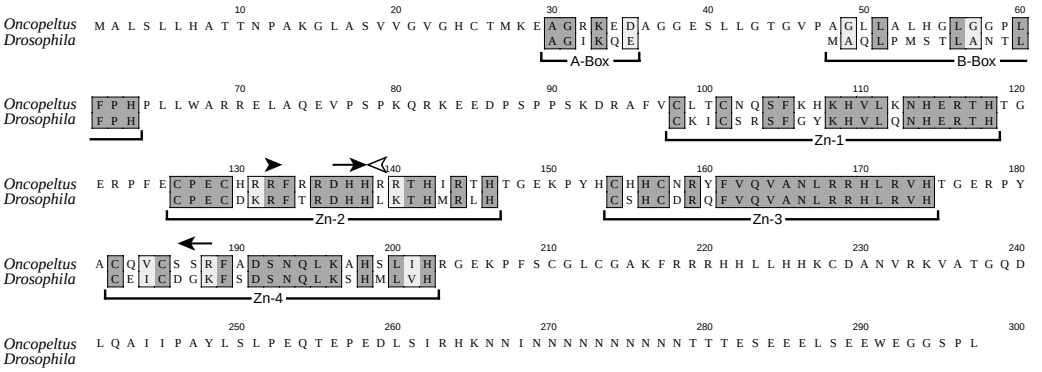

Fig. 2. Predicted amino acid sequence for *Oncopeltus Krüppel* aligned with homologous regions of *Drosophila Krüppel*. Locations of putative zinc fingers, A-box, and B-box are marked. Filled arrowhead denotes beginning of large 3′ fragment used in RNAi and for synthesis of in situ probes. Solid arrows demarcate the short 150 bp 3′ fragment used in RNAi. Open arrowhead shows the end of the 5′ fragment used in RNAi.

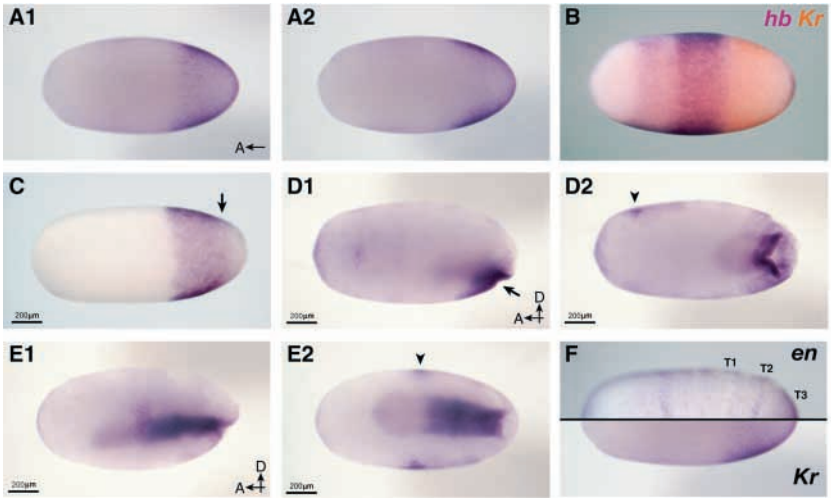

Fig. 3. *Oncopeltus fasciatus Krüppel* blastoderm expression. (A1) View of dorsal aspect of 28-32 hour blastoderm stained for *Kr* RNA. *Kr* transcript accumulates in the posterior 33.5% of the blastoderm surface. (A2) Ventral aspect of same embryo as in A1. Note lack of expression on ventral surface. (B) 28-32 hour blastoderm stained for *hunchback* (purple) and *Kr* (orange). Very little, if any, overlap can be seen for these two genes. (C) *Kr* in situ hybridization of 32-36 hour blastoderm. *Kr* transcript clears from posterior pole (arrow). (D1,D2) Lateral and ventral aspects, respectively, of 36-40 hour embryo undergoing germband invagination. Blastoderm cells expressing the *Kr* gap domain gene are migrating towards the site of invagination, marked by arrow in D1, and are contributing to the germband (visible beneath the yolk). Arrowhead in D2 marks an anterior patch of *Kr* expression. (E1,E2) Lateral and ventral aspects, respectively of 36-40 hour embryo during germband invagination. This embryo is at a later stage than in (D1,D2). *Kr* gap domain cells have now fully invaginated and made their contribution to the germband. Arrowhead in E2 marks position of the same anterior *Kr* patch indicated in D1. These *Kr*-expressing cells have now migrated to a more central position in this later embryo. (F) Montage consisting of two different embryos stained for *engrailed* (top) and *Krüppel* (bottom) aligned to show that *Krüppel* is expressed approximately from pT1-T3. Scale bars: 200 μm.

of the blastoderm at the initiation of germband invagination. Shortly thereafter, this patch has migrated to the middle portion of the blastoderm, coincident with the germband increasing in length (see Fig. 3D1-E2). The movement of this patch of *Kr* expression highlights the movement of the blastoderm cells across the yolk mass.

As described above, in late blastoderms *Krüppel* expression retreats from the posterior pole just before germband invagination begins. This clearing is evident in very early germbands still undergoing invagination (Fig. 3E3). Just after the completion of germband invagination, *Kr* is expressed in the central region of the germband and is excluded from the posterior growth zone. The exclusion is maintained throughout germband growth (Fig. 4A-G). Thus this lack of *Kr* expression in the germband growth zone is an apparent continuation of the posterior clearing seen in late blastoderms.

After completion of invagination, in the early germband *Of'Kr* transcript is expressed in a central domain while it is absent from both the gnathal regions in the anterior and the growth zone in the posterior (Fig. 4B,H,I). This central expression domain is a continuation of expression from the blastoderm stage and is maintained in the ectoderm at least through germband invagination, but subsequently fades (see Fig. 4A2,B2). In order to establish the segmental register of this central domain, we performed double in situ hybridization with probes made to *Kr* and *en*. The double staining shows that in the early germband, *Kr* expression spans the labial through third thoracic segments (Fig. 4H). However, *Kr* expression in the blastoderm only spans the first to third thoracic segments. Therefore, it would at first appear that *Krüppel* expression in the early germband encompasses a *larger* region than that covered by the previous blastoderm expression. However, as we describe below, this increase in the expression domain is probably due to the initiation of underlying mesodermal expression rather than an expansion of accumulation in the ectoderm.

