The Evolution of Gap Gene Orthologues

Dissertation

der Fakultät für Biologie der
Ludwig-Maximilians-Universität München

vorgelegt im März 2002 von
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Tag der mündlichen Prüfung: 28.11.2002

Acknowledgments

First of all, I would like to thank Martin for his constant support. He always took his time to discuss good ideas and bad results and vice versa. But he always let me do things my way and accepted that not everything can be done at once by one student... I am thankful to have spent some time in Diethard Tautz´ lab and to have learned so much about the diverse fields he is interested in. In addition, I thank him for his help with phylogenetic sequence analysis.

I am deeply indebted to Gabi Büttner. Without her, the stainings would not have been as beautiful, the results of the mapping not that convincingly negative for most and thus unexpectedly positive for one gene. I also want to thank Sigrid Richter for some egg sieving and cuticle preparations for the parental RNAi experiment – she even accepted to make ”Überzeit” in order to perform the work. John Baines searched the Drosophila genome until he found CG4575 - and shared his findings with me. Thanks also to Gregor Gilfillan, Barbara Wimmer and John Baines for reading the manuscript and improving its english.

I want also to thank my collegues in *Coleoptera* (in order of appearance): Andreas Berghammer for always knowing who did what in the institute; Christoph Eckert for introducing me to the pains of a 60er fan and sharing his good mood when they won – rare enough as it was. I would like to thank companero Johannes Scholten for his inclination to discuss the matters of science, politics, journalism and the meaning of the universe anytime over a cup of tea. Hilde Wohlfrom not only provided me with mutants from time to time – she also turned out to be an excellent ”molli”. Markus Weber silently developed into a constant in the lab and was always happy to share his exitement about his favourite animals (not beetles, though). Xiaohui enriched us with her friendly manner and turned us into an international lab. Finally, Alex Cerny took over *jaws* project and had as much success in the first months as I wished to have in my first year.

I should also thank the collegues that shared the lab but not the fondness for beetles: Barbara Wimmer with her never ending interest for her collegues and her ability to talk to everybody about everything (with incredible speed) - often enough she was the one to share a cup of tea and problems. Wim Damen would never appreciate the importance of arthropods with less than 4 pairs of legs. Nevertheless he gave me invaluable technical tips regarding the design of redundant primers, the screening of libraries and much more. Bianka Hartung helped with her good mood and the positive inclination she would keep towards everybody in the lab. Barbara Wigand - besides expert technical assistance - kept the mutants alive while they tended not to survive my treatments. I also want to thank Reinhard Schröder who introduced me with so much patience to Tribolium handling and constantly shared know-how and results. Thanks also to all the other lab members that shared bench and beers with me.

I am deeply indebted to my parents for their constant support during my studies and the PhD work. Finally I want to thank Bärbel – she shared my happiness during the ups and supported me during the downs in these years. And she reminded me from time to time that there is more in life than beetles.

Abbreviations:

aa	amino acid
bp, kb	base pairs, kilo base pairs
°C	degrees Celsius
cDNA	DNA complementary to mRNA
Dm'xxx	*Drosophila melanogaster* gene xxx
DNA	deoxyribonucleic acid
dsRNA	double stranded RNA
GA-1	*Tribolium* wildtype strain *Georgia-1*
h	hours
mM	milli molar
min	minutes
mg	milligram
ml	milliliter
nl	nanoliter
ng	nanogram
mRNA	messenger RNA
oligo	oligonucleotide
PCR	polymerase chain reaction
phenotype	is due to a mutation in a gene
phenocopy	is due to knocking down gene function by RNAi
pRNAi	parental RNAi
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNAi	dsRNA mediated interference
SB	*Tribolium* wildtype strain *San Bernardino*
Tc'xxx	*Tribolium castaneum* gene xxx
Tiw1	*Tribolium* wildtype strain from India
ul	microliter

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Summary

Early pattern formation is well understood in *Drosophila melanogaster*. In this so-called long germ insect, all segments are specified during the blastoderm stage, where diffusion of transcription factors is unimpeded by cell walls. Gap genes play a crucial role in subdividing the blastoderm into broad domains and in activating the primary pair rule genes. The expression of these pair rule genes in seven stripes is the first sign of subdivision of the embryo into repetitive units and is the basis for metamerization into parasegmental units.

Most other insects, however, develop by the so-called short germ embryogenesis. Here, only the anterior-most segments are patterned during the blastoderm, while the posterior segments are specified one after the other in an anterior to posterior growth and patterning process. How segmentation proceeds in such a cellular environment is currently investigated in the red flour beetle *Tribolium castaneum*. Comparing the function of orthologues genes in both insects will ultimately reveal, which changes on the level of segmentation genes accompanied the evolution of these two different modes of development.

One way to investigate segmentation in *Tribolium* is by cloning of orthologous genes. By the recently developed RNA interference method (RNAi) these genes then can be knocked down, and phenotypes corresponding to different levels of residual gene activity can be investigated. Only in the nematode *Caenorhabditis elegans*, however, it was possible to knock down zygotic gene function in offspring embryos by injecting the parent. This procedure simplifies RNAi analysis and made possible genome-wide screens in *Caenorhabditis*. In the first part of this thesis I show that injection of double stranded RNA into *Tribolium* pupae also leads to zygotic gene knock down in the offspring. We named this finding parental RNAi (pRNAi). In Tribolium, pRNAi significantly reduces the labour required to analyze RNAi embryos for changes in the expession pattern of other genes. Moreover, the presence of such a mechanism in a nematode and an insect suggests that pRNAi is an ancient phenomenon that potentially might work in all arthropods (see project 1).

Previously, several orthologues of *Drosophila* segmentation genes had been cloned and their expression patterns described. While segment polarity and pair rule genes appear to be expressed in conserved patterns, the situation is less clear for orthologues of abdominal gap genes. In order to investigate whether abdominal gap gene orthologues play a similar role in segmentation of a short germ embryo, I cloned the *Tribolium giant* orthologue *(Tc'giant).* I could show that its anterior expression domain is conserved but that the posterior domain is shifted five segments towards anterior. RNAi analysis revealed that in the head, *Tc'giant* is involved in homeosis, while in thorax and abdomen, segmentation of is severely affected. The segmentation defects indicate, however,

that *Tribolium giant* functions differently from its *Drosophila orthologue*. This result indicates major changes in the genetic network responsible for the patterning of abdominal segments (see project 2).

Previously, patterning mutants had been isolated to identify genes that are crucial for segmentation in *Tribolium*. Their phenotypes led to speculations about the class of genes they might belong to, but it was not known, which genes are affected in these mutants. To identify some of these genes, I attempted to map all segmentation mutants relative to the candidate genes *Tc'even-skipped, Tc'hairy, Tc'runt and Tc'Krüppel*. I could show that most mutations are not linked to any of these genes, while the mutant *krusty* maps in moderate distance to *Tc'runt*. Intriguingly, my results also indicate that *jaws* may be a *Tc'Krüppel* allele. The *jaws* phenotype consists of anterior homeotic transformations without segmentation defects while segmentation of the whole abdomen is disturbed (see project 3). This phenotype is very different from that of *Dm'Krüppel*. Together with the *Tc'giant* data, this suggests that subdivision of the *Tribolium* embryo differs significantly from Drosophila. A model for evolution of abdominal gap gene orthologues is presented in the general discussion.

A word on the structure of this thesis:

The "parental RNAi" work has been accepted for publication, and the results of the "*giant*" project will be submitted soon. The manuscripts of these projects have been included into the thesis with minor changes. The third project, i.e. the "mapping" experiment is described in a third chapter. Both the "mapping" and the "*giant*" chapter have their own introduction, results and discussion sections. The materials and methods of all three projects have been combined. In addition, the general introduction gives an overview of the field, the model system *Tribolium castaneum,* and the questions that motivated the projects of this thesis. The general discussion at the end expands the discussion on abdominal pattern formation provided in the *giant* chapter to include the result of the mapping experiment. It also elaborates some speculations that may guide the design of future experiments.

List of publications

Maderspacher, F., G. Bucher, M. Klingler (1998). "Pair-rule and gap gene mutants in the flour beetle Tribolium castaneum." Dev Genes Evol **208**(10): 558-68

Berghammer, A., G. Bucher, F. Maderspacher, M. Klingler (1999). "A system to efficiently maintain embryonic lethal mutations in the flour beetle Tribolium castaneum." Dev Genes Evol **209**(6): 382-9

Bucher, G., J. Scholten, M. Klingler (2002). "Parental RNAi in Tribolium (Coleoptera)." Curr. Biol. **12**(3): R86-87

Bucher, G. and M. Klingler (2002). "Expression and function of the gap gene giant in Tribolium suggest divergent patterning mechanisms in long and short germ insects." in preparation.

General introduction

Evolution and ontogeny

The major driving force behind evolution is natural selection acting on phenotypic variation. Advantageous variations will be positively selected for and may eventually become fixed within a population (Darwin 1859). Cumulation of such changes in a subpopulation can result in reproductive isolation and speciation. Importantly, variation arises in the population mainly on the genetic level, while selection acts on the phenotype. Regarding the morphology of an organism, the relationship between genotype and phenotype is, however, not trivial. It is laid down during ontogeny, that is, the development of the organism from the fertilized egg to the adult. It comprises highly complex cellular processes governed by developmental genes that eventually lay the foundations for the body plan. Since the late 19th century, the ontogeny of many organisms throughout the animal kingdom has been described. This revealed a variety of ways how embryos are formed, and some developmental principles were uncovered by experimental manipulations of embryos. Technical constraints, however, restricted the depth of these functional analyses and left the ultimate genetic regulatory level as an unknown "black box". Only in the last decades, genetic and molecular advances have allowed for the analysis of the genes that govern pattern formation. In other words: the relationship between genotype and morphology can now be investigated.

From an evolutionary developmental point of view, this allows for tracking down the changes in genotype that accompanied the evolution of different body plans. On the other hand, genes or genetic interactions that are conserved between taxa provide information about the situation in the last common ancestor. To this end, different animal taxa are being analyzed and the roles of homologous genes and their interactions are compared. The basis for such comparisons is the thorough analysis of a small number of model species, which are especially amenable to genetic and molecular manipulations. Such model systems are, for instance, *Drosophila melanogaster* for insects, *Caenorhabditis elegans* for nematodes, the Zebrafisch *Danio rerio* and the mouse *Mus musculus* for the vertebrate clade. Using these systems as a reference, less profound comparative studies in related taxa can identify relevant changes in developmental mechanisms that allow answering questions about the evolution of body plans.

The gene network that governs segmentation in the fruit fly *Drosophila melanogaster* is well

understood (St Johnston and Nüsslein-Volhard 1992). This provides a perfect reference system for comparative studies with other insects and arthropods.

Drosophila embryogenesis: long germ development

All insects have a blastoderm stage, where one layer of undifferentiated cells surrounds the central yolk mass (Sander 1976). In *Drosophila melanogaster*, all segments are specified during this stage. Importantly, a great part of pattern formation occurs at a time when the nuclei are not yet surrounded by cell membranes and transcription factors are free to diffuse, obviating the need for cell-cell communication. Because all segments are formed at that stage, a complete fate map of the larval body can be mapped onto the blastoderm. This feature defines the so-called "long germ mode" of development (Sander 1976).

Drosophila segmentation has been shown to follow a hierarchical principle, where positional information is successively refined through subsequent levels of a pattern formation cascade (Tautz 1992; Ingham 1988; Klingler and Tautz 1999). The maternal coordinate genes whose products are localized in the egg by the mother during oogenesis provide the initial positional information. The most important maternal signal for segmentation comes from the anteriorly localized *bicoid* mRNA. Its gene product forms an anterior-posterior gradient that acts upon genes in the next level of the hierarchy - the gap genes. The *Bicoid* gradient activates the zygotic gap genes in broad domains. Repressive interactions between gap genes further refine their expression pattern. The gap gene products are thought to act by the formation of short range gradients emanating from their expression domains (Hülskamp and Tautz 1991) (Rivera-Pomar and Jäckle 1996). Accordingly, the patterning defects observed in gap mutants are gaps that extend their respective expression domains on both sides. The mentioned mutual repression is strongest between nonadjacent gap genes as, for instance, between *Krüppel* and *giant* (Kraut and Levine 1991).

The non-repetitive pattern of gap gene expression is used to initiate the double segmental expression of the next level, the primary pair rule genes *hairy*, *even-skipped (eve), runt* and *fushi tarazu (ftz)*. Each pair rule stripe is regulated by an enhancer element that integrates different concentrations of several gap gene products. Subsequently, the overlapping stripes of primary pair rule expression are further refined by interactions among themselves. These repetitive stripes are used to define the secondary pair rule stripes, which eventually regulate the last level of the cascade, the segment polarity genes. Their segmentally reiterated domains determine the segmental compartments on the level of gene expression.

The Hox genes, finally, define the identities of thus specified segments. They initially depend on gap gene regulation, but pair rule genes also modulate their expression.

Tribolium castaneum: short germ development

The red flour beetle *Tribolium castaneum* has become an important model system for evolutionary developmental questions because many molecular and genetic methods are now available. These include methods to screen for embryonic lethal mutations, transgenic approaches, and efficient gene knock down by RNAi and Morpholino oligonucleotides (Berghammer, Bucher et al. 1999; Berghammer, Klingler et al. 1999; Brown, Mahaffey et al. 1999). The phylogenetic position of *Tribolium* is at the basis of holometabolous insects, i.e. insects that undergo complete metamorphosis. It is thought to represent the ancestral mode of insect development, the so-called short germ embryogenesis (Tautz, Friedrich et al. 1994). In short germ insects, only the head and anterior thoracic segments are determined during the blastoderm stage (Sander 1976). Thus, in contrast to the fly, the blastoderm fate map lacks all posterior segments. These are added at later stages one after the other from a posterior growth zone. This occurs in a cellular environment, where transcription factors are not able to form gradients by free diffusion as in the fly. Understanding *Tribolium* segmentation should show whether ancestral insect metamerisation is hierarchical as in *Drosophila* or relies on another principle. In any case, the comparison of *Tribolium* and *Drosophila* segmentation should reveal the genetic changes that led from short to long germ development.

