Divergent segmentation mechanism in the short germ insect *Tribolium* revealed by *giant* expression and function

Gregor Bucher and Martin Klingler*,†

Department for Biology II, Ludwig-Maximilian-University Munich, Luisenstraße 14, 80333 Munich, Germany
*Present address: Institut für Zoologie, Friedrich-Alexander-Universität Erlangen, Staudtstraße 5, 91058 Erlangen, Germany
†Author for correspondence (e-mail: klingler@biologie.uni-erlangen.de)

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Summary

Segmentation is well understood in *Drosophila*, where all segments are determined at the blastoderm stage. In the flour beetle *Tribolium castaneum*, as in most insects, the posterior segments are added at later stages from a posteriorly located growth zone, suggesting that formation of these segments may rely on a different mechanism. Nevertheless, the expression and function of many segmentation genes seem conserved between *Tribolium* and *Drosophila*. We have cloned the *Tribolium* ortholog of the abdominal gap gene *giant*. As in *Drosophila*, *Tribolium giant* is expressed in two primary domains, one each in the head and trunk. Although the position of the anterior domain is conserved, the posterior domain is located at least four segments anterior to that of *Drosophila*. Knockdown phenotypes generated with morpholino oligonucleotides, as well as embryonic and parental RNA interference, indicate that *giant* is required for segment formation and identity also in *Tribolium*. In *giant*-depleted embryos, the maxillary and labial segment primordia are normally formed but assume thoracic identity. The segmentation process is disrupted only in postgnathal metamers. Unlike *Drosophila*, segmentation defects are not restricted to a limited domain but extend to all thoracic and abdominal segments, many of which are specified long after *giant* expression has ceased. These data show that *giant* in *Tribolium* does not function as in *Drosophila*, and suggest that posterior gap genes underwent major regulatory and functional changes during the evolution from short to long germ embryogenesis.

Key words: *giant*, Short germ, Long germ, Segmentation, *Tribolium*, Gap gene, Abdomen, *Krüppel*, *jaws*, *Drosophila*, Evolution, Morpholino, Parental RNAi

Introduction

Most insect embryos pass through a blastoderm stage where different fates are inscribed into the cells by the activity of embryonic patterning genes. The ‘extended germ band’ stage, when all body segments and major organ systems have been determined, is also similar among different insect orders, and has therefore been termed the phylotypic stage (Sander, 1983). Remarkably, the developmental processes leading from the blastoderm to the phylotypic stage vary fundamentally in different insect taxa. Most insects develop as short germ embryos, where only the anteriormost segments are specified at the blastoderm stage. The more posterior segments are formed in an anterior to posterior succession from a posterior growth zone. This mode of segmentation is believed to be ancestral (Davis and Patel, 2002; Tautz et al., 1994). By contrast, long germ insects specify all segments during the blastoderm stage. At the molecular level, segmentation is well understood only in the long germ insect *Drosophila melanogaster* (Pankratz and Jackle, 1990). Here, gap genes play a crucial role during pattern formation. They are activated in broad domains by maternal gradients, and diffusion of gap gene products results in overlapping short range gradients (Hulskamp and Tautz, 1991; Rivera-Pomar and Jackle, 1996). These short-range gradients then serve to position the stripes of primary pair rule genes (Klingler and Tautz, 1999; Small and Levine, 1991). In parallel, gap genes also provide positional information for the expression of Hox genes, which assign identities to the specified segments (McGinnis and Krumlauf, 1992).

The red flour beetle, *Tribolium castaneum*, is a short germ insect amenable to functional analysis via genetic, transgenic and RNAi approaches (Beeman et al., 1989; Berghammer et al., 1999; Brown et al., 1999; Bucher et al., 2002; Maderspacher et al., 1998; Sulston and Anderson, 1998). A number of segmentation genes have been isolated from this species. The segment polarity genes *engrailed* and *wingless* (Brown et al., 1994b; Nagy and Carroll, 1994), as well as several pair-rule genes (Brown et al., 1994a; Patel et al., 1994; Sommer and Tautz, 1993) were shown to be expressed in corresponding patterns in *Tribolium* and *Drosophila*, suggesting that their functions are largely conserved. Although conservation of pair rule activity within arthropods is a matter of ongoing debate (Davis and Patel, 2003), mutant phenotypes indicate pair rule action in *Tribolium* (Maderspacher et al., 1998; Sulston and Anderson, 1996). In addition, several homologs of gap genes have been cloned from *Tribolium*. *Orthodenticle* (*Tc’otd-1*), *hunchback*, *Krüppel* and *tailless* are expressed in the blastoderm in a similar anterior to posterior order (Li et al., 1996; Schroder et al., 2000; Sommer and Tautz,

1993; Wolff et al., 1995). While the gap genes *Tc'otd-1* and *Tc'hunchback* are active in similar segment primordia as in *Drosophila*, they appear to play a more prominent role in anterior specification than in *Drosophila* (Schroder, 2003). The function of the posterior *Tc'tailless* domain, however, is not conserved, as abdominal segments arise at a time when Tc'Tailless protein has long disappeared (Schroder et al., 2000). The apparent conservation of pair rule functions and the non-conservation of at least some gap genes prompted us to investigate the role of the abdominal gap gene *giant*.

giant is a transcription factor of the basic leucine zipper family which so far has been investigated only in *Drosophila* (Capovilla et al., 1992; Hewitt et al., 1999; Strunk et al., 2001). *Dm'giant* expression appears during the early blastoderm in two broad domains. The anterior domain subsequently resolves into several stripes, the most posterior of which is located in the maxillary segment (Eldon and Pirrotta, 1991; Kraut and Levine, 1991b; Mohler et al., 1989). Also at later stages of development, *Dm'giant* remains expressed in a complex pattern in the embryonic brain. The posterior domain initially covers the posterior pole of the blastoderm embryo, but later retracts and covers the primordia of abdominal segments 5-7. Shortly after cellularization, this domain disappears. In mutant embryos, the labial *engrailed* stripe is deleted leading to a fusion of the labial with the first thoracic segment in cuticles (Petschek and Mahowald, 1990; Petschek et al., 1987). In addition, the *engrailed* domains of abdominal segments 5-7 fuse. In cuticles, the anterior compartments of these segments are deleted while the remnants fuse (Petschek and Mahowald, 1990). *Dm'giant* exerts repressive functions on gap, pair rule and Hox genes. Mutual repression of *giant* and *Krüppel* has been shown to be crucial for the refinement of their expression domains (Capovilla et al., 1992; Kraut and Levine, 1991b). The patterns of pair rule genes are disturbed in head as well as in abdominal regions in *Dm'giant* mutant embryos (Langeland et al., 1994; Petschek and Mahowald, 1990; Small et al., 1991). Direct interaction of *Dm'giant* with one of its pair-rule target genes, *even-skipped* (*eve*), has been studied in great detail (Small et al., 1992; Wu et al., 1998). Bound to regulatory DNA, Giant functions as a short range repressor that acts over distances of 100-150 bp (Gray et al., 1995). The protein contains an interaction domain for the co-repressor *CtBP* but exerts its repressive function in part through *CtBP* independent mechanisms (Strunk et al., 2001).

In this work, we describe the isolation and analysis of *Tribolium giant*, the first ortholog of *giant* in a species outside of Diptera. Similar to *Drosophila* gap genes, *Tc'giant* functions in both segmentation and Hox gene regulation. However, expression and functional analysis of *Tc'giant* clearly show that it plays a role that fundamentally differs from the well understood function of its *Drosophila* ortholog.