Of'Kr expression in mesodermal and neural domains

Shortly after the germband finishes invagination and during elongation, segmentally reiterated mesodermal expression of *Of'Kr* begins (Fig. 4). This mesodermal expression begins in the thoracic segments, underlying the ectodermal expression and during germband extension, expands both anteriorly and posteriorly to encompass more of the germband than the previous ectodermal gap-like domain. This anterior expansion can be seen as expression of *Kr* first in the labial then maxillary and finally in the mandibular segment (arrowheads in Fig. 4H-K). Although the *en* stripes of this region are already present, this anterior expansion of the *Kr* domain may reflect the anterior and posterior morphological differentiation of segments that starts from a region called the differentiation center, which in many insects is located in the presumptive thorax (Krause, 1939). In some insects, thoracic *en* stripes do indeed appear before the gnathal expression of this gene (Patel et al., 1989). As noted, this anterior expansion of mesodermal *Of'Kr* expression is the reason that the early germband expression extends further than the earlier blastoderm expression. In sum, continuation of the blastoderm pattern results in ectodermal expression corresponding to the first through third thoracic segments and additional *Kr* transcript accumulates in the underlying mesoderm of the thorax with this mesodermal expression expanding anteriorly as development proceeds.

During the formation of the abdominal segments, mesodermal *Kr* expression also expands posteriorly (arrows in Fig. 4H-K), but is absent from the overlying ectoderm (Fig. 4D1-E2). However, the posterior boundary of ectodermal *Kr* expression remains in the third thoracic segment, consistent with the pattern established during the blastoderm stage. This abdominal mesodermal expression proceeds in an anterior to posterior progression and probably reflects the anterior to posterior maturation of the abdominal segments – chronologically older (more anterior) segments express *Kr* mesodermally, while younger segments (closer to the growth zone) do not. Inspection of this mesodermal expression reveals no apparent regional differences in the abdomen, and it merely

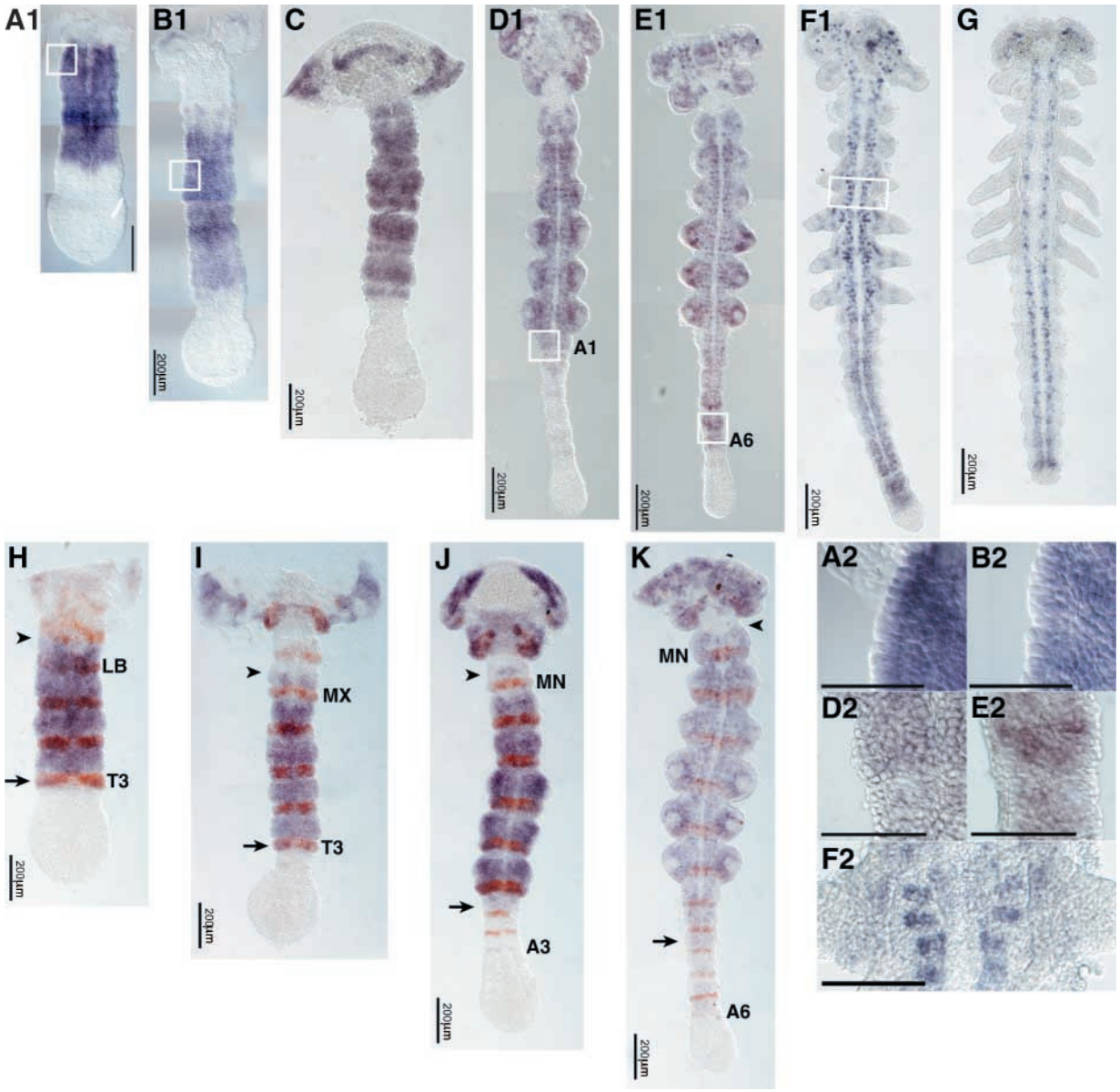

Fig. 4. *Krüppel* germband expression. (A-G) Developmental series of successively older germband stage embryos stained for *Krüppel*. *Kr* expression begins in an ectodermal domain in the central portion of the germband. Expression in the mesoderm and in neural domains then develops in older germbands. (A1,B1) *Kr* expression in the germband begins as a continuation of the previous blastoderm domain and accumulates in the central region of the germband. (A2,B2) Higher magnification of boxed regions in A1 and B1 respectively. Note that ectodermal expression fades, but mesodermal expression remains strong. (D2,E2) Higher magnification of boxed regions in D1 and E1, respectively. Abdominal expression appears mesodermal and not ectodermal. (E1-G) In addition to the mesodermal expression, dots of neural expression also accumulate. Note the anterior to posterior maturation of the neural pattern. (F2) Higher magnification image of boxed region in F1 showing neural expression. (H-K) Embryos double stained for *Kr* (purple) and *en* (orange). Anterior limit of *Kr* expression is marked by arrowheads, posterior limit by arrows. By comparing the boundaries of mesodermal expression in successively later staged embryos, it is apparent that *Kr* mesodermal expression expands both anteriorly and posteriorly. Scale bars: 200 μm (A-G,H-K); 100 μm. (A2-F2) LB, labial segment; MN, mandibular segment; MX, maxillary segment; T1-T3, thoracic segments; A1-A6, abdominal segments.

appears in a segmentally reiterated pattern (compare Fig. 4D2 and E2). Thus developing germbands express *Kr* in an ectodermal gap-like pattern in the central portion of the germband reflecting continued expression of the blastoderm domain. *Kr* is also expressed mesodermally beginning with the central portion of the germband and expanding to eventually encompass the mesoderm throughout the entire germband. This mesodermal expression of *Kr* seems to be shared with other insect species (Gaul et al., 1987; Sommer and Tautz, 1991).