Orthologues of *Drosophila* segmentation genes in *Tribolium*

A number of orthologues of *Drosophila* segmentation genes have been isolated from *Tribolium*. The segment polarity genes *engrailed* and *wingless* are expressed in similar intra-segmental positions as in *Drosophila* (Brown, Patel et al. 1994; Nagy and Carroll 1994), and limited functional data suggests that their interactions are conserved (Oppenheimer, MacNicol et al. 1999). Also the primary pair rule genes *Tc'eve*, *Tc'hairy* and *Tc'runt* have been found to be transcribed in similar frames as in *Drosophila* (Brown, Parrish et al. 1997; Sommer and Tautz 1993; Brown, Parrish et al. 1994). Their expression reflects, however, the differences between short and long germ embryogenesis: In the blastoderm, only three stripes appear, corresponding to the fate map. The more posterior stripes are formed one after the other near the posterior growth zone. From their conserved expression it has been suggested that pair rule genes have conserved functions during segmentation. This

interpretation is corroborated by the pair-rule phenotype of the two mutants *itchy (icy)* and *scratchy (scy)*. An exception is the orthologue of the pair rule gene *fushi tarazu (ftz):* it is expressed in more diffuse stripes than in *Drosophila* and deletion of the homeotic complex including the *Tc'ftz* locus has no overt segmentation phenotype. This indicates that *Tc'ftz* is probably not required for segmentation (Brown, Hilgenfeld et al. 1994). On the gap gene level, *Tc'hunchback* expression appears to be conserved apart from an additional domain in the serosa (Wolff, Sommer et al. 1995). *Tc'Krüppel* expression starts in the blastoderm as a cap covering the growth zone, and subsequently develops into a broad domain assumed to be at a conserved position (Sommer and Tautz 1993). A clear difference has been shown, however, for the posterior *Tc'tailless* expression domain: it is expressed at the posterior pole of the blastoderm as in *Drosophila*. However, it can not regulate the same set of posterior pair rule stripes as in *Drosophila*, because *Tc'Tll* protein fades long before these segments are formed. It has therefore been suggested to determine terminal cells but not abdominal segments (Schroder, Eckert et al. 2000).

The projects of this thesis

As mentioned above, one major difference between long and short germ embryogenesis is the ontogeny of abdominal segments. Both, segment polarity and pair rule genes, appear to have largely conserved functions in *Tribolium* and *Drosophila*. Thus, the question arises whether evolutionary changes in abdominal gap genes correlate with the morphological differences in segmentation. To answer this question, an orthologue of the gap gene *giant* was cloned and its function investigated (project 2).

Comparing expression patterns of orthologous genes allows for some speculations on their conservation. Nevertheless, functional data are required for answering this question conclusively. One approach currently under way is the analysis of patterning mutants. Several embryonic lethal mutants have been isolated from two independent screens (see introduction of project 3) (Maderspacher, Bucher et al. 1998; Sulston and Anderson 1996). The phenotypes of some mutants have been described and speculations raised as to what gene class they might belong to. The identity of the mutated genes, however, remains elusive. In order to identify the genes affected in these mutants, a genetic mapping approach is presented in this work: all segmentation mutants were tested for linkage with several candidate genes (project 3).

Another technique used to assess gene function is the recently developed RNA interference (RNAi) that knocks down gene function in many organisms (Fire, Xu et al. 1998). Also in *Tribolium*,

injection of dsRNA into eggs efficiently blocks gene function in the zygote (Brown, Mahaffey et al. 1999). It has been technically difficult, however, to generate sufficient RNAi embryos for staining them for expression of other genes. In the next chapter (project 1) I show that dsRNA injection into pupae leads to efficient gene knock down in the offspring (parental RNAi, pRNAi). This eases functional studies of cloned genes, and significantly improves analysis of gene expression in RNAi treated embryos.

Project 1:
Parental RNAi in *Tribolium* (Coleoptera)

RNA interference (RNAi) allows for rapid and straightforward analysis of gene function. RNAi can be applied with special ease in *C. elegans* where injection of dsRNA into the body cavity, or application of dsRNA via ingestion, leads to gene inactivation in offspring embryos (Fire *et al 1998,* Timmons and Fire 1998). This parental RNAi effect made possible efficient genome-wide functional screens in this organism (Fraser et al 2000). Here we show for the flour beetle *Tribolium castaneum* that injection of double stranded RNA (dsRNA) into the mother's haemocoel results in knock down of zygotic genes in offspring embryos ("parental RNAi"). This suggests that transfer across cell boundaries is an ancient feature of the RNAi pathway, and opens up new applications in functional genomics and in the study of developmental evolution.

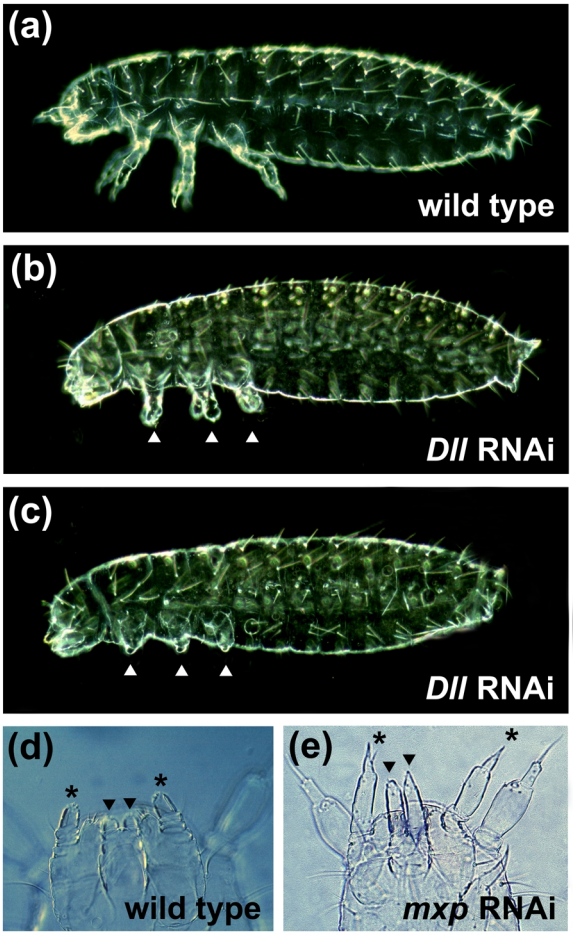


Figure 1
Tribolium *beetles injected as pupae with dsRNA produce offspring displaying gene-specific phenotypes. (a) Wild type first instar larva. (b, c) Parental RNAi (pRNAi) for* Tc'Dll *results in truncated legs (arrowheads) and head appendages; the embryo in (b) represents an intermediate, that in (c) a strong phenotype. (d) Ventral view of wild type head. Maxillary and labial palps are indicated by stars and arrowheads, respectively. (e) Parentally induced phenocopy of* mxp (= Tc'pb)*: maxillary and labial palps are transformed to legs.*

We tested parental RNAi in *Tribolium* for two genes of known function, the leg gene *Distalless* (*Tc'Dll*) (Beermann et al 2001) and the homeotic gene *maxillopedia* (*mxp*), a *Tribolium* homologue of *proboscipedia* (*pb*) (Shippy TD et al. 2000). In addition, we applied this method to investigate the function of a newly isolated segmentation gene, the *Tribolium giant* homologue (*Tc'gt*). For injection, female *Tribolium* pupae were affixed

to microscope slides with a drop of rubber cement (Fixogum, Marabu) at their posterior abdomen. Using a simple micromanipulator setup, approximately 0.15 µl of dsRNA solution (0.2 – 2 mg/ml in injection buffer (Fire et al 1998)) were injected per pupa, at a ventro-lateral position between abdominal segments 3 and 4. After completion of metamorphosis, these females were mated to untreated males, and eggs were harvested in weekly time intervals. For all three genes, nearly all offspring larvae (665 of 669) displayed gene-specific phenotypes one week after injection. Buffer-injected females and dsRNA-injected males gave rise to normal offspring. For *Tc'Dll* and *mxp,* the phenotypes obtained by parental RNAi (Fig. 1b,c,e) were similar to those of partial or complete loss of function mutants (Beermann et al 2001, Shippy TD et al 2000). This shows that parental RNAi faithfully reproduces reduction or inactivation of gene function in *Tribolium.* Since neither *Tc'Dll* nor *mxp* are maternally provided, it is evident that parental RNAi can knock-down zygotic gene expression in offspring embryos. In case of *Tc'giant,* parental RNAi phenotypes were obtained similar to those generated by injecting embryos with dsRNA or with morpholino oligonucleotides designed to inhibit translation of *Tc'giant* mRNA (not shown). The congruence of these data indicates that also the observed parental *Tc'gt* phenotype is a gene-specific effect.

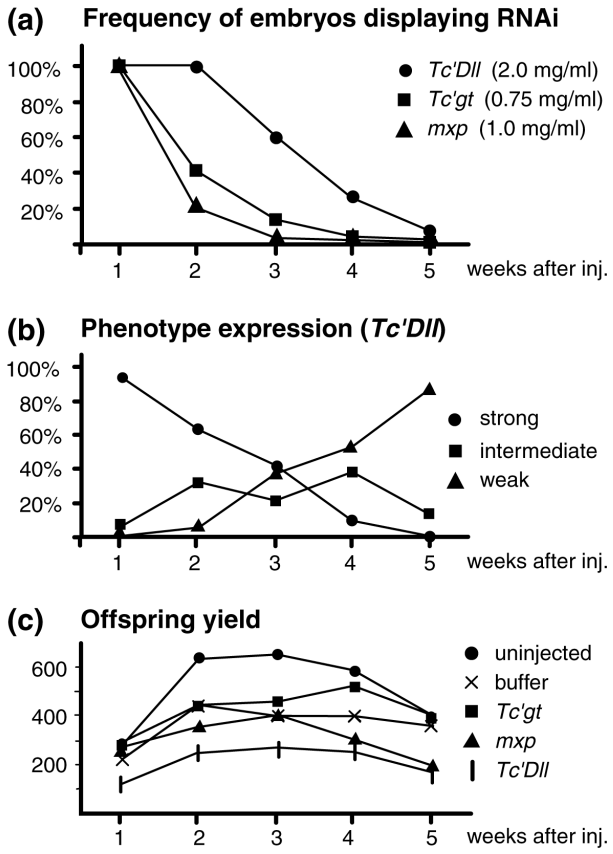


Figure 2

Time course of parental RNAi efficacy. (a) Relative frequency of offspring embryos displaying RNAi effects at different time points after their mother was injected with dsRNA. For all three genes tested, nearly all embryos displayed specific embryonic defects one week after injection. During subsequent weeks, the percentage of embryos with knock down phenotypes declined continually. (b) Also the strength of RNAi phenotypes decreases with time. Strong phenotypes were defined by loss of femur, tibia and claw, intermediate phenotypes by loss of tibia and claw, and weak phenotypes by fusion of femur and tibia. Note that only embryos deviating from wild type are represented in this figure. (c) Number of offspring obtained from females which were either uninjected, injected with buffer alone, or injected with different dsRNA solutions (concentrations as in (a)). Each time point represents the number of larvae produced by 30 females in a 3 day interval (only eggs were counted that completed development, i.e. differentiated a larval cuticle).

Parental RNAi is highly efficient in *Tribolium:* in all three experiments, nearly 100% of embryos in the first egglays after injection displayed RNAi phenotypes (Fig. 2a). In subsequent egglays, the portion of embryos exhibiting phenotypes declined, as did the strength of phenotype expression (Fig. 2a,b). This gradual loss of RNAi activity may reflect

continuous depletion of introduced dsRNA. The fecundity of injected females (dsRNA or buffer only) was reduced by about 50% relative to untreated animals, but still yielded ample material for developmental analysis (Fig. 2c). Over 90% of injected pupae survived the treatment, which is in contrast to embryonic RNAi experiments (or embryonic morpholino experiments) where only about 20-40% of the injected eggs completed development and differentiated a cuticle.

The mechanism by which dsRNA (or some processed form of it) enters the oocytes is not clear. Mechanical damage of ovaries during injection we consider unlikely to be responsible since pupae were injected unilaterally, and the high frequency of RNAi phenotypes indicates that oocytes in both ovaries are similarly affected. To see if *Tribolium* ovaries have an unspecific import propensity, we injected morpholino oligonucleotides also into pupae. No offspring displayed a *Tc'gt* phenotype, arguing that these modified nucleic acids do not enter oocytes in significant amounts. We speculate that either a specific cellular uptake mechanism for dsRNA, or secondary amplification of small amounts of incidentally incorporated dsRNA, could underlie the parental RNAi phenomen.