Materials and methods

Cloning of *Tc'giant*

Based on alignments of Dm'Giant (sp|P39572|) with related leucine-zipper genes [*Drosophila melanogaster*, PAR domain protein (gb|AAF04508.1|); *Caenorhabditis elegans*, similar to BZIP transcription factor (gi|2291143|) and Cell death specification Protein 2 (sp|Q94126|CES2_CAEEL); *Gallus gallus*, vitellogenin gene-binding protein VBP (pir||S50109); and *Homo sapiens*, Hepatic leukemia factor (ref|NP_002117.1|)], we designed a nested set of three redundant primers (see Fig. 1B for primer position and a comparison of Dm'Giant and Hs'HLF). The sequences of these guessmers were GAR MGN MGN MGN AAR AAY AA (gt-5′), ARN WVN ATR TTY TSN CKY TCN AG (gt-3′a) and GCN CKD WKN GCN ADY TSN TCY TCY T (gt-3′b). As template for RT-PCR we prepared total RNA from staged embryos (0-24 hours at 33°C, containing all segmentation stages) following standard procedures (Sambrook et al., 1989). cDNA was prepared with the SuperScriptTM Preamplification System (GibcoBRL) using polyT primers. 3 μl of this cDNA was used as template for 'touch down' PCR using primers gt-5′ and gt-3′a. PCR conditions were: denaturation for 5 seconds at 94°C; annealing for 1 minute in all cycles, at 53°C in first 5 cycles, 51°C during the next 5 cycles, and 47°C in the remaining 20 cycles; elongation was 15 seconds at 72°C for all cycles. Of this reaction, 0.5 μl were used as template for a nested PCR with primer gt-5′ and gt-3′b (same PCR conditions). After the second PCR, a 78 bp fragment was detected in a 2% NuSieve GTG low melting agarose gel (FMC BioProducts), which was cloned into pZErOTM-2 (Invitrogen). Twenty-one independent inserts were sequenced, all of which turned out to represent the same amplified 36 bp (excluding primers) sequence (EMBL Accession Number AJ606487).

To obtain a complete transcript, this fragment was labeled with alpha [^{32}P]dCTP using the Random Primer DNA Labeling System (GibcoBRL), with random primers supplemented by our PCR primers gt-5′ and gt-3′b. Using this probe, a lambda ZAP cDNA library (Wolff et al., 1995) was screened employing HighBond-XL filters (Amersham). Five independent cDNA clones with identical sequence were isolated which represent four independent reverse transcription events. In addition, we used RACE (rapid amplification of cDNA ends) in order to complete the transcript and to identify additional splice products. For this experiment, the Marathon Kit (Clontech) and the following primers were used: ATC CTC TTT AGC TCT TCT GGC ATC TCT G (first 5′race PCR), CTC TGG ATC TTT TCG CCG CTT CGT TG (nested 5′race PCR), AAC GAA GCG GCG AAA AGA TCC AGA GA (first 3′race PCR) and GCG AAA AGA TCC AGA GAT GCC AGA AGAG (nested 3′race PCR). All 5′ and 3′ RACE products concurred with our cDNA sequences.

Sequence analysis

Alignment of Dm'Giant and Tc'Giant was done using Clustal W 1.5 (Higgins et al., 1992) using default settings except for a gap open penalty of 30 and a gap extension penalty of 0.1. For the phylogenetic analysis we conducted a BLAST (Altschul et al., 1997) search with the leucine zipper domain of Tc'Giant to identify all closely related sequences in the database. Of these, a representative range of species was selected and these sequences were aligned by the Clustal W program (BLOSUM matrix, default values). The PUZZLE algorithm (Strimmer and von Haeseler, 1996) as implemented in PAUP 4.0 (Swofford, 1998) was then used for a phylogenetic analysis using default settings. Bootstrap analysis was performed with PAUP 4.0, using standard settings and 500 replicates. A search in the Conserved Domain Database (CDD) at NCBI did not identify any conserved protein motives apart from the leucine zipper.

Histology

Whole-mount in situ hybridization was performed according to established protocols (Tautz and Pfeifle, 1989). For double staining, fluorescein- and digoxigenin-labeled probes were detected using alkaline phosphatase and β-galactosidase, the latter after signal enhancement via biotin deposition (Prpic et al., 2001). A detailed protocol is available from the authors. For in situ hybridization of injected RNAi embryos, embryos were removed from the microscope slide using a fine brush soaked with PEMS buffer. Fixation was done as usual, but embryos were devitellinized manually. As extended exposure of embryos to room temperature resulted in mRNA degradation, the bulk of the embryos were refrigerated, while small

batches were devitellinized using fine insect needles (Original EmilCarlt Insect Pins 0.1mm).

RNAi

For embryo injections, sense and antisense RNAs were synthesized from a full-length *Tc'giant* cDNA plasmid using the T7 Megascript Kit (Ambion), using T7 RNA polymerase (Ambion) and T3 RNA polymerase (LaRoche). Annealing was performed in injection buffer (potassium phosphate 20 mM, sodium citrate 3 mM pH 7.5) (Fire et al., 1998). Different concentrations of resulting dsRNA (*Tc'giant*: 2000 ng/µl, 750 ng/µl, 75 ng/µl and 7.5 ng/µl, *Tc'dll*: 2 µg/µl) were supplemented with Phenol Red to 0.05% (Sigma) and filtered (Ultrafree 0.45 um, Millipore) prior to injection. *Tribolium* eggs were collected for 1 hour at 25°C and kept for another hour at 33°C to improve injection survival. The embryos were then dechorionated using 'Klorix' bleach, washed in water and mounted on microscope slides without applying glue. They were injected in air at an intermediate anteroposterior position to minimize damage to egg poles where maternal morphogens may be localized and where the growth zone will develop. After injection, embryos were allowed to develop for four days at 33°C in a humid chamber. Fully differentiated embryos/larvae were embedded in Hoyer's medium and cleared at 65°C. Thirty-two percent of the injected eggs differentiated cuticles, and of these, 56% displayed *Tc'giant* phenotypes. Of the three concentrations tested, the two higher ones resulted in similar frequencies of RNAi phenotypes, while the lowest concentration produced mostly wild-type cuticles. As a control, we injected dsRNA from a gene of known function, *Tc'distalless* (Beermann et al., 2001). The resulting embryos displayed *distalless*-specific leg defects with high frequency, while segmentation defects were not observed.

For parental RNAi, mature female pupae were fixed to a microscope slide, ventral side up using rubber cement ('Fixogum', Marabu). To avoid interference with eclosion, only the posteriormost portion of the abdomen was allowed to contact the rubber cement. We generated dsRNA from a PCR template whose primers had T7 promoter sequences at both ends. After precipitation with NaAc/ethanol the dsRNA was dissolved in injection buffer. Approximately 0.15 µl of dsRNA (2000 ng/µl and 750 ng/µl) was injected between abdominal segments three and four, at a ventrolaterally position (in order not to damage the CNS). About 30 eclosed females were mated to untreated males, and eggs were collected beginning 1 week after injection. All embryos in the first egg-lay displayed *Tc'giant* phenotypes in cuticle preparations. During the following 2 weeks, eggs were fixed for histochemistry using standard procedures. Three weeks after injection, the portion of embryos displaying *Tc'giant* phenotypes dropped to 40% and egg collection was discontinued. Therefore, at least 40% of embryos used for histochemistry were expected to display *Tc'giant* phenotypes.

Morpholino oligonucleotides

A morpholino oligo (Gene-Tools) was designed to cover both possible starting ATGs (5′C**CAT**CGCAAATTCTGCTTTTTC**CAT**-3′). Injection of 1 mM and 0.66 mM concentrations (in injection buffer) resulted in premature termination of development of all embryos. With lower concentrations (0.4 and 0.2 mM) the proportion of fully differentiated embryos (32%) and cuticles displaying phenotypes (42% of differentiated embryos) was similar to our embryonic RNAi experiments. Morpholino injections essentially gave the same results as with RNAi experiments. However, in only 9% (n=32) was the transformation of maxilla to T1 complete. Morpholinos stoechiometrically compete with translation, whereas dsRNA is thought to involve an enzymatic reaction. Probably, the enzymatic RNAi mechanism knocks down gene function more effectively, resulting in residual *giant* function in morpholino-injected animals.

Results

Identification of a *giant* ortholog in *Tribolium*

As no ortholog of *Dm'giant* was known at the time, we chose a nested primer PCR strategy to isolate any gene belonging to a broad subgroup of the leucine zipper transcription factor family (see Materials and methods). Using the amplified 78 bp product as a probe, several concordant cDNAs were isolated from a lambda library. An identical sequence was obtained by extending the sequence through 5′ and 3′ RACE (EMBL Accession Number, AJ606487). These data suggest that only a single transcript is produced by this leucine zipper gene during early embryogenesis. According to BLASTP, the gene most similar to the *Tribolium* Giant protein is the putative *Anopheles* Giant followed by *Drosophila* Giant (Blast similarity value: 6e-17), and several vertebrate leucine zipper proteins with values of 2e-13 and lower. The *Drosophila CG4575* gene has a leucine zipper almost identical to *Drosophila giant* but lacks any similarity in the N-terminal region. It is derived from a genomic duplication with a breakpoint within the *giant*-coding sequence (J. Baines, personal communication). Because *CG4575* lacks important sequences, it is unlikely to be a functional duplicate of *giant*. Within the 54 amino acids leucine-zipper domain, *Tc'giant* and *Dm'giant* share 34 positions (63%) (large box in Fig. 1A). This similarity is only moderately higher than that among other proteins of the leucine zipper family. PUZZLE analysis based on the leucine zipper domain (not including the other conserved residues) resulted in a star-like branching pattern where *Tc'giant* was positioned with *Dm'giant* with moderate support (data not shown). However, additional N-terminal sequences are highly conserved between *Tribolium*, *Anopheles* and *Drosophila* Giant but not other leucine zipper genes. In *Dm'giant*, this region is believed to promote transcriptional repression through protein-protein interactions with co-repressors (Strunk et al., 2001). Similar to other *Tribolium* transcription factors, which are usually shorter than their *Drosophila* cognates (Schmid and Tautz, 1999), *Tc'Giant* is half the size of its *Drosophila* counterpart. Together, sequence analysis suggests that the single leucine zipper gene that we isolated from *Tribolium* is indeed a *giant* ortholog. Expression and functional data support this interpretation (see below).

