Dev Genes Evol (1998) 208:558–568

ORIGINAL ARTICLE

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Pair-rule and gap gene mutants in the flour beetle *Tribolium castaneum*

Received: 8 May 1998 / Accepted: 17 June 1998

Abstract Early pattern formation in the *Drosophila* embryo occurs in a syncytial blastoderm where communication between nuclei is unimpeded by cell walls. During the development of other insects, similar gene expression patterns are generated in a cellular environment. In *Tribolium*, for instance, pair-rule stripes are transiently expressed near the posterior end of the growing germ band. To elucidate how pattern formation in such a situation deviates from that of *Drosophila*, functional data about the genes involved are essential. In a genetic screen for *Tribolium* mutants affecting the larval cuticle pattern, we isolated 4 mutants (from a total of 30) which disrupt segmentation in the thorax and abdomen. Two of these mutants display clear pair-rule phenotypes. This demonstrates that not only the expression, but also the function of pair-rule genes in this short-germ insect is in principle similar to *Drosophila*. The other two mutants appear to identify gap genes. They provide the first evidence for the involvement of gap genes in abdominal segmentation of short-germ embryos. However, significant differences between the phenotypes of these mutants and those of known *Drosophila* gap mutants exist which indicates that evolutionary changes occurred in either the regulation or action of these genes.

Keywords Evolution of development · Short-germ embryo · Long-germ embryo · Segmentation · Mutagenesis

Introduction

Segmented animals are found in a number of phyla, most notably arthropods, annelids and chordates. One mechanism for generating a metameric body plan during embryogenesis has been elucidated in *Drosophila*. The phenotypes of the major mutant classes that affect segmentation in this organism, i.e. gap, pair-rule and segment-polarity mutants, suggested that during this pattern formation process, the embryo is subdivided in three steps into increasingly smaller units, until the segmental primordia are eventually generated (Nüsslein-Volhard and Wieschaus 1980). This concept was corroborated by the molecular analysis of the genes involved which are expressed in corresponding patterns of increasing detail (Ingham 1988). In the meanwhile, many mechanistic aspects of this pattern refinement process have been clarified (Pankratz and Jäckle 1993).

Although some homologs of *Drosophila* segmentation genes appear to be involved in somitogenesis in vertebrates (Holland et al. 1997; Müller et al. 1996), it is questionable if the mechanism of this pattern formation process is conserved even within insects. *Drosophila* as a paradigm for insect development is problematic since this species belongs to a derived phylogenetic group, the diptera, with a mode of development not typical for insects in general. *Drosophila* develops as a long-germ embryo, i.e. the embryo is large relative to the egg size, and all segments become defined by the blastoderm stage. In contrast, more ancestral insects have short-germ embryos which initially occupy a small portion of the egg and only in the course of development grow to fill up the entire egg (Sander 1976). Only anterior segments are determined during the blastoderm stage in short-germ embryos, whilst posterior segments arise from a growth zone at the rear end of the germ band. This growth zone generates cells which are then recruited into the newly arising segments. In this respect, short-germ embryos are more similar to chordate embryos which also generate the majority of somites from a posterior growth zone, the tail bud. Pattern formation in a growth zone could be very different from that in the syncytial blastoderm where transcription factors can diffuse and form long-reaching gradients. Whilst the fundamental principle of pattern formation in *Drosophila* segmentation can be summarized as the successive interpretation of positional information provided by such gradients, segmentation in a

Edited by M. Akam

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growing field of cells, like in the abdomen of short-germ embryos, may instead be dominated by very different mechanisms, for example temporal regulation, as has been proposed for somitogenesis in the chick embryo (Palmeirim et al. 1997).

The flour beetle *Tribolium castaneum* is a short-germ insect that can be easily bred in the laboratory and is amenable to genetic analysis (Beeman 1987; Sulston and Anderson 1996). Various homologs of *Drosophila* segmentation genes have been studied in *Tribolium* (Brown and Denell 1996; Brown et al. 1994b; Li et al. 1996; Nagy and Carroll 1994; Patel et al. 1994; Sommer and Tautz 1993; Wolff et al. 1995). The expression of these homologs reflects and illustrates the short-germ situation. Expression domains located in the anterior half of the *Drosophila* blastoderm embryo are also present in the *Tribolium* blastoderm, for example those of the gap genes *orthodenticle* (*otd*), *hunchback* (*hb*) and *Krüppel* (*Kr*; Li et al. 1996; Sommer and Tautz 1993; Wolff et al. 1995). Similarly, only the two or three anteriormost stripes of pair-rule genes are formed during the blastoderm stage whilst more posterior pair-rule stripes form, one after the other, during later growth of the germ band (Sommer and Tautz 1993). It is conceivable that the anterior pair-rule stripes are formed as in *Drosophila*, i.e. by the integration of short-range gap gene gradients. The manner by which the posterior stripes of short-germ embryos are formed is an open question. Attempts to clone *Tribolium* homologs of the posterior gap genes *knirps* (*kni*) and *giant* (*gt*) have failed so far. Therefore it is uncertain if this class of segmentation genes also functions in the growing germ band. Whilst the expression patterns of pair-rule and segment-polarity genes suggest that they have a role in the formation of the posterior segments, it remains to be elucidated whether they do so in the same manner as in *Drosophila*.

Recently, three segmentation mutants were identified in *Tribolium* (Sulston and Anderson 1996). Two of these mutants were classified as members of the segment-polarity and pair-rule mutant classes, respectively, whilst the third mutant combines apects of homeotic as well as gap gene phenotypes. None of these phenotypes is very similar to any known *Drosophila* phenotype. In this paper, we describe four additional segmentation mutations which we identified in an ongoing screen for embryonic patterning mutants in *Tribolium*. The phenotypes of these mutants are more easily interpretable and suggest that a class of genes with "pair-rule" function exist in short-germ insects as well as long-germ insects like *Drosophila*.

Materials and methods

Mutant screen

As wild-type strain in the mutagenesis we used the San Bernardino strain (established by Alexander Sokoloff) which we obtained from Klaus Sander's laboratory in Freiburg. A number of different mutagenesis protocols were used. In the following we describe what seemed to work best. Male beetles were starved in single confinement (microtiter wells) for 4–5 days at 30°C until about one-fifth of the animals had died. They then were incubated over night at room temperature in 90-mm petri dishes with one layer of Whatman paper soaked with 2 ml 50 mM ethyl methanesulfonate = methanesulfonic acid ethyl ester (EMS) in sugar water (Beeman et al. 1989). Subsequently, the mutagenized males were mated to virgin females for 2 days and discarded afterwards. F1 males from this cross were singly mated to seven untreated virgin females each. After 1 week the males were separated from the females and retained in vials with flour and bulgur topping (all crosses were done at 25 to 32°C). From their offspring, 20 F2 virgins were collected from each line and crossed back to the corresponding F1 male (i.e. their father). From these crosses, eggs were collected and processed for cuticle preparations.