Tribolium is not the only animal outside the nematodes where parental RNAi has been observed: Arendt and Wittbrodt recently found that parental RNAi also functions in the polychaete worm *Platynereis dumerilii* (personal communication). Together, their and our findings suggest that transmission across cell membranes is a conserved feature of the RNAi response, and that parental RNAi may function in many metazoan taxa. Parental RNAi greatly facilitates the analysis of gene function, since large numbers of RNAi embryos can be readily obtained and histochemically analysed using standard procedures. This is a significant improvement even for species where embryonic RNAi already is available, but is a crucial step forward for many non-model-system species whose eggs are not accessible or do not survive microinjection. Parental RNAi should allow for large-scale RNAi screens in many additional animal species, and will greatly facilitate functional approaches in the burgeoning field of evolutionary developmental biology.

Acknowledgments

We thank D. Arendt and J. Wittbrodt for communication of unpublished results and comments on this manuscript. A. Beermann and S. Brown, and their collaborators, we thank for *Dll* and *mxp* plasmids. Johannes Scholten provided a phenotypic series of *Tc'dll* RNAi embryos (embryonic injection) that could be compared with the parental RNAi results.

Project 2: Expression and function of the *Tribolium giant* gene suggest divergent patterning mechanisms in long and short germ insects

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 different mechanisms. Nevertheless, the expression and function of many segmentation genes seem conserved between *Tribolium* and *Drosophila*. We cloned, from *Tribolium*, the first orthologue of the abdominal gap gene *giant*. Like *Drosophila giant* (*Dm'gt*), *Tribolium giant* (*Tc'gt*) is expressed in two domains, i.e. in head and trunk, respectively. While the anterior domain is conserved in position, the posterior domain is located five segments more anteriorly than in *Drosophila*. Knock-down of *Tc'gt* function using morpholino oligonucleotides as well as embryonic and parental RNA interference (RNAi), indicates that the head domain is required for homeosis but not for segment formation: in *Tc'gt* knock-down embryos, the maxillary and labial segments are normally formed but assume thoracic identity. The posterior domain, however, is essential for segmentation. Surprisingly, segmentation defects in embryos with reduced *Tc'gt* activity are not restricted to a limited domain (as in *Drosophila*) but extend to all thoracic and abdominal segments, many of which are specified long after *Tc'gt* expression has ceased. These data show that *Tc'gt* does not function like *Dm'gt*, and they suggest that posterior gap genes underwent major changes during the transition from short to long germ embryogenesis.

Introduction

Most insect embryos pass through a blastoderm stage where all somatic cells still look alike while different fates are inscribed into them by the activity of embryonic patterning genes. Also the "extended germ band" stage, when all body segments and major organ systems have been formed,

is strikingly similar among all insect orders. It has therefore been called the phylotypic stage of insect development (Seidel 1960). Remarkably, the morphological 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 in the blastoderm stage. The more posterior segments are formed in an anterior to posterior succession by a growth zone located at the posterior end of the growing germ band. This mode of segmentation is believed to be ancestral (Sander 1976; Tautz, Friedrich et al. 1994). Long germ insects, in contrast, specify all segments during the blastoderm stage already. On the molecular level, segmentation is well understood only in the long germ insect *Drosophila melanogaster* (Pankratz and Jäckle 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 is believed to assist the formation of overlapping short range gradients (Hülskamp and Tautz 1991; Rivera-Pomar and Jäckle 1996). These short range gradients then serve to position the stripes of primary pair rule genes (Small and Levine 1991). In parallel, gap genes also provide positional information to demarcate the expression domains of Hox genes, which assign identities to the segments specified by pair-rule and segment-polarity genes (Klingler and Tautz 1999).

The red flour beetle, *Tribolium castaneum*, is well suited for studying pattern formation in short germ insects since it is amenable to functional studies via genetic, transgenic and RNAi approaches (Beeman, Stuart et al. 1989; Sulston and Anderson 1998; Maderspacher, Bucher et al. 1998; Berghammer, Klingler et al. 1999; Brown, Mahaffey et al. 1999; Bucher, Scholten et al 2002). A number of segmentation genes has been isolated from this species. The segment polarity genes *engrailed (en)* and *wingless (wg)* (Brown, Patel et al. 1994; Nagy and Carroll 1994), as well as several pair-rule genes (Sommer and Tautz 1993; Patel, Condron et al. 1994; Brown, Parrish et al. 1994) were shown to be expressed in corresponding patterns in *Tribolium* and *Drosophila*, suggesting that these genes serve similar functions in *Tribolium* and *Drosophila*. Also mutant phenotypes support the view that these genes fulfill principally similar roles in long and short germ embryos (Maderspacher, Bucher et al. 1998). Also several homologues of gap genes have been cloned from *Tribolium*. The domains of *orthodenticle (Tc'otd), hunchback (Tc'hb), Krüppel (Tc'Kr)* and *tailless (Tc'tll)* are arranged in a similar anterior to posterior order as in *Drosophila* (Li, Brown et al. 1996; Wolff, Sommer et al. 1995; Sommer and Tautz 1993; Schroder, Eckert et al. 2000). While the anterior gap genes *Tc'otd and Tc'hb* are located at similar positions relative to segment primordia in the blastoderm, this is not the case for *Tc'Krüppel*, which is expressed in the thorax only. In *Drosophila*, in contrast, it extends up to abdominal segment 5. Also the posterior *Tc'tll* expression has changed: *tll* is expressed at the posterior pole in *Tribolium*, as in *Drosophila*, but the segments for whose formation *tll* is required in *Drosophila* form much later in the short germ of *Tribolium*, at a time

when *Tll* protein has long disappeared (Schroder, Eckert et al. 2000). Therefore, expression of *tll* in *Tribolium* is not compatible with a conserved function of this posterior gap gene, suggesting that major differences in long and short germ segmentation are to be found for other abdominal gap genes as well.

The posterior gap gene *giant* (*Dm'gt*) is a transcription factor of the basic leucine zipper family (Capovilla, Eldon et al. 1992) which so far only has been described for *Drosophila melanogaster*. *Dm'gt* expression arises at 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 and labial segments (Mohler, Eldon et al. 1989; Kraut and Levine 1991; Eldon and Pirrotta 1991). Also at later stages of development, *Dm'gt* 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 from the pole and extends over abdominal segments 5 through 7. Shortly after completion of cellularization this domain disappears. *Dm'gt* functions in segmentation of both, head and abdomen: in mutant first instar larvae, the labial *engrailed* stripe is deleted (Petschek, Perrimon et al. 1987; Petschek and Mahowald 1990), and the *engrailed* domains of the abdominal segments 5 through 7 become fused (Petschek and Mahowald 1990). Several target genes of *Dm'gt* have been identified, and it has been shown that this gap gene exerts repressive functions on gap, pair rule and Hox genes. Mutual repression of *giant* and *Krüppel* has been shown to be crucial for refinement of both their gap expression domians (Kraut and Levine 1991; Capovilla, Eldon et al. 1992). The patterns of pair rule genes are disturbed both in head and abdominal regions in *Dm'gt* mutant embryos (Petschek and Mahowald 1990; Small, Kraut et al. 1991; Langeland, Attai et al. 1994). However, the initially severe pair rule defects are subsequently repaired to some extent, such that the terminal phenotype of *Dm'gt* mutants is less severe than that of other gap genes (Klingler and Gergen 1993). Direct interaction of *Dm'gt* with one of its pair-rule target genes, *even-skipped (eve)* has been shown in great detail (Small, Blair et al. 1992; Wu, Vakani et al. 1998).

In this work we describe the isolation of *Tribolium giant*, the first orthologue of *giant* in a species other than *Drosophila*. Similar to *Drosophila* gap genes, *Tc'gt* functions in both segmentation and segment identity. However, expression analysis as well as functional analysis in *Tribolium* clearly show that the head and trunk domains of *Tc'gt* play roles that fundamentally differ from the well understood functions of these domains in *Drosophila*.

Results

Identification of a giant orthologue in Tribolium

Based on limited sequence similarity of *Dm'gt* with other leucin zipper proteins (see Methods), we designed three redundant primers that should amplify a 78 bp fragment of the leucin zipper domain (Fig. 1A). Nested PCR with embryonic cDNA as template indeed yielded a 78 bp product (36 bp novel sequence plus primer sequences) which we found to comprise a single sequence species (see Methods). Using this fragment as probe, several concordant cDNAs were isolated from a lamda library. An identical sequence was obtained by extending the 36 bp sequence through 5' and 3' RACE. These data suggest that during early embryogenesis a single transcript is produced by this leucine zipper gene.

Using the full cDNA sequence we employed the BLASTP program to search the available databases for related genes. According to BLAST, the gene most closely related to this *Tribolium* protein is the *Drosophila Giant* protein (Blast similarity value: $6e^{-17}$). This similarity score suggests that the single leucine zipper gene that we isolated from *Tribolium* embryos is a *giant* orthologue, i.e. *Tc'gt*. Expression and functional data support this interpretation (see below) and alignment of both proteins reveals additional extensive amino acid similarity N-terminal of the DNA-binding domain (Fig. 1A). Conservation of these N-terminal amino acids is the main argument for *Tc'gt* representing a true *giant* ortholog, since no other proteins in the database share these sequences with *Tc'gt* and *Dm'gt*. As is the case for a number of other *Tribolium* genes (Schmid and Tautz 1999), also the predicted *Tc'gt* protein is much shorter than *Dm'gt* (222 versus 448 amino acids).

Tc'gt and *Dm'gt* share 34 identical amino acids within the leucin zipper domain of about 54 amino acids (63%). We undertook a more extensive phylogenetic analysis based on leucine zipper domains alone. PUZZLE analysis of a representative set of leucine zipper domains related to *Tc'gt* is shown in Fig. 1C. This analysis identifies a *Drosophila* gene, CG4575, very similar to *Dm'gt*. CG4575 is a putative gene identified by the *Drosophila* genome project, which appears to have originated from a recent genomic duplication event: only the leucin zipper and several kb downstream noncoding sequences are highly conserved, while the N-terminal portion of the coding region and upstream sequences are not (John Baines, personal communication). Therefore, a *giant*-like role of CG4575 in segmentation is unlikely.

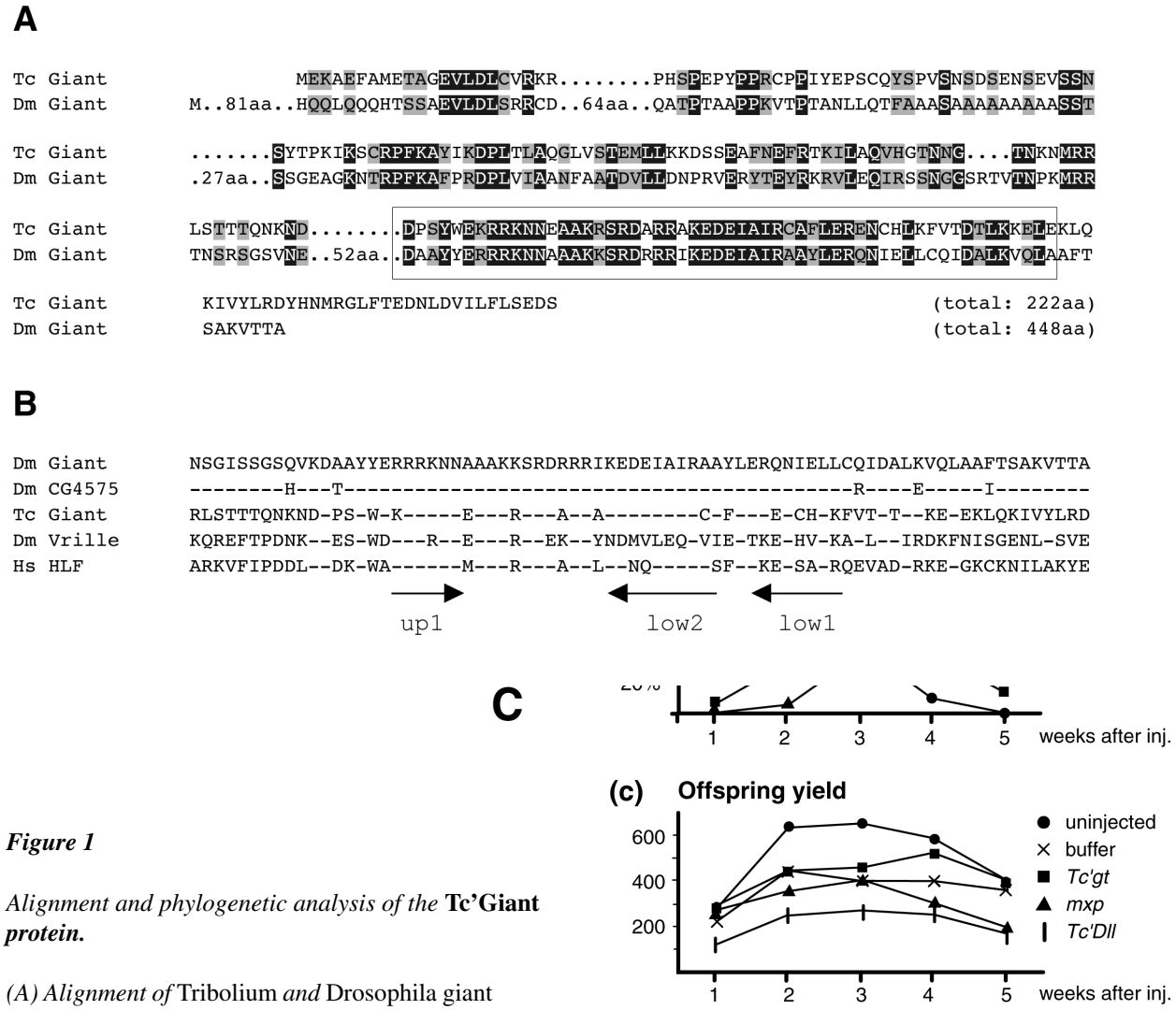


Figure 1

Alignment and phylogenetic analysis of the **Tc'Giant *protein.***

(A) Alignment of Tribolium *and* Drosophila giant *using Clustal W (see methods for details). Identical and similar amino acids are highlighted in black and grey, respectively. Long sequence stretches without homology were omitted from the* Dm'Giant *sequence (see dots; the number of omitted amino acids is indicated). The DNA binding domain (frame) has 63% identical and 15% similar amino acids. Additional conserved motifs upstream of the zipper corroborate homology between the genes, because they have not been found in other leucin zipper genes.*