Tc'giant expression during embryogenesis

We used in situ hybridization to see if the expression of *giant* is conserved in *Tribolium* (Fig. 2). In freshly laid eggs, putative maternal transcripts are distributed homogeneously throughout the syncytial blastoderm. Later, expression retracts from both poles and intensifies along the posterior edge of this domain (Fig. 2A-C). Eventually a circumferencial stripe is formed, which persists into the germ rudiment stage (Fig. 2D,F), while the intensity of the remaining domain decreases. A second *Tc'giant* domain arises de novo at the posterior pole of the embryo at the posterior pit stage (Fig. 2D). Cells lining the posterior pit express *Tc'giant*, while cells in the center of the invaginating pit remain unstained (Fig. 2E). In the germ rudiment, *Tc'giant* staining becomes more intense at the anterior boundary of this posterior domain (Fig. 2F). During early germ band elongation, this domain splits into two stripes (Fig. 2G) while expression in the head stripe ceases. As the

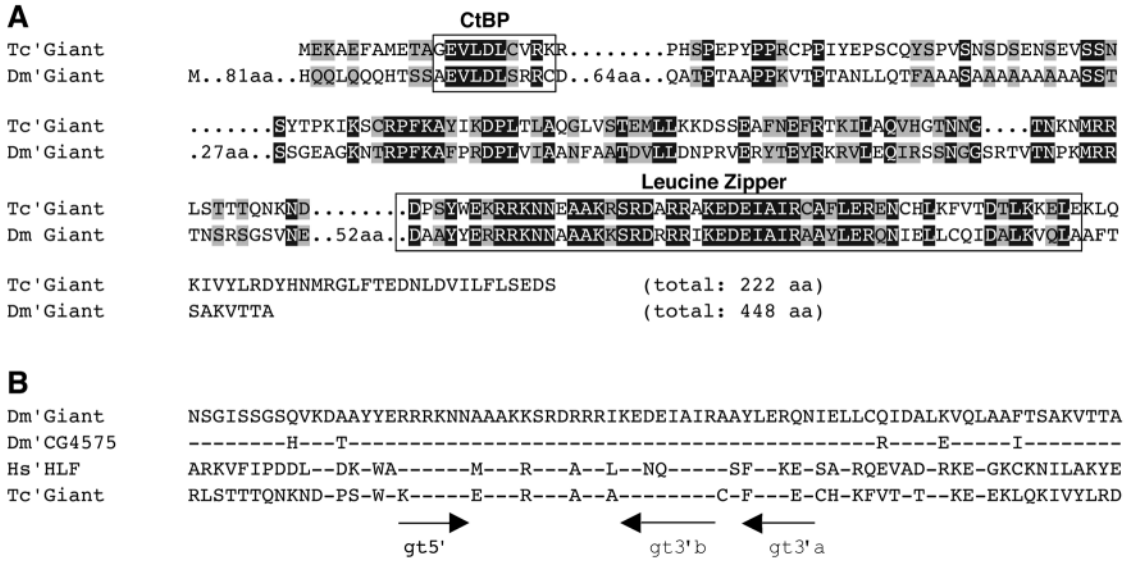

Fig. 1. Sequence of the *Tribolium* Giant ortholog. (A) Alignment of *Tribolium* and *Drosophila* Giant using Clustal W. Identical and similar amino acids are highlighted in black and grey, respectively. Longer sequence stretches without homology were omitted from the Dm'Giant sequence (indicated by dots, the number of omitted amino acids is indicated). The DNA-binding leucine zipper (large box) is 63% identical and 78% similar between both insects. The binding domain of the co-repressor *CtBP* (small box) is highly conserved, as are a number of additional motifs of unknown function. These conserved motifs upstream of the leucine zipper substantiate the orthology of Tc'Giant and Dm'Giant, as they are not present in other leucine zipper genes. Note that Dm'Giant is twice as large as the predicted beetle protein. (B) Alignment of the leucine zippers of Tc'Giant, Dm'Giant, Dm'CG4575 and Hs'HLF, the Human hepatic leukemia factor. Dashes indicate sequence identity. The position of the three nested PCR primers used for isolation of *Tc'giant* is given below.

germ band continues to grow, the first of the posterior stripes also fades, followed somewhat later by the second. Meanwhile, the low-level expression in the head condenses into a complex and dynamic pattern of brain cell clusters (Fig. 2I-K). *Tc'giant* expression ceases altogether before the germ band has fully elongated, and no staining was detected in subsequent embryonic stages.

Position of *Tc'giant* domains relative to other segmentation genes

To determine the relative position of the *Tc'gt* domains, we performed double in situ stainings with other segmentation genes. *Tc'giant* expression appears to be conserved anteriorly while its posterior domain has dramatically shifted. The *Tc'eve* gene marks odd-numbered parasegments in *Tribolium* and *Drosophila* (Brown et al., 1994a). In *Tribolium*, each double-segmental *eve* stripe splits into segmental stripes that exactly coincide with *engrailed*-expressing cells. Double stainings of *Tc'giant* and *Tc'eve* reveal that the anterior *Tc'giant* stripe coincides with the maxillary segment: The first *eve* stripe resolves into segmental stripes overlaying mandibular and maxillary *engrailed* stripes. *Tc'giant* expression abuts the mandibular segmental eve stripe (1a) and overlaps the maxillary one (1b) sharing its posterior boundary (Fig.

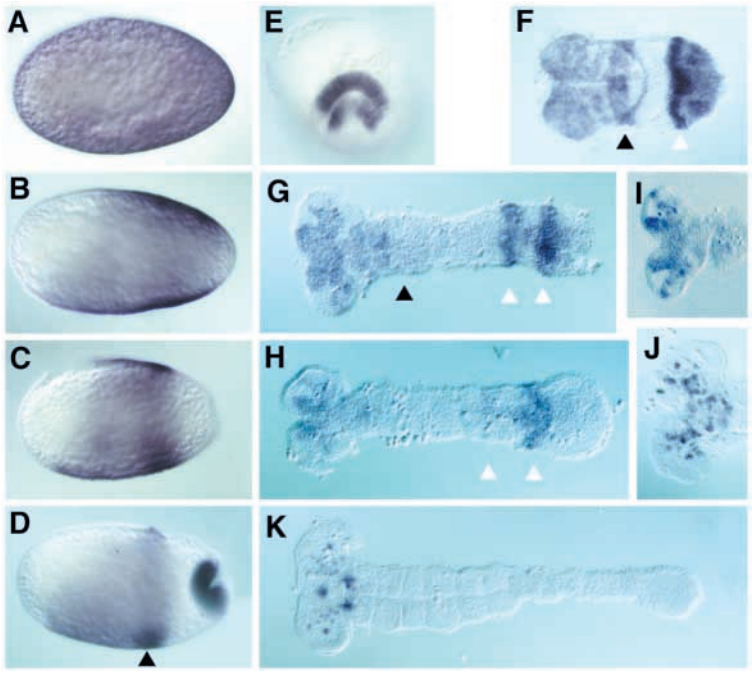

Fig. 2. Expression of *Tc'giant* in successive developmental stages. (A-D,F-K) Embryos are oriented anterior towards the left; (E) embryo viewed from the posterior pole. Blastoderm stages (A-E) are oriented dorsal side upwards. Germ band embryos (F-K) were dissected from the yolk and are shown in ventral view. (A-C) After initial ubiquitous maternal expression, *Tc'giant* forms an anterior domain comprising brain and gnathal segments, but excluding the anteriorly located extra-embryonic serosa. (C,D,F) In the maxilla, expression becomes stronger (black arrowhead) before anterior expression condenses (G-K) in a complex pattern in the brain. (D,E) A second domain arises de novo at the posterior pole of the late blastoderm and later splits in two domains that cover T3 and A2 (white arrowheads). See text for further details.

