Breakdown of abdominal patterning in the *Tribolium Krüppel* mutant *jaws*

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**Summary**

During *Drosophila* segmentation, gap genes function as short-range gradients that determine the boundaries of pair-rule stripes. A classical example is *Drosophila Krüppel (Dm’Kr)* which is expressed in the middle of the syncytial blastoderm embryo. Patterning defects in *Dm’Kr* mutants are centred symmetrically around its bell-shaped expression profile. We have analysed the role of *Krüppel* in the short-germ beetle *Tribolium castaneum* where the pair-rule stripes corresponding to the 10 abdominal segments arise during growth stages subsequent to the blastoderm. We show that the previously described mutation *jaws* is an amorphic *Tc’Kr* allele. Pair-rule gene expression in the blastoderm is affected neither in the amorphic mutant nor in *Tc’Kr* RNAi embryos. Only during subsequent growth of the germ band does pair-rule patterning become disrupted. However, only segments arising posterior to the *Tc’Kr* expression domain are affected, i.e. the deletion profile is asymmetric relative to the expression domain. Moreover, stripe formation does not recover in posterior abdominal segments, i.e. the *Tc’Kr* phenotype does not constitute a gap in segment formation but results from a breakdown of segmentation past the 5th *eve* stripe. Alteration of pair-rule gene expression in *Tc’Kr* mutants does not suggest a direct role of *Tc’Kr* in defining specific stripe boundaries as in *Drosophila*. Together, these findings show that the segmentation function of *Krüppel* in this short-germ insect is fundamentally different from its role in the long-germ embryo of *Drosophila*. The role of *Tc’Kr* in Hox gene regulation, however, is in better accordance to the *Drosophila* paradigm.

Key words: *Krüppel*, Giant, Even-skipped, *Dfd, Scr, Antp, Ubx*, Short germ, Long germ, segmentation, Gap gene, Abdomen, *jaws*, *Tribolium castaneum*, *Drosophila*, Evolution, Parental RNAi

**Introduction**

Anteroposterior patterning in *Drosophila* is controlled by regulatory elements that measure the local concentrations of transcription factors and convert them into new expression profiles. In several steps, this machinery translates flat initial gradients spanning most of the egg length into expression domains of increasing detail and precision. At least for the formation of interacting gradients at the maternal and gap gene domains of increasing detail and precision. At least for the maternal and gap gene levels, transcription factor diffusion is thought to be essential, which suggests that the *Drosophila* segmentation machinery can work only in a system unimpeded by cell walls, i.e. in a syncytial blastoderm.

How is anteroposterior patterning accomplished in fully cellularized organisms? Somitogenesis in vertebrates has been shown to rely on temporal regulation for the generation of repeating units along the anteroposterior axis, based on a segmentation clock involving components of the *Notch* signalling pathway (Pourquié, 2001). A segmentation clock involving the *Notch* system appears to function in basal arthropods, i.e. spiders (Schoppmeier and Damen, 2005; Stollewerck et al., 2003) and a clock mechanism may function in centipedes as well (Chipman et al., 2004). Also in these taxa, as in many insects including *Tribolium*, the majority of segments arise by posterior addition of cells to a growing germ band, similar to vertebrate embryos. In contrast to vertebrates, many orthologs of *Drosophila* pair-rule and segment-polarity genes are expressed in stripes also in these short-germ arthropods (Chipman et al., 2004; Damen et al., 2000; Patel et al., 1994; Sommer and Tautz, 1993). It has been suggested, therefore, that the segmentation clock is an ancient mechanism to pattern posteriorly growing embryos, and that pair-rule and segmentation genes originally served to transmit the primary clock signal to the growing and differentiating segments (Tautz, 2004). In the evolutionary line leading to *Drosophila*, the regulation of stripe genes then may have come under the control of spatial regulation provided by those genes that, in *Drosophila*, represent the upper levels of the segmentation hierarchy, i.e. gap genes and maternal genes (Peel and Akam, 2003).

In the short-germ beetle *Tribolium*, the embryo elongates by
posterior growth similar to spider and myriapod embryos. However, the Notch pathway appears not to be involved in anteroposterior patterning in this insect (Tautz, 2004). Pair-rule genes are expressed and function in double-segmental units in Tribolium (Brown and Denell, 1996; Maderspacher et al., 1998), and an analysis of the Tc’hairy regulatory region provided evidence for stripe-specific regulation (Eckert et al., 2004). In addition to pair-rule genes, homologues of gap genes are also expressed during germ-band growth in Tribolium, and in other short-germ insects (Bucher and Klingler, 2004; Liu and Kaufman, 2004a; Liu and Kaufman, 2004b; Mito et al., 2005; Patel et al., 2001; Schröder et al., 2000; Sommer and Tautz, 1993; Wolff et al., 1995). Functional studies using RNAi in these species have led to diverse interpretations of how similar the role of these short germ gap genes are compared with Drosophila gap genes.

One problem with RNAi studies is that the true null phenotype of the genes investigated remains unknown. Unlike many other evo-devo systems, in Tribolium, developmental genes can be identified and analysed through the isolation of embryonic lethal mutants. Albeit more laborious, the mutagenesis approach has the potential of providing more defined, and less variable, lack of function situations. In addition, this classical genetics approach allows us to identify short-germ-specific genes that have been lost in long-germ dipteran species, the sequence of which evolves very fast, or which in Drosophila are not involved in segmentation. Screens for embryonic lethal genes identified several putative gap and pair-rule mutations (Maderspacher et al., 1998; Sulston and Anderson, 1996). Most of these phenotypes differ substantially from those of known Drosophila mutants. In order to determine if any of the segmentation genes already molecularly identified in Tribolium is affected in one of these mutants, we tested putative gap gene mutations for linkage to gap gene orthologues.