In addition to the ectodermal and mesodermal expression domains described above, *Oncopeltus Kr* transcript also appears to accumulate in a neural-like pattern. In mid-germband stage embryos, an orderly grid-like pattern of dots

appears in the middle region of each segment (Fig. 4F1,F2) and this pattern is consistent with known *Kr* expression and function in the *Drosophila* nervous system (Gaul et al., 1987; Isshiki et al., 2001). Like the mesodermal expression described above, this neural-like expression also progresses in an anterior to posterior direction, most likely reflecting progressive maturation of each body segment (Fig. 4E-G).

Morphological analysis of *Kr* RNAi

In order to determine the functional role of *Kr* in *Oncopeltus* segmentation, we used RNA-mediated gene interference (RNAi) to deplete *Kr* transcript and assayed the resulting embryos for the knockdown phenotype. RNAi is a technique that has been used in many organisms, which allows specific suppression of gene function via the introduction of double-stranded RNA (dsRNA) corresponding to the gene of interest into the developing embryos (Brown et al., 1999; Fire et al., 1998; Hughes and Kaufman, 2000; Miyawaki et al., 2004; Schoppmeier and Damen, 2001). It has been reported previously that direct injection of dsRNA into early embryos, termed embryonic RNAi (eRNAi) and also injection of dsRNA into the abdomens of mothers, termed parental RNAi (pRNAi) yield knockdown phenotypes in *Oncopeltus* (Bucher et al., 2002; Liu and Kaufman, 2004). Since pRNAi does not produce any injection artifacts, we largely used pRNAi in our analysis but also included eRNAi as a confirmation of the phenotype. We injected three different dsRNA fragments corresponding to different regions of the *Of'Kr* transcript: a 460 bp 5′ fragment, a small 150 bp 3′ fragment, and a larger 1.7 kb 3′ fragment, which completely spans the smaller 3′ piece and also includes an additional 250 bp of 3′ untranslated sequence (Fig. 2). All of these dsRNA fragments yielded the same qualitative phenotype. Injection of dsRNA at different concentrations resulted in embryos that ranged in phenotypic severity from strongly affected to completely wild type (Table 2). This hypomorphic series of *Kr* depletion allowed us to categorize the embryos into three phenotypic classes based on final embryo morphology. All affected phenotypic classes showed a gap phenotype that included the thorax and anterior abdomen, with milder classes showing more limited regions of deletion than the more severe phenotypic classes. *Oncopeltus* embryos seem to be very sensitive to *Kr* depletion, as mothers injected with the high concentration of dsRNA solution (2 μg/μl) did not give class I or wild-type embryos. It was only upon injection with very low dsRNA concentrations (0.004 μg/μl) that we were able to obtain the full range of severity (Table 2).

By examining the hypomorphic series for *Kr* function, we found that the anterior abdomen is most sensitive to *Kr* depletion. As the RNAi defect increases in severity, the deleted region expands both anteriorly and posteriorly to encompass more of the thorax and abdomen. In the mildest phenotypic class (class I), embryos show a small deletion of one or two abdominal segments that are frequently associated with defective segmentation in the adjacent anterior segments of the remaining abdomen (Fig. 5D2). The third thoracic leg is present but often defective and is occasionally fused with the second thoracic leg (Fig. 5D2,D3). In the more strongly affected class II embryos, the third thoracic segment is absent and the second thoracic leg is deformed. Additionally, the abdomen is visibly shortened and lacks even more segments than class I embryos (Fig. 5E1,E2). The strongest phenotypic class (class III) shows a large deletion of the central portion of the embryo. In fully developed class III individuals, the entire animal is visibly shortened and it is apparent that the second and third thoracic segments are deleted along with several segments of the anterior abdomen (Fig. 5F1,F2). In these animals, the prothoracic leg is deformed but retains at least partial leg identity, as it retains a tarsal claw (arrow in Fig. 5F2). Anterior segments appear unaffected as normal antenna, mandibular and maxillary stylets (arrows and arrowheads, respectively in Fig. 5F4), and a normal labium can be seen (arrowhead in Fig. 5F3). In some class III individuals, the labium is deformed, but in these animals the first thoracic segment is still present, albeit with a deformed leg (not shown).

Analysis of *Of'Kr* RNAi germbands

Since it is difficult to determine the precise number of missing segments by examination of fully developed embryos, we fixed and performed in situ hybridization on germband stage RNAi depleted embryos. *engrailed* is a convenient segmental marker, and in situ hybridization with *Of'en* probe allowed us to compare segmentation of affected individuals with wild-type embryos. Owing to the weak blastoderm expression of *en* even in wild-type embryos, *en* expression in *Kr* RNAi blastoderms was inconclusive and all analysis was performed on germband stage embryos.

