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’Krjaws* 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’Krjaws* 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 level, transcription factor diffusion is thought to be essential, which suggests that the *Drosophila* segmentation machinery can work only in a system unimpended 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 (Pourquie, 2001). A segmentation clock involving the *Notch* system appears to function in basal arthropods, i.e. spiders (Schoppmeier and Damen, 2005; Stollewerk 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 segment-polarity 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 Kriippel mutant, and provide the first detailed analysis of a gap gene null phenotype in a short-germ embryo. This amorphic Tc’Kr phenotype, as well as weaker phenotypes generated by RNAi, clearly differ from those of Drosophila Kriippel (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 GGCCACCTGAGAACACTGC and 3’ primer GCAGTCTGGTCCA- GTGGGCC. To complete the sequence, additional RACE reactions were performed using 5’RACE primer CACGGCGATGTTGGTCT-TGAGGTT and 3’RACE primers GTTGAATTGATAGCGAGTT- CCTCC and TTGGCCAGCAGCGATAGGGGACC. 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 ACGACTTGCGGTTAATG and TACGAAATAGGC-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 germband 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 (Sommer 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 (Sommerr 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 germband 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,jaws, is similar to an amorphic Krüppel mutation identified in Drosophila: in the Dm'Kr° 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 Tc'Krjaws indeed does fully inactivate the Tc'Kr locus.

Phenotypic series caused by Tc'Kr loss or depletion

The identification of jaws as a Tc'Kr allele is also supported by RNAi evidence. Injecting dsRNA representing 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 Tc'Krjaws.

In Tc'Krjaws 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 Tc'Krjaws 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 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 Tc'Krjaws 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 maxillas). 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 severe.

**Fig. 2.** jaws is a mutation in the first Zn finger of Tc'Kr. (A) After repeated outcrossing of the jaws mutation (induced in a GA-1 background) with the SB wild-type strain, all adult beetles that were jaws mutant carriers are also heterozygous for polymorphisms at the Tc'Kr locus, indicating close linkage between the mutation and the polymorphism. The polymorphism ‘SB’ represents a Tc'Kr 3’UTR sequence specific to the SB strain that can be detected as a 205 bp RFLP band. The polymorphism ‘Tiw’ is specific to the Tc'Kr copy of the jaws-carrying chromosome and results in a 141 bp RFLP (this polymorphism originally had been identified in the Tiw-1 wild-type strain). Depicted is an agarose gel with 11 samples (of 80 total). As controls, Ase I-digested DNA amplified from a SB animal (SB/SB), a Tiw-1 animal (Tiw/Tiw) and an animal that resulted from a cross between Tiw-1 and SB parents (SB/Tiw) are shown. (B) Tc'Kr gene structure; the four Zn fingers are shown as black boxes. (C) Sequence of the first Zn finger of Tc'Kr. In the jaws mutant, the 2nd histidine is altered to a tyrosine.

**Fig. 3.** Phenotypic series for Tc'Kr: ventral views of first instar larvae (confocal image projections based on cuticle autofluorescence). (A,B) Wild-type first instar larva (A). (B) Enlarged view of the ventral head, with left maxilla and labium outlined in white. (C-E) Tc'Kr RNAi embryos of increasing phenotypic strength. (F) Tc'Krjaws mutant embryo. Appendages resembling maxilla are labelled with arrows, those resembling labial palps with arrowheads. In some embryos, the urogomphi and pygopodes, corresponding to the 9th and 10th abdominal segments, respectively, are also indicated. For detailed phenotypic description see Results. The embryo in F is at a higher magnification than those in A-E. mx maxilla; lb labium; T1 to T3, thoracic segments 1 to 3; A1 to A5, abdominal segments 1 to 8; pp, pygopod; ug, urogomphi.
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’Kr jaws a null-allele?
The sequence alteration in Tc’Kr jaws 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 (Muller, 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’Kr jaws is the same as that of larvae with additional RNAi induced depletion of the mutant Tc’Kr transcript, then we can conclude that Tc’Kr jaws represents the strongest possible loss-of-function phenotype. We performed Tc’Kr RNAi knock-down in a Tc’Kr jaws-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’Kr jaws larvae. These homozygously mutant larvae did not show a stronger phenotype than Tc’Kr jaws larvae (Fig. 3F; Fig. 4A). From this experiment, we conclude that Tc’Kr jaws 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’Kr jaws.

The homeotic effect of Tc’Kr jaws 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’Kr jaws 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’Kr jaws homozygous animals showed a phenotype very similar to that of Tc’Kr jaws alone (Fig. 4B). They differed only from the normal Tc’Kr jaws 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’Kr jaws, i.e. the segmentation phenotypes of these experimental larvae corresponds to a superposition of Tc’gt RNAi and Tc’Kr jaws. However, the homeotic transformations caused by Tc’Kr jaws 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’Kr jaws and Tc’gt RNAI embryos
The striking homeotic transformations in Tc’Kr jaws 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 already has shown that the Hox gene proboscipedia (Tc’pbd) is ectopically expressed in Tc’Kr jaws mutant embryos (Sulston and Anderson, 1998). However, Tc’pbd 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’Kr jaws phenotype.