In this paper, we identify the previously identified Tribolium mutant jaws (Sulston and Anderson, 1996) as an amorphic Krüppel mutant, and provide the first detailed analysis of a gap gene null phenotype in a short-germ embryo. This amorphic Krüppel mutant, and provide the first detailed analysis of a gap gene null phenotype, as well as weaker phenotypes generated by RNAi, clearly differ from those of Drosophila Krüppel (Dm’Kr) mutations (Wieschaus et al., 1984), suggesting a principally different role for this gap gene orthologue in the short-germ embryo of Tribolium.

Materials and methods
Cloning, RACE and sequence analysis
The Tc’Kr-coding region was initially amplified applying 5’ and 3’ RACE (Gene-Racer, Invitrogen). RACE primers were designed using the Tc’Kr zinc-finger fragment available (GenBank Accession Number L01616) (Sommer and Tautz, 1992), using 5’ primer GGCCACCTGGACGAACTGC and 3’ primer GCAGTTGTCCCA-GGTGCC. To complete the sequence, additional RACE reactions were performed using 5’RACE primer CAGCCGCATGGGTGACTTGAGGTTGAGGT and 3’RACE primers GTTGAAGTTGATAGCGAGTTCCTCC and TTGGCGGACGCGAGATAGGGGCC. The presumed ATG is at position 147-149 of the cDNA. One intron between position 182 and 183 of the cDNA separates amino acids 11 and 12. In the Tribolium genome sequence (as of March 2005), the Tc’Kr cds is covered by the contigs 6872 (bp 1-180) and 5054 (bp 181-1259), whereas the 3’ untranslated region is contained in contigs 5054 and 1669. The Accession Number for the Tc’ Kr cDNA is AF236856. The predicted peptide sequence is given in Fig. S1 in the supplementary material.

Mapping of jaws relative to Tc’Kr
Sequence polymorphisms in candidate genes were identified by amplifying and sequencing non-coding fragments (5’ UTR, 3’ UTR or intronic DNA) from adult beetles of GA-1, SB and Tiw-1 wild-type strains. Identified sequence polymorphisms could either be scored directly as PCR fragments on an agarose gel or were converted into RLFPs. For Tc’Kr, a polymorphism in the 3’UTR was identified. This polymorphism was amplified as a 205 bp fragment by primer sequences ACGACTTGGCGTTAAATG and TACGAAAGTGG-CACACAAC. In Tiw-1, but not in SB, this fragment is cleaved by AseI into subfragments of 141 and 64 bp (Fig. 2) that were visualized on a 2.5% NuSieve Agarose gel (Cambrex Bio Science). For mapping, DNA was isolated from single beetles that had been identified as mutant carriers by scoring the offspring from single matings for presence of mutant larvae. Detailed protocols concerning our general mapping strategy, and DNA extraction from beetles and larvae can be provided on request.

Parental RNAi
Parental RNAi was performed as described (Bucher et al., 2002). As template for in vitro transcription, PCR-products with T7 sequences at both ends were amplified from cDNA plasmids or genomic Tribolium DNA. For injection, dsRNA was used at a concentration of 1-4 μg/μl.

Harvest of mutant jaws embryos
In order to obtain jaws mutant embryos in large numbers, offspring from 40 identified jaws/+ parents were sexed as pupae, and virgin females were crossed to their fathers. One-sixth of the eggs produced by this father/daughter population will be homozygous for the mutants. Similarly, to obtain the Tc’gt/jaws ‘double mutant’ phenotype, Tc’gt dsRNA was injected into the same offspring pupae and eclosed females then were crossed to identified jaws carrier males.

Confocal images
First instar larvae were cleared in lactic acid/10% ethanol overnight at 60°C. After washing with lactic acid, cuticles were mounted on a slide under a cover-slip that was supported with rubber gum. This allowed manual positioning to a ventral-up orientation. Cuticular autofluorescence in the 520 to 660 nm range was detected on a Leica confocal microscope by excitation at 488 nm and maximum projection images were generated from image stacks.

Expression analysis
Single (Tautz and Pfeifle, 1989) and double label (Prpic et al., 2001) whole-mount in situ hybridisations were carried out as described. Tc’Kr-RNAi germ-band stage embryos are particularly fragile and were manually devitellinized on double sticky tape: 12- to 18-hour-old embryos were transferred to ethanol and then gently attached to a double-sided sticky tape. After replacing ethanol with water, the vitelline membrane tightly adheres to the tape and embryos can be manually devitellinized using diminutive insect needles. In order to avoid RNA degradation, devitellinized embryos were promptly transferred to methanol and stored at –20°C.

Results
Reanalysis of Tc’Kr expression
A fragment from the Tc’Kr-coding sequence had been identified previously and used for expression analysis (Sommmer and Tautz, 1993). We extended the molecular analysis of Tc’Kr in order to identify non-coding sequences carrying
polymorphisms useful for mapping, and to obtain a complete cDNA suitable for more comprehensive RNAi knock down as well as more sensitive in situ hybridization (see Materials and methods).