Putative class I germband stage embryos stained for *en* show a small deletion of the anterior abdomen along with defects in meso- and metathoracic segmentation (Fig. 6B), consistent

Table 2. Results of parental and embryonic *Krüppel* RNAi

	dsRNA*	Concentration (mg/ml)	Non-specific *n* (%)†,§	Wild type *n* (%)	Class I *n* (%)†,‡	Class II *n* (%)†,‡	Class III *n* (%)†,‡	Totals *n*
Kr pRNAi	5′	2.0	56 (27.5)	0 (0)	0 (0)	103 (50.5)	45 (22.1)	204
	3′ small	2.0	7 (8.5)	0 (0)	0 (0)	0 (0)	75 (91.5)	82
	3′ large	2.0	31 (11.7)	0 (0)	0 (0)	36 (13.6)	198 (74.7)	265
	3′ large	0.02	73 (12.9)	0 (0)	12 (2.1)	268 (47.5)	211 (37.4)	564
	3′ large	0.004	35 (12.1)	101 (34.8)	44 (15.2)	95 (32.8)	15 (5.2)	290
Kr pRNAi Totals			202 (14.4)	101 (7.2)	56 (4.0)	502 (35.7)	544 (38.7)	1405
Kr eRNAi	3′ large	2.0	30 (19.0)	3 (1.9)	0 (0)	8 (5.1)	117 (74.1)	158
Buffer pRNAi			14 (6.8)	191 (93.2)	0 (0)	0 (0)	0 (0)	205

*The 5′, 3′ small, or 3′ large dsRNAs refer to different fragments of the *Krüppel* gene product and correspond to different regions of the transcript. See Fig. 1.
†Percentages may not add up to 100 because of rounding.
‡Phenotypic classification was determined based on severity of phenotype, with class III being the most severe.
§The non-specific category includes embryos where some embryonic development had occurred, but was uninterpretable.

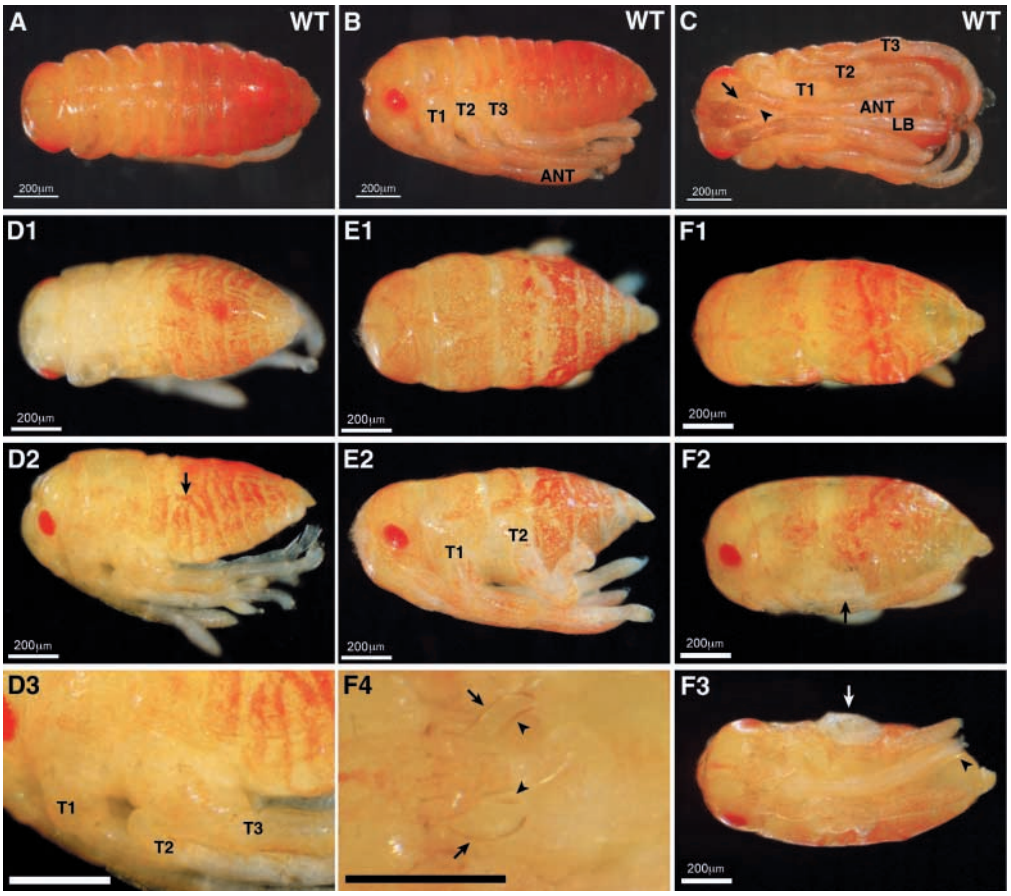

Fig. 5. *Krüppel* RNAi phenotype. (A-C) Uninjected embryos just prior to hatching. (A) Dorsal view. (B) Lateral view. (C) Ventral view. Antenna (ANT) and thoracic legs (T1-T3) are marked. Arrow in C indicates mandibular stylet; arrowhead indicates maxillary stylet; LB, labium. (D1-D3) Representative class I embryo. (D1) Dorsal view, abdomen is visibly shortened. (D2) Lateral view showing defective segmentation in the abdomen (arrow), and fusion of second and third thoracic segments. (D3) Higher magnification image of same embryo, showing fusion of second and third thoracic legs. (E1,E2) Class II embryo with deletion of third thoracic segment and more deleted abdominal segments resulting in shorter abdomen. Also note that second thoracic segment is present, but with a reduced leg. (F1-F4) Class III embryo. Second and third thoracic segments are deleted and the first thoracic leg is highly reduced (arrow in F2 and F3). Labium is unaffected (arrowhead in F3). (F4) High magnification image showing presence of mandibular (arrows) and maxillary (arrowheads) stylets. Scale bars: 200 µm in all images except F4,which is 100 µm.

with the late-stage morphological phenotype. In stronger RNAi embryos, this region of defect expands to include more thoracic and abdominal segments. In putative class II germband stage embryos, *en* expression shows that thoracic segments are more defective and fewer abdominal segments are present than in the class I embryos (Fig. 6D). The weaker phenotypic classes show that the anterior abdominal segments are most sensitive to *Kr* depletion and as *Kr* function is further suppressed, the gap expands in both anterior and posterior directions. By counting the number of *en* stripes on class III germband stage embryos, we determined that severely affected RNAi embryos lack a total of six segments (compare Fig. 6A and D) and that the deleted region seems spans the mesothoracic through fourth abdominal segments.