Maintainance of embryonic lethals

We keep embryonic lethals by mating one male heterozygous for the mutation with eight to ten wild-type females. These beetles are transferred to fresh food every 3 weeks at 25°C. Within this time period the offspring from the cross is still small enough to fall through the 0.80-mm sieve used to separate beetles and flour, such that the single adult male is not replaced by a son which may not carry the mutation. The flour with eggs and young larvae is incubated until most animals are mature, and then kept at 18°C as a backup. Since male beetles live longer than females, additional wild-type females are added occasionally. After 6–9 months the lines need to be re-established because the male carriers die or their fertility declines. To again identify carriers of the mutation from the offspring of the original male, single-male egglays are set up each consisting of one male and three virgin females. If a line has died out, single pair egglays are established with non-virgin adults from the 18°C backup stocks. In most cases, 24 single-male egglays are sufficient to identify at least 4 egglays where both the male and at least one of the females carry the mutation and thus produce mutant offspring which is identified in cuticle preparations. From these newly identified male carriers, new lines are established by adding wild-type females. As a safeguard to loss or contamination, each mutation is kept in three to four parallel lines.

Egg collections and microscopic preparations

For cuticle preparations, eggs were collected from populations of heterozygous beetles whose genotype had been previously affirmed in single pair matings. The beetles were allowed to lay eggs for 3 days at 30°C. Eggs then were separated from the flour using a 0.30-mm sieve, and incubated on polyamid gauze of mesh size 0.25 mm for 4 days. Freshly hatched larvae display geotropism and tend to escape through the sieve such that the eggs retained above are enriched with mutant embryos. The eggs were cleaned from attached flour by washing in undiluted commercial bleach (Klorix), and then in water, and mounted on a microscope slide in a 1:1 mixture of Hoyer's medium and lactic acid. After clearing overnight at 60°C, cuticles were photographed using dark-field optics. For histochemistry, eggs were harvested from larger populations of beetles which were the offspring of heterozygous parents. Since only two-thirds of these beetles are heterozygous, one-ninth of the eggs layed by these populations are homozygous for the mutation. In situ hybridization and immunohistochemistry were performed as described in Wolff et al. (1995).

Results

Identification of four new segmentation mutants in *Tribolium*

Tribolium is an emerging new insect system for studying development by genetic means (Beeman 1987; Sulston

and Anderson 1996). However, only part of the genome is covered by chromosomal rearrangements useful as balancers. Moreover, there are ten chromosomes in *Tribolium*, which makes it impractical to set up parallel screens for each chromosome as was done in *Drosophila* (Nüsslein-Volhard et al. 1984). Therefore, we utilized a screening scheme similar to that of Sulston and Anderson (1996), in which mutations in genes located on any autosome are identified after only two generations (see Materials and methods). To improve the efficiency of the screening procedure, we designed a block system which allows 24 lines to be processed in parallel at each step. This system, which will be described in detail elsewhere, resulted in an about twofold improvement in the screening efficiency when compared to the technique used previously (Sulston and Anderson 1996). More importantly, this system is also used for stockkeeping (see Materials and methods). Stockkeeping is a major problem for lethals not covered by balancers when working with species not amenable to cryopreservation or other long-term storage techniques. With our method up to 100 embryonic lethals can be maintained by one person, which is a significant improvement upon previous methods and makes work with embryonic lethals in *Tribolium* feasible on a larger scale.

To date, we have screened 2500 lines from which we obtained 30 mutants with cuticle phenotypes sufficiently interesting to warrant maintaining the stocks. Of these, 6 mutants affect segmentation of thoracic or abdominal segments. Here we describe 4 of these, 2 pair-rule and 2 gap mutations.

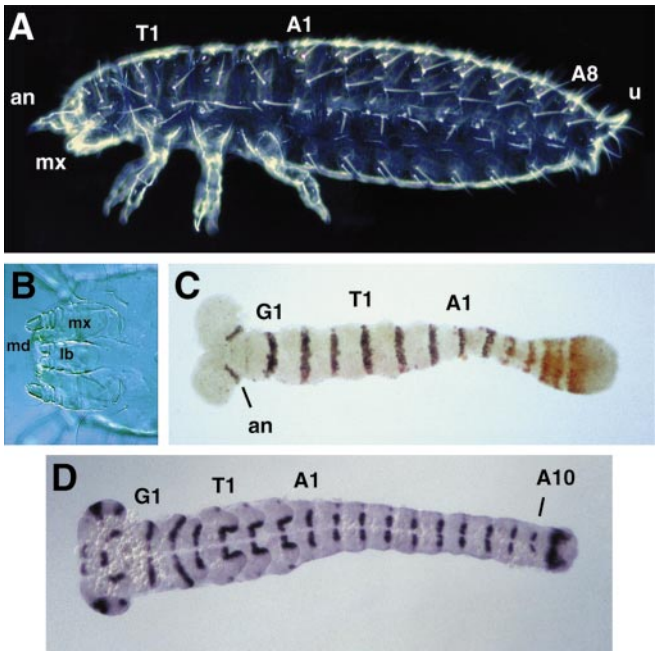

Fig. 1A–D Cuticular and molecular markers in wild-type *Tribolium*. **A** Lateral view of a differentiated *Tribolium* larva with head, thorax, abdomen and telson (anterior to *left*, dorsal *up*; dark field optics). In the head, the left antenna (*an*) and maxilla (*mx*) are in the focal plane. In the telson (which forms during development as a fusion of abdominal segments A9 to A11), the dorsal urogomphi are labelled (*u*). A stoma in T2 can be seen as a circle above the leg, and smaller stomata are also visible in all abdominal segments. **B** Ventral view of the mouth parts (Nomarski optics). The labium (*lb*) in the centre is flanked on both sides by the maxillae (*mx*). Somewhat out of focus, the mandible can be seen (*md*). **C** Ventral view of a germ band preparation doubly stained for *engrailed* (*en*; *black*) and *even-skipped* (*eve*) protein (*brown*). During germ band growth, *en* is expressed in segmental stripes, and *eve* in double-segmental stripes near the posterior growth zone (*right*). A short distance behind the growth zone the double-segmental *eve* stripes split into segmental stripes. In the head lobes (*left*), *en* expression is also detected in the antennal primordia. **D** Expression of *wingless* (*wg*) mRNA in a fully extended germ band. In addition to segmental stripes, *wg* is also expressed in several domains in the head and in a broader band in the growth zone area. *G1, T1, A1, A8* and *A10* label the correspondingly numbered gnathal, thoracic and abdominal segments

itchy (*icy*) is required for even-numbered abdominal *en* stripes

As a reference, a cuticle preparation of a wild-type first instar larva is depicted in Fig. 1A,B. The gnathal (G1 to G3) and thoracic segments (T1 to T3) are distinguished by their appendages, and in the pre-gnathal head labrum and antenna can be recognized. In the abdomen eight segments are evident (A1 to A8), each with bilateral stomata (in the thorax, only T2 carries a pair of stomata). During earlier germ band stages, ten complete abdominal segments are formed, of which segments A9 and A10 fuse with the telson during germ band shortening.