While Dm’Kr is expressed in the centre of the blastoderm embryo, in the Tribolium blastoderm this domain appears at the posterior pole (Fig. 1A). Relative to the segment primordia, however, this position is roughly conserved, as Tribolium is a short-germ embryo (Sømmer and Tautz, 1993). We used Tc’even-skipped (Tc’eve) as an additional marker to map the position of the gap domain precisely (Fig. 1B-E). During germ rudiment formation, Tc’Kr remains expressed in a broad central domain. In early germ band stages (Fig. 1B,C), the anterior border of Tc’Kr lies within the 2nd stripe of Tc’eve (‘eve2’). When eve2 splits into segmental stripes, eve2a and eve2b [corresponding to labial and first thoracic segments (Patel et al., 1994)], the Tc’Kr domain abuts the posterior border of eve2a (1D). At this time, Tc’Kr also fades from the growth zone and a posterior border forms just anterior to the eve4 stripe as it arises near the growth zone (Fig. 1C,D). As the segmental stripes eve3a and eve3b form, the posterior boundary of the Tc’Kr gap domain coincides with eve3b (Fig. 1E). Accordingly, in germ band embryos the Tc’Kr gap domain overlaps very precisely the three thoracic segments – which is more anterior than in Drosophila, where the Tc’Kr domain is centered over the primordia of segments T2 to A2 (Myasnikova et al., 2001).

During later stages of development, the gap-domain of Tc’Kr disappears. A second phase of rather homogenous expression emerges in all segments, excluding recently formed segments close to the growth zone (Fig. 1F,G). This signal later intensifies in the appendages and extends to anterior and posterior gut primordia. Additionally, a dynamically changing pattern of Tc’Kr expression is observed in the head region. These late expression aspects probably relate to possible functions during mesoderm development, gut development and neurogenesis as described for other Krüppel orthologues (Gaul et al., 1987; Liu and Kaufman, 2004a).

**jaws is closely linked to the Tc’Kr locus**

The jaws mutation was originally induced in a GA-1 background (Sulston and Anderson, 1996). Preliminary experiments suggested that this mutation had been induced in a chromosome carrying a RFLP polymorphism in the Tc’Kr gene (‘Tiw-1 specific polymorphism’) that differs from the corresponding sequence in the SB wild-type strain (‘SB specific polymorphism’). In order to test for close linkage between jaws and Tc’Kr, we made use of the fact that a jaws mutant strain had been kept in our laboratory by recurrent outcrossing to SB females for over six generations [for stock-keeping of embryonic lethal mutations see Berghammer et al. (Berghammer et al., 1999)]. Therefore, in our stock collection, most of the genome in the jaws strain must have been replaced by SB-specific alleles. Only loci very close to jaws are likely to still be represented by GA-1-specific alleles, because presence of the jaws mutant had been selected for in every generation. When we scored 80 adult beetles from our stock collection that carried one copy of the jaws mutation, we found that every one of these animals was heterozygous for both polymorphisms at the Tc’Kr locus (Fig. 2A). This shows very close linkage between the jaws mutation and the Tc’Kr gene and suggested that jaws is a mutation in the Tc’Kr gene. As a control, we also tested 20 of these animals for polymorphisms in the Tc’eve gene and found, as expected for a locus not linked to jaws, that they all were homozygous for a SB-specific Tc’eve polymorphism.

**The first zinc finger of Tc’Kr is altered in jaws**

To confirm the identity of the jaws and Tc’Kr loci, we isolated genomic DNA from homozygous jaws-mutant larvae and PCR-amplified three fragments from the Tc’Kr locus that cover both exons. Sequence comparison with control amplificates from the SB and GA-1 strains revealed an amino acid replacement in the Tc’Kr-coding sequence of mutant animals. This transition changes the second histidine of the first zinc finger to a tyrosine (Fig. 2B,C). As the Cys-Cys-His-His Zn-finger motive is essential for the correct structure of the DNA-binding domain, a missense mutation in such a key amino acid is likely to inactivate the Tc’Kr gene. In this respect, the jaws mutation, now to be termed Tc’Kr<sup>jaws</sup>, is similar to an amorphic Krüppel mutation identified in Drosophila: the Dm’Kr<sup>j</sup> allele, one of the crucial Zn-finger cysteines is converted to serine, completely abolishing Dm’Kr function (Redemann et
Below, we provide additional evidence that \textit{Tc'Kr}^{jaws} indeed does fully inactivate the \textit{Tc'Kr} locus.

**Phenotypic series caused by \textit{Tc'Kr} loss or depletion**

The identification of \textit{jaws} as a \textit{Tc'Kr} allele is also supported by RNAi evidence. Injecting dsRNA representing \textit{Tc'Kr} cDNA or genomic sequences (see Materials and methods) resulted in various homeotic and segmentation phenotypes (Fig. 3C-E) which – in some injection experiments – included phenotypes very similar or identical to \textit{Tc'Kr}^{jaws}.

In \textit{Tc'Kr}^{jaws} embryos (Fig. 3F), the head is differentiated as in wild type. The next four segments (thoracic and 1st abdominal) develop gnathal structures such that the regular maxillary (mx) and labial (lb) segments are followed by two additional pairs of maxillary and labial segments. Including the normally developed mandible (md), this results in a total of seven gnathal segments (md-mx-lb-mx-lb-mx-lb). Posteriorly, these gnathal segments are followed by one segment of abdominal morphology, and the posterior end of the embryo is formed by terminal structures similar to wild type, including urogomphi and pygopodes, the derivates of the 9th and 10th abdominal segments. Hence, the total of gnathal, thoracic and abdominal segments in \textit{Tc'Kr}^{jaws} embryos is 10 compared with 16 in wild type, i.e. six segments are deleted, while four segments are homeotically transformed (Sulston and Anderson, 1996). This phenotype differs from that of strong \textit{Dm'Kr} mutants where the thoracic and the first four abdominal segments are deleted and no homeotic transformations are evident in differentiated mutant larvae. The ectopic maxillary structures of \textit{Tc'Kr}^{jaws} mutant embryos deviate somewhat from normal maxillae in that they lack endites (the mala) and sometimes possess distal claws rather than the sensory structures characteristic of maxillary palps (this is especially the case for the most posterior pair of maxillae). In addition, the ectopic labia (as well as the endogenous labium) are abnormal in that they usually do not fuse ventrally. Weaker phenotypes obtained by RNAi support the interpretation that these imperfect gnathal segments in fact are of mixed gnathal and thoracic character (Fig. 3D,E).