The Deformed (Tc’Dfd) gene is expressed in the mandibular and maxillary segments (Brown et al., 1999). In Tc’Kr jaws 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\textsuperscript{jo} 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\textsuperscript{jo} 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\textsuperscript{jo} 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\textsuperscript{jo} 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\textsuperscript{jo} 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\textsuperscript{jo} 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\textsuperscript{jo} 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'\textsuperscript{em}) and the pair-rule genes Tc'\textsuperscript{eve} and Tc'\textsuperscript{runt} are altered in Tc'Kr\textsuperscript{jo} (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'\textsuperscript{eve} exactly are affected by the Tc'Kr\textsuperscript{jo} 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\textsuperscript{jo} 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\textsuperscript{jo} embryos, the posterior domain of Tc'gt is absent, while an additional stripe of expression appears; A.C., unpublished). We find that
the first three *Tc’eve* stripes arise and split into segmental stripes in *Tc’Kr*mutant embryos exactly as in wild type (Fig. 6A–E,G). 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’Kr*mutant (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 patterning observed in *Tc’Kr*mutants 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’Kr*mutant 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’Kr*mutant (Fig. 7G–I) (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’Kr*mutant embryos. Several more posterior *Tc’wg* stripes arise but are fragmentary, weakly expressed or only present on one side of the embryo (Fig. 7J–K). As with *Tc’eve* stripes, no additional stripes re-emerge at later stages in *Tc’wg*. 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’Kr*mutant 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.

![Image of developing Tribolium embryo](https://example.com/tribolium_embryo.png)
The expression of homeotic genes in \textit{Tc'Kr}^{jawnz} embryos is consistent with the morphological transformations observed (Fig. 3F, Fig. 5). Our results with \textit{Tc'Dfd}, \textit{Tc'Scr}, \textit{Tc'Antp} and \textit{Tc'Ubx} confirm and extend earlier findings for \textit{Tc'pb} and \textit{Tc'UBX/Tc'ABD-A} expression (Sulston and Anderson, 1998). Notably, the complementary double-segmental expression of \textit{Dfd} and \textit{Scr} in \textit{Tc'Kr}^{jawnz} embryos explains the phenotype of alternating maxillary and labial segments. As summarized in Fig. 8, these expression patterns indicate that the posterior limit of \textit{Tc'Dfd} and \textit{Tc'Scr} domains is set through inhibition by \textit{Tc'Kr}. In this respect, \textit{Tc'Kr} fulfils a function similar to \textit{Drosophila} gap genes.

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

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

\textbf{\textit{Tc'Kr} does not function as a canonical gap gene during segmentation}

In \textit{Drosophila}, \textit{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 \textit{Tribolium} blastoderm, only one such gradient is present as the \textit{Tc'Kr} domain covers the posterior pole (Sommer and Tautz, 1993). When the germ rudiment has formed, the \textit{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 \textit{Tc'Kr} domain covers more anterior segment primordia (and more anterior pair-rule stripes) than does its \textit{Drosophila} counterpart.

Both boundaries of the \textit{Dm'Kr} expression domain...
Development clearly show that the Severe deviations from the wild-type pattern only become first four stripes of compared with wild type (Fig. 6). The same is the case for the posterior border of the Tc’Kr becomes free of significant role in generating those primordia that arise within the first abdominal segment (Figs 6, 7). In addition, the first Tc’en expression (Figs 5, 8), it is not required for the formation of boundary evidently is used for limiting gnathal Hox gene expression in Tc’Krjaws through activation, whereas the anterior border of Tc’Ubx depends on its larval phenotype, the abdominal domain of Tc’hairy and Tc’hairy (Mito et al., 2005) and Tc’gt function (Maderspacher et al., 1998). In contrast to the earlier report, we interpret the progression of the en/ wg pattern in Tc’Krjaws 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’Krjaws mutant 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 segmental stripe 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’Krjaws 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’Krjaws 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’Krjaws 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 Kräppel 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’h b (Mito et al., 2005) and Of’h b (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
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 expression in 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 appear 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'Kr RNAI, 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 (Chippman et al., 2004; Stollewerk et al., 2003) raises the issue of when in the evolutionary line leading to the diptera this clock was replaced by the hierarchical mode of Drosophila segmentation. Although at present no evidence is available for a segmentation clock functioning in Tribolium, it is conceivable that a modified clock is installed at the posterior end of the blastoderm embryo. Tc'Kr could have a role in initiation of this clock machinery. Alternatively, it could be required for its continued function. Because the number of abdominal segments is constant in insects, some type of counting principle would be required to stop the clock once the last segment has formed. Such a counting mechanism could be provided, for example, by a series of abdominal ‘gap gene’ activities (including the posterior domains of Tc'gt and Tc'hb), the last of which would shut off the clock. In this view, abdominal ‘gap genes’ would have a permissive rather than a positionally instructive function during abdominal segmentation of short germ embryos.

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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


Role of Tc'Kr in abdominal patterning