In *Oncopeltus*, this deleted region spans fewer segments than *Kr* null mutations in *Drosophila*. This may either reflect

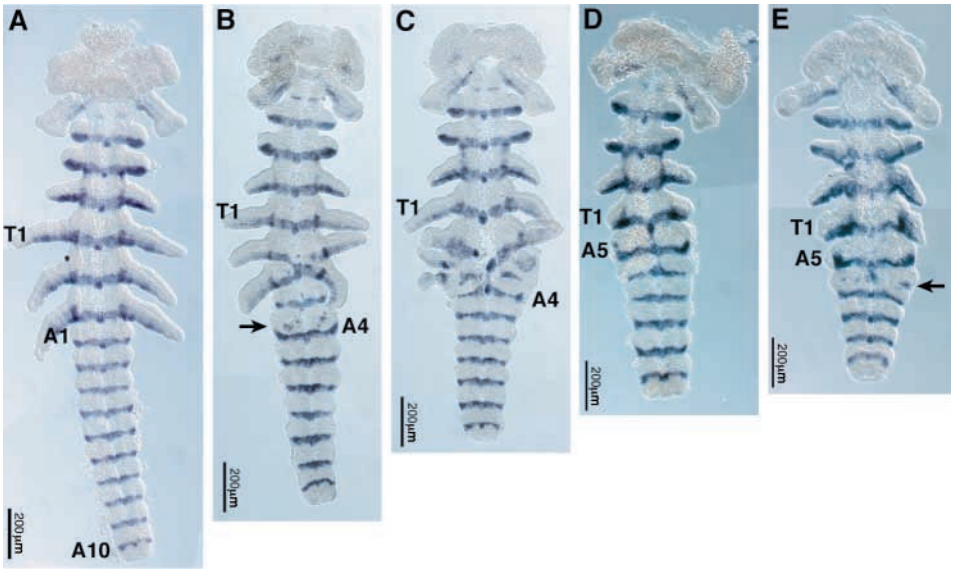

Fig. 6. Wild-type (A) and *Krüppel* RNAi (B-E) embryos stained for *engrailed*. (B) Putative class I RNAi embryo with a small deletion of anterior abdomen, possibly of the first two abdominal segments. All thoracic segments are present, but second and third thoracic segments show defective *engrailed* expression. Also note 'skipping' of defect, indicated by an arrow. (C) Putative class II embryo, with larger abdominal deletion and stronger thoracic segmentation defects. Second and third thoracic segments are strongly affected, but first thoracic segment appears normal. (D) Class III embryo, with full *Kr* deletion of mesothoracic through fourth abdominal segments. (E) Class III embryo, with 'skipping' of the sixth abdominal *en* stripe, indicates by an arrow. T1, first thoracic segment; A1-A10, abdominal segments. Scale bars: 200 µm.

differences in *Krüppel* function between the two insects or be a consequence of incomplete RNAi suppression of *Kr* activity. Extrapolating from the hypomorphic series, it may be that if further depletion were possible, the deleted region in *Oncopeltus* would expand to encompass the same segments as in *Drosophila*. The relatively large number of the 'uninterpretable' class of embryos (Table 2) may be individuals that had complete knockdown of *Kr* activity. However, they were so strongly disrupted in their embryogenesis that the resulting embryo did not undergo any segmentation at all. At any rate, in the class III RNAi animals, there is a large gap phenotype and the remaining segments appear to be morphologically normal. In the anterior, the mandibular, maxillary and labial segments all appear unaffected, while the prothoracic legs are defective, consistent with the terminal phenotype at the hatching stage. In the posterior, segmentation of the remaining six abdominal segments also appears to be largely normal.

In some RNAi depleted embryos, defects in segmentation were seen to be discontinuous – abnormal *en* expression seemed to 'skip' segments. This 'skipping' is reminiscent of discontinuous defects produced by weak alleles of *Kr* in *Drosophila* (Wieschaus et al., 1984). This discontinuity in *Oncopeltus Kr* action was seen in all phenotypic classes and was not associated with any particular segment. Figure 6E shows an example of a putative class III embryo where the usual central gap of the mesothoracic through fourth abdominal segment is associated with an additional partial loss of the sixth abdominal *en* stripe. Figure 6B shows an example of a putative class I embryo with a defective third abdominal *en* stripe that is bounded by apparently normal *en* stripes.

Hox gene expression in *Kr* RNAi embryos

We wished to extend our analysis of the *Kr* RNAi embryos by confirming the identity of the remaining segments using molecular markers. The homeotic (Hox) genes are a group of genes that are expressed in, and are thought to be required for, segmental identity in all arthropods including insects (for a review, see Hughes and Kaufman, 2002). Thus they make convenient molecular markers for segmental identity in *Oncopeltus*. In wild-type embryos, the *Oncopeltus* Hox gene *Deformed* (*Of'Dfd*) is expressed in the mandibular and maxillary segments and associated limb buds (Fig. 7A). In class III RNAi embryos, the mandibular and maxillary expression is normal, confirming the identity of these segments. However, *Of'Dfd* is ectopically expressed in the first thoracic legs (Fig. 7B). This suggests that in *Oncopeltus*, *Kr* represses *Of'Dfd* expression posterior to its normal domain. *Oncopeltus proboscipedia* is expressed in the labium [but not in the maxillae as in most insects (Rogers et al., 2002)] in wild-type embryos. This expression appears intact in RNAi embryos, confirming the identity of this segment (Fig. 7C,D). In the case of the *Tribolium jaws* mutation, which is most likely a lesion in the *Tribolium* homolog of *Kr*, *proboscipedia* (*maxillopedia* in *Tribolium*) is ectopically expressed in the thorax (Bucher, 2002; Sulston and Anderson, 1998). In contrast, we do not detect any ectopic expression of *proboscipedia* in *Oncopeltus Kr* RNAi embryos.

In wild-type animals, *Oncopeltus Sex combs reduced* (*Of'Scr*) is expressed in the labium and in the mesoderm of the first thoracic leg (Fig. 7E). The labial expression appears unaffected in RNAi embryos, and the mesodermal expression in the prothoracic leg is still present but has a spotty expression pattern (Fig. 7F). This modulation of *Of'Scr* expression may be due either to direct regulation by *Kr* or to a secondary effect such as the ectopic expression of either *Deformed* or *abdominal-A* in *Kr* knockdown embryos (see below).

We also examined the expression of the posterior Hox genes, *Ultrabithorax* (*Of'Ubx*) and *abdominal-A* (*Of'abd-A*) in *Kr*-depleted animals. In wild-type animals, *Of'Ubx* is strongly expressed throughout the first abdominal segment and weakly in a neural-like pattern in the entire abdomen (Fig. 7G). This neural-like expression remains intact in RNAi depleted animals, but the segmental expression is not detectable (Fig. 7H). Since the neuronal expression is weak relative to the segmental expression, detection of the neuronal expression suggests that the lack of segmental expression is not merely the result of lack of sensitivity of the in situ hybridization technique. Rather, the lack of segmental expression along with the *en* expression described above indicates that at least the first abdominal segment is deleted. *Of'abd-A* is normally expressed in the abdomen, from the posterior of the first abdominal segment and extending posteriorly through the remainder of the abdomen (Fig. 7I). *Krüppel* RNAi depleted animals show fewer *abd-A*-expressing abdominal segments, consistent with a large deletion of the anterior abdomen (Fig. 7J,K). Additionally, weak ectopic patches of *Oncopeltus abd-A* can be seen in the labial and first thoracic segments (Fig. 7K). This ectopic expression of *abd-A* suggests that in *Oncopeltus*, *Krüppel* normally represses *abd-A* in anterior segments. Thus in *Oncopeltus*, *Krüppel* acts to repress expression of both anterior and posterior Hox genes in the central portion of the animal.