In intermediate strength and weak RNAi phenotypes (Fig. 3C,D), more abdominal segments remain and the transformation of thoracic segments towards gnathal fate is less...
pronounced. Frequently, the first and third thoracic segments still differentiate legs in embryos whose second thoracic segment already is transformed into labium. This indicates that higher levels of Tc'Kr activity are required for inhibiting labial fate than for repressing maxillary fates. In addition, the additionally present abdominal segments in these embryos usually display homeotic transformations towards a more anterior, i.e. thoracic or gnathal, fate (Fig. 3D). In these abdominal segments, there is also a tendency for alternating maxillary and labial fates, and small irregular appendages can sometimes be observed. In conclusion, the weak Tc'Kr RNAi phenotypes also differ significantly from those of weak Dm'Kr mutants, displaying additional homeotic transformations of abdominal segments towards more anterior fates.

**Is Tc'Krjaws a null-allele?**

The sequence alteration in Tc'Krjaws is no definite proof that Tc'Kr activity is entirely abolished in mutant embryos. In *Drosophila*, a mutation is regarded as ‘amorphic’ if the mutant allele (mut) in trans over a deficiency (Df) for the locus displays the same phenotype as in the homozygous condition. The rationale behind this test is that if some gene activity remains in the mutation, then mut/Df embryos would possess only half as much activity for the gene in question than mut/mut embryos, and therefore should display a discernibly stronger phenotype (Mueller, 1932). No deletion for the Tc'Kr locus is available in *Tribolium*. However, by combining the mutation with RNAi knockdown allows for a similar test: if the phenotype of larvae homozygous for Tc'Krjaws is the same as that of larvae with additional RNAi induced depletion of the mutant Tc'Kr transcript, then we can conclude that Tc'Krjaws represents the strongest possible loss-of-function phenotype. We performed Tc'Kr RNAi knock-down in a Tc'Krjaws-mutant background using a moderate concentration of dsRNA such that we could distinguish mutant and RNAi embryos. Beside intermediate strength Tc'Kr RNAi phenocopies, this experiment also yielded Tc'Krjaws larvae. These homozygously mutant larvae did not show a stronger phenotype than Tc'Krjaws larvae (Fig. 3F; Fig. 4A). From this experiment, we conclude that Tc'Krjaws is an amorphic Tc'Kr allele that is functionally equivalent to a null mutation. In order to understand the homeotic and segmentation phenotypes of this mutant, we analysed the expression of potential target genes in Tc'Krjaws.

**The homeotic effect of Tc'Krjaws is epistatic over that of Tc'gt RNAi**

Interestingly, RNAi knock-down of the Tc'giant gene (Tc'gt) leads to a homeotic phenotype opposite to that caused by Tc'Kr inactivation. In Tc'gt RNAi embryos, the maxillary and labial segments are transformed towards thoracic identity (Bucher and Klingler, 2004) (see also Fig. 4C). We wondered which of these transformations would prevail in a ‘double-mutant’ situation. To this end, we performed Tc'gt RNAi knock-down in a Tc'Krjaws mutant background (see Materials and methods). In this experiment, we obtained Tc'gt knock-down phenotypes in the majority of embryos while a fraction corresponding to Tc'Krjaws homozygous animals showed a phenotype very similar to that of Tc'Krjaws alone (Fig. 4B). They differed only from the normal Tc'Krjaws phenotype in that they lacked one or two additional segments. This is to be expected, because in Tc'gt RNAi embryos, thoracic and abdominal segments can be deleted that are not affected in Tc'Krjaws, i.e. the segmentation phenotypes of these experimental larvae corresponds to a superposition of Tc'gt RNAi and Tc'Krjaws. However, the homeotic transformations caused by Tc'Krjaws are clearly epistatic over those produced by Tc'gt RNAi knock-down. This suggests that the homeotic transformation of gnathal segments into thorax in Tc'gt RNAi embryos is an indirect effect (see Discussion).

**Expression of homeotic genes in Tc'Krjaws and Tc'gt RNAi embryos**

The striking homeotic transformations in Tc'Krjaws larvae could either be due to misregulation of homeotic genes, or could indicate a direct role of Tc'Kr in specifying segmental fates. Previous work has already shown that the Hox gene *proboscipedia* (Tc'pb) is ectopically expressed in Tc'Krjaws mutant embryos (Sulston and Anderson, 1998). However, Tc'pb becomes active relatively late during development, and only in the maxillary and labial palps, not in complete segments. Thus, we asked how the expression of Hox genes early active in the maxillary, labial and thoracic segments would relate to the Tc'Krjaws phenotype.