3B). Thus, the anterior *Tc'giant* stripe covers exactly the maxillary segment. At earlier stages, however, *Tc'giant* expression is somewhat shifted posteriorly relative to the first *eve* stripe (Fig. 3A). The first *Tc'hairy* stripe only partially overlaps the anterior *Tc'giant* stripe (Fig. 3E), which is consistent with the known phasing of *eve* and *hairy* stripes in *Tribolium* (Brown et al., 1994a).

The posterior domain of *Tc'giant* forms near the posterior pole, well behind the 2nd *eve* and *hairy* stripes (Fig. 3B,E). When the third *eve* stripe is formed, it partially overlaps the posterior *Tc'giant* domain (Fig. 3B). As the germ band grows, the posterior *Tc'giant* domain breaks up into two stripes which partially overlap the 3rd and 4th *eve* stripes, respectively (Fig. 3C). As the pattern matures, the segmental *eve* stripes 3a and 4a abut both posterior *Tc'giant* stripes anteriorly, while *eve* 3b and 4b overlap with them posteriorly. Therefore, the posterior *Tc'giant* stripes can be mapped precisely to the 3rd thoracic and 2nd abdominal segments at this stage (shown for the second posterior stripe in Fig. 3D). All three *giant* stripes mature exactly in the same relative position to the respective *Tc'eve* stripes. Our expression analysis reveals that the anterior *giant* domain is approximately conserved in position, i.e. it covers the maxillary segment in both *Tribolium* and *Drosophila*. The posterior domain, however, is shifted towards anterior by at least four segments in *Tribolium*: *Tc'giant* expression overlaps the 3rd and 4th *eve* stripes (Fig. 3D) but not stripes 6 and part of stripe 7 as in *Drosophila* (Myasnikova et al., 1999).

In *Drosophila*, the gap gene *Krüppel (Dm'Kr*) is positioned exactly between the two *giant* domains and negatively interacts with both of them (Capovilla et al., 1992; Kraut and Levine, 1991a). *Tribolium Krüppel* appears at the posterior pole at a time when the anterior *Tc'giant* domain has just retracted from this region and well before the posterior *Tc'giant* domain emerges. Therefore, *Tc'giant* and *Tc'Krüppel* are expressed mutually exclusively prior to the posterior pit stage (Fig. 3F) as in *Drosophila*. In the germ rudiment, *Tc'Krüppel* becomes restricted to a sharply demarcated band initially covering segment primordia T2 and T3 and eventually extending into T1 (data not shown). The posterior *Tc'giant* domain arises within this *Krüppel* domain, overlapping in the T3 segment during the whole course of expression (Fig. 3G,H). This data indicates that the posterior *Tc'giant* domain is not negatively regulated by *Krüppel* as in *Drosophila*.

Tc'giant determines the identity of gnathal segments

To investigate the function of *Tc'giant* during segmentation, we applied both embryonic and parental RNAi and morpholino oligos to reduce *Tc'giant* activity (Bucher et al., 2002; Brown et al., 1999; Fire et al., 1998). Although the strength of the phenocopies depends on the amount of injected dsRNA and morpholino oligos (Figs 4, 6), the majority of embryos share three characteristics: (1) the total number of body segments is reduced; (2) the number of segments with thoracic morphology (i.e. leg-bearing segments) is increased to four or five; and (3) the gnathal appendages maxilla and labium are missing. Other head structures, i.e. antenna, labrum and mandible, are not affected.

As *giant* mutants in *Drosophila* affect formation of the labial segment (Petschek and Mahowald, 1990; Petschek et al., 1987), it is tempting to interpret the loss of maxilla and labium

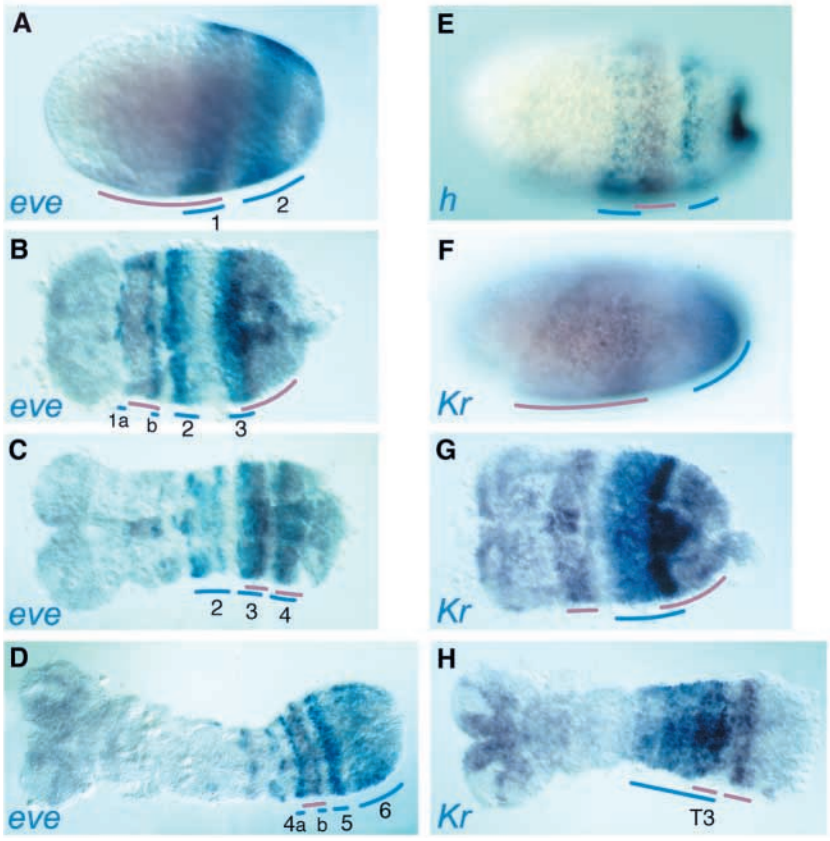

Fig. 3. Double in situ hybridization with *Tc'giant* in brown and *Tc'eve* (A-D), *Tc'hairy* (E) and *Tc'Krüppel* (F-H) in blue. *eve* stripes are numbered, with a/b, indicating the secondary segmental stripes derived from the respective double-segmental primary stripe. (A-D) The *Tc'giant* stripes in maxilla, T3 and A2 coincide roughly with the first, third and fourth *Tc'eve* stripes, respectively, and mature in the same relation to each other: early *Tc'eve* stripes are shifted slightly anterior to the *giant* stripes. As the pattern matures, the stripes successively coincide (see stripe 3 in B and C). (E) *Tc'hairy* is expressed in a frame roughly complementary to *Tc'eve*. Its posterior expression borders initially overlap the *Tc'giant* stripes but the overlap fades with time and the borders eventually abut each other. The anterior borders of the *hairy* stripes always remain separated from *Tc'giant* stripes. (F) *Tc'Krüppel* appears at the posterior pole of the blastoderm within *giant* free tissue (compare with C in Fig. 2). (G,H) The posterior *Tc'giant* domain-arises right within the *Tc'Krüppel* domain and the genes remain co-expressed in the third thoracic segment (T3 in H). Strong mutual repression as described for the *Drosophila* orthologs seems unlikely for this region. In addition, this staining shows that *Tc'Krüppel* is not expressed posteriorly to T3.

in *Tc'giant* RNAi larvae as a defect in head segmentation. However, the occurrence of additional thoracic segments in most of these larvae indicates that either abdominal or gnathal segments are transformed towards thoracic identity. Fortunately, in a small number of larvae (six out of 154) a full complement of 14 visible segments was still formed, the same number as in wild-type larvae (which have three gnathal, three thoracic and eight abdominal segments). These RNAi larvae display a mandible, five thoracic segments and eight abdominal segments (Fig. 4B). As no abdominal segments are missing in these larvae, it is evidently the maxillary and labial segments that are transformed towards the thorax. Moreover, of 52 RNAi embryos stained for *engrailed* expression, all displayed perfectly formed gnathal *engrailed* stripes (Fig. 5, see also following section). Therefore, even in embryos with strong segmentation defects, it is the identity, not the formation of gnathal segments that is affected.