(B) Alignment of the DNA binding domain including some adjacent amino acids for representative leucin zipper proteins. Dashes indicate sequence identity. The position of the redundant primers that were used to clone Tc'giant *is given below.*

(C) PUZZLE tree using the leucin zipper of the most related genes in the database. All branches of the tree have a PUZZLE support of >90 except for the indicated ones. The length of the branches represents the number of amino acid changes (see bar at bottom). A tree based on a bootstrap analysis resulted in essentially the same tree but did not cluster Dm CG7786 *and* Dm'vrille. *The leucin zipper of* Dm'giant *is significantly closer related* to Dm CG4575 *than to its* Tribolium *homologue, and the PUZZLE support for the split from* Dm'vrille *is slightly less than 90. There are, however, additional conserved domains upstream the leucin zipper between the* giant *orthologues (see alignment in A) but not in* CG4575 *or* Dm'vrille. HLF *human hepatic leukemia factor;* Dm Drosophila melanogaster*;* Ce Caenorhabditis elegans*;* Tc Tribolium castaneum*;* Hs Homo sapiens

Tc'giant expression during development

We used in situ hybridization to assess the conservation of *giant* expression (Fig. 2). In freshly laid eggs, transcripts are distributed homogenously throughout the syncytial blastoderm likely by maternal expression. Somewhat later, *Tc'gt* expression retracts from both poles. Anteriorly, the primordium of the extraembryonic serosa soon is cleared from *Tc'gt* staining (Fig. 2B,C). Subsequently, transcript abundance intensifies along the posterior edge of the domain (Fig. 2C) and eventually forms a distinct circumferencial stripe during gastrulation (Fig. 2D) and in the germ rudiment (Fig. 2E). This stripe covers the primordium of the maxilla (see below). A second *Tc'gt* domain arises *de novo* at the posterior pole of the embryo at the posterior pit stage. Cells lining the posterior pit express *Tc'gt* in a ring (Fig 2D), while cells in the centre of the invaginating pit remain unstained. In the germ rudiment (Fig. 2E), *Tc'gt* staining becomes more intense at the anterior boundary of this posterior domain, and during early germ band elongation, the posterior domain splits into two stripes (Fig. 2F). These stripes coincide (as shown below) with the metathoracic primordium (T3) and the second abdominal segment (A2). Around this time, expression of the maxillary stripe ceases. As the germ band continues to grow, the stripe in T3 fades, and somewhat later also the remaining A2 stripe. In the meantime, head expression condenses into a complex and dynamic pattern of brain cell clusters (Fig. 2I-K) which will not be discussed here further. *Tc'gt* expression ceases altogether before the germ band has fully elongated, and no staining was detected in subsequent embryonic stages.

Anterior **Tc'giant** *expression is conserved but the posterior domain is shifted by five segments*

To assess the exact position of these expression domains relative to segment primordia, we performed double in situ stainings with other segmentation genes. The *even-skipped (eve)* gene marks odd-numbered parasegments in *Tribolium* as in *Drosophila* (Brown, Parrish et al. 1994). While the pattern is dynamic in both species, the anterior border of eve stripes always coincides with the anterior boundaries of odd-numbered parasegments. In *Tribolium*, each double-segmental *eve* stripe splits into segmental stripes before the stripe fades. Double stainings of *Tc'gt* and *Tc'eve* show that the anterior *Tc'gt* stripe in the germ rudiment coincides with the maxillary segment: at this stage, the first *eve* stripe (parasegment 1) has resolved into narrow stripes coincident with the mandibular and maxillary *engrailed* stripes. The anterior *Tc'gt* stripe abuts the anterior one of these segmental *eve* stripes, and it overlaps the posterior one, sharing its posterior boundary (Fig. 3B). Also at earlier stages, when this *Tc'gt* stripe emerges from the initial broad domain, its posterior border

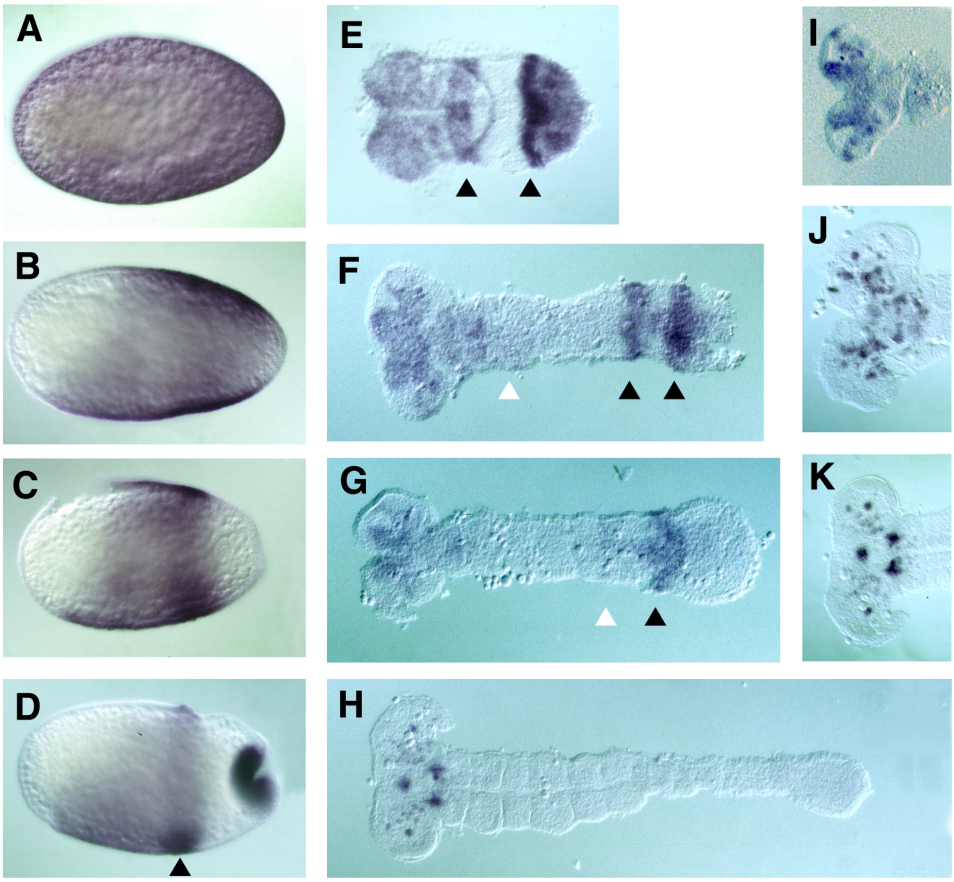


Figure 2

Expression of Tc'giant. *In all embryos, anterior is to the left. Blastoderm stages are in lateral views with the ventral side down (A-D). Yolk was removed from the germ bands; they are shown from ventral (E-K).*

(A) Putatively maternal message in early blastoderms is distributed homogeneously.

(B-C) Expression retracts from both poles. Anteriorly, the extraembryonic serosa becomes devoid from Tc'giant *staining.*

(D) Late blastoderm with beginning invagination of the posterior growth zone. Expression becomes stronger in a stripe in the future maxillary segment (arrowhead); a second domain arises de novo at the posterior pole at the posterior pit stage.

(E) The germ band is formed, but the extraembryonic serosa does not yet envolve the embryo completely (see ring in the anterior half of the germ band). Expression is equivalent to (D): the whole anterior part of the germ is staining, with stronger expression in the maxilla (arrowhead), and a posterior domain covers the growth zone. At the anterior rim of the posterior domain, a stripe begins to condense (arrowhead).

(F-G) The anterior domain has retracted from the maxilla (white arrowhead) but remains in the mandible and in the brain. The posterior domain has resolved into two stripes in T3 and A2 (black arrowheads). The anterior stripe fades before the T3 engrailed *stripe is formed (white arrowhead in G), as does the posterior stripe a bit later.*

(H) Posterior expression has ceased while the germ still grows.

(I-K) Head expression condenses into domains and later into cell clusters that will probably contribute to neural tissue.

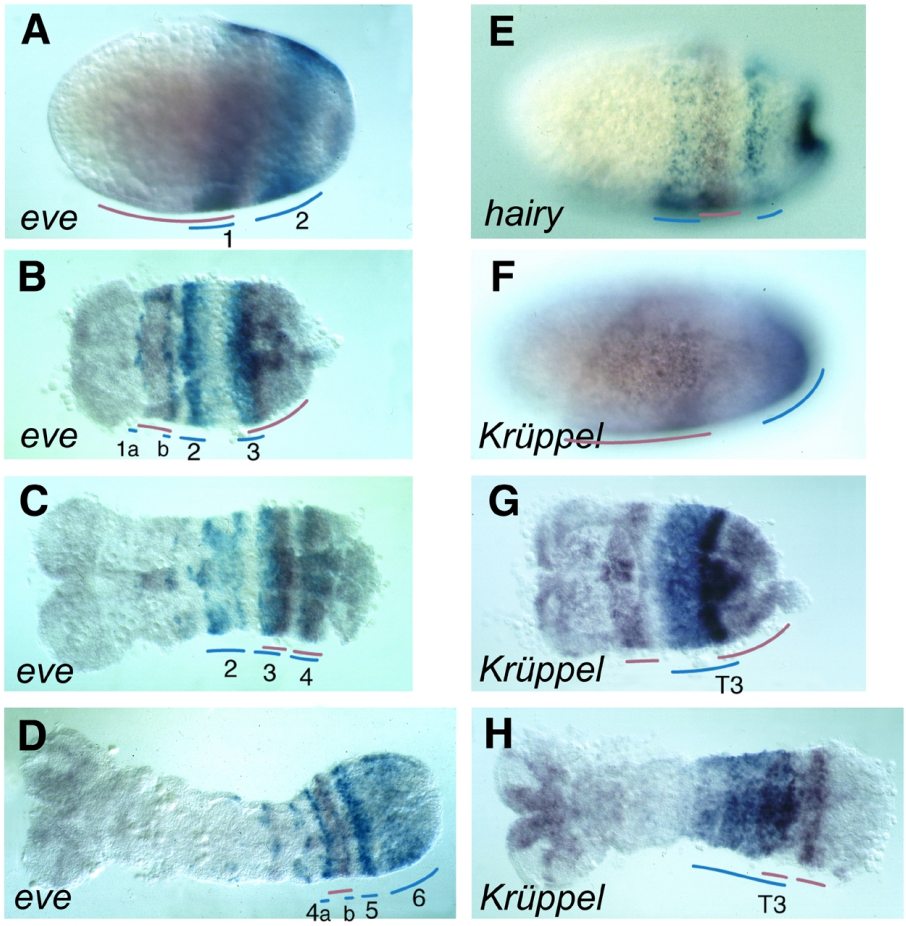


Figure 3

Double in situ hybridization with Tc'giant *in brown and* Tc'even-skipped (Tc'eve) *(A-D),* Tc'hairy *(E) and* Tc'Krüppel *(F-H) in blue. Orientation of the embryos is as in figure 2.*

(A-D) The Tc'giant *stripes in maxilla, T3 and A2 coincide roughly with the 1st, 3rd and 4th* Tc'eve *stripes, respectively. Interestingly, they all mature in the same relation to each other: Early* Tc'eve *stripes extend some cells anteriorly to the* giant *stripes. As the pattern matures, they come to coincide almost (see stripe 3 in B and C). Each* Tc'eve *stripe splits into secondary stripes which later express* engrailed. *The posterior secondary stripe coincides with the posterior rim of* Tc'giant *stripes, while the anterior one lies anteriorly adjacent (see 4 a and b in D).*

(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 stripes become adjacent. The anterior borders of the hairy stripes remain separated from* Tc'giant *stripes by some cells .*

(F-H) In Drosophila, giant *and* Krüppel *are expressed in mutually exclusive domains and they strongly repress each other. (F) In* Tribolium, Tc'Krüppel *arises at the posterior pole of the blastoderm within* giant *free tissue (compare with C in figure 2). In early stages both genes appear as nonoverlapping opposing gradients which could indicate conserved negative interaction as in* Drosophila. *(G-H) The posterior* Tc'giant *domain, however, arises right within the* Tc'Krüppel *domain and the genes remain coexpressed in the 3rd thoracic segment (T3 in G and H). Strong mutual repression seems therefore unlikely for this region. In addition, this staining shows, that in contrast to* Drosophila, Tc'Krüppel *is not expressed posterior to T3 .*

approximately coincides with that of the first *eve* stripe (Fig. 3A).