The Deformed (Tc'Dfd) gene is expressed in the mandibular and maxillary segments (Brown et al., 1999). In Tc'Krjaws embryos, two strong and one weak additional Tc'Dfd domains
are observed that are separated from each other by gaps approximately one segment wide (Fig. 5A-D). The two strongly expressing ectopic domains correspond to the first and third thoracic segments that, in Tc'Kr<sup>Δw</sup> mutant larvae, develop maxillary characteristics. The Sex combs reduced (Tc'Scr) gene is active in the ectoderm of the second parasegment in *Tribolium* (Curtis et al., 2001), which largely corresponds to the labial segment (Fig. 5E-G; Tc'Scr expression is also present in the mesoderm of additional segments). In Tc'Kr<sup>Δw</sup> embryos (Fig. 5H-J), ectopic activity of Tc'Scr is present in the primordia that correspond to the second thoracic and first abdominal segments of wild-type animals, i.e. in those segments that differentiate labial palps in mutant larvae. Therefore, the gnathal Hox genes Tc'Dfd and Tc'Scr are active in complementary double-segmental frames in Tc'Kr<sup>Δw</sup> mutant embryos, which is consistent with the phenotype of differentiated mutant larvae.

Concomitant with the expanded expression of gnathal Hox genes, the Tc'Ubx gene, the anterior expression boundary of which lies in the thorax, is shifted posteriorly in Tc'Kr<sup>Δw</sup> mutants (Fig. 5C,D). This may explain, at least in part, why in weak Tc'Kr RNAi phenocopies anterior abdominal segments are transformed towards thorax (Lewis et al., 2000). The anterior boundary of Tc'Antp, however, is similar as in wild type (Fig. 5H-J). This is consistent with our interpretation that the ectopic maxillary structures in Tc'Kr<sup>Δw</sup> are incompletely transformed and retain some thoracic characteristics. We also investigated the expression of Tc'Scr and Tc'Antp in Tc'gt RNAi embryos (Fig. 5K,L) as they display homeotic transformations opposite to those in Tc'Kr<sup>Δw</sup> mutants. Indeed we find that Tc'Antp expands towards anterior by two segments whereas Tc'Scr expression is largely abolished in these embryos (which lack maxillary and labial differentiation).

Together, these data show that the Tc'Kr<sup>Δw</sup> homeotic phenotype can be explained by defective Hox gene regulation, and they suggest inhibition of Tc'Dfd and Tc'Scr by Tc'Kr, whereas Tc'Ubx positively depends on Kr activity. In addition, the double-segmental appearance of ectopic gnathal Hox expression domains suggests that the Hox genes Tc'Dfd and Tc'Scr also are under strict pair-rule control.

**Function of Tc'Kr in regulating segmentation genes**

Previous work has already revealed that the pattern of the segment-polarity gene engrailed (Tc'en) and the pair-rule genes Tc'eve and Tc'runt are altered in Tc'Kr<sup>Δw</sup> (Sulston and Anderson, 1998). We repeated and extended this work in order to relate the defects observed with what we now know about the spatial expression of the gene that is inactivated in this mutant.

We first attempted to identify which stripes of Tc'eve exactly are affected by the Tc'Kr<sup>Δw</sup> mutation. To distinguish pair-rule stripes arising in the growing germ band, we performed double staining with segment polarity genes, and to identify Tc'Kr<sup>Δw</sup> mutant embryos at stages before morphological differences to wild types become evident, Tc'giant was included as an additional marker in some experiments (in Tc'Kr<sup>Δw</sup> embryos, the posterior domain of Tc'gt is absent, while an additional stripe of expression appears; A.C., unpublished). We find that

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**Fig. 5.** Hox gene expression in wild-type, Tc'Kr<sup>Δw</sup> and Tc'gt RNAi embryos. (A-D) Tc'Dfd (purple) and Tc'Ubx (blue) in situ double staining in stage-matched wild-type (A,B) and Tc'Kr<sup>Δw</sup> mutant (C,D) embryos. In the mutant (D), the anterior boundary of the Tc'Ubx domain recedes towards the posterior. Tc'Dfd is expressed in three strong and one weak domain of double segmental periodicity. The two ectopic domains with stronger expression correspond to the ectopic maxillary structures in Tc'Kr<sup>Δw</sup> (E,J) Tc'Scr (purple) and Tc'Antp (blue) staining of wild-type (E-G) and Tc'Kr<sup>Δw</sup> mutant (H-J) embryos. Tc'Scr is also strongly expressed in two ectopic domains with double-segmental periodicity, corresponding to the two ectopic labial segments (J). In contrast to Tc'Ubx, Tc'Antp expression is not shifted in Tc'Kr<sup>Δw</sup> germ bands (H-J). (K,L) Tc'Scr (brown) and Tc'Antp (blue) in Tc'gt RNAi embryos. Tc'Antp expands towards anterior by two segments, which correlates with the fact that the maxillary and labial segments attain thoracic appearance in Tc'gt RNAi embryos. High-level expression of Tc'Scr in the labial segment is repressed in these embryos. Weak Tc'Scr expression in the maxillary segment of older embryos (L) probably corresponds to the weak expression seen in the prothoracic segment in wild type (G).
the first three Tc’eve stripes arise and split into segmental stripes in Tc’Krjaws mutant embryos exactly as in wild type (Fig. 6A-C, E-H). In addition, a stripe of eve4 is formed in the growth zone as a distinct band with sharp boundaries. Although this stripe arises just posterior to the Tc’Kr domain, Tc’Kr apparently has no role in defining its anterior boundary. However, segmentation defects become evident at subsequent stages: while eve4 does split into segmental stripes 4a and 4b, these segmental stripes (particularly eve4b) appear somewhat irregular. The anterior boundary of eve5 also forms perfectly in Tc’Krjaws (Fig. 6G, H), very similar to wild type. However, this stripe never progresses into segmental stripes 5a and 5b (Fig. 7A-C); instead, its expression becomes irregular in shape and then decreases in strength and fades away (Fig. 7G-I). The defects of Tc’eve patterning observed in Tc’Krjaws differ strongly from the situation in Drosophila gap gene mutants, where negative regulation of stripe-specific elements results in widened stripes.