In *icy* mutants, the number of segments is reduced in comparison to wild type. This results in significantly shorter embryos (Fig. 2A). The pregnathal head and the posterior terminus remain unaffected. Mandible and maxilla are deleted whilst the labium is present in most mutant embryos. In the thorax only one segment is retained. Since T1 and T3 do not have tracheal openings, the presence of a stigma appears to identify this segment as T2, i.e. the first and third thoracic segments seem to be deleted in this mutant. Since the identity of abdominal segments cannot be determined unequivocally, only the number of missing segments was counted. About 40% of the mutant larvae have four segments less than the wild type. A similar proportion lack three segments, and another 20% of the larvae lack only two abdominal segments (Fig. 3). Hence, there is some variation in the abdomen whilst the phenotype is quite consistent in the gnathal and thoracic segments. The deletion of maxilla, T1, T3 and some abdominal segments hint at pair-rule periodicity. However, the deletion of the mandible does not fit this pair-rule deletion profile.

To determine how this cuticular pattern correlates with changes in segmentation gene expression, we monitored the expression of two segment-polarity genes and one pair-rule gene, i.e. the *Tribolium* homologs of *engrailed* (*en*), *wingless* (*wg*), and *even-skipped* (*eve*), in mutant embryos. The distribution of *eve*-protein in *icy* embryos is not obviously altered in comparison to wild type (Figs. 1C and 2B; this also pertains to earlier stages

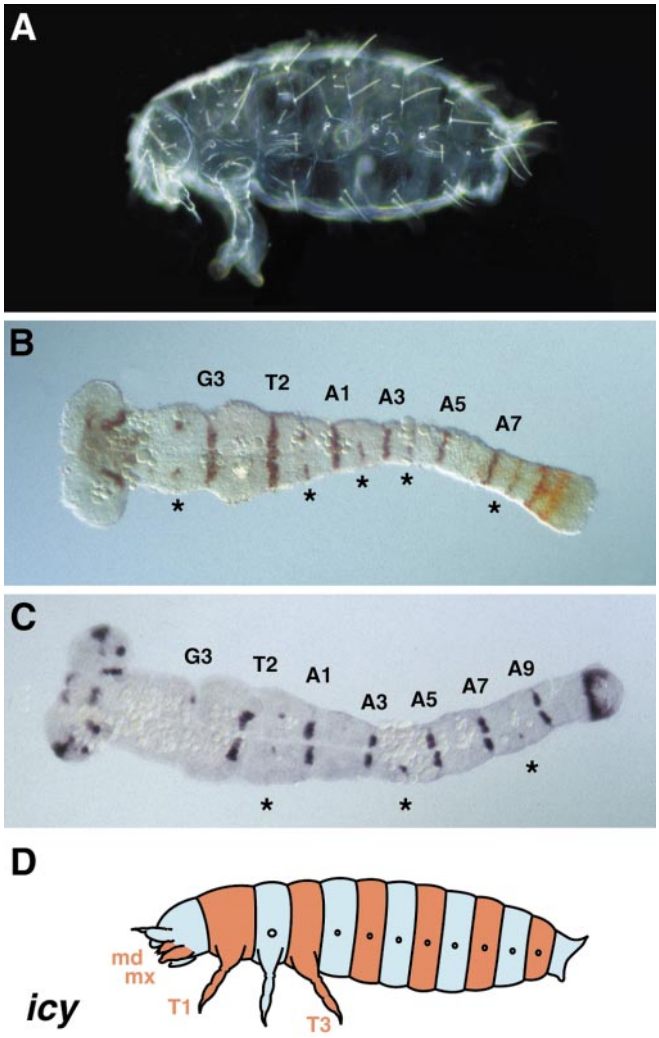

Fig. 2A–D Phenotype of *itchy* (*icy*). **A** The head of mutant larvae appears smaller because the mandibular and maxillary structures are missing. Only one thoracic segment is present which bears a stoma. In this larva, five abdominal segments are formed. The telson is not affected. **B** Mutant germ band stained for *en* (*black*) and *eve* (*brown*). In alternating segment primordia, the *en* stripe is absent or reduced (residual stripes are labelled by *asterixes*). The *eve* pattern remains unaffected. **C** Mutant germ band stained for *wg*. The effect of *icy* on *wg* expression is similar to *en*, with the exception of the labial segment (*G3*) where *wg* is severely affected but *en* is not. **D** Sketch of epidermal areas affected by *icy*. Note that the segmental deletions shown in *red* are approximate only – the precise intra-segmental borders of deletion domains cannot be specified with the markers available

of development, when those segments are formed which are more severely affected by the mutation; not shown). In contrast, the expression of segment-polarity genes is clearly disturbed. In mutant embryos there is only one *en* stripe of a wing-shaped appearance as is characteristic for thoracic stripes (Fig. 2B). Anterior to this stripe a single gnathal stripe is formed that probably corresponds to the labial segment primordium as judged from the cuticle phenotype. In most mutant embryos in the fully extended germ band stage, five complete *en* stripes are visible in the abdominal region. At earlier stages there frequently are weak *en*-expressing domains, often confined to single cells, located in-between these complete stripes. This alternation of partially deleted and fully intact stripes clearly shows that the double-segment characteristic of this phenotype extends into the abdomen. The distribution of *wg* RNA in mutant embryos closely mimicks the *en* pattern (Fig. 2C). Only the single gnathal *wg* stripe is usually weaker than the corresponding *en* stripe, and residual abdominal stripes are seen less frequently.

Taken together with the cuticle deletion pattern, it is evident that the *icy* mutation leads to the deletion of alternating segments across the entire embryo. The frame of the deletions is such that in the abdomen even-numbered stripes are affected. Evidently, *icy* can be classified as a pair-rule gene. Only the deletion of the mandibular segment does not conform to pair-rule periodicity and may indicate a separate function of this gene (see Discussion).

Odd-numbered *en* stripes are affected by *scratchy* (*scy*)

The cuticles of *scy* mutants lack the mandibular and labial segments, whereas the maxilla and pregnathal head appear normal. Only two thoracic segments remain, the posterior of which carries a stigma and therefore appears to be the T2-segment (Fig. 4A). However, the expression of *en* and *wg* clearly show that this intact thoracic segment must in fact be T3 (see Discussion). In the abdomen, the *scy* phenotype displays a higher variability than *icy*: only one to three abdominal segments are usually deleted (Fig. 3), and approximately 20% of mutant embryos have no abdominal defects at all. In the terminus, in many larvae one or both of the urogomphi is missing.