The posterior domain of *Tc'gt* forms within the growth zone, posterior of the 2nd *eve* and *hairy* stripes (Fig. 3 B/E). When the third *eve* stripe forms at the germ rudiment stage, it appears at the anterior border of the posterior *Tc'gt* domain, partially overlapping with it (Fig. 3B). Subsequently, the posterior *Tc'gt* domain breaks up into two stripes which overlap the 3rd and 4th *eve* stripes, respectively (Fig. 3 C). As the pattern matures and the *eve* stripes split into segmental stripes, both posterior *Tc'gt* stripes are anteriorly abutted, and posteriorly overlapped, by segmental *eve* stripes. Therefore, the late *Tc'gt* stripes can be mapped precisely to the 3rd thoracic and 2nd abdominal segments. In *Drosophila*, in contrast, the posterior expression border is five segments more posterior at abdominal segment 5. *Tc'gt* mRNA is not detectable in the cells where the 5th *eve* stripe formes (Fig. 3D). We conclude that the posterior expression of *Tc'gt* could be involved in determining the position of *eve* stripes 3 and 4, but is unlikely to be involved in directly specifying *eve* stripes 5 to 8. This is in marked contrast to *Drosophila*, where the posterior *giant* domain is directly required for proper formation of *hairy* stripes 5 and 6 (Langeland, Attai et al. 1994) and *eve* stripe 5 (Fujioka, Emi-Sarker et al. 1999). We conclude, therefore, that the anterior *Tc'gt* domain is approximately conserved in position, while the posterior domain is shifted towards anterior by 5 segments.

Double stainings with other segmentation genes confirm these interpretations. The first *Tc'hairy* stripe overlaps with the anterior *Tc'gt* stripe (Fig. 3E), which corroborates that this stripe coincides with the maxillary segment primordium (Brown, Parrish et al. 1994). *Krüppel* (Kr) in *Drosophila* is a gap gene that negatively interacts with *giant* such that *Dm'Kr* and *Dm'gt* domains are expressed in adjacent but non-overlapping domains (Kraut and Levine 1991) (Capovilla, Eldon et al. 1992). In *Tribolium*, the *Krüppel* domain arises at the posterior extreme of the blastoderm once the early *Tc'gt* expression has retracted from the pole, and before the posterior *Tc'gt* domain has formed. Prior to the posterior pit stage, therefore, *Tc'gt* and *Tc'Kr* are mutually exclusively expressed, as in *Drosophila*. Subsequently, however, the posterior *Tc'gt* domain arises within the *Krüppel* domain. In the germ rudiment, *Tc'Kr* becomes restricted to a sharply demarcated band initally covering segments T2 and T3 and then also extending into T1 (own observation). This domain overlaps with the posterior *Tc'gt* domain in a stripe corresponding to the 3rd thoracic segment (Fig. 3G, H). It is clear from these data that the posterior *Tc'gt* domain is not negatively regulated by *Kr* as is the posterior *gt* domain in *Drosophila*.

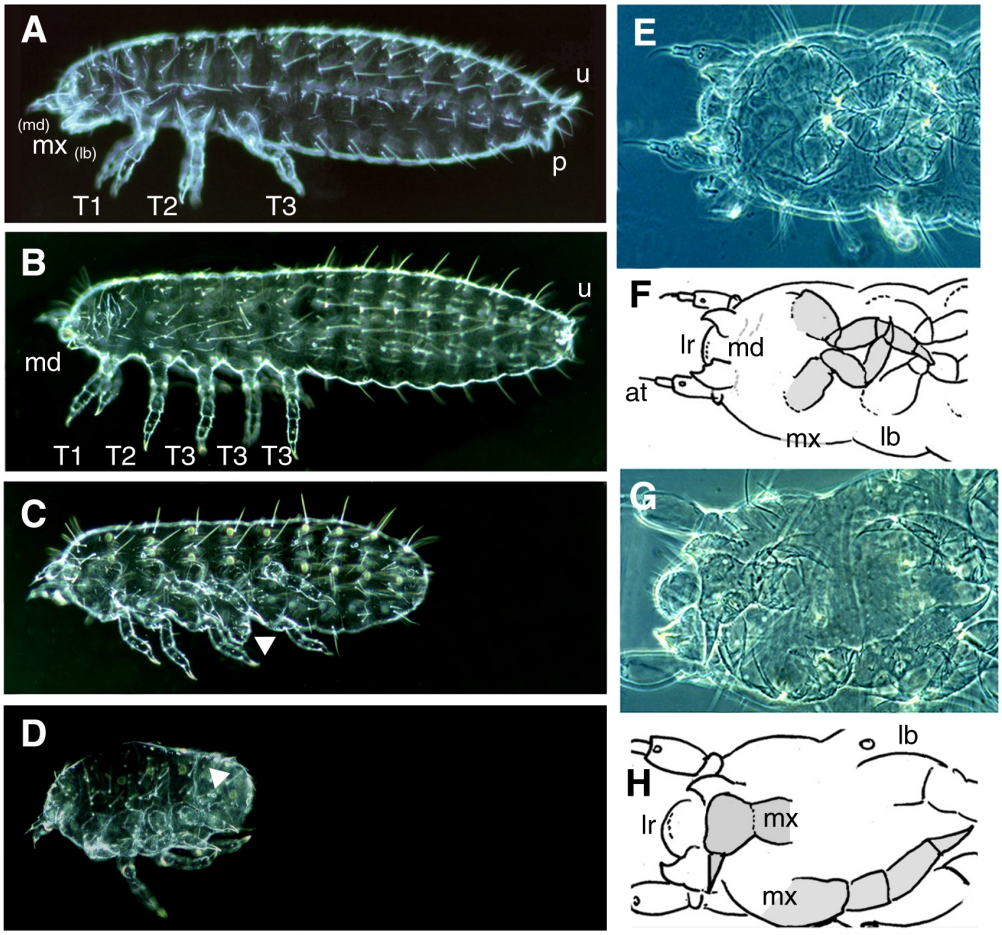


Figure 4

Effect of RNAi gene knock down on first instar larval cuticles. All larvae are shown with anterior to the left.

(A) Wildtype larva with three leg bearing thoracic segments (T1-T3) and eight discernible abdominal segments. The urogomphi (u) are outgrowths of the telson but are derived from A9. In this lateral view, the mandibles (md) and the labium (lb) cannot be seen because they are covered by the maxilla (mx).
In almost all cuticles, maxilla and labium were transformed to T1 and T2 respectively. (B) In weak phenocopies the transformation of gnathal segments is not accompanied by segmentation defects. Intriguingly, the thorax is shifted coordinately such that the mandible is followed by T1, T2 and T3. The posterior thoracic segments have an identity ranging between T3 and abdominal.
(C) Most Tc'giant *phenocpies have segmentation defects in addition to homeotic transformations. In this specimen, five thoracic and four residual abdominal segments are formed, the urogomphi are missing. Thus, five segments are deleted. Because abdominal segments have identical cuticle patterns, 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 segments with thoracic identity are left and the presence of only one pair of stomata (white arrowhead) indicates that only one abdominal segment is formed. Also in such severely disturbed cuticles, the terminal pygopods are 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) is a schematic representation of (E) with the transformed maxillary appendages highlighted in grey.
(G-H) In some cuticles, the transformation of the maxillary segment is not complete. Here, the lower appendage is transformed to leg, while the other adopted an intermediate identity. Partial transformations were only observed in the maxillary but not the labial segment, and in no case were thoracic identities shifted only one segment towards anterior.

Analysis of Tc'gt function by RNAi, parental RNAi, and morpholino oligonucleotides

To investigate the function of *gt* during segmentation, we injected double stranded RNA (dsRNA) into *Tribolium* eggs in order to reduce *Tc'gt* activity by RNA interference (Fire, Xu et al. 1998) (Brown, Mahaffey et al. 1999). The RNAi treatment elicited homeotic transformations as well as variable segmentation defects (Fig. 4). The phenotypic strength of the segmentation defects

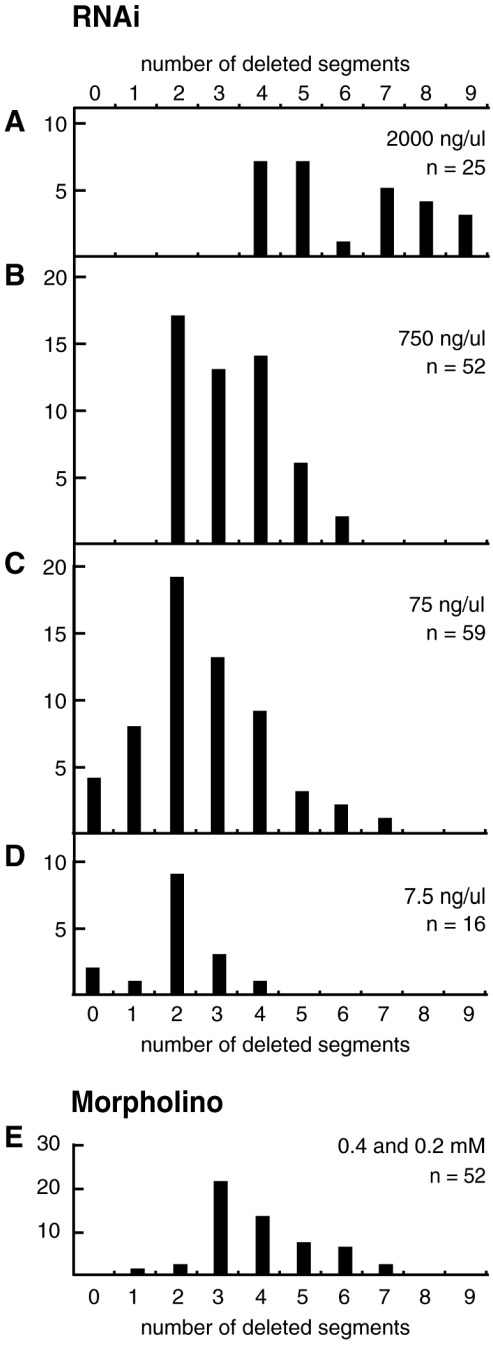


Figure 5

Distribution of cuticular segmentation defects for different concentrations of dsRNA (A-D) and morpholino oligos (E). Given is the absolute number of cuticles that lacked a certain number of segments. Only individuals were counted that had been injected with an effective dose of dsRNA/ morpholino as judged by the presence of anterior transformations. The dsRNA concentrations ranged within several orders of magnitude (2000 ng/ul in A through 7,5 ng/ ul in D); nevertheless, the observed dosage effect was relatively mild (A-D). Although morpholino oligos inhibit gene function by a different mechanism and are chemically distinct, a similar range of deletions was observed (E).

The portion of injected embryos that developed cuticles decreased with dsRNA concentration: ~20% with 2000 and 750 versus ~50% with 75 and 7.5 ng/ul, respectively. Moreover, the portion 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/ul, respectively. Injecting low amounts of the lowest concentration resulted in 80% wildtype cuticles, suggesting that the minimal requirement for dsRNA was approached with 7.5 ng/ul.

correlated (albeit not linearly) with the concentration of dsRNA (Fig. 5A-D).

Since we worried if some of the observed segmentation defects might result either from mechanical damage during injection, or from unspecific disturbances of development by dsRNA as observed in the zebrafish (Zhao, Cao et al. 2001), we performed a number of positive and negative controls. First, we injected dsRNA from a gene of known function, *Tc'Dll* (Beermann, Jay et al. 2001). In these experiments, embryos displaying *Dll*-specific leg defects were observed with high frequency (not shown), while similar segmentation defects were not observed. This demonstrates that dsRNA does not usually result in unspecific disturbance of segmentation or germ band growth.

However, many of the injected eggs failed to complete embryonic development, probably due to injury by injection. We recently could show that dsRNA injection into *Tribolium* pupae leads to zygotic gene inactivation in the offspring (Bucher et al. 2002).We also applied this "parental RNAi" technique to knock down *Tc'gt* function. In this experiment, a range of embryonic phenotypes (not shown) was observed similar to that obtained by embryo injection. This confirms that the observed segmentation defects are not due to mechanical injury, since in parental RNAi experiments the eggs are not directly penetrated by the capillary. Moreover, most embryos completed development in the parental RNAi experiment, which suggests that artifacts due to unspecific effects of dsRNA also are not responsible for these phenotypes.

Finally, we sought to confirm these functional data through an independent method. To this end, we injected morpholino oligonucleotides complementary to sequences around the start codon of *Tc'gt* (see methods). By this means we obtained larval phenotypes very similar to those generated by RNAi (Fig. 5E) Morpholinos are structurally different from dsRNA, and are thought to knock down gene function by a different mechanism (probably by sterically blocking the ribosomal entry site). This is strong evidence that the RNAi results described below are indeed specific to reduced *Tc'gt* activity.

Tc'gt determines the identity of gnathal segments

Most RNAi embryos share three phenotypic characters: (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 segments maxilla and labium are missing. Other head structures, i.e. antenna, labrum and mandible develop normally. In *Drosophila gt* mutants the labial segment is missing (Petschek, Perrimon et al. 1987; Petschek and Mahowald 1990). In contrast

to that, several observations support the view that in *Tc'gt* knock-down embryos the maxillary and labial segments are patterned normally, but are transformed to thoracic identity. First, the two leg-bearing segments posterior to the mandible do never show traces of segmentation defects (Fig. 4B-F) - quite in contrast to the posterior segments (see below), which are often associated with partial deletions. Secondly, in some RNAi embryos, we observed abnormally shaped legs in the anteriormost segment which appear to be partial transformations of gnathal appendages towards legs (Fig. 4G,H). These intermediate phenotypes show that at least one gnathal segment is transformed towards thorax in these embryos. Thirdly, four percent of all embryos lacking maxilla and labium (6 embryos out of 154) developed a full complement of 14 segments, the same number as in wild type larvae (which have 3 gnathal, 3 thoracic and 8 abdominal segments). These RNAi embryos displayed 1 gnathal segment (the mandibular segment), 5 thoracic segments and 8 abdominal segments. This suggests that the maxillary and labial segments, but no abdominal segments, were transformed towards thorax. Finally, of 52 embryos stained for *engrailed* expression after injection with *Tc'gt* dsRNA, all displayed perfectly formed *engrailed* stripes in the gnathocephalon (Fig. 6, see also following section).