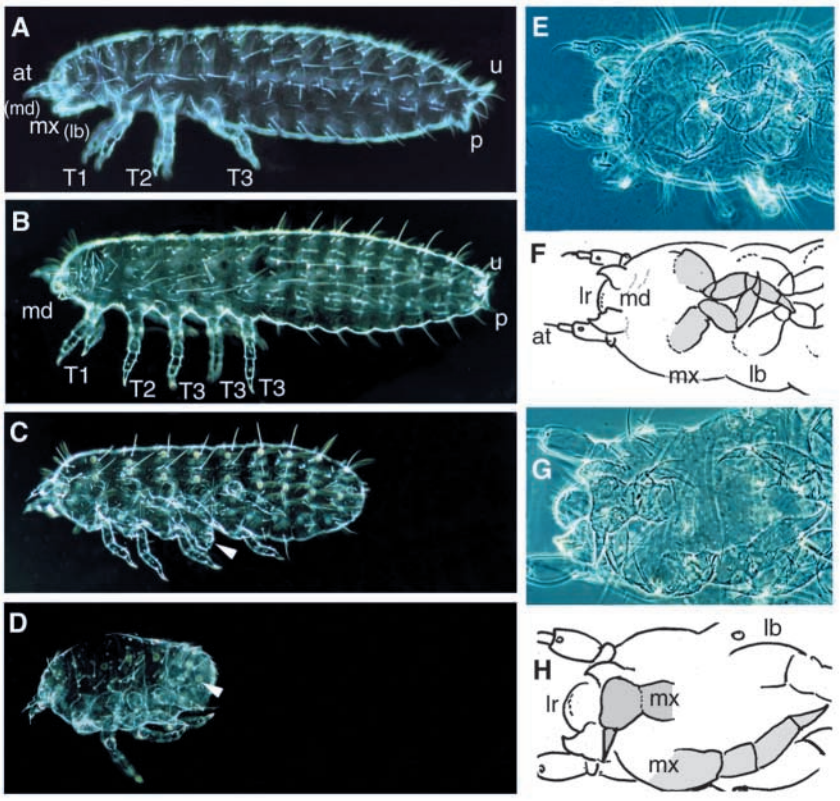

Fig. 4. Effect of RNAi gene knock-down on first instar larval cuticles. All larvae are shown anterior towards the left. (A) Wild-type larva with three leg-bearing thoracic segments (T1-T3) and eight abdominal segments. Two additional abdominal segments (A9 and A10) are fused to the telson and bear the urogomphi (u) and pygopods (p). In this lateral view, the mandibles (md) and the labium (lb) cannot be seen because they are covered by the maxilla (mx). (B-D) In all RNAi embryos, maxilla and labium are transformed to T1 and T2, respectively. Intriguingly, the thorax is shifted coordinately such that the mandibula is followed by segments displaying T1, T2 and T3 identity. The following two thoracic segments also have T3 identity. (B) In weak phenocopies, the transformation is not accompanied by segmentation defects. (C) Most *giant* phenocopies also display segmentation defects. In this specimen, five thoracic and four residual abdominal segments are formed, and the urogomphy are missing. Together, five segments are deleted. Because abdominal segments have identical cuticle pattern, it is not possible to determine which segments are missing. Often, the penultimate pair of legs is less well patterned or homeotically specified than the most posterior one (white arrowhead). (D) In this strong phenocopy, nine segments are deleted. Three thoracic segments are left and the presence of a pair of stomata (white arrowhead) indicates the presence of one abdominal segment. Even in such severely disturbed larvae, the terminal pygopods are usually present. (E,F) The gnathal transformation in a ventral view: antenna (at), labrum (lr) and mandibles (md) are not affected, but maxilla (mx) and labium (lb) are completely transformed to thorax. (F) Schematic representation of E; transformed maxillary appendages highlighted in grey. (G,H) In a few larvae, the transformation of the maxillary segment was not complete. Here, the lower appendage is transformed to leg, while the other appendage adopts an intermediate identity. Note that partial transformations are rarely observed in the maxillary but never in the labial segment. In no case were thoracic identities shifted only one segment towards anterior.

Intriguingly, the transformation consists of a coordinated shift of three segment identities (T1, T2 and T3) across two segment widths. The identity of the following two segments range between T3 and abdomen. This interpretation is based on the extent of appendage formation and the transversal distance of segmental pairs of dorsal bracts. By this distance, T1, T2 and T3 can be discriminated from each other as well as from abdominal segments. Interestingly, the coordinated homeotic shift is usually complete: either both gnathal segments are transformed, or both are normal. Only rarely did we observe partially transformed maxillae (one example is shown in Fig. 4G-H), and partially transformed labia were not observed. We also note that homeosis of the maxillary and labial segments is more sensitive to *Tc'giant* reduction than the segmentation defects in more posterior segments, because we never observed embryos with segmentation defects combined with normal gnathal region. Evidently, the homeotic effect of *Tc'giant* involves a tight threshold mechanism.

The role of *Tc'giant* in homeotic segment specification could be either direct, similar to the function of the non-Hox homeotic gene *spalt* or it could indicate a role in regulating homeotic genes. One potential target Hox gene is *maxillopedia* (*mxp*), the *Tribolium* ortholog of *Dm'proboscipedia* (Shippy et al., 2000). Loss of this gene leads to the transformation of maxillary and labial palps into legs. To see if *mxp* expression is indeed regulated by *Tc'giant*, we used parental RNAi (Bucher et al., 2002) to generate embryos with reduced *Tc'giant* activity and stained them for *mxp* by in situ hybridization. Indeed we find that expression of *mxp* in the appendages of the maxillary and labial segments is reduced or absent (see Fig. 5I,J). This confirms that *giant* is involved in Hox gene regulation in the gnathocephalon. However, the homeotic phenotype of *Tc'giant* probably involves mis-regulation of additional homeotic genes, because only the palps are transformed in *mxp*-null mutants, not the complete maxillary and labial segments as in *Tc'giant* RNAi embryos. A detailed analysis of the Hox genes involved in the transformation will be published elsewhere.