Discussion

We have cloned the homolog of the *Drosophila* gap gene *Krüppel* from an intermediate germband insect, *Oncopeltus fasciatus*. *Oncopeltus* undergoes distinct blastoderm and germband phases of segmentation and we report the transcript expression pattern during both of these stages of development and find both similarities and differences with other insect species. We also report the first functional analysis of *Kr* in a short or intermediate germband insect and outside of *Drosophila*. Surprisingly, we find that the role of *Kr* is largely conserved in milkweed bugs despite the fundamental differences in segmentation between *Drosophila* and *Oncopeltus*.

Kr expression shows both conserved and divergent aspects

During the blastoderm stage, *Oncopeltus Kr* is expressed in a broad domain in the posterior one third of the blastoderm which corresponds roughly to the posterior of the first thoracic through third thoracic segments. This segmental register is maintained during germband invagination and results in the continuation of this thoracic pattern in the ectoderm of early germbands. During germband elongation, *Kr* is also detected in the mesoderm underlying the thoracic ectoderm. As germband elongation proceeds, this mesodermal expression expands both anteriorly and posteriorly to eventually encompass the mesoderm of the entire body.

The segmental register of *Krüppel*'s central gap-like pattern differs from its expression in *Drosophila melanogaster*. In fruit flies, *Kr* is expressed in the blastoderm from the mesothoracic segment to approximately the third abdominal segment (Gaul and Jäckle, 1989; Knipple et al., 1985). In *Oncopeltus*, the gap-like domain of *Kr* covers the posterior of the first through third thoracic segments but does not extend into any abdominal ectoderm. Thus relative to the fruit fly, the posterior boundary of *Oncopeltus Kr* is shifted anteriorly by about three segments. This expression domain is more similar to that of the red flour beetle *Tribolium castaneum*, where *Kr* is expressed in only the thoracic segments and not in the anterior abdomen (Bucher and Klingler, 2004; Sommer and Tautz, 1993). Given the similarities between the *Oncopeltus* and *Tribolium* expression patterns, this pattern would appear to represent the ancestral state for *Kr* expression at least within the paraneopteran insects.

Krüppel expression clears in the very posterior of the late blastoderm just before germband invagination. This may represent either loss of activation or the initiation of suppression of *Kr* in these cells. This posterior clearing of *Krüppel* expression is maintained through germband invagination and is manifested in the early germband as a growth zone devoid of *Kr* expression. This exclusion from the