It is important to point out that the homeotic shift always includes two segments or none at all. We did not observe embryos where the maxilla is followed by a complete thorax (which would be a one segment shift) nor were there phenocopies where maxilla and labium were transformed into T2 and T3 (which would be a three segment shift). In some embryos, however, the maxilla was only partly transformed to a thoracic segment followed by perfect T2 and T3 (e.g. transformed labium and T1, respectively. Since at least these posterior thoracic segments clearly represent a two segment shift, these embryos fall into the same class despite the incompletely transformed maxilla.

From these observations we conclude that the anterior *Tc'gt* domain functions in homeosis but not segmentation. This role in homeosis could either indicate that the head domain of *Tc'gt* fulfills similar functions as canonical homeotic genes like *Deformed* or *spalt*. Alternatively, this phenotype could indicate a function of *Tc'gt* in the regulation of homeotic genes. Maxillary and labial palps are transformed into legs also in mutants of one homeotic gene of *Tribolium*, *maxillopedia (mxp)*, which is the *Tribolium* orthologue of *Dm'proboscipedia* (Shippy, Guo et al. 2000). To see if *mxp* expression is regulated by *Tc'gt*, we used parental RNAi (Bucher et al. 2002) to generate embryos with reduced *Tc'gt* activity, and stained them for *mxp* by in situ hybridization. Indeed we find that ectodermal expression of *mxp* in the appendages of the maxillary and labial segments (now transformed to thorax) is reduced or absent (see Fig. 6I, J). This confirms that *giant* is involved in Hox gene regulation in the gnathocephalon. However, the homeotic phenotype of

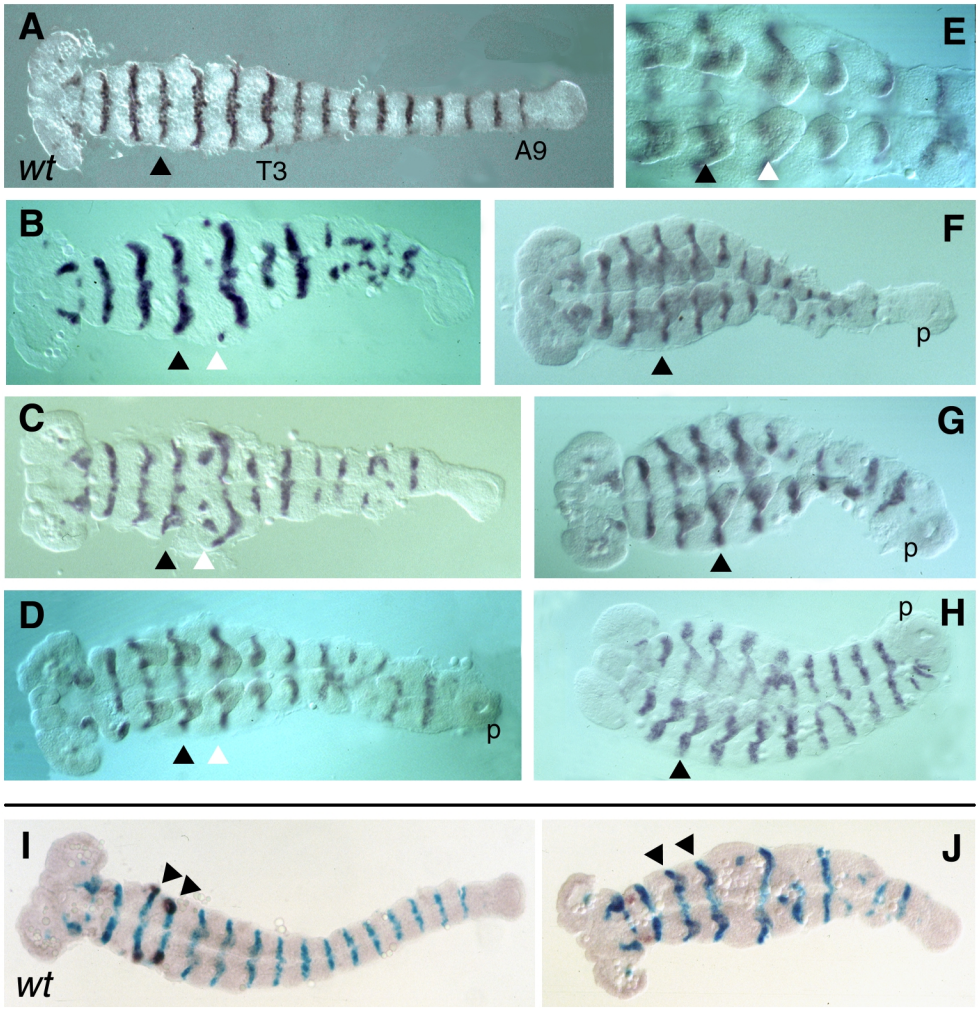


Figure 6

In situ detection of engrailed *(B-J) and the Hox gene* maxillopedia *(J) in RNAi treated germ bands. An arrowhead in A-H indicates the labial segment. The proctodeum (p) indicates that embryos in D through H had finished segmentation.*

(A) Wildtype germ band shortly before generating the last abdominal engrailed *stripe. 10 abdominal, 3 thoracic and 3 gnathal segments are specified in wildtype on the* engrailed *level, totaling 16 stripes.*

(B-H) Segmentation is disturbed between T1 and A9 in a variable pattern. In all germ bands analyzed, the first three segments were patterned correctly, suggesting that in the gnathocephalon Tc'giant *acts only in homeosis. The T1 stripe was often disturbed or deleted in young embryos (white arrowheads in B and C), leading to an enlarged segment. By the end of segmentation similar defects in T1 were not observed any more (D-H), suggesting, that the embryo corrects for these early patterning defects. In some cases, cells of the enlarged T1 segment 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 any more, suggesting further correction. In germ bands with proctodeum (p), the number of deleted segments can be determined (D: 7; F and G: 8; H: 4)*

(I-J) The Hox gene maxillopedia *is expressed in the appendages of the maxillary and labial segments (arrowheads in I). In* Tc'giant *RNAi embryos, this expression is reduced or absent (arrowheads in J), confirming that* Tc'giant *knock down interferes with proper Hox gene regulation. However, the observed coordinated shift of three thoracic identities likely requires mis-expression of additional Hox genes (see discussion).*

Tc'gt likely involves mis-regulation of additional homeotic genes, since in null-mutants of *mxp* only the palps but not the complete maxillary and labial segments are transformed (see discussion).

The posterior Tc'giant *domain has a long-reaching function in segmentation*

Depending on dsRNA concentration, up to 9 body segments are missing in *Tc'gt* RNAi embryos (Fig. 5A-D). Using morpholino oligonucleotides to knock down *Tc'gt* gene activity, we achieved deletions of up to 7 segments (Fig. 5E). While some of these larvae have clear-cut deletions of whole segments, others display an irregular pattern with some segments being deleted only in part. The most anterior larval defect is a rarely observed disturbance of the dorsal cuticle in the region of T1. Partial or complete deletion of T2 and T3 are more frequent: 17% and 7% of *Tc'gt* RNAi embryos have only four or three pairs of legs due to defects in thoracic segments. In the case of abdominal segments it is more difficult to determine which segments are affected, since all abdomial segments are of similar morphology. Only in very weak phenotypes, where at least remnants of all eight abdominal segments can still be recognized, is it possible to determine which segments exactly are affected. In such rare embryos, the segments T2, A2, A6 or A7 have been found to be partially deleted (once in each case), suggesting that pattern formation is affected more or less evenly in all of the abdomen. The posterior limit of the segmentation defects, however, can be determined quite precisely. Two pairs of terminal structures, the dorsal urogomphi and the ventral pygopods (e.g. Fig. 4A), are formed by the 9th and 10th abdominal segment, respectively. The respective segments later fuse with the telson. The urogomphi are missing in 70% of all embryos displaying RNAi phenotypes. This identifies A9 as a segment very sensitive to lack of *Tc'gt* activity. The pygopods, on the other hand, are never affected. By these observations, the region requiring *Tc'gt* for segmentation can be delimited to the 12 segments from T1 through A9. However, we never obtained a larva lacking all these segments.

To better understand the nature of the segmentation defects, we examined the expression of the segment-polarity gene *engrailed* in embryos injected with *Tc'gt* dsRNA (Fig. 6). These expression data we also confirmed in embryos whose *giant* gene had been depleted by parental RNAi. As was already mentioned above, none of 51 injected and stained embryos showed any disturbance of gnathal segments e.g. the anteriormost three stripes. Partial or complete deletions of *engrailed* stripes were frequently observed throughout thorax and abdomen. To determine the number of affected stripes, we analysed 28 germ bands that had completed segmentation (as indicated by the presence of an invaginated proctodeum). Most of these (61%) lacked four to six segments, and a sizable fraction (18%) was missing 7 to 8 segments. This share of severe defects is somewhat higher than that seen

in cuticle preparations. It is possible, however, that some of the early patterning defects can be repaired during subsequent development. Alternatively, the most severely affected embryos may die before differentiating a cuticle. Another discrepancy between early and late phenotypes we observed in the prothorax. The first thoracic segment is disturbed or deleted in 39% of stained embryos, but appears to be rarely affected in cuticles of 1st instar larvae. In many *engrailed*-stained embryos, the T1 stripe is more severely affected than the T2 stripe, and quite frequently the absence of the T1 stripe is only apparent from an increased distance between the last gnathal and the first thoracic *engrailed* stripe (which propably represents the T2 stripe). It is likely, therefore, that in many cases where T1 appears unaffected in 1st instar larvae, the apparent T1 segment actually is formed by the T2 stripe.

By and large, however, the analysis of *engrailed* expression in *Tc'gt* RNAi embryos confirms our interpretations based on cuticle preparations, i.e. it confirms that all abdominal segments except for the last (i.e. A10) can be malformed in embryos with reduced *Tc'gt* activity. Furthermore, *engrailed* expression analysis shows that formation of all thoracic segments requires *Tc'gt* function.

Discussion

We have cloned the first non-dipteran orthologue of the *Drosophila giant* gene (*Dm'giant*). Phylogenetic analysis of the leucin zipper domain shows that *Tc'giant* is more closely related to *Dm'giant* and GC4575 than to any other gene in the database. But only with *Dm'giant* it shares several additional conserved motivs upstream of the zipper domain. This and some conserved aspects of *Tc'giant* expression suggest homology with *Dm'giant*: Anteriorly, both *Tc'giant* and *Dm'giant* are expressed in a dynamic pattern in the brain and in a stripe in the maxilla. Moreover, both genes have a second expression domain more posteriorly. These similarities strongly suggest that *Tc'giant* is indeed the true orthologue of *Dm'giant* although expression and function of both genes differ in many respects, as discussed below.

The anterior Tc'giant *domain functions in homeosis*

In *Drosophila*, the anterior *giant* domain is involved in positioning pair rule stripes. Especially its direct input in defining the anterior border of *eve* stripe 2 has been shown in detail (Small, Blair et al. 1992), and lack of *giant* function leads to loss of the labial *engrailed* stripe (Eldon and Pirrotta 1991). In *Tribolium*, in contrast, we could not detect segmentation defects in the gnathal segments, only in the thorax and abdomen. Since thorax and abdomen are close to, or arise from within, the posterior domain, it appears that the anterior domain is not involved in segmentation.

In addition to its function in segmentation, *Drosophila giant* is also involved in regulating Hox gene expression, for example it defines the anterior border of the thoracic Hox gene *Antennapedia* (Reinitz and Levine 1990). Also *Tribolium giant* is required for homeosis: In *Tc'giant* RNAi embryos the maxillary and labial segments are transformed to thorax. Intriguingly, all three thoracic identities are shifted towards anterior in a coordinated manner, suggesting that *Tc'giant* is required for regulation of several Hox genes, which together define at least three distinct identities.

The homeotic code for gnathal and thoracic segments in *Tribolium* is not known completely. We have shown that in RNAi phenocopies, the expression of the orthologue of the *Drosophila proboscipedia* gene, *maxillopedia (mxp),* is abolished or reduced in maxilla and labium. However, this finding explains the transformation only in part, because in *mxp* mutants only the appendages of maxilla and labium are transformed to legs - the maxillary endite and the overall segment

organisation remain unchanged (Shippy, Guo et al. 2000). For the complete transformation observed in *Tc'giant* phenocopies, additional Hox genes must be involved. RNAi with *Tc'Deformed* affects the endite of maxilla while the telopodite remains normal (Brown, Mahaffey et al. 1999) suggesting involvement of *Tc'Dfd* in the transformation event. Because of the coordinated transformation to thoracic identities, lack of *Tc'giant* probably also results in ectopic expression of thoracic Hox genes in the gnathum. Taking the *Drosophila* situation as reference, the *Tribolium* orthologue of *Antennapedia (Antp)* is likely to be involved: *Antp* defines thoracic identities in *Drosophila* (Carroll, Laymon et al. 1986) and *Dm'giant* is involved in defining its anterior border (Reinitz and Levine 1990). In the posterior thorax of *Tc'giant* phenocopies, the situation is less clear: The posterior two pairs of legs often adopt a mixed identity between T3 and abdomen. Thus, also Hox genes responsible for specifying the border between thorax and abdomen are probably disturbed, i.e. *Tc'Ubx* and *Tc'abdominal-A* (Bennett, Brown et al. 1999; Stuart, Brown et al. 1993). Unfortunately, the homeotic situation in the posterior thorax is blurred by segmentation defects also affecting this region.