We did not observe re-establishment of Tc’eve stripes at later stages, i.e., posterior of a gap-like deletion zone. At the time when eve7 and eve8 form in wild-type embryos (Fig. 7D-F), Tc’eve expression in Tc’Krjaws already has ceased (Fig. 7J-L). The pattern of the segmental marker Tc’wg confirms that the gnathal and thoracic segments form normally in the Tc’Krjaws mutant (Fig. 7G-L) (Sulston and Anderson, 1996). In contrast to this earlier analysis, however, in the pattern of a segment-polarity gene we also find no evidence for re-establishment of stripe formation. Using the dynamic Tc’wg head expression as marker for developmental time, we find that after six or seven normally formed gnathal and thoracic Tc’wg stripes, the pattern becomes irregular in Tc’Krjaws embryos. Several more posterior Tc’wg stripes arise but are fragmentary, weakly expressed or only present on one side of the embryo (Fig. 7I-K). As with Tc’eve stripes, no additional stripes re-emerge at later stages in Tc’Krjaws. Instead, the initially irregular and fragmentary stripes reorganize themselves later on into a more orderly pattern, such that older embryos can display a very regular pattern of typically 10 gnathal, thoracic and abdominal Tc’wg stripes, corresponding to the number of segments differentiated in mutant larvae (Fig. 7L). Such pattern repair phenomena also are observed in other Tribolium segmentation mutants and RNAi embryos (Bucher and Klingler, 2004; Maderspacher et al., 1998).

### Discussion

Our description of Tc’Kr phenotypes represents the first definite functional analysis of an insect gap gene orthologue outside the diptera. This was possible by combining the complementary advantages of RNAi and a chemically induced mutation (Sulston and Anderson, 1996) that appears to represent a null situation given that its phenotype is not further enhanced by parental RNAi (Fig. 4A).

### Regulation of homeotic genes by Tc’Kr

The most obvious difference between the phenotypes of Krüppel in Tribolium and Drosophila are the homeotic transformations in Tc’Krjaws and Tc’Kr RNAi larvae that are not evident in Dm’Kr mutants. Such transformations are not entirely unexpected given that in Drosophila the expression boundaries of Hox genes are also set by gap genes, including Dm’Kr. However, in Drosophila gap mutants all segments that would be transformed because of misregulation of homeotic genes usually also suffer segmentation defects and fail to develop. By contrast, Tribolium segment primordia anterior of, and within, the Krüppel expression domain do differentiate, such that homeotic transformations can manifest themselves in the differentiated larva.
The expression of homeotic genes in Tc'Kr\textsuperscript{jaws} embryos is consistent with the morphological transformations observed (Fig. 3F; Fig. 5). Our results with Tc'Dfd, Tc'Scr, Tc'Antp and Tc'Ubx confirm and extend earlier findings for Tc'pb and Tc'UBX/Tc'ABD-A expression (Sulston and Anderson, 1998). Notably, the complementary double-segmental expression of Dfd and Scr in Tc'Kr\textsuperscript{jaws} embryos explains the phenotype of alternating maxillary and labial segments. As summarized in Fig. 8, these expression patterns indicate that the posterior limit of Tc'Dfd and Tc'Scr domains is set through inhibition by Tc'Kr. In this respect, Tc'Kr fulfills a function similar to Drosophila gap genes.

The homeotic phenotype of Tc'gt RNAi embryos (Bucher and Klingler, 2004) could suggest a similar function in Hox regulation for Tc'gt. Indeed we find Tc'Antp anteriorly expanded and gnathal Hox genes (Tc'Scr) repressed in Tc'gt RNAi embryos, consistent with the expansion of thoracic fates found in differentiated Tc'gt RNAi larvae. These transformations are just opposite to those of Tc'Kr\textsuperscript{jaws} larvae. Interestingly, in embryos that lack Tc'Kr and at the same time have reduced Tc'gt activity, the homeotic effect of Tc'Kr\textsuperscript{jaws} clearly is epistatic (Fig. 4B). This shows that the ectopic Tc'gt stripes in the Tc'Kr mutant do not contribute to the Tc'Kr phenotype. However, this experiment suggests that the homeotic transformation of gnathal segments into thorax in Tc'gt RNAi embryos is indeed an indirect effect and comes about through misregulation of Tc'Kr in these embryos. This interpretation is supported by our finding that the Tc'Kr expression domain expands anteriorly in Tc'gt RNAi embryos (A.C.C. and M.K., unpublished). Evidently, it is expansion of Tc'Kr that results in repression of gnathal Hox genes in maxilla and labium of Tc'gt RNAi embryos, not loss of gnathal Hox gene activation. Similarly, expansion of Tc'Antp in Tc'gt RNAi larvae could be due to activation by anteriorly expanded Tc'Kr. However, as Antp is not significantly reduced in Tc'Kr\textsuperscript{jaws}, it seems more likely that Tc'gt acts directly to define the anterior boundary of the Tc'Antp domain (stippled arrow in Fig. 8).