Mutant germ bands stained for the *en* protein (Fig. 4B, C) display only one complete gnathal and two complete thoracic stripes. Often, mutant embryos still show residual expression of the affected stripes (these residual stripes are exceptionally strong in the germ band in Fig. 4B; the embryo in Fig. 4C is a more typical example). This allows identification of the fully formed *en* stripes as corresponding to the maxillary and the T1 and T3 segment anlagen. In the posterior abdomen, the affected stripes can approach the strength of intact stripes. With increasing developmental age, the residual stripes tend to disappear in gnathal and thoracic regions, and to weaken further in the abdomen. Interestingly, the register of deleted segments in *scy* is opposite to that observed for *icy*, i.e. odd-numbered abdominal *en* stripes are affected. Thus, *scy* also leads to full or partial deletion of alternating segments. The deletion of one or both urogomphi in *scy* mutant larvae conforms to this frame, since this structure develops from segment A9. Analysis of *wg* expression confirms the observations obtained for *en* (Fig. 4D). As in *icy*, the *eve* pattern was not obviously altered in *scy*$^-$ embryos (data not shown).

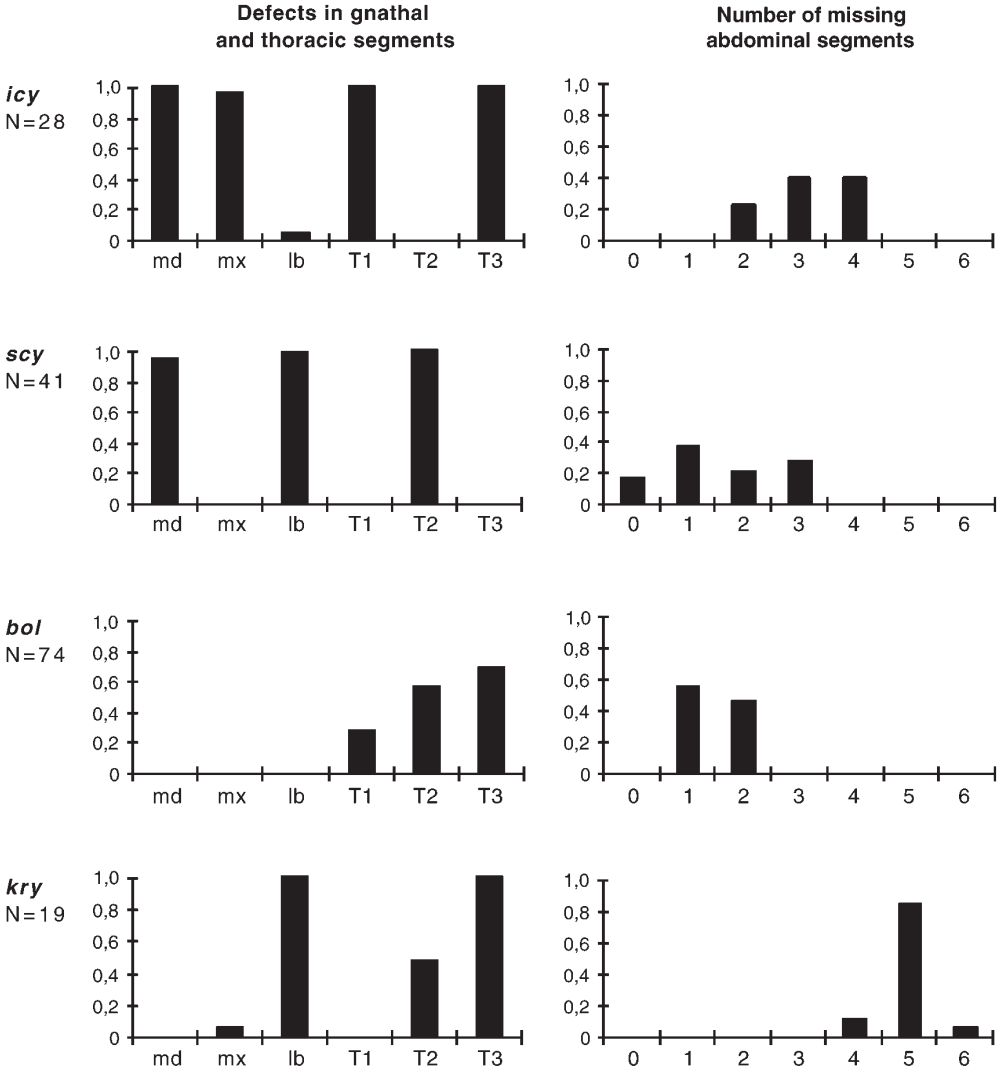

Fig. 3 Phenotypic variation of *ichy*, *scratchy* (*scy*), *bollig* (*bol*) and *krusty* (*kry*) cuticle phenotypes. In the *left column*, defects in individual gnathal and thoracic segments are depicted. The *bar graphs* indicate the relative frequency of defects in a particular segment. In the case of *kry*, for example, the T2 segment is deleted in about 50% of all mutant embryos. In the *right column*, the total number of deleted abdominal segments is given instead, since abdominal segments cannot be unambiguously identified by specific landmarks. Partially missing segments were weighted with the factor 0.5. For *kry*, for instance, the corresponding graph indicates that in 80% of all mutant embryos five abdominal segments were missing, whilst in the remaining embryos either four or six segments were absent

A group of adjacent segments is affected by *bollig* (*bol*)

bol mutant larvae display a variable segmentation phenotype in thorax and abdomen (Fig. 5A). Pregnathal and gnathal regions are usually intact. In the thorax, defects increase towards the posterior, i.e. T1 is affected in approximately 10%, T2 in 40% and T3 in 60% of mutant larvae (Fig. 3). Most sensitive is the first abdominal segment, which is absent in over 90% of larvae displaying segmentation defects. Also, the second abdominal segment is often deleted whilst segments posterior to A2 usually remain intact. In a small number (<10%) of larvae with segmentation defects in thorax or anterior abdomen, an additional defect in the telson can be seen that consists of the formation of additional urogomphi (not shown). This alteration resembles that of previously published homeotic mutants which were described as a transformation of A10 towards A9 (Beeman et al. 1989). This latter defect was never found in wild-type sibling larvae and therefore appears to be linked to the *bol* mutation and not to be caused by some other mutation in this strain. The segmentation phenotype is temperature sensitive; the numbers in Fig. 3 correspond to 32°C; at 25°C the frequency of thoracic defects is reduced to about half. Probably, the phenotype is not fully penetrant since it is displayed by only about 19% of differentiated embryos obtained from heterozygous parents, instead of 25% (300 first instar larvae were scored). Frequently, branched legs can be seen in *bol* mutant larvae, i.e. legs with two or more distal portions. Similar leg phenotypes have also been observed for *godzilla* (*god*); these are probably secondary effects of the segmentation phenotype, i.e. the disruption of *en* and *wg* stripes leads to pattern rearrangements which define new areas of distal outgrowth (Sulston and Anderson 1996).

