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 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 (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 Krüppel 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 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 G GCCACCTGGACGGCTGC and 3' primer GCAGTTCGTCCAAGTGTCG. To complete the sequence, additional RACE reactions were performed using 5' RACE primer CAGCCGCATGTGGGTCTTTGAGGTG and 3' RACE primers GTTGATTGGTATGCGAGTTGCAGCCGCATGTGGGT. 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 AGCAATGGCCGTTAAATG and TACGAAAGTACGCCAACAAC. 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 (Somer 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 DmKr 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 (Sommers 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 excludes recently formed segments close to the growth zone (Fig. 1F,G). This signal later intensifies in the appendages and excludes recently formed segments close to the growth zone (Patel et al., 1994)] 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.

**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: in the DmKr<sup>9</sup> allele, one of the crucial Zn-finger cysteines is converted to serine, completely abolishing DmKr 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 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

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* 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* 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’pb*) is ectopically expressed in *Tc’Kr* jaws 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’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
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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 \textit{Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}}} mutant larvae, develop maxillary characteristics. The \textit{Sex combs reduced} (\textit{Tc'Scr}) gene is active in the ectoderm of the second parasegment in \textit{Tribolium} (Curtis et al., 2001), which largely corresponds to the labial segment (Fig. 5E-G; \textit{Tc'Scr} expression is also present in the mesoderm of additional segments). In \textit{Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}}} embryos (Fig. 5H-J), ectopic activity of \textit{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 \textit{Tc'Dfd} and \textit{Tc'Scr} are active in complementary double-segmental frames in \textit{Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}}} mutant embryos, which is consistent with the phenotype of differentiated mutant larvae.

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

Together, these data show that the \textit{Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}}} homeotic phenotype can be explained by defective Hox gene regulation, and they suggest inhibition of \textit{Tc'Dfd} and \textit{Tc'Scr} by \textit{Tc'Kr}, whereas \textit{Tc'Ubx} positively depends on \textit{Kr} activity. In addition, the double-segmental appearance of ectopic gnathal Hox expression domains suggests that the Hox genes \textit{Tc'Dfd} and \textit{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 \textit{engrailed} (\textit{Tc'en}) and the pair-rule genes \textit{Tc'eve} and \textit{Tc'runt} are altered in \textit{Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}}} (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 \textit{Tc'eve} exactly are affected by the \textit{Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}}} mutation. To distinguish pair-rule stripes arising in the growing germ band, we performed double staining with segment polarity genes, and to identify \textit{Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}}} mutant embryos at stages before morphological differences to wild types become evident, \textit{Tc'giant} was included as an additional marker in some experiments (in \textit{Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}}} embryos, the posterior domain of \textit{Tc'gt} is absent, while an additional stripe of expression appears; A.C., unpublished). We find that

![Fig. 5. Hox gene expression in wild-type, Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}} 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'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}} 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'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}} (E-J) Tc'Scr (purple) and Tc'Antp (blue) staining of wild-type (E-G) and Tc'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}} 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'M{	extquoteright}Kr\text{\textsubscript{in\textsuperscript{vs}}} 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).](image-url)
the first three Tc’eve stripes arise and split into segmental stripes in Tc’Krjawsmutant embryos exactly as in wild type (Fig. 6A–C,G–I). 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’Krjawsmutant (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 pattern of the segmental marker Tc’wg, normally formed gnathal and thoracic segment primordia anterior of, and within, the Tc’Kr jaw domain, this stripe never progresses into segmental stripes 5a and 5b and within, the expression domain do differentiate, 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’Krjawsmutant (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’Krjawsmutant 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’Krjawsmutant 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’Krjawsmutant (Fig. 7G–L) (Sulston and Anderson, 1996). In contrast to this earlier analysis, however, in the pattern of a segment-polarity gene we 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’Krjawsmutant embryos. Several more posterior Tc’wg stripes arise but are fragmentary, weakly expressed or only present on one side of the embryo (Fig. 7L–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 Triboli um 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 Kriippel in Triboli um and Drosophila are the homeotic transformations in Tc’Krjawsmutant 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, Triboli um segment primordia anterior of, and within, the Kriippel expression domain do differentiate, such that homeotic transformations can manifest themselves in the differentiated larva.
The expression of homeotic genes in Tc’Krantz 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*Krantz 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’Krantz larvae. Interestingly, in embryos that lack Tc’Kr and at the same time have reduced Tc’gt activity, the homeotic effect of Tc’Krantz 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’Krantz, 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*Krantz 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...
Development