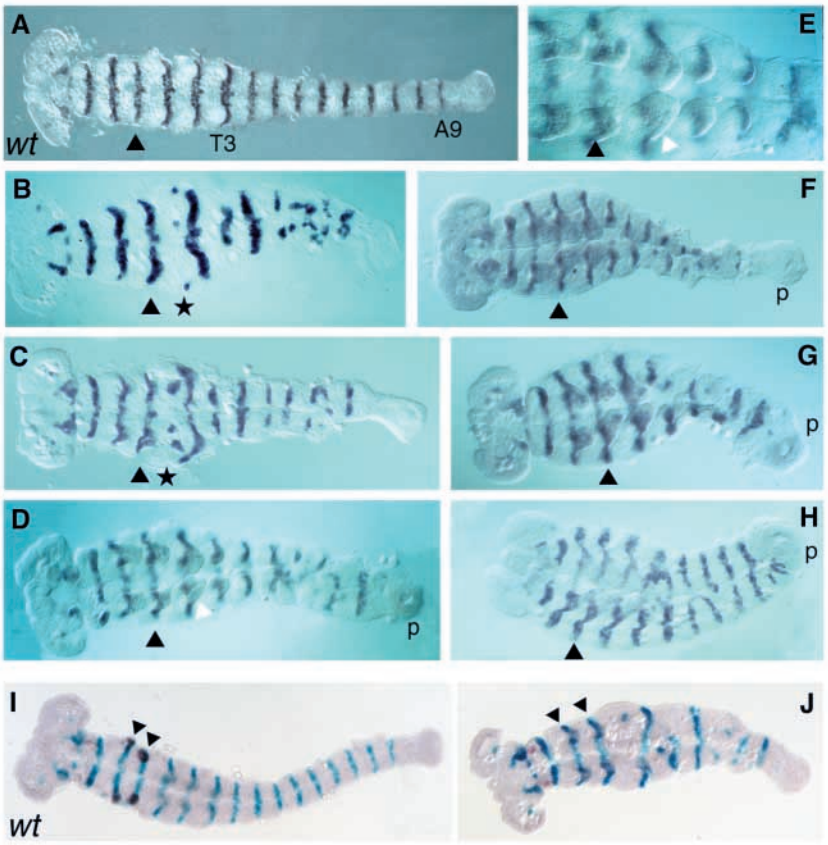

Fig. 5. In situ detection of *engrailed* and *maxillopedia* (*mxp*, ortholog of *Dm'probiscipedia*) in wild type (A,I) and in embryos depleted for *Tc'giant* by RNAi (B-H,J). Black arrowheads in A-H indicate the labial segment. The proctodeum (p) indicates completion of segmentation in D-H. (A) Wild-type germ band shortly before formation of the last (tenth) abdominal *engrailed* stripe. (B-H) In all germ bands analyzed, the first three segments were unaffected, suggesting, that in the head *Tc'giant* has a homeotic function. The T1 stripe was often disturbed or deleted in young embryos (stars in B and C), leading to an enlarged segment. By the end of segmentation, no defects are evident in the anterior thorax (D-H), suggesting that the embryo corrects for these early patterning defects. In some cases, the superfluous cells became assigned to the appendages that then appeared enlarged (white arrowheads in D and the close-up E). In cuticles, enlarged appendages were not observed, suggesting further correction. Segmentation is disturbed in a variable pattern in the region between T1 and A9. In germ bands with proctodeum formed (p), the number of deleted segments can be determined (D, 7; F and G, 8; H,4). (I) The Hox gene *maxillopedia* (red) is expressed in the appendages of the maxillary and labial segments (arrowheads). (J) In *Tc'giant* RNAi embryos, this expression is reduced or absent (arrowheads), confirming that *Tc'giant* knock down interferes with proper Hox gene regulation.

Tc'giant is required for segmentation of thorax and abdomen

Depending on dsRNA concentration, up to nine body segments are deleted in *Tc'giant* RNAi embryos (Fig. 6A-D). Using morpholino oligonucleotides to knock down *Tc'giant* gene activity, we achieved similar phenotypes with deletions of up to seven segments (Fig. 6E). As morpholinos are structurally different from dsRNA, and are thought to knock down gene function by a different molecular mechanism, the RNAi results outlined below are indeed specific for reduced *Tc'giant* activity. Although the abdomen is affected in most RNAi larvae injected with high concentrations of dsRNA, leg bearing segments are deleted only in 24% of these larvae: 17% and 7% have four and three leg bearing segments, respectively, instead of five. In the following, we will refer to particular segments according to their wild-type identity and not the identity resulting from homeotic transformation.

Owing to the uniform morphology of abdominal segments, it is difficult to ascertain which segments exactly are deleted in a given larva. Only in larvae displaying very weak phenotypes, where remnants of all segments are still present, is it possible to unambiguously identify the affected segments. In four such embryos, segments T2, A2, A6 or A7 were found to be partially deleted, respectively. This suggests that sensitivity to *Tc'giant* depletion is distributed rather evenly throughout the thorax and abdomen. The last two abdominal segments bear pairs of specialized appendages (which later during development fuse with the telson): the dorsal urogomphi (segment A9) and the ventral pygopods (A10, see Fig. 4A). Of these, the urogomphi are missing in 70% of all RNAi embryos. This identifies A9 as the posteriormost affected segment that additionally appears to be very sensitive to lack of *Tc'giant* activity. The pygopods, however, are usually not affected.

The anterior limit of *Tc'giant* requirement for segmentation is T1. This is not obvious from the inspection of larval cuticles, but can be clearly seen in the pattern of the segment-polarity gene *engrailed* in RNAi embryos (Fig. 5). As mentioned earlier, none of 52 such embryos showed any patterning disturbance in gnathal segments. However, partial or complete deletions of *engrailed* stripes were frequently observed throughout thorax and abdomen. Notably, the T1 segment is reduced or deleted in 39% of these embryos. In many *engrailed*-stained embryos, the T1 stripe is more severely affected than the T2 stripe, and quite frequently the T1 stripe is deleted completely, leaving an increased distance between the last gnathal and the first thoracic *engrailed* stripe. Later, however, cells are rearranged within the fused segment such that the labial and T2 engrailed stripes reach wild-type distance while the segment broadens laterally. Some of the excess cells contribute also to enlarged appendage primordia (Fig. 5E, white arrowhead; compare with more anterior appendages that are at the same stage of development or slightly older, black arrowhead). During subsequent development, this defect is further corrected for such that no disturbance in the first thoracic segment is apparent on the cuticular level. However, this segment actually represents a fusion of T1 and T2.

This regulative propensity of the embryo indicated that analysis based on cuticles might underestimate the defects elicited by RNAi. To determine the number of affected

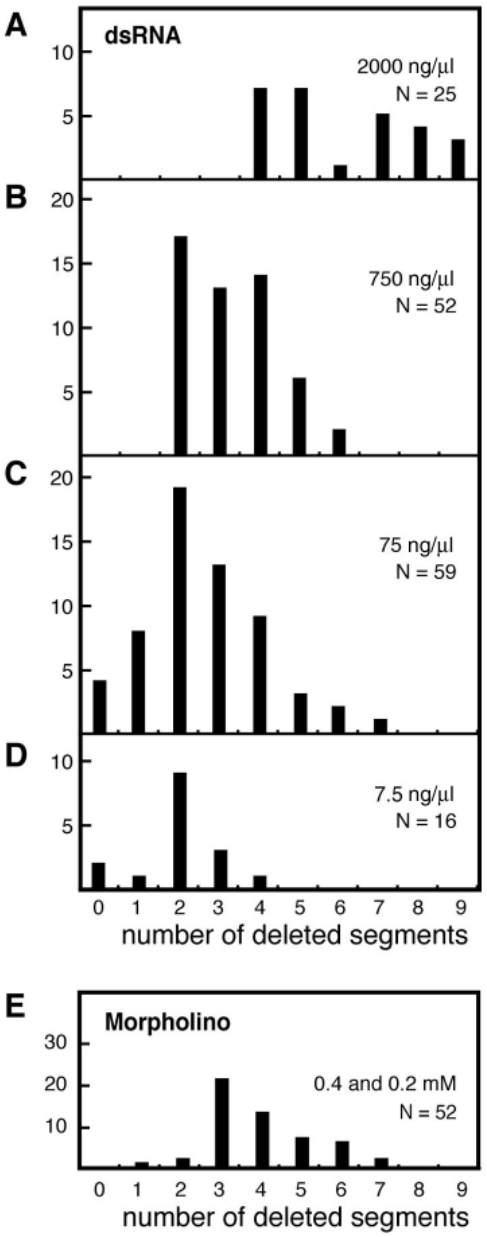

Fig. 6. The distribution of cuticular segmentation defects for different concentrations of dsRNA (A-D) and morpholino oligos (E) are shown. Given is the absolute number of cuticles that lacked a certain number of segments. Only individuals that had been injected with an effective dose of dsRNA/morpholino were counted (as judged by the presence of anterior transformations). The dsRNA concentrations ranged within several orders of magnitude (2000 ng/µl in A to 7.5 ng/µl in D). Nevertheless, the observed dose effect was relatively mild (compare A through D). The proportion of injected embryos that developed cuticles decreased with dsRNA concentration: ~20% with 2000 and 750 versus ~50% with 75 and 7.5 ng/µl, respectively. Additionally, the proportion of cuticles that produced a phenotype increased with higher concentrations of dsRNA: ~75% with 2000 and 750 versus ~50% with 75 and 7.5 ng/µl, respectively. Injection of low amounts of the lowest concentration resulted in 80% wild-type cuticles, suggesting that the minimal requirement for dsRNA was approached with 7.5 ng/µl. (E) Although morpholino oligos inhibit gene function by a different mechanism and are chemically distinct, a similar range of deletions was observed.

engrailed stripes prior to repair or elimination of partially deleted segments, we analyzed 28 germ bands just after they had completed segmentation, as indicated by the presence of an invaginated proctodeum. Most of these (61%) lacked four to six segments, and in a sizable fraction (18%) seven to eight segments were missing. This share of severe defects is indeed somewhat higher than in cuticle preparations. Taken together, the analysis of germband and cuticle phenotypes indicates that *Tc'giant* is pertinent for formation of 12 body segments, i.e. T1 through A9. Although we never obtained a larva lacking all these segments, these 12 segments all have a certain probability to be missing in *Tc'giant* RNAi larvae. In *Drosophila*, no more than four contiguous segments are affected in *giant* mutations. Thus, *Tc'giant* appears to have a different, and potentially much more central role in segment patterning than *Dm'giant*.