One straightforward model for explaining the coordinated shift of three thoracic segment identities is that *Tc'giant* protein forms a morphogenetic gradient. The *Giant* protein would emanate from its source in the maxillary segment and the gradient would be read directly by several Hox genes: The more distant thoracic segments would be defined by lower levels than the closer gnathal ones. However, according to this model, a homeotic shift of one segment width should occur when the morphogen is knocked down to an intermediate level. This prediction is in contrast to our observation that the shift always encompasses two complete segments.

Taking into account the observed two-segment-shift, we prefer an alternative model that includes pair rule gene action in homeotic specification. The model assumes two steps: First, mutual repression of *Tc'giant* with a posterior factor restricts their respective expression to mutually exclusive domains. In this first step, presence of *Tc'giant* specifies gnathal fate while presence of the posterior factor determines thorax. During the second step, pair rule genes modulate Hox expression within the *Tc'giant* domain to specify maxilla versus labium. Similarily, they distinguish between T1 and T2 within the domain of the posterior factor. In *Tc'giant* phenocopies, the thoracic factor would expand anteriorly, because its repressor *Tc'giant* is lacking. Together with the unaffected pair rule stripes it would specify T1 and T2 instead of maxilla and labium. In this model, the involvement of pair rule genes explains the shift across two or none segments: The (probably unaltered) presence of pair rule activity for instance in the labium does not allow a transformation to the adjacent T1 identity, because T1 relies on input of a pair rule gene of the opposite frame. Therefore, depending on the level of *Tc'giant* versus the posterior factor, the unaltered pair rule information in the labial segment will either define labium or T2.

We also observed embryos where a partially transformed maxilla is followed by T2 and T3. These latter identities are shifted by two segment widths as is the case in fully transformed specimen. The partial transformation of the maxilla can be explained, if one assumes that residual *Tc'giant* protein is still present in that segment (where its expression is strongest in wildtype animals): The amount of *Tc'Giant* protein may not be enough for restricting the posterior factor to the thorax, enabling thoracic fates in the gnathum. But enough *Tc'giant* may still be present in the maxilla to repress the posterior fate to some extent.

The mutant *jaws* provides additional evidence for the involvement of pair rule genes in homeosis: In *jaws* mutant embryos, all thoracic and the first abdominal segment are transformed to alternating maxillary and labial segments (Sulston and Anderson 1996). Interestingly, this double segmental phenotype is complementary to the transformation observed in *Tc'giant* phenocopies. *jaws* is thus a good candidate for the hypothetical posterior factor of the above presented model (see general discussion for a model including the result that *jaws* is a *Krüppel* allele).

Expression and function of the posterior Tc'giant *domain deviates from* Drosophila

The expression pattern of the posterior *Tc'giant* domain shows several marked differences to the *Drosophila* situation. First, *Drosophila giant* is activated by maternal genes (Kraut and Levine 1991) while *Tc'giant´*s posterior domain arises *de novo* in the late blastoderm, which suggests zygotic rather than maternal activation. Secondly, the posterior expression domain is shifted by about five segments: *Drosophila giant* is expressed in, and required for, proper segmentation of segments A5-A7 (Kraut and Levine 1991; Petschek and Mahowald 1990) where it is involved in pair rule gene regulation (Langeland, Attai et al. 1994; Nibu and Levine 2001). *Tribolium giant,* in contrast, is expressed in segments T3 through A2. Therefore, direct input of *Tc'giant* on posterior pair rule stripes is hardly possible. Finally, *Drosophila Krüppel* and *giant* are strong mutual repressors in the posterior blastoderm (Kraut and Levine 1991) while their *Tribolium* orthologues overlap in the third thoracic segment during the whole course of expression, arguing against strong negative interactions. Together, these expression data suggests that in *Tribolium* neither gap nor pair rule genes are regulated by *giant* in the same way as in *Drosophila*.

The segmentation defects in *Tc'giant* RNAi embryos further suggest major changes in *giant* function. The *Drosophila* gap genes are thought to function by providing short range morphogenetic gradients emanating from their expression domains (Hülskamp and Tautz 1991). Accordingly, the

deletion domains in the respective mutants exceed the expression domains only by several cell diameters. In strong *Tc'giant* phenocopies, in contrast, segmentation is disturbed in a region comprising twelve segments, ranging from T1 through A9. Only the anterior defects from T1 through A3 could rely on similar short-range function as in *Drosophila*, because *Tc'giant* is expressed in T3-A2. The more posterior segments are disturbed although they are formed more than four segments apart from *Tc'giant* activity and even after *Tc'giant* expression has ceased. Such long reaching effects can hardly be explained by a short-range morphogenetic gradient.

At this point, we can only speculate about alternative mechanisms of segmentation in *Tribolium* and the role, which *Tc'giant* plays in the process. One possibility relates to the early *Tc'giant* expression in the growth zone. This early expression could be involved in establishing the growth zone by inducing growth zone specific genes, e.g. making its cells competent for signalling, proliferation and/or movement. It might also be involved in determining the polarity of the growth zone or its internal structure. Lack of *Tc'giant* function would then result in aberrant cellular behavior during growth and/or patterning, and thereby indirectly disturb the segmentation process. In this model, *Tc'giant* does not provide spatial cues and has no function in directly regulating abdominal pair rule stripes.

A second model assumes that *Tc'giant* and *Tc'Krüppel* could be involved in establishing a "prepattern" of the whole abdomen in the growth zone, which would unfold later during germ band extension. Specific (narrow) anterior-posterior positions defined in the growth zone would correspond to (broader) positions in the expanded germ band. Lack of *Tc'giant* would disturb the narrow growth zone patterning, which would later lead to long reaching effects in the whole extended germ band. This model requires that early positional information is stored in growth zone cells and remembered later during elongation. A more difficult requirement of the model is that the prepattern has to remain stable despite complex cell movements and cell divisions.

The third class of models assumes that early *Tc'giant* is involved in setting up and/or starting a segmentation machinery that subsequently patterns the abdomen autonomously. Improper set up would lead to a break down of the whole patterning machinery and thus explain defects in segments that lie distant from the *Tc'giant* domain and that are formed only after its expression has ceased. Different autonomous patterning machineries can be assumed: First, segmentation could rely on a chain of induction mechanism involving gap genes (Meinhardt, 1982). *Tc'giant* expression could be required for activation of more posterior gap genes, which in turn would activate even more posterior genes and so on. Lack of *Tc'giant* could lead to breakdown of the whole induction chain. The anterior to posterior sequence of gap gene activation in *Drosophila* (Hülskamp and Tautz 1991)

could be reminiscent of such an ancestral process. As *Tc'giant, Tc'Krüppel* and *Tc'tailless* are shifted anteriorly in *Tribolium* (see also general discussion), such a model would require the existence of several additional gap genes in the *Tribolium* abdomen. A variant of this model assumes a repetitive chain of induction. In contrast to the gap gene model, the same set of genes would be activated repeatedly. As pair rule genes play a crucial role in patterning *Drosophila* and have conserved expression patterns in *Tribolium*, such a repetetive chain of induction may well work on the level of pair rule genes. In addition to the spatial chain of induction mechanisms proposed above, also a temporal regulation is possible. It is believed that somitogenesis of vertebrates relies on a clock mechanism that works in the posterior part of the presomitic mesoderm (Palmeirim 1997). *Tc'giant* could be required for setting up and/or starting a similar mechanism in *Tribolium*. There is, however, no indication for a clock mechanism in *Tribolium* so far.

Finally, an autonomous patterning process might involve a diffusion reaction system of gene regulation that flips between different states, depending on the distance of the last segment from the posterior extreme of the germ band: One state with a certain number of stripes (for instance two pair rule stripes) is stable with a relatively short growth zone. As the posterior end elongates, a critical length is reached and the system flips into a second state where one additional interstripe splits one of the stripes. Establishment of a new segment by, for example, segment polarity genes could form a new border that lies more posteriorly and thereby shortens the distance again, leaving the system again in the first state. The setting up of the inital state during the blastoderm might depend on *Tc'giant* function, while the following flips would not require its input any more.

There are some problems with all models assuming an autonomous process established by *Tc'giant*. First, it remains elusive how any repetitive mechanism can count the rounds and stop after the correct number of segments has been formed. Furthermore, some abdominal segments are still patterned in most *Tc'giant* RNAi phenocopies. In contrast to that, an autonomous process is likely either to be set up properly or to break down almost completely. However, there may be redundancies in setting up the segmentation machinery, buffering for reduction of *Tc'giant* function to some extent. Moreover, the *Tribolium* embryo is able to correct for early segmentation defects: Severely disturbed T1 stripes were frequently observed in young RNAi treated germ bands, while at germ band retraction, these stripes had been either repaired or deleted completely such that the T2 stripe adopted T1 identity. It could, for instance, be that proper establishment of segments relies on an autonomous process, while downstream genes (for instance pair rule and segment polarity genes) have the role to fine tune this initial pattern. This would require some regulative capacity on the lower genetic levels, which could lead to the *de novo* formation of some stripes even in the absence of any upstream signal. And indeed, it is known from Drosophila that the pair rule gene network has pattern

refinement and pattern maintanance capacity. Also segment polarity genes can autonomously correct for some early patterning defects.

Future experiments will have to distinguish between these models. In any case, our results show that the segmentation hierarchy known from *Drosophila* is not conserved on the level of abdominal gap genes. Interestingly, these changes correlate with the major developmental difference between *Drosophila* and *Tribolium,* namely the formation of the abdomen in a growing germ band. It is therefore likely that the observed genetic changes were necessary for the evolution of different modes of development, i.e. the short and long germ embryogenesis.

Additional evidence for a fundamental change of abdominal segmentation comes from the finding that other abdominal gap genes have also changed their position on the fate map (see general discussion) and that *jaws* is a *Krüppel* allele (see next chapter). These data are discussed in the general discussion and a model for the evolution of abdominal gap genes is presented there.

Project 3:
Mapping segmentation mutants relative to candidate genes

Introduction

Segmentation mutants in Tribolium

In order to compare the segmentation machinery of *Tribolium* with that of *Drosophila*, orthologues of fly segmentation genes have to be functionally analyzed in the beetle (see general introduction). Mutants in patterning genes provide such functional data and, in contrast to RNAi studies, genetic screens also can identify novel genes. Therefore, two genetic screens for embryonic lethal mutations have been carried out and several segmentation mutants have been isolated and described (Sulston and Anderson 1996; Maderspacher, Bucher et al. 1998). Comparisons of their phenotypes (see figure 1) with *Drosophila* mutants led to speculations to what gene class they might belong: *itchy* (*icy*) and *scratchy* (*scy*) are clear pair rule mutants with complementary deletion frames. *tigerente* (*tig*) was identified independently from *scy*, but I found them to be allelic. Because *scy* was the first allele identified, its name will be used in the following (*tig* was renamed to $scy^{tig)}$. Also *godzilla* (*god*) has been suggested to be a pair rule mutant because it affects segments along the whole body length. However, an overt double segmental periodicity of the defects was not observed. Both *krusty* (*kry*) and *bollig* (*bol*) lack a group of adjacent segments at the border between thorax and abdomen and have therefore been described as gap mutants. Also *jaws* has severe abdominal segmentation defects, but in addition it shows anterior transformation of the thoracic to gnathal segments. Because of this combination, it has been suggested to be a gap or a Polycomb/Trithorax group gene. *hintenrum* (*rum*) is a mutant where the posterior part of the abdomen is not formed. Because of its low penetrance and temperature sensitivity, it is probably a hypomorphic mutation (unpublished observation).

For a thorough gene by gene comparison of the segmentation mechanism, it is necessary to identify the genes affected in those mutants. Several approaches to identify mutated genes in *Tribolium* are presented in the following and the rational for taking the mapping approach is discussed.

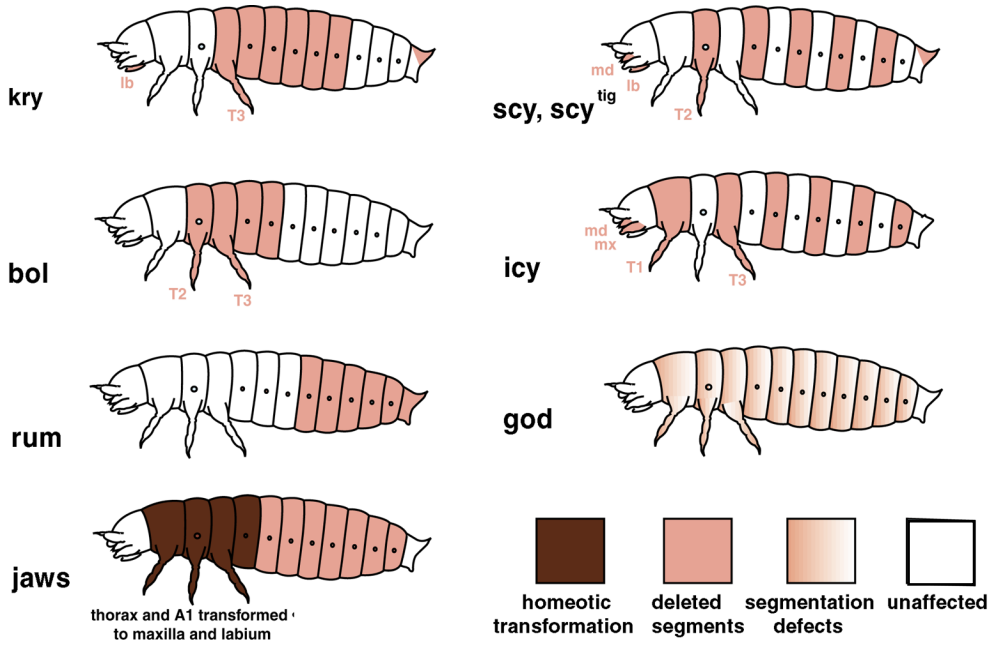


Figure 1
Segmentation phenotypes of the mutants that I mapped relative to candidate genes. Mutants lacking a group of adjacent segments are shown on the left, mutants that are considered to be pair rule mutants on the right side. Segments highlighted in red are entirely or partly deleted in first instar larval cuticles. The dark red color in the thorax of the jaws *mutant indicates homeotic transformation without segmentation phenotype.* godzilla *has segmentation defects along the whole body axis, but it is not possible to assign them to specific segments - all segments are therefore shown in* red/*white*

Approaches for identifying genes affected in patterning mutants

Positional cloning

Positional cloning is a lengthy procedure that identifies the gene affected by a mutation. First, by genetic linkage to molecular markers, the mutation is mapped down to a small region in the genome. The thus identified genomic region is then analyzed for open reading frames by chromosomal walking and mutations in there are identified, for example, by sequencing. Crucial for positional cloning is that a genetic map with many markers is available. A genetic map is being constructed for *Tribolium* but the average distance between the markers of this map is still in the range of 3,8 cMorgan (Beeman and Brown, personal communication). Positional cloning in *Tribolium* would therefore require the development of many additional markers and is thus still an extremely time and resource consuming approach.