The expression of *en* is disturbed in *bol* embryos in a manner consistent with the cuticle phenotype. Thoracic and anterior abdominal stripes are fully or partially fused or deleted, with T3 and A1 being affected most frequently (Fig. 5B,C). In the gnathal region and posterior abdomen the pattern is usually not affected. Together with the cuticle phenotype this shows that *bol* is a gap gene required for a group of adjacent segments in thorax and an-

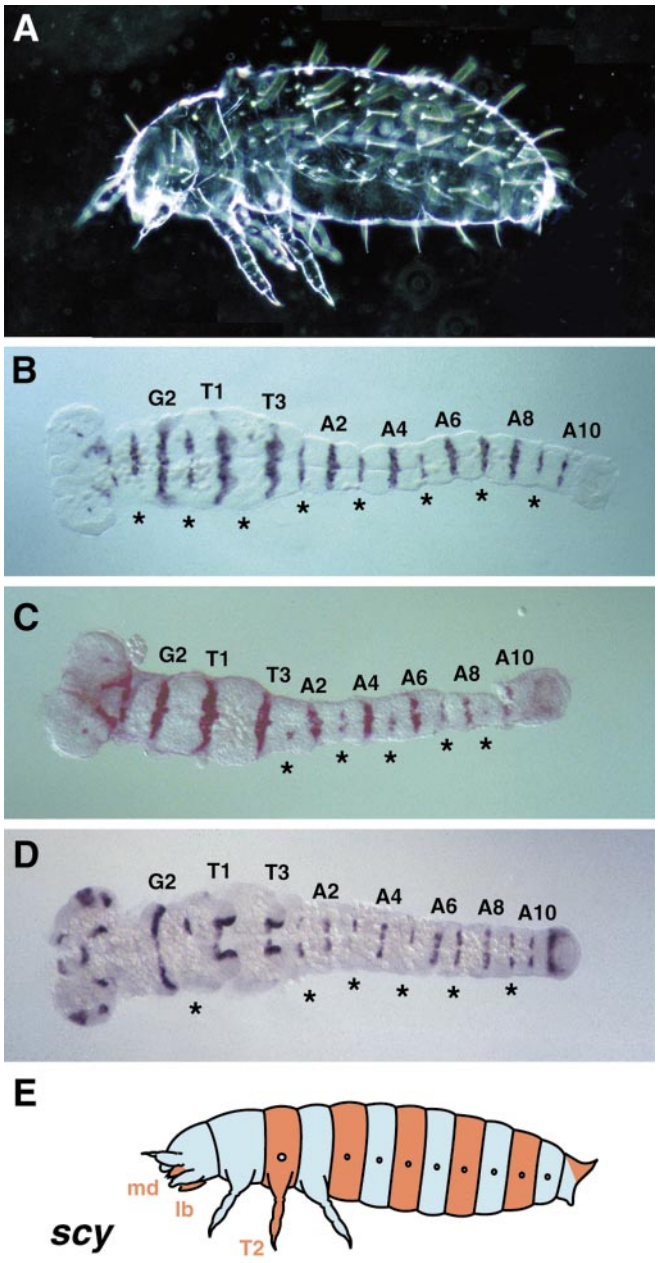

Fig. 4A–E Phenotype of *scratchy* (*scy*). **A** The head of *scy* mutant larvae is reduced in size due to the missing mandibular and labial segments. Only two segments are usually formed only in the thorax. In the larva depicted, three segments are missing in the abdomen, and the urogomphi (which derive from segment A9) are partially deleted. **B**, **C** *en* expression in mutant germ bands. Alternating segments are affected in a frame complementary to that observed in *icy*. **D** Germ band stained for *wg*. **E** Sketch of our interpretation of the *scy* phenotype. The urogomphi in the telson, which derive from segment A9, are also affected

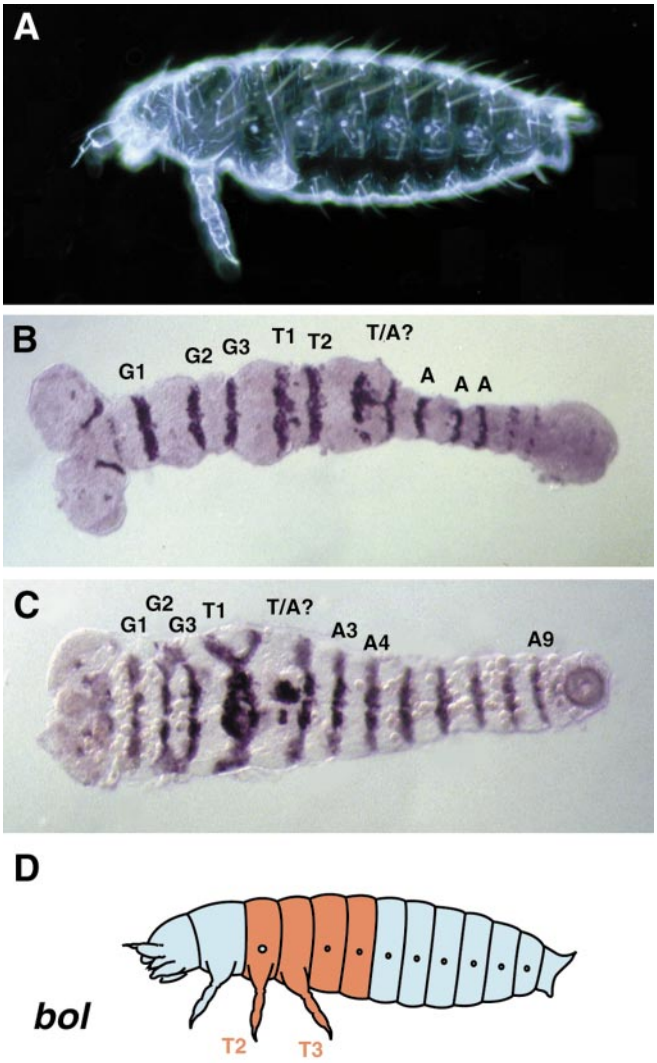

Fig. 5A–D Cuticle and germ band defects in *bollig* (*bol*) mutants. **A** Head and telson are unaffected in *bol*$^{-}$; in the larva depicted, one thoracic segment is missing, and another one is only partially formed (the stoma of T2 is present but the appendage is severely malformed). In the abdomen, only six segments are present. **B**, **C** Mutant germ bands stained for *en*. Severe defects are observed in the thoracic and anterior abdominal region. Minor irregularities also can extend into the gnathal region, like in the embryo in **B** where the distance between G2 and G3 stripes is reduced. (the lateral fusion of G2 and G3 stripes in **C** is similar to wild type at this stage of development). **D** Summary of the *bol* cuticle phenotype

terior abdomen. Rare disturbances in the telson may hint at an additional function of *bol* in this region.

Gap mutations should also alter the expression pattern of pair-rule genes. Therefore we examined the expression of *eve* in the *bol*$^{-}$ background. However, we were unable to demonstrate a clear and distinct alteration of the *eve* pattern. In a few embryos, the third and fourth *eve* stripe seem not to be fully separated, but this alteration was not consistently detected. Therefore, we cannot ascertain a hierarchical relation of *bol* and *eve* as to be expected between a gap and pair-rule gene. We also note, however, that some *Drosophila* gap gene mutations (i.e. *gt* mutations) do not severely disrupt pair-rule gene patterns, although they display conspicuous cuticle defects.