Discussion

In this study we present the first functional analysis of a non-*Drosophila* ortholog of the gap gene *giant*. Both sequence analysis and the similarity of some aspects of expression are strong evidence for orthology. However, other expression aspects deviate significantly, and our functional data show that the role of *Tribolium giant* in segmentation differs crucially from its *Drosophila* ortholog. These differences particularly concern the posterior *giant* domain, which is involved in segmentation of abdominal segments in *Drosophila*. Thus, divergent *giant* function implies different molecular mechanisms in patterning of posterior segments in long and short germ insects.

giant expression is conserved in the head but not in the abdomen

Expression of *Tc'giant* reveals both conserved and diverged aspects. In *Tribolium* and in *Drosophila*, *giant* is active in the maxillary segment, and later in a highly dynamic pattern in the brain. Therefore, this expression was probably present in the last common ancestor of all holometabolous insects. Another similarity is the appearance of a second expression domain in the posterior blastoderm. However, although in *Drosophila* both domains appear simultaneously, the posterior domain appears later than the anterior one in *Tribolium*. This could simply reflect the anterior to posterior sequence of segment formation in the beetle. However, relative to emerging segment primordia, this domain is located five segments more anterior. In *Drosophila*, the abdominal segments A5 to A7 arise right under the posterior *giant* domain (Kraut and Levine, 1991b; Petschek and Mahowald, 1990) while in *Tribolium* the anlagen of segments T3 through A2 are covered by the posterior *Tc'giant domain*. This shift in expression must reflect a fundamental change in gene function: either the *Tribolium* and *Drosophila giant* orthologs function by different mechanisms to pattern the same segments, or alternatively, if they act through a similar short range gradient mechanism, they must specify different segments.

The *Tc'giant* expression pattern also indicates divergent interactions with other segmentation genes. As the posterior domain arises in the late blastoderm, it is probably under zygotic control. This is in contrast to the situation in *Drosophila*, where the maternal genes *caudal* and *bicoid* cooperate to activate posterior *giant* expression (Rivera-Pomar

et al., 1995). Second, the posterior *Tc'giant* domain appears right within the *Krüppel* domain, and co-expression of both genes is observed in segment T3 for an extended time period. This again is in contrast to *Drosophila*, where strong mutual repression with *Krüppel* is crucial for regulation and proper function of *giant* (Kraut and Levine, 1991a). However, inhibitory interactions between *Tc'Krüppel* and the anterior *giant* domain could still be conserved, since these domains are mutually exclusive also in *Tribolium*. Finally, we note another intriguing feature: maturation of all three *Tc'giant* stripes (maxialla, T3 and A2) occurs in identical relation to the pair-rule gene register. In fact, the split of the posterior domain into distinct stripes concurs with pair rule patterning rather than preceding it (Fig. 3B,C). This raises the possibility that in later stages *Tc'giant* may be regulated by pair rule genes. In contrast to this, *Dm'giant* expression precedes pair rule activation and the gene unambiguously acts on a higher hierarchical level. Evidently, it is not only the position, but also many aspects of the regulatory network involving *giant* that differ between *Tribolium* and *Drosophila*. Our functional data confirm this, and in addition reveal that the function of the anterior domain has diverged in both insects.

Required for identity but not formation of head segments?

Like other *Drosophila* gap genes, *Dm'giant* functions in positioning pair-rule stripes, and its role in defining the anterior border of *eve* stripe 2 has been studied in much detail. Lack of *giant* function leads to expansion of this stripe (Arnosti et al., 1996; Small et al., 1992; Small et al., 1991) and to concomitant loss of the labial *engrailed* stripe (Eldon and Pirrotta, 1991; Petschek and Mahowald, 1990). By contrast, we did not detect any defects in head segmentation, and the labial *engrailed* stripe was unaffected in RNAi embryos. The most anterior segmentation defect that we observed was the deletion of the T1 *engrailed* stripe. The primordium of this stripe arises at a distance of one segment width to the posterior but two segment widths to the anterior *Tc'giant* domain. It seems more likely, therefore, that formation of this segment depends on the posterior rather than the anterior domain, which may not be involved in segmentation at all.

However, a crucial role of the anterior *Tc'giant* domain in homeotic specification is established by our experiments. *Tc'giant* RNAi larvae display a coordinated two-segment shift of all thoracic identities towards anterior. Generally, maxillary and labial segments are fully transformed towards T1 and T2 respectively, while the T1 segment adopts T3 identity, followed by two segments with identities ranging between T3 and abdomen. The shift of several segment identities implies that *Tc'giant* directly or indirectly regulates several Hox genes (i.e. those required for the identities of at least maxilla, labium, T1 and T2). Homeotic function of *Tc'giant* is not surprising, because *Drosophila* gap genes are known to regulate homeotic genes, and *Dm'giant*, specifically, defines the anterior border of *Antennapedia* (Reinitz and Levine, 1990). However, these functions in the regulation of homeotic genes are usually not evident from *Drosophila* gap gene phenotypes, as the homeotically affected regions are missing in the developed embryo because of the segmentation defects.

The homeotic two-segment shift in *Tc'giant* RNAi embryos follows 'all or nothing' kinetics: We never observed a homeotic shift across one segment width. This argues against a simple mechanism where a gradient of Tc'Giant protein emanating from the anterior domain would directly position gnathal and thoracic Hox genes. In addition, partial transformation of maxilla or labium is extremely rare – even though RNAi or morpholino knockdown experiments should produce many intermediate levels of residual gene function. Therefore, the coordinated regulation of several Hox genes by *Tc'giant* appears to rely on a mechanism involving tight thresholds. Interestingly, the phenotype of *jaws* (Sulston and Anderson, 1996), a mutant in the *Tc'Krüppel* gene (A. Cerny, G.B. and M.K., unpublished) displays a homeotic transformation that is opposite to *Tc'giant* phenocopies. In *jaws* larvae, thoracic and anterior abdominal segments are transformed to alternating pairs of maxillary and labial segments, while in *Tc'giant* RNAi embryos, maxilla and labium are transformed to T1 and T2, respectively. This suggests that *Tc'giant* and *Tc'Krüppel* have opposing functions in regulating the same set of thoracic and gnathal Hox genes. This may indicate mutual inhibition of *Krüppel* and the anterior *giant* domain as in *Drosophila* (but in contrast to the posterior *Tc'giant* domain). In addition, the homeotic phenotypes of both *Tc'Krüppel* (*jaws*) and *Tc'giant* display double segmental effects, suggesting the involvement of pair-rule genes in homeotic segment specification.

In *Tribolium*, *giant* has a long-range effect on abdominal patterning

Even though our RNAi and morpholino knock down experiments may not have achieved complete inactivation of the *Tc'giant* gene product, we frequently obtained segmentation phenotypes much more severe than those of *Dm'giant* null-mutations. In *Dm'giant* mutant embryos, the loss of the posterior domain results in a fusion of the *engrailed* stripes corresponding to segments A5 to A7, which are the segment primordia covered by this domain (Petschek and Mahowald, 1990; Langeland et al., 1994). *Drosophila* gap genes are expressed in domains whose diffuse boundaries function as short-range morphogenetic gradients that position pair-rule stripes (Hulskamp and Tautz, 1991; Rivera-Pomar and Jackle, 1996). Accordingly, both *Dm'giant* domains regulate pair-rule stripes in this manner (Langeland et al., 1994; Reinitz and Sharp, 1995; Small et al., 1991; Wu et al., 1998). However, the rather severe patterning defects observed at the pair-rule level are to some extent repaired during later stages of development (Klingler and Gergen, 1993), resulting in a less serious larval phenotype. By contrast, in *Tribolium* embryos displaying strong *Tc'giant* phenocopies, segmentation is disturbed in a region comprising twelve segments, ranging from T1-A9.

Intriguingly, the phenotype of *Tc'giant* knock-down larvae is not only stronger than that of *Dm'giant* mutants, but it also differs in the spatial and temporal relationships between expression domain and affected segments. The posterior domain of *Tc'giant* appears at the posterior pole of the blastoderm embryo at a time when the primordia of the first thoracic segments are patterned in this region. By this time, cellularization has most likely occurred (Handel et al., 2000). Thus, if thoracic and anterior abdominal defects of *Tc'giant* RNAi larvae reflect a short-range regulation comparable to that of *Dm'giant*, diffusion of the *Tc'Giant* protein across cell membranes would be required. However, the secondary

Tc'giant stripes actually resemble pair-rule stripes in width and spacing, in addition to the way they arise near the growth zone (see. Fig. 2F-H, Fig. 3B-D). It is therefore possible that these two stripes regulate pair-rule stripes in a manner more typical of pair-rule interactions in *Drosophila*, i.e. by direct activation and repression within precise boundaries.