In addition to gap gene input, Drosophila Hox genes also receive input from pair-rule genes. The near-pair-rule pattern of Tc'Dfd and Tc'Scr in Tc'Kr\textsuperscript{jaws} embryos reveals an important role of pair-rule genes also in defining Tribolium Hox domain boundaries. It seems likely that regulation of Tc'Dfd and Tc'Scr by pair-rule genes is responsible for the precision of their expression boundaries in wild-type Tribolium embryos, while input from gap genes defines the broad region where a particular Hox gene can become active (Fig. 8).

**Tc'Kr does not function as a canonical gap gene during segmentation**

In Drosophila, Krüppel is expressed in a bell-shaped profile centered over the primordia of segments T2 to A3 (Gaul and Jäckle, 1987; Myasnikova et al., 2001). In the Tribolium blastoderm, only one such gradient is present as the Tc'Kr domain covers the posterior pole (Sommer and Tautz, 1993). When the germ rudiment has formed, the Tc'Kr domain retracts from the posterior end and forms a distinct domain overlapping the three thoracic segment primordia (Fig. 1). At this stage, therefore, the Tc'Kr domain covers more anterior segment primordia (and more anterior pair-rule stripes) than does its Drosophila counterpart.

Both boundaries of the Dm'Kr expression domain

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**Fig. 7.** Segment formation in Tc'Kr\textsuperscript{jaws} is not re-established during later stages of development. Embryos are doubly labelled for Tc'eve (brown) and Tc'wg RNA (blue). Numbers relate to canonical Tc'wg stripes, i.e. starting with the mandibular segment. (A-F) Wild-type embryos of increasing developmental age, stage-matched to Tc'Kr\textsuperscript{jaws} embryos (G-L; staging is based on the dynamic Tc'wg head expression). Compared with wild type (A-C), Tc'eve expression becomes irregular and prematurely fades in Tc'Kr\textsuperscript{jaws} embryos (G-I). Initially, only the seven anteriormost wg stripes (md to A1) are properly formed in Tc'Kr\textsuperscript{jaws}. Posterior to A1, several irregular or fragmentary wg stripes form (G-K), which then reorganize such that in more mature embryos (L) normal Tc'wg stripes 8-10 are frequently observed, consistent with the 10 differentiated segments in Tc'Kr\textsuperscript{jaws} larvae. No new Tc'eve or Tc'wg activity is evident at later stages, when in wild-type embryos the last Tc'wg stripes form (15 and 16 in our notation, which correspond to abdominal segments A9 and A10) (D-F). A non-segmental Tc'wg domain is present in the growth zone throughout development. This domain represents terminal fates and eventually becomes part of the proctodeum.
Development probably set by posterior border of the becomes free of Tc'Kr reach of its blastoderm expression domain. The first four stripes of Tc'hairy through activation, whereas the anterior border of periodicity. Tc'Kr expands of the region where the gap domain normally resides. No such gap mutants the corresponding pair-rule stripes expand towards in for pair-rule genes. In stripes. We argue that also the posterior boundary of the syncytial blastoderm. However, although this anterior border of Krüppel in turn acts as general posterior repressor for domain delimits the anterior giant gene domain, the abdominal domain of Krüppel in abdominal patterning (Maderspacher et al., 1998). In contrast to the earlier report, we interpret the progression of the en/wg pattern in Tc'Krmutant embryos as reflecting a breakdown of segmentation, not a temporal gap in the sequence of abdominal segment additions. While the 9th and 10th abdominal segments usually are present in Tc'Krmutant and Tc'Kr RNAi larvae and give rise to urogomphi and pygopods, we conclude from the time series in Fig. 7 that these structures actually derive from the fragmentary stripes formed immediately after the anterior seven unaffected stripes have been generated. This implies that the remnants of middle-abdominal segments later on differentiate as posterior abdominal segments in Tc'Krmutant mutant embryos. To explain the specification of earlier formed segments as A9 and A10, we speculate that after completion of germ band growth, a signal emanates from the posterior terminalia and instructs the next two segments to fuse with the telson and to form urogomphi and pygopods. In addition, non-segmental terminal structures are present in Tc'Krmutant embryos. These primordia are known to arise early in the blastoderm, posterior of the growth zone proper (reviewed by Anderson, 1972). One marker for terminal structures is the posterior terminal domain of Tc'wg (Nagy and Carroll, 1994), which is formed and maintained in Tc'Krmutant embryos similar to wild type (Fig. 7). In addition, the cuticle lining of the hindgut is present in mutant larvae (e.g. Fig. 4A).

The role of Krüppel in short germ insects

As the growth zone is a patterning environment very different from the syncytial blastoderm, it was expected that segmentation genes in short germ embryos would play similar roles as in Drosophila during early stages, while abdominal segmentation was predicted to be fundamentally different. It is surprising that knock-down of several short germ gap gene homologues, i.e. Tc’gt (Bucher and Klingler, 2004), Tc’Kr, Gb’hb (Mito et al., 2005) and Of’hb (Liu and Kaufman, 2004a), results mainly in homeotic transformations in those segments that form during the blastoderm. This also pertains to Tc’hb (Schröder, 2003), where homeotic transformations occur in addition to segmentation defects (A.C. and R.S. unpublished). That so many of these gap gene homologues do not seem to have strong roles in the formation of anterior segments raises the possibility that the original role of gap genes early during arthropod evolution may have been to boundaries of abdominal pair-rule stripes were directly specified by Tc’Kr. Because at least one posterior segmentation gene domain, the abdominal domain of Tc’hb, does expand anteriorly in Tc’Krmutant (A.C.C. and M.K., unpublished), the lack of expanding pair-rule stripes is probably not due to the particular situation of the growing germ band but indeed reflects a genuine difference in the way that pair-rule stripes depend on Krüppel function in Tribolium versus Drosophila.