Three domains are impaired in *krusty* (*kry*) mutants

In the gnathal region of *kry* mutant larvae, the labial segment is always absent. Mandible, maxilla, and the pregnathal head are usually unaffected. All larvae lack segment T3, and in about half the second thoracic segment (as identified by the presence of a stoma) is also partially or completely deleted (Fig. 6A). In the abdomen, three segments remain intact in most larvae, whereas about 15% have either two or four abdominal segments (Fig. 3). In addition to these defects, the urogomphi, which derive from segment A9, are missing in most *kry*$^-$ larvae.

Mutant germ bands stained for *en* display two properly formed stripes in the gnathal region, which correspond to the mandibular and maxillary segment primordia. The following *en* stripe is usually as strong as in wild-type embryos, but often with altered morphology, i.e. bent or fused with more posterior stripes (Fig. 6C). Appendages in older embryos identify this stripe as thoracic, i.e. it represents T1 (Fig. 6D). More posteriorly, two or three *en* stripes can often be seen within the deletion domain. These stripes usually are well-formed laterally but not complete in the medial region. In the posterior abdomen, i.e. posterior to the area of disrupted segmentation, three *en* stripes again are perfectly normal (Fig. 6D). These observations suggest that in *kry*$^-$ embryos a continuous stretch of segments forms improperly or incompletely which extends from the thorax into the abdomen. Outside this region, two additional segments are affected, i.e. the labium and A9. Therefore, *kry* is classified as a gap mutation with additional functions in G3 and A9 (see Discussion).

The effect of *kry* on the expression of *eve* was also assessed. At stages when the segmentally disrupted parts of the germ band are generated by the growth zone, *eve* protein is detected ectopically in a highly disordered manner. *eve* protein is found in a single broad domain covering much of the affected area, and this expression domain is not demarcated by clear boundaries (Fig. 6B). At later stages, when the posterior abdominal segments are being formed, the normal *eve* pattern of double-segmental and segmental stripes becomes re-established (Fig. 6C). This clearly argues for a region-specific effect of *kry* on the spatial regulation of *eve* and hence confirms its gap-like character.

Since the deletions in *bol* mutant embryos largely overlap with those observed in *kry*, we considered that these mutants could represent alleles of the same locus. Therefore, we tested these mutants for complementation by crossing males heterozygous for *kry* (as detected in previous crosses) to virgin females from the *bol* strain,

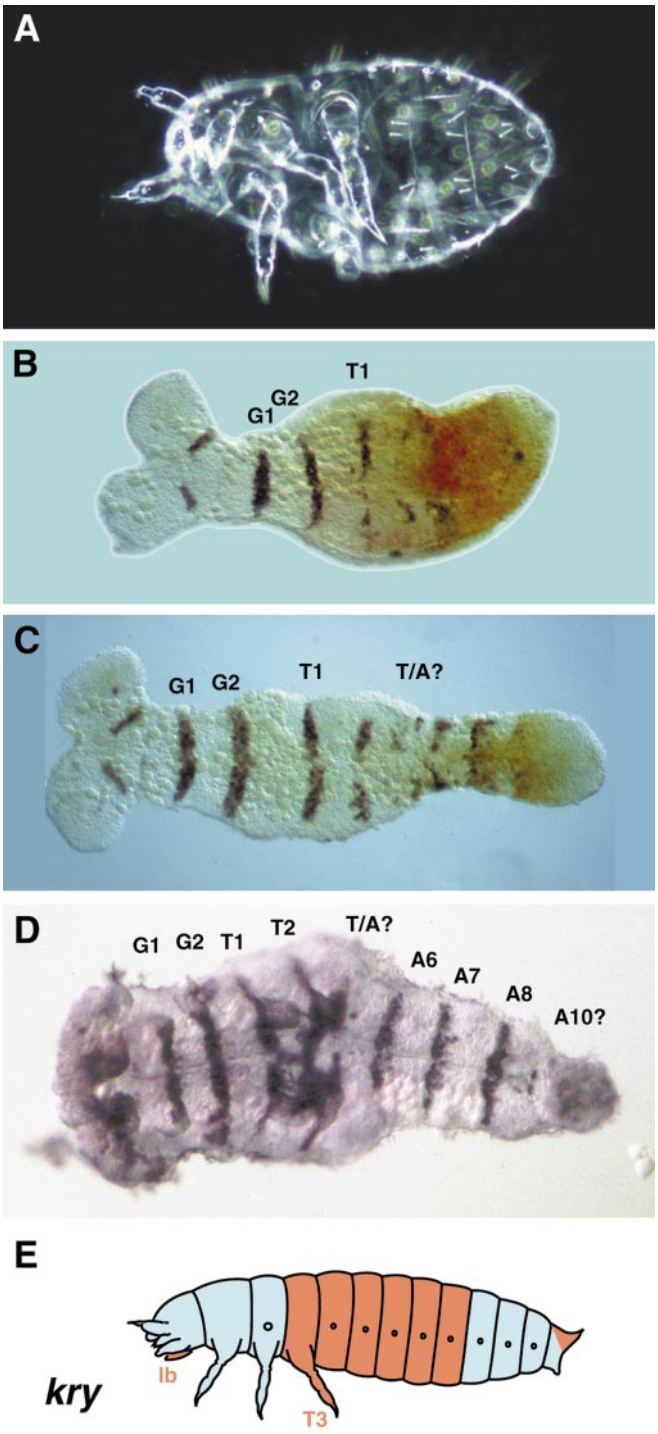

Fig. 6A–E Phenotypic effects of the *krusty* (*kry*) mutant. **A** Ventral view of a mutant larva. One gnathal (labium) and one thoracic segment are missing. A stigma characteristic of T2 is visible, indicating that the missing thoracic segment in this larva is T3. In the abdomen, only three segments remain, and in the telson the urogomphi are absent. **B, C** Mutant germ bands doubly stained for *en* (*black*) and *eve* (*brown*). The *eve* pattern is severely disrupted in the embryo in **B**. The germ band in **C** has progressed to a later stage of development when the posterior abdominal segments are formed. At this stage, the *eve* pattern has regained its distinct stripes. **D** *en* expression in a fully extended germ band. Two normal *en* stripes are visible in the gnathal area, and three normal stripes in the posterior abdomen. Whilst the G3 stripe is absent, two thoracic segments have been formed as indicated by the formation of limb buds, despite the severe pattern disruptions in the thorax seen at earlier stages. The three posterior *en* stripes have been labelled *A6 to A8* since the cuticle phenotype indicates deletion of A9. A10 is represented by a very small *en* stripe, as in wild type. **E** Summary of the *kry* cuticle phenotype

and vice versa. Five parallel crosses were set up each comprising one male and five females, and eggs layed by these beetles were scored for mutant phenotypes. Of several hundred eggs, only one displayed a phenotype similar to *bol*. To control if mutant carriers were properly represented amongst the virgin females, the males were then exchanged such that males and females from the same mutant background were combined. After 1 week (to allow sperm replacement), embryos were collected. In both cases, many mutant embryos now were encountered, confirming that a sufficient number of virgin females had been of the correct genotype. This experiment shows that *kry* and *bol* represent two separate loci. The one embryo with a phenotype similar to *bol* probably diplays an unspecific defect which by chance ressembled one of the phenotypes involved.