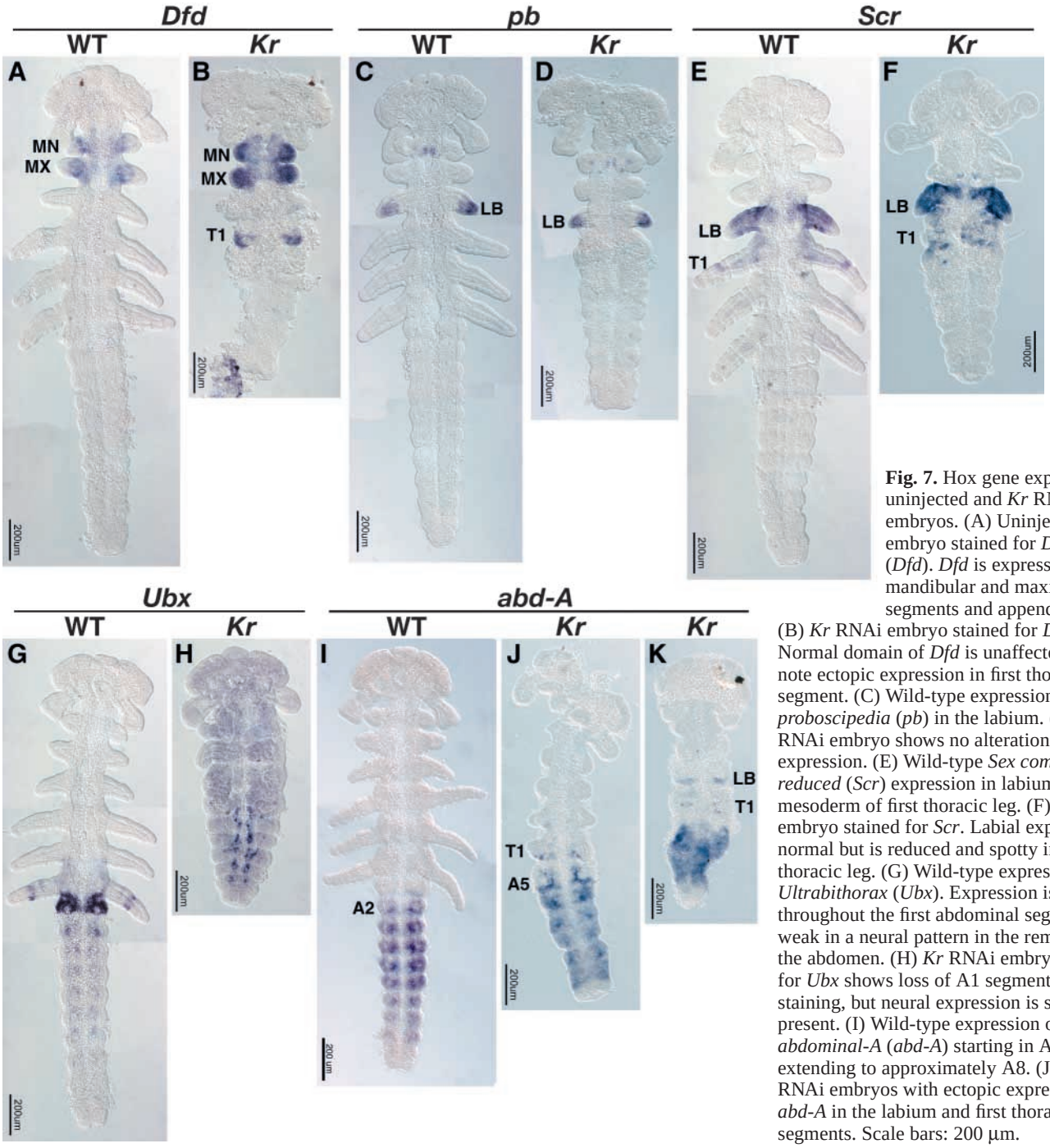

Fig. 7. Hox gene expression in uninjected and *Kr* RNAi embryos. (A) Uninjected embryo stained for *Deformed* (*Dfd*). *Dfd* is expressed in the mandibular and maxillary segments and appendages. (B) *Kr* RNAi embryo stained for *Dfd*. Normal domain of *Dfd* is unaffected, but note ectopic expression in first thoracic segment. (C) Wild-type expression of *proboscipedia* (*pb*) in the labium. (D) *Kr* RNAi embryo shows no alteration of *pb* expression. (E) Wild-type *Sex combs reduced* (*Scr*) expression in labium and mesoderm of first thoracic leg. (F) *Kr* RNAi embryo stained for *Scr*. Labial expression is normal but is reduced and spotty in first thoracic leg. (G) Wild-type expression of *Ultrabithorax* (*Ubx*). Expression is strong throughout the first abdominal segment, and weak in a neural pattern in the remainder of the abdomen. (H) *Kr* RNAi embryo stained for *Ubx* shows loss of A1 segmental staining, but neural expression is still present. (I) Wild-type expression of *abdominal-A* (*abd-A*) starting in A2 and extending to approximately A8. (J,K) *Kr* RNAi embryos with ectopic expression of *abd-A* in the labium and first thoracic segments. Scale bars: 200 μm.

growth zone is preserved throughout germband elongation. Thus with regard to *Kr* expression, the posterior growth zone appears special in some way. Indeed, other segmentation genes are specifically *expressed* in the growth zone in both *Oncopeltus* and other short and intermediate germ insects (Dearden and Akam, 2001; Liu and Kaufman, 2004; Patel et al., 1992; Wolff et al., 1995). While the characteristics of the insect growth zone are not well understood, the fact that several segmentation genes are expressed in or excluded from this region suggests that there is something special about this portion of the germband. *Krüppel*'s exclusion from the growth zone can be directly traced to the posterior clearing first seen in the blastoderm and suggests that this region may be specified *before* the actual formation of the germband and may actually begin to acquire its unique identity during late blastoderm and seems consistent with observations in other short/intermediate germ insects (Schroder et al., 2000).

Spatial discrepancy between the *Krüppel* expression pattern and its phenotype

Since the *Kr* gap expression pattern covers only the thoracic segments and not the anterior abdomen, deletion of part of the abdomen raises the important issue of discrepancy between the ectodermal gap expression pattern and phenotype. A possible explanation for this discrepancy may lie in technical limitations of determining the precise extent of gap gene products. For example, *Drosophila Krüppel* protein expression was initially reported to span 54-39% of egg-length, but using more sensitive techniques, was later found to be larger and span 60-33% of egg-length (Gaul and Jäckle, 1987; Gaul and Jäckle, 1989). In fact, based on genetic evidence, the *Drosophila* Kr protein gradient may extend even further still (Pankratz et al., 1989). Thus our failure to detect transcript in the anterior abdominal ectoderm may be because of experimental limitations rather than be a reflection of biological significance. However, *Krüppel* expression in another short germ insect, *Tribolium castaneum*, has been examined and has also been found to accumulate in the thorax but not in the abdomen (Bucher and Klingler, 2004; Sommer and Tautz, 1993). If *Oncopeltus Kr* was indeed expressed in the ectoderm of the milkweed bug abdomen, this would further imply that *Kr* expression differs significantly even between two short/intermediate germ insects and changes in its expression would have to be highly evolutionarily labile. Since the *Oncopeltus* blastoderm becomes cellularized very early in development (around 17 hours after egg lay), it seems unlikely that a *Krüppel* protein gradient can be utilized to specify the anterior abdominal segments. Rather it may be that the gap genes pattern the germband-derived segments via cell-cell signaling or via some other long-range effect (Bucher and Klingler, 2004; Davis and Patel, 1999; Eckert et al., 2004).

It is a formal possibility that the mesodermal expression, which extends into the abdomen, has a direct function in segmentation. However, *Krüppel* RNAi resulted in deletion of only the thoracic and anterior abdomen. If the mesodermal expression is important for formation of the anterior abdominal segments, it would require segmentation function to be limited to only these segments and not the rest of the body. This region-specific function seems unlikely and since we detect no qualitative differences between the mesodermal expression of the anterior abdomen as compared with the rest of the body, it seems probable that the ubiquitous mesodermal expression is not involved in segmentation. For these reasons, we attribute the segmentation role to the ectodermal domain in the thorax with the implication that the anterior abdominal segments are deleted as a result of a long-range requirement for *Kr* in these segments.

Oncopeltus Kr is a bona fide gap gene

We have shown that strong *Kr* RNAi depletion results in deletion of the mesothoracic through fourth abdominal segment. This gap phenotype is in contrast to the RNAi phenotype of the *Oncopeltus* homolog of another gap gene *hunchback* (Liu and Kaufman, 2004). *hb* RNAi results in a terminal phenotype in which the segments of the head are followed by several segments with abdominal identity. By analysis of the RNAi hypomorphic series, it was apparent that instead of a true gap phenotype, the *hb* phenotype is really due to a combination of anterior homeosis towards abdominal identity coupled with defective segmentation of the posterior germband resulting in posterior compaction. Our analysis of a *Kr* RNAi hypomorphic series shows that in mildly affected animals, the anterior abdominal segments are deleted, and the remaining segments are intact with no overt evidence of homeosis or posterior compaction. As the RNAi depletion becomes more severe, the deleted region expands both anteriorly into the thorax and posteriorly to cover more of the abdomen until the terminal phenotype is reached. In strongly affected animals this results in a large gap spanning the second thoracic through fourth abdominal segments, but leaving behind normal anterior and posterior segments. This hypomorphic series in *Oncopeltus* is similar to the phenotypes obtained in a *Krüppel* allelic series in *Drosophila* where weak and moderate alleles delete a smaller region of the body than amorphic alleles (Wieschaus et al., 1984). The lack of homeosis or posterior compaction, along with the gap-like ectodermal expression pattern suggests that in *Oncopeltus*, *Kr* is a bona fide gap gene. Interestingly, a probable mutation in the *Tribolium Kr* homolog, *jaws*, has already been isolated and mutant embryos show a homeotic transformation of the thorax and first abdominal segment towards gnathal identity as well as a large deletion of almost the entire remaining abdomen (Bucher, 2002; Sulston and Anderson, 1996). Therefore, although the expression pattern of *Oncopeltus Kr* is more similar to the *Kr* expression in *Tribolium* than in *Drosophila*, the *Oncopeltus Kr* phenotype is more similar to the *Kr* phenotype in *Drosophila* than in *Tribolium*. Figure 8 shows a comparison of the expression domains and loss-of-function phenotypes of the *Kr* homologs in *Oncopeltus*, *Tribolium* and *Drosophila*.