Compared with the classical gap phenotype of Dm’Kr mutants, the segmental defects in Tc’Krmutant are shifted towards posterior. Based on its larval phenotype, Tc’Krmutant has been described as a gap gene, in that most abdominal segments are deleted while gnathal and thoracic segments, as well as the most posterior abdominal segments (A9 and A10), remain intact (Sulston and Anderson, 1996). However, when analysing pair-rule and segment-polarity expression, we did not observe resumption of stripe formation posterior of a defect zone (Figs 6, 7) as is observed for the mutation krusty, for example (Maderspacher et al., 1998). In contrast to the earlier report, we interpret the progression of the en/wg pattern in Tc’Krmutant embryos as reflecting a breakdown of segmentation, not a temporal gap in the sequence of abdominal segment additions.

Fig. 8. Regulation of Hox genes by Krüppel in Tribolium. The repressor activity of the anterior giant domain delimits the anterior border of Tc’Kr. Tc’Kr in turn acts as general posterior repressor for Tc’Dfd and Tc’Sscr. The precise boundaries of these gnathal Hox genes are defined by pair-rule genes. Therefore, ectopic gnathal Hox gene expression in Tc’Krmutant is interrupted with double-segmental periodicity. Tc’Kr also determines the anterior border of Tc’Ubx through activation, whereas the anterior border of Tc’Antp is probably set by Tc’gt directly.

have been shown to serve as short-range gradients that provide positional information to define the margins of pair-rule stripes (Klingler et al., 1996; Langeland et al., 1994; Small et al., 1991). A similar function should have been expected at least for the anterior boundary of Tc’Kr, which already forms during the syncytial blastoderm. However, although this anterior boundary evidently is used for limiting gnathal Hox gene function, its larval phenotype, Tc’hairy and Tc’runt, do not seem to have strong roles in the formation of anterior segments as A9 and A10, we speculate that after completion of germ band growth, a signal emanates from the posterior terminalia and instructs the next two segments to fuse with the telson and to form urogomphi and pygopods. In addition, non-segmental terminal structures are present in Tc’Krmutant embryos. These primordia are known to arise early in the blastoderm, posterior of the growth zone proper (reviewed by Anderson, 1972). One marker for terminal structures is the posterior terminal domain of Tc’wg (Nagy and Carroll, 1994), which is formed and maintained in Tc’Krmutant embryos similar to wild type (Fig. 7). In addition, the cuticle lining of the hindgut is present in mutant larvae (e.g. Fig. 4A).
regulate Hox genes, but not to directly regulate pair-rule genes (G. Bucher, PhD thesis, Ludwig-Maximilians-Universität, München, 2002) (Liu and Kaufman, 2004a). In Tribolium, however, some blastoderm pair-rule stripes are affected by gap gene orthologues other than Kr (A.C.C. and M.K., in preparation), and there is good evidence for stripe-specific elements driving at least the first two Tc'hairy stripes (Eckert et al., 2004).

Our results for Tc'Kr deviate from those obtained for Krüppel in Oncopeltus fasciatus (Liu and Kaufman, 2004b). In this short-germ insect, knock-down of Kr also results in mis-expression of Hox genes, although the effects are more limited as only one ectopic Of'Dfd domain is detected. Interestingly, expression of Of'en in such embryos seems to indicate a clear gap phenotype, i.e. perfect segmental stripes reappear posterior to a region of segmental disruption. Incomplete inactivation of Of'Kr could be responsible for this difference; we note, however, that weak Tc'Kr RNAi situations do not result in obvious gap phenotypes (see Fig. S2 in the supplementary material). Rather, in such embryos the segmentation process simply breaks down somewhat later than in Tc'KrRNAi, i.e. the additional segments present in weak Tc'Kr RNAi embryos appear to represent anterior abdominal rather than posterior (post-gap) abdominal segments. Oncopeltus is sometimes denoted an intermediate-germ insect, because a few more segments are formed already in the blastoderm than, for example, in Tribolium. It will be interesting to see if the ‘next posterior’ gap gene in Oncopeltus will also display a ‘gap’ phenotype, and to find out whether pair-rule gene expression in Of'Kr RNAi embryos indicates a role in the regulation of specific stripes boundaries.

If our interpretation is correct that Tc'Kr does not directly specify pair-rule stripes during abdomen formation, what could its function be in this process? All abdominal cells derive from progenitors that expressed Tc'Kr at the blastoderm stage. Therefore, regulation of later-acting abdominal expression domains (e.g. the posterior domains of Tc'gt and Tc'hb), may depend on Tc'Kr activity in the blastoderm, rather than on its activity at later stages when its domain forms a distinct posterior boundary. In this way, the long-ranging action of Tc'Kr could be explained through a temporal persistence rather than a spatial diffusion mechanism. Later acting genes depending on Tc'Kr activity then could have a role in regulating pair-rule genes.

However, the discovery that a segmentation clock appears to pattern lower arthropods (Chipman et al., 2003; Stollewerk et al., 2003) raises the issue of when in the evolutionary line to pattern lower arthropods (Chipman et al., 2004; Stollewerk et al., 2004). This system could be explained through a temporal persistence rather than a spatial diffusion mechanism. Later acting genes depending on Tc'Kr activity then could have a role in regulating pair-rule genes.

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**Supplementary material**

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/24/5353/DC1

**References**