Discussion

Role of pair-rule genes in short-germ embryos

In *Tribolium*, pair-rule genes are expressed in stripes of double-segmental periodicity during growth of the germ band, suggesting a segmentation function for these genes also in this short-germ insect (Brown and Denell 1996; Patel et al. 1994; Sommer and Tautz 1993). However, in a situation where pattern formation occurs in a field of cells that grows at one end, loss of a pair-rule gene might have phenotypic consequences very different from those in *Drosophila*. One could conceive, for example, that pair-rule genes were involved in regulation of germ band growth to ensure termination of cell proliferation after formation of all segments; if so, one would expect growth disturbances up to complete loss of the abdominal region in a pair-rule mutation. In another conjecture, pair-rule genes in short-germ embryos could be part of a clock mechanism which ensures periodic activity of these genes in the growth zone. In that case, a mutation in any one pair-rule gene might interrupt the clock altogether, blocking all anterior-posterior pattern formation in the abdomen. That pair-rule mutants in short-germ embryos could indeed result in phenotypes different from those in *Drosophila* was suggested by a previously published *Tribolium* mutation, *godzilla* (*god*). This mutation has been classified as a pair-rule mutant (Sulston and Anderson 1996) because the pattern of segment-polarity genes is altered over the whole length of the germ band, which indicated that *god* functions at a higher level of the hierarchy but is not a gap gene. However, the pattern of *en* in *god* mutant embryos is highly irregular with little evidence of double-segmental periodicity. For this reason it is uncertain if this mutant does indeed represent a pair-rule gene and not some other more general cellular function.

In contrast to *god*, the *icy* and *scy* mutants display classical pair-rule phenotypes where every other segment is affected. Thus, at least some pair-rule genes in *Tribolium* can mutate to generate phenotypes with clear double-segmental deletions. Evidently, segment-polarity genes in this short-germ embryo are regulated by pair-rule genes in a way similar to *Drosophila*, with odd- and even-numbered stripes being dependent on different sets of pair-rule genes. Whilst these two mutants suggest that the function of pair-rule genes in *Tribolium* is in general similar to *Drosophila*, we should point out the possibility that other pair-rule mutants in *Tribolium* could still result in dramatically different phenotypes. It is possible that *icy* and *scy* are hypomorphic mutations, or that they both affect secondary pair-rule genes only. Both phenotypes are somewhat variable, the deletion of *en* and *wg* stripes is often not complete, and the expression of *eve* is not severely affected in both mutants – which would be consistent with either of these two possibilities. Therefore, we cannot exclude that some pair-rule genes in the growing germ band situation could have functions quite different from those in *Drosophila*. However, in combination with our evidence for the existence of gap gene functions in the *Tribolium* abdomen (as discussed below), it now seems unlikely that the role of pair-rule genes in short-germ insects like *Tribolium* is radically different from that in long-germ embryos.

Some pecularities of *icy* and *scy* warrant a more detailed discussion of their phenotypes. In *icy* mutant embryos two adjacent segments are affected, i.e. the mandibular and maxillary segment. Only the latter of these conforms to the pair-rule frame of deletions in this mutant. This is remniscent of the *sloppy-paired* (*slp*) gene in *Drosophila* which is thought to function both as a pair-rule gene and a head gap gene (Grossniklaus et al. 1994). Such a dual function may also be exerted by *icy*. However, in *slp* mutant embryos the antennal segment is deleted (which is present in *scy*$^-$), and the odd-numbered set of *en* stripes is affected, whilst *icy* is required for the even-numbered set. Given these phenotypic differences, it is unlikely that *icy* and *slp* represent homologous genes. Therefore, the *icy* phenotype also points to evolutionary differences between *Drosophila* and *Tribolium*, because no known *Drosophila* gene has a phenotype identical to *icy*. Either *icy* represents a gene not known or not present in *Drosophila*, or it represents a gene common to both organisms which has acquired some functional differences during evolution.

Our interpretation of the *scy*$^-$ phenotype also requires a comment. The analysis of *en* expression in this mutant indicates that the second thoracic segment (T2) is deleted. However, the remaining segment T3 carries a stigma which is characteristic for T2, not T3. It appears that T3 is transformed to T2 identity in *scy*$^-$. This transformation could be due to misexpression of homeotic genes in pair-rule mutants. Alternatively, the cells forming the stigma in this segment could actually be derived from T2, if the disappearence of the *en* / *wg* stripes in T2 would lead to a deletion of posterior T2 and anterior T3, such that the anterior portion of the T2 segment becomes fused to the posterior portion of T3. Since the stomata are formed at a fairly anterior position within each segment, the stoma of the fused segment would then still be of T2 identity.

In the case of *icy*, however, such a fusion appears not to occur, because in this mutant only T2 remains in the thorax, which still carries a stoma. This would not be the case if this segment was a fusion of anterior T1 and posterior T2. Therefore, the presence of T2 stomata in both mutants may indicate that their deletions in the epidermis are not exactly complementary.

Another peculiarity of both mutants is that their phenotypic consequences in head and thorax are more severe than in the abdomen. This could indicate a somewhat different role of these genes in different body regions. However, we think that it may just reflect the timing of stripe formation, or, in other words, the relative age of *en* and *wg* stripes at the time of epidermal differentiation. In *Drosophila*, some pair-rule mutants affect the *en* pattern initially in a fairly mild manner; only with developmental time do the pattern distortions become more severe. For example, in *slp* mutants, all *en* stripes are formed during gastrulation, but alternating stripes disappear entirely later during the extended germ band stage (Cadigan et al. 1994). In short-germ embryos, anterior *en* stripes have to be maintained over a longer time period than posterior stripes, and this may well make them more sensitive to reduced initial activation by pair-rule genes.

For a concise comparison of pair-rule functions in short- and long-germ embryos it will be necessary to see how the phenotypes of corresponding homologous genes in *Tribolium* and *Drosophila* match. In *Drosophila*, each pair-rule gene has an unique phenotype in that the precise borders of the double-segmental deletions vary from case to case. Unfortunately, the cuticle of *Tribolium* larvae displays landmarks quite unlike those of *Drosophila* larvae. This hinders the identification of the gene affected in a specific *Tribolium* mutant based on detailed phenotypic analysis with *Drosophila* mutants. Also the molecular information available, i.e. the deletion of either even- or odd-numbered *en* stripes, still leaves several candidate genes for each of our mutants. Therefore, we are currently using recombination mapping to determine whether any of the segmentation genes that have been cloned in *Tribolium* so far are linked to either the *icy* or *scy* mutations.