In any case, the *Drosophila* paradigm cannot explain why very posterior abdominal segments require *giant* function in *Tribolium*, as these segments are formed at a large distance (spatially and temporally) from the posterior *Tc'giant* domain(s). The segment A9, for example, is frequently deleted in *giant* RNAi larvae, but arises six segments posterior to *Tc'giant* expression and long after expression has ceased (Fig. 2K). At this point, we can only speculate how *Tc'giant* exerts this long-range effect. For example, *Tc'giant* could be involved in setting up and/or starting a segmentation process in which a 'chain of induction' mechanism (involving gap or pair-rule genes) would pattern the growing abdomen (Meinhardt, 1982). Alternatively, *Tc'giant* may jump-start an oscillator machinery analogous to that underlying somitogenesis in vertebrates (Pourquie, 2001). In both cases, loss of *Tc'giant* would lead to improper setup and subsequent breakdown of the machinery, which could then result in defects in distant segments. However, one could also envisage the role of *Tc'giant* to be a rather general one. *Tc'giant* expression in the early growth zone may simply be required for making a proper growth zone, and reduction of *Tc'giant* activity may result in aberrant behavior of the affected cells during later growth, leading to segmentation defects in an indirect way. Evidently, more data are needed to distinguish between these disparate possibilities, including data about other posterior gap genes.

Gap genes in long and short germ embryos

The *Drosophila* blastoderm is evenly covered by seven overlapping gap gene domains, which provide ample positional information for the regulation of pair-rule stripes (see Fig. 7A). Our findings on *giant*, together with data for several other *Tribolium* genes, suggest that the positions of gap gene domains are conserved anteriorly, but have changed fundamentally in posterior body regions (compare Fig. 7A with 7B,C). For example, *Tc'otd-1* and *Tc'hunchback* are expressed in and are required for the formation of head and thoracic segments in both *Drosophila* and *Tribolium* (Finkelstein et al., 1990; Hulskamp and Tautz, 1991; Li et al., 1996; Royet and Finkelstein, 1995; Schroder, 2003; Wolff et al., 1995). In addition, *Tc'tailless* is expressed in similar head regions as in *Drosophila* (Mahoney and Lengyel, 1987; Schroder et al., 2000; Weigel et al., 1990), and we have shown in this paper that the anterior stripe of *Tc'giant* covers the maxillary segment in both beetle and fly. Anterior conservation is also observed in more basal insect taxa: In the grasshopper *Schistocerca*, *hunchback* is also expressed in gnathal and thoracic segments (Patel et al., 2001). At the level of the head gap genes, *otx/otd* similarities are observed even between *Drosophila* and vertebrates, suggesting conserved principles of head patterning among distantly related bilaterian animals (Reichert and Simeone, 1999).

Posteriorly, by contrast, only the abdominal *Tc'hunchback* domain is expressed in similar segment primordia as in *Drosophila* (Wolff et al., 1995) and *Schistocerca* (Patel et al., 2001). In the latter, there is an additional abdominal domain in A4/A5, which is not present in *Tribolium* or *Drosophila*. All other gap genes that affect the abdomen in *Drosophila* and have been investigated in *Tribolium* cover different segment primordia in these two species (compare fate maps in Fig. 7A with 7B,C). The posterior border of *Tc'Krüppel* expression, for example, is shifted about three segments towards anterior, as revealed by our double stainings (Fig. 3). Thus, it is active in thoracic segments only, whereas *Drosophila Krüppel* extends well into A3 (Hulskamp and Tautz, 1991). More dramatically, expression of the posterior *Tc'giant* domain has shifted by four to five segments. Finally, the posterior *Tc'tailless* domain is probably not involved in abdominal segmentation at all. This gene is expressed at the posterior pole of the blastoderm as in *Drosophila*. However, because of the short germ mode of development, only terminal structures, posterior thoracic

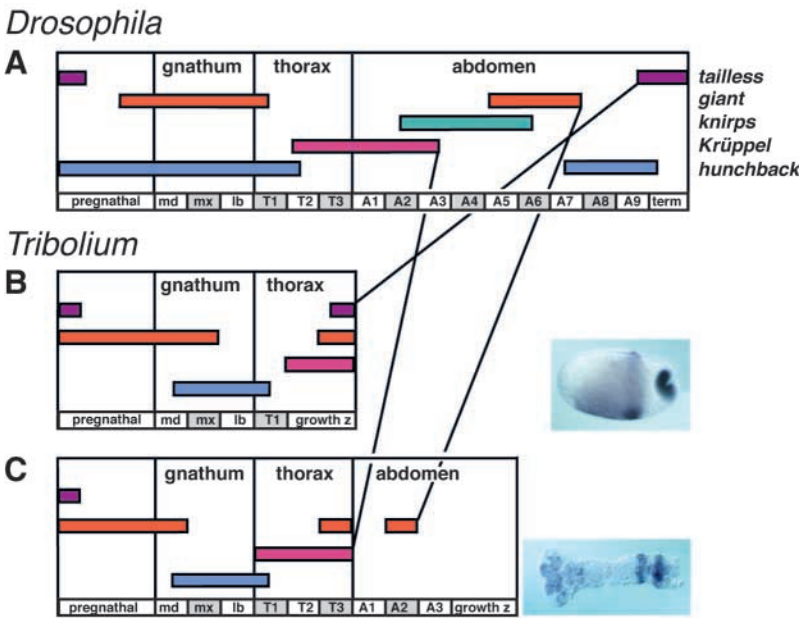

Fig. 7. The expression domains of *Drosophila* and *Tribolium* gap genes. Head, thorax and abdomen are separated by vertical bars. (A) Fate map and gap gene expression in the *Drosophila* blastoderm. (B,C) Late blastoderm and early germ band stages in *Tribolium* (the photos to the right illustrate the stages represented by B and C). (B) In the *Tribolium* blastoderm only head and thoracic segments are specified. The posterior pole comprises the growth and patterning zone (growth z) and probably includes terminal cells (not shown). Extra-embryonic tissue has been omitted for simplicity. (C) During germ band growth, the posterior *giant* domain splits into stripes located in segments T3 and A2, far anterior of the segment primordia covered by *Dm'giant* (A). Although anterior expression domains of gap gene orthologs appear largely conserved (compare anterior domains of *tailless*, *giant* and *hunchback*), the posterior gap domains in *Tribolium* are shifted relative to the segment primordia. See discussion for further details.

segments and the growth zone are formed at this time, whereas abdominal segments are formed long after Tc'*Tailless* expression has faded (Schroder et al., 2000). Thus, given the absence of *tailless* and the shifted domain of *giant*, there is a shortage of gap domains in the *Tribolium* abdominal region. Even if a *Tribolium knirps* gene should exist and be expressed in the abdomen, it is difficult to conceive how two gap domains alone (*knirps* and posterior *hunchback*) could pattern all abdominal segments from A3 to A10. Therefore, if an abdominal patterning mechanism similar to that in *Drosophila* would work in *Tribolium*, it would require additional gap genes in order to provide sufficient positional information for 10 abdominal segments. The *Tribolium* mutants *bollig* and *krusty* have deletions of several adjacent segments and were therefore characterized as gap mutants (Maderspacher et al., 1998). Molecular mapping showed they are not *Tc'Krüppel* alleles (G.B., unpublished), and their phenotypes are different from the range of *Tc'giant* RNAi phenotypes. Thus, either of these two mutants may represent *Tc'knirps* or an additional gap gene. However, other classes of mutations could also lead to gap-like phenotypes, for example regulatory mutants deleting pair-rule enhancer elements. Therefore, the issue of whether additional gap genes are required for short germ segmentation remains unresolved.

Although the expression and function of pair-rule genes in the *Tribolium* abdomen appears to be largely conserved (Brown and Denell, 1996; Maderspacher et al., 1998; Sommer and Tautz, 1993), we show that for gap gene orthologs this is not the case. If the beetle mode of short germ embryogenesis indeed represents the ancestral mode (Tautz et al., 1994), our data suggest that it is changes in the abdominal gap gene system that lie at the heart of the evolution from short to long germ embryogenesis. Although the exact role of the *Tribolium* gap gene orthologs remains to be elucidated, they have clearly experienced more evolutionary change than the pair-rule and segment polarity networks.

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