probably set by clearly show that the apparent beginning with the 5th Severe deviations from the wild-type pattern only become Tc’hairy first four stripes of compared with wild type (Fig. 6). The same is the case for the becomes free of the reach of its blastoderm expression domain. Tc’Antp through activation, whereas the anterior border of periodicity. four pair-rule stripes of Tc’eve the first abdominal segment (Figs 6, 7). In addition, the first Tc’en gnathal or thoracic segments. The segment polarity genes expression (Figs 5, 8), it is not required for the formation of boundary evidently is used for limiting gnathal Hox gene development.(or Maderspacher et al., 1998). In contrast to the earlier report, we interpret the progression of the en/wg pattern in Tc’Kr

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’Scr. The precise boundaries of these gnathal Hox genes are defined by pair-rule genes. Therefore, ectopic gnathal Hox gene expression in Tc’Kr

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 expression (Figs 5, 8), it is not required for the formation of gnathal or thoracic segments. The segment polarity genes Tc’en and Tc’wg are expressed normally in all segments up to the first abdominal segment (Figs 6, 7). In addition, the first four pair-rule stripes of Tc’eve show little or no change compared with wild type (Fig. 6). The same is the case for the first four stripes of Tc’hairy and Tc’runt (data not shown). Severe deviations from the wild-type pattern only become apparent beginning with the 5th Tc’eve stripe. These data clearly show that the Krüppel domain in Tribolium has no significant role in generating those primordia that arise within the reach of its blastoderm expression domain. Shortly after germ rudiment formation, the growth zone becomes free of Tc’Kr transcript, and this newly arising posterior border of the Tc’Kr domain could, in principle, provide positional information to regulate posterior pair-rule stripes. We argue that also the posterior boundary of the Tc’Kr domain is unlikely to function as a direct instructive gradient for pair-rule genes. In Drosophila, gap genes usually define pair-rule stripe boundaries through repression. Accordingly, in gap mutants the corresponding pair-rule stripes expand towards the region where the gap domain normally resides. No such expansion of Tc’eve (or Tc’runt or Tc’hairy) stripes is observed in Tc’Kr

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’Kr

Compared with the classical gap phenotype of Dm’Kr mutants, the segmental defects in Tc’Kr

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
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 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., 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

Dev. Genes Evol. 208, 558-568.


MPLLQETTPK RDMLDSQEKT PLSSVSYPMS FTPSQLLMAS HLMAASRLSL
PTNPAFFHPG LLPLAWQANS PSPPAPSEL PALKSRKLNN NNVSSTNQE
IRGPKRKTWK VEEDSPSPTS SVSPEVKDSS RDRPFTCEVC NRSFGYKHVL
QNHERTHTGE KPFECEQECHK RFRDHDHLKT HMRLHHTGERP YRCEHCDRQF
VPVANLRRRL RVHTGERPYG CEHCSMKFSD SNQLKAHVL1 HTNEKPFEC
KCRGRFRRRH HLVHHKCGGE EEAERAPAPA VRAAGDAAAA RGAARADGAG
GPLHDHRPAL AQQRRVAAVQ VPQLAGGGRR GGPGRSSRRH LPAHLVAGTC
FVQLIGMRVP PEMMQAGDYC