Gap genes in the *Tribolium* abdomen

In the *Drosophila* blastoderm, gap gene products are expressed in domains of bell-shaped concentration profiles. The flanks of these domains form gradients which provide positional information for the generation of pair-rule stripes (Hülskamp and Tautz 1991). In *Tribolium*, several gap genes have been identified that are expressed in the blastoderm (Li et al. 1996; Sommer and Tautz 1993; Wolff et al. 1995). These genes could function during blastoderm patterning similar to in *Drosophila*. So far, it has not been clear if this class of genes is also involved in posterior segmentation in short-germ embryos. Diffusion of transcription factors is probably instrumental in generating gap gene gradients in *Drosophila*. However, in the abdomen of short-germ embryos diffusion should be limited as pattern formation here occurs in a cellularized environment. Hence, one could envisage that mechanisms independent of gap genes would be used for the formation of pair-rule stripes in *Tribolium* embryos. Such novel mechanisms could involve positional information provided by signals of low molecular weight which can diffuse within a cellularized tissue. Alteratively, a mechanism based on temporal regulation rather than spatial regulation may be used, obviating the need for long-reaching diffusion (Meinhard 1982; Palmeirim et al. 1997).

However, *bol* and *kry* display attributes of classical gap gene mutants, i.e. they delete or disturb a group of adjacent segments, whilst segments located more anteriorly and posteriorly are properly formed. Their phenotypic ranges are overlapping but shifted relative to each other, *bol* affecting predominantly segments T2 to A2, and *kry* segments T3 to A5. In addition, *kry* also affects the labial segment, whilst T1 (and often T2) are unaffected. Such a complex deletion profile is not unprecedented for gap genes, since in *Drosophila* both *hb* and *gt* affect more than one region of the larval epidermis. Although other interpretations are possible (for example, *bol* and *kry* could be involved in the production of a small molecule that diffuses into the growing germ band), at this point it appears that gap genes probably function in segmentation of the growing germ band of short-germ embryos as well as in the blastoderm.

To corroborate this interpretation, it will be necessary to molecularly identify the genes affected by these mutants and to compare their expression with the phenotypes observed. In *Drosophila*, defects in the area of thorax and abdomen are caused by mutations in four different gap genes: *gt*, *hb*, *Kr* and *kni*. *bol* may well be an allele of the *Tribolium Kr* homolog (*Tc'Kr*), since *Kr* in *Drosophila* (*Dm'Kr*) affects segments T1 to A5, similar to *bol* which affects T2 to A2. Given that the *bol* phenotype is quite variable, temperature sensitive, and has little effect on the *eve* pattern, this mutation is likely to be a hypomorphic allele. Therefore, complete loss of *bol* function might result in deletions even more similar to *Dm'Kr*.

In the case of *kry* the comparison is more difficult since no *Drosophila* gap gene affects three distinct regions of the larval epidermis. *Tc'hb* is a possible candidate gene for *kry*, if we assume that the *Tc'hb* gap domain affects two separate regions, i.e. labium on the one hand and thorax / anterior abdomen on the other hand. This expression domain arises in the posterior half of the *Tribolium* blastoderm (the anterior domain is extra-embryonic and unlikely to affect segmentation). In contrast to the corresponding domain in *Drosophila*, which covers the anterior pole and therefore has one border only, the *Tribolium* domain has two borders both of which may serve as instructional gradients for the regulation of downstream genes, i.e. the anterior border could regulate pair-rule stripes in the gnathal area, whilst the posterior

border would do so for stripes in the thoracic and abdominal region. The third domain of *Tc'hb* appears later during germ band growth and could be responsible for *kry* function in segment A9. The only problem with this interpretation is that we cannot explain at this point how the posterior blastoderm domain of *Tc'hb* could exert such a long-ranging effect on the first five abdominal segments. Therefore we should not discount the possibility that other, more posteriorly expressed gap genes, i.e. the *Tribolium* homologs of *kni* or *gt*, could be mutated in *kry*. In that case the expression pattern of the respective gene probably will deviate quite a bit from its *Drosophila* homolog in order to account for the different phenotypic effects of *kry*.

Regardless of which gene *kry* will turn out to be, there are most certainly major differences in function and/or expression between the *Tribolium* and *Drosophila* homologs, since the *kry* phenotype does not fit well into the phenotypic series of any *Drosophila* gap gene (Lehmann and Nüsslein-Volhard 1987; Nauber et al. 1988; Petschek et al. 1987; Wieschaus et al. 1984). Whilst our finding that pair-rule genes function in short-germ embryos similar as in long-germ embryos leaves little room for alternative interpretations, we view our evidence for gap gene functions in the *Tribolium* abdomen still as preliminary. The ongoing genetic and molecular analysis of *Tribolium* segmentation will show if the role of *bol* and *kry* in abdominal segmentation is indeed comparable to *Drosophila* gap gene functions.

Implications for other segmented animals

The pair-rule mutants described in this paper show for the first time that this gene class indeed has similar functions in short- and long-germ embryos. As a holometabolous species, *Tribolium* is more closely related to *Drosophila* than are most other groups of short-germ insects. Therefore, the question may be posed whether the findings in *Tribolium* pertain to more ancestral species like the grasshopper, *Schistocerca*. Homologs of *fushi-tarazu* (*ftz*) and *eve* are not expressed in double-segmental patterns in *Schistocerca*, which led to the conclusion that the segmental expression of *en* in that species is accomplished by some other mechanism (Dawes et al. 1994; Patel et al. 1992). However, the recent discovery of pair-rule patterns in vertebrates (Müller et al. 1996) suggests that the temporary establishment of double-segmental units could be an ancestral characteristic of segmented animals. *Schistocerca* either may have evolved another mechanism secondarily (Tautz et al. 1994), or the function of *eve* and *ftz* in this species has been taken up by other genes, similar to what probably happened with *ftz* in *Tribolium* (Brown et al. 1994a; Stuart et al. 1991). Therefore, we think that *Tribolium* is indeed representative of short-germ insects in general, and that our findings can be extended to other insects, possibly to other arthropods and maybe even to vertebrates. For example, even though there is molecular evidence for pair-rule gene functions in zebra fish embryos, no pair-rule phenotypes were identified in screens for patterning mutants in this species (Driever et al. 1996; Haffter et al. 1996). The view that the phenotypic effects of such mutants in embryos that sequentially add segments at the posterior end would result in defects not recognizable as pair-rule phenotypes, now seems less likely.

The phenotypes of *bol* and *kry* provide the first strong evidence for the involvement of the gap genes class in segmentation of a growing germ band. To date, there only was clear molecular evidence for a blastoderm function of *otd*, *Kr* and *hb* in *Tribolium* (Li et al. 1996; Sommer and Tautz 1993; Wolff et al. 1995). The manner in which abdominal gap genes exert their function, how their pattern is generated, whether they interact with each other, and by which means they manage to regulate their down-stream genes (via diffusion gradients?) remain central questions for understanding the evolution of segmentation from short- to long-germ insects.

Acknowledgements We wish to thank R. Beeman, S. Brown, S. Haas, I. Sulston and K. Anderson for invaluable advice essential for initiating genetic work with *Tribolium* in our laboratory; N. Patel, K. Zinn and L. Nagy for antibodies and molecular probes; D. Tautz for continuous support, and A. Berghammer, M. Kania and D. Tautz for suggestions on the manuscript. This work was funded by the Deutsche Forschungsgemeinschaft.

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