Given the biphasic nature of *Oncopeltus* segmentation – with anterior segments formed via allocation of the blastoderm and abdominal segments from germband growth – we were surprised by the large gap phenotype spanning both blastoderm-derived and germband-derived segments. Although these segments develop via different embryological processes, our results show that patterning of these two regions share molecular underpinnings with each other as well as with *Drosophila*. It is unlikely however, that segmentation between *Oncopeltus* and *Drosophila* is conserved in all of its details. Some segmentation genes that have been analyzed at the functional level in short germ insects have been shown to play

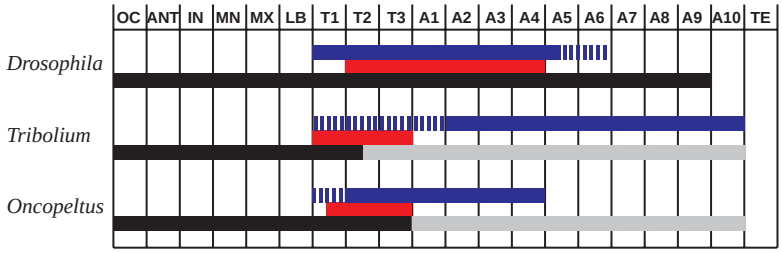

Fig. 8. Summary of *Krüppel* expression (red bars) and function (blue bars) in *Drosophila*, *Tribolium* and *Oncopeltus*. Solid blue bars denote deletions of affected segments while broken blue bars denote defects other than deletions. Black and gray bars denote body regions specified during blastoderm stage and from growth of the posterior growth zone, respectively. *Drosophila Kr* null mutations result in a deletion that spans the first thoracic through the fourth abdominal segment, with the fifth abdominal segment partially deleted, and with an enlargement of the sixth abdominal segment. The *Drosophila Krüppel* protein is expressed in a gradient from the mesothoracic through fourth abdominal segments. *Tribolium jaws* is probably the *Krüppel* homolog (see text) and mutants show a transformation of the thorax and first abdominal segment towards gnathal identity along with a deletion of most of the remaining abdomen. *Tribolium Krüppel* expression accumulates in the thorax. *Oncopeltus* RNAi results in a deletion of the mesothoracic through fourth abdominal segments with reduction, but not deletion of, the prothoracic appendage. *Oncopeltus Kr* transcript accumulates from the posterior of the first through third thoracic segments.

different roles in flies (Bucher and Klingler, 2004; Liu and Kaufman, 2004) (G. Bucher, PhD thesis, Ludwig-Maximilians-Universität München, 2002). Therefore, gap gene function is surprisingly labile and a comparative approach spanning several insect taxa and modes of segmentation must be considered in order to understand these genes in patterning the insect body plan.

The *Krüppel* phenotype and the evolution of insect segmentation

We would like to make explicit three observations that may have implications for the evolution of insect segmentation. First, as we discussed above, some of the molecular underpinnings may be shared between blastoderm-derived and germband-derived segments. However, in *Tribolium*, another short germ insect, the abdominal gap gene *giant* does not act as a canonical gap gene. Instead, *Tribolium giant* may have a more general role in segmentation, suggesting that some aspects of abdominal segmentation have diverged (Bucher and Klingler, 2004). Nevertheless, *Oncopeltus Kr* does act as a true gap gene, suggesting that at least some of the mechanisms underlying segment formation may be shared between the milkweed bug blastoderm and germband as well as with *Drosophila*.

Secondly, *Kr* RNAi embryos ectopically express *Dfd* in a posterior domain and *abd-A* in an anterior domain. *Oncopeltus Kr* is not only required for the formation of segments in the middle portion of the embryo, but also regulates anterior and posterior genes. *Kr* may directly regulate these Hox genes or instead, *Kr* may regulate other gap genes as is the case in *Drosophila* and these may in turn regulate the downstream Hox genes (Jäckle et al., 1986; Kraut and Levine, 1991; Mohler et al., 1989). Thus in *Oncopeltus*, *Kr* seems to act as a sort of ‘spacer’ both to specify central segments and to prevent central expression of anterior and posterior genes.

Lastly, although loss of *Krüppel* function results in a deletion of anterior abdominal segments, posterior abdominal segments appear normal. Since all abdominal segments are normally produced through elongation of the posterior germband, presence of normal posterior abdominal segments in *Kr* RNAi embryos means that for these segments, the segment *formation* function of the growth zone was not disrupted. Although called a ‘growth zone’, this region has not yet been well studied in insects, and it has yet to be shown to share characteristics with the growth zones of other arthropods such as spiders (Stollewerk et al., 2003). Nevertheless, our results imply that the segment *formation* function by the growth zone can, to some degree, be decoupled from the actual *number* of segments that it produces.

The above observations suggest a possible (and admittedly speculative) mechanism for transition between short, intermediate and long germ forms of segmentation. For instance, our results show that alterations in activity of a gap gene can change the number of segments that are normally specified at the blastoderm stage. Evolutionarily, this can perhaps be accomplished by decreasing the width of the *Krüppel* domain on the blastoderm while maintaining the number of segments that it specifies. This would allow more posterior genes to be expressed on the blastoderm and would serve to pack more gap domains (and therefore body regions) on the blastoderm fate map. We have shown that although the *number* of segments the growth zone produces can increase or decrease, the segment *formation* ability of the growth zone seems to be largely independent, and the remainder of the posterior segments would be generated as usual, via germband growth. This would result in shifting the relative number of segments generated at the blastoderm stage versus the germband stage – in effect converting a shorter germ insect into a longer germ insect. The above scenario is highly speculative and no doubt overly simplistic but is attractive because it offers a mechanism for evolving the mode of segmentation. At this point, it is clear that further work needs to be done. Functional analysis of the segmentation genes in short germ insects has only begun but should provide a greater understanding of these questions and conundrums in insect segmentation.

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