Candidate gene approaches

A number of genes has been cloned from *Tribolium* and for a few the phenotype is known. In the case of *Distalless* the phenotype looks similar to its *Drosophila* orthologue (Beermann, Jay et al. 2001). It may therefore be possible to determine candidate genes for segmentation mutants by analogy to the *Drosophila* phenotypes, and to test these genes for mutations. The candidate gene approaches presented below can potentially reveal mutated genes more rapidly than positional cloning but novel genes will, of course, not be identified by this strategy.

A mutation can be molecularly identified, after a candidate gene has been determined by guess, guided by the expression pattern and/or analogy with its phenotype in *Drosophila*. Sequencing reveals mutations in the coding region, southern analysis tests for genomic rearrangements, while in situ hybridization in mutant embryos can reveal absence of the transcript. There are, however, some restrictions to this approach: not for all genes the complete coding sequence is available so far and only for very few the regulatory region is cloned. Secondly, if no mutation is detected molecularly, the possibility remains that the mutation lies outside the known sequences, for instance in a distant regulatory element. Thirdly, molecular differences may not be unambiguous. A mutation that changes an amino acid could also be a silent polymorphism and not cause the phenotype. Finally, the guess of what candidate gene could be mutated comes from analogy with the function in *Drosophila*. Homologues that underwent major evolutionary changes between both species might therefore not be recognised as candidates.

Another candidate approach involves RNAi analysis. Phenotypic series resulting from RNAi with all known segmentation genes could be compared with the phenotypes of mutants. Similarities would lead to the hypothesis that the respective gene is mutated and this could then be confirmed by molecular identification as described above. This approach potentially gives a fast indication on what candidate genes may be mutated. False negative and false positive guesses are not unlikely, however: First, a mutation might affect only part of the regulation of a gene, e.g. one region specific element. The phenotype will then match only one aspect of the RNAi phenocopy and might hence not be considered. Secondly, a mutation might affect only one splice variant of a gene while RNAi would knock down all variants and thus produce a different phenotype. Thirdly, mutations in different genes of the same pathway can have similar phenotypes - in that case, all genes in the pathway would have to be analyzed. Finally, knocking down the respective gene cannot reproduce gain of function mutations. Nevertheless, this is a potentially interesting approach. It was not considered for our mutants, however, because when the project was initiated at the beginning of this dissertation, RNAi had not been discovered yet.

Finally, a straightforward strategy to identify a mutated gene is the mapping of mutations relative to candidate genes. This experiment answers the question, whether a candidate gene is genetically linked to the mutation. Linkage indicates that the candidate gene itself might be mutated. Because also a nearby gene could be affected, such a hypothesis has subsequently to be further supported by molecular identification of a mutation in the gene. The power of the approach lies in the second case: if the candidate gene is not linked to the mutation, it can be excluded conclusively. By excluding all candidate genes, those mutations can be determined that affect novel genes with high probability and should therefore be considered for positional cloning. We decided to take this approach, because a subset of genes can be identified quickly and because the same material can later also be used for positional cloning. The procedure will be explained in more detail in the following section.

Candidate gene mapping - a strategy to assess linkage of mutations and candidate genes

Polymorphisms and strains

A population is not genetically uniform. Single base substitutions or small deletions/insertions within noncoding DNA are present in subpopulations. These polymorphisms are mostly silent mutations and thus are supposed to evolve neutrally (Kimura 1979). If the population is divided into isolated subpopulations, different alleles of the polymorphisms may by chance become fixed in the two populations. In addition, new polymorphisms may arise in separated populations by de novo mutation and fixation. The first experimental step for mapping a cloned gene is the identification of a polymorphism that lies close to a candidate gene and that has different fixed alleles in the two populations. The alleles of these polymorphisms must be molecularly distinguishable. In our case, one strain was *San Bernardino* (*SB*, American descendence, genotype: *SB/SB*) in which the Munich screen for mutations had been carried out. The strain used for outcrossing was *Tiw1* (derived from India, genotype: *Tiw1*/*Tiw1*).

Crossing scheme for candidate gene mapping

A mutation carrier male with *SB* background is crossed to a wildtype female of strain *Tiw1* (see figure 2). The resulting F1 generation is heterozygous for all loci in the genome (SB/*Tiw1*). A male mutation carrier of the F1 is again identified (see methods) and backcrossed with wildtype females of strain *Tiw1*. Recombination occurs during spermatogenesis in the male and “shuffles” both genomes. Every locus in the genome of the resulting F2 offspring has a 50% probability to be *SB*/*Tiw1* or *Tiw1*/*Tiw1* (Mendel 1865).

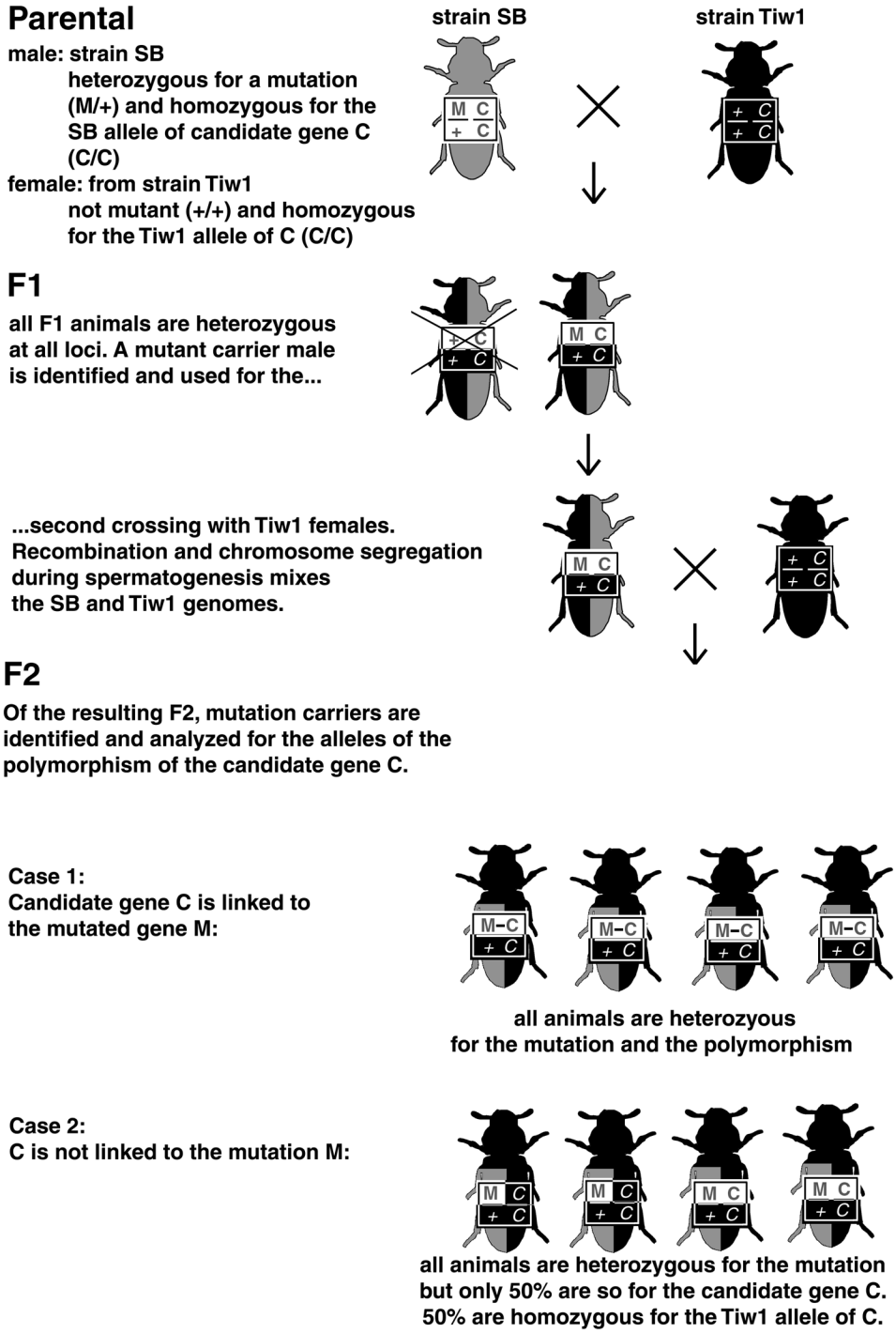


Figure 2
Crossing scheme used in the mapping experiment. The colors of the beetles indicate their genetic background (black = Tiw1*; grey* = SB*). See text for more details.*

This probability changes, when only F2 animals are regarded that carry the mutation. These individuals all carry the mutated allele *SB,* because the mutagenesis has been carried out in strain *SB*. Consequently, the other allele must be the *Tiw1* allele derived from the wildtype crossing partner. Therefore, in all animals identified as mutation carriers, the genomic region close to the mutation is heterozygous for *SB/Tiw1*. All loci that are not located close to the mutation are either *SB/Tiw1* or *Tiw1/Tiw1,* each with a 50% probability. Consequently, analyzing the F2 mutation carriers for the alleles of a polymorphism, two extreme cases are expected:

First case: all animals are heterozygous for SB/*Tiw1*. This indicates that the polymorphism lies close to the candidate gene. Possibly (though not necessarily), the candidate gene itself is mutated. Second case: half of the F2 animals are SB/*Tiw1* and *Tiw1/Tiw1*, respectively. This outcome shows that the mutation is not linked to the polymorphism (for instance lying on another chromosome). This result excludes the candidate gene from being affected in this mutant. If the portion of heterozygotes is significantly higher than 50% but does not reach 100%, the mutation and the candidate gene are genetically linked at a certain distance. The higher the portion of heterozygotes, the closer the mutation lies to the polymorphism.

Results

Identification of polymorphisms

To identify polymorphisms near candidate genes, noncoding DNA (3'UTR or introns) was sequenced from genomic DNA of 10 pooled beetles of the *SB* and *Tiw1* strains, respectively. By aligning the resulting sequences, polymorphisms were identified. The two strains turned out to be highly diverged: On average, a polymorphism was detected every 110 base pairs. One third of these were, however, present with both alleles in at least one strain and could therefore not be used. For some others, detection procedures were developed to distinguish the different alleles. These were then tested with 18 wt animals of each strain as well as animals heterozygous for the genome of the two strains (not shown). Only those polymorphisms were used that had the expected allele in all animals tested. The location and nature of the polymorphisms used are depicted in figure 3. In the case of *Tc'eve*, *Tc'hairy* and *Tc'runt,* small insertions/deletions were found in introns. PCR amplification of the region and subsequent separation of the different fragment lengths allows direct detection by electrophoresis in high concentration agarose gels. In the *Tc'Krüppel* 3´UTR, a single base substitution within an *Ase1* restriction site was found. It is detected by a restriction fragment length polymorphism (see methods for details on the detection reactions). In the case of *Tc'hunchback*, two polymorphisms were identified in the 3´ and 5´ UTR but both turned out to be present with both alleles in one or the other strain (not shown). They could therefore not be used for the mapping.

Crossing and mapping

In figure 4, the number of identified mutation carriers of the F2 generation is given for each mapping experiment (n). It ranges between 69 and 98. Therefore, a genetic resolution of about 1 to 1,5 cMorgan can be achieved (1/n *100 = genetic distance in cMorgan). The mutants *scy* and scy^{tig} had been found to be allelic in the meanwhile, so that n adds to 186, resulting in a maximal genetic resolution of 0,54 cMorgan for *scy*. For the mapping experiments, those individuals with most mutant offspring were chosen.

For all mappings, every polymorphism was initially checked in about 18 animals (plus positive and negative controls). Only when this first result strongly deviated from the 50% ratio, additional animals were scored. In figure 5 the detection gels are shown for *Tc'eve* and *Tc'hairy*, in figure

6 for *Tc'runt* and *Krüppel*. The numbers of heterozygotes and homozygotes for all experiments are summarized in figure 7 and the portion of heterozygotes in % is given below. As expected, in most cases approximately 50% of the F2 were heterozygous for the polymorphism. This shows that these mutations do not affect the respective candidate genes (all non-highlighted values in figure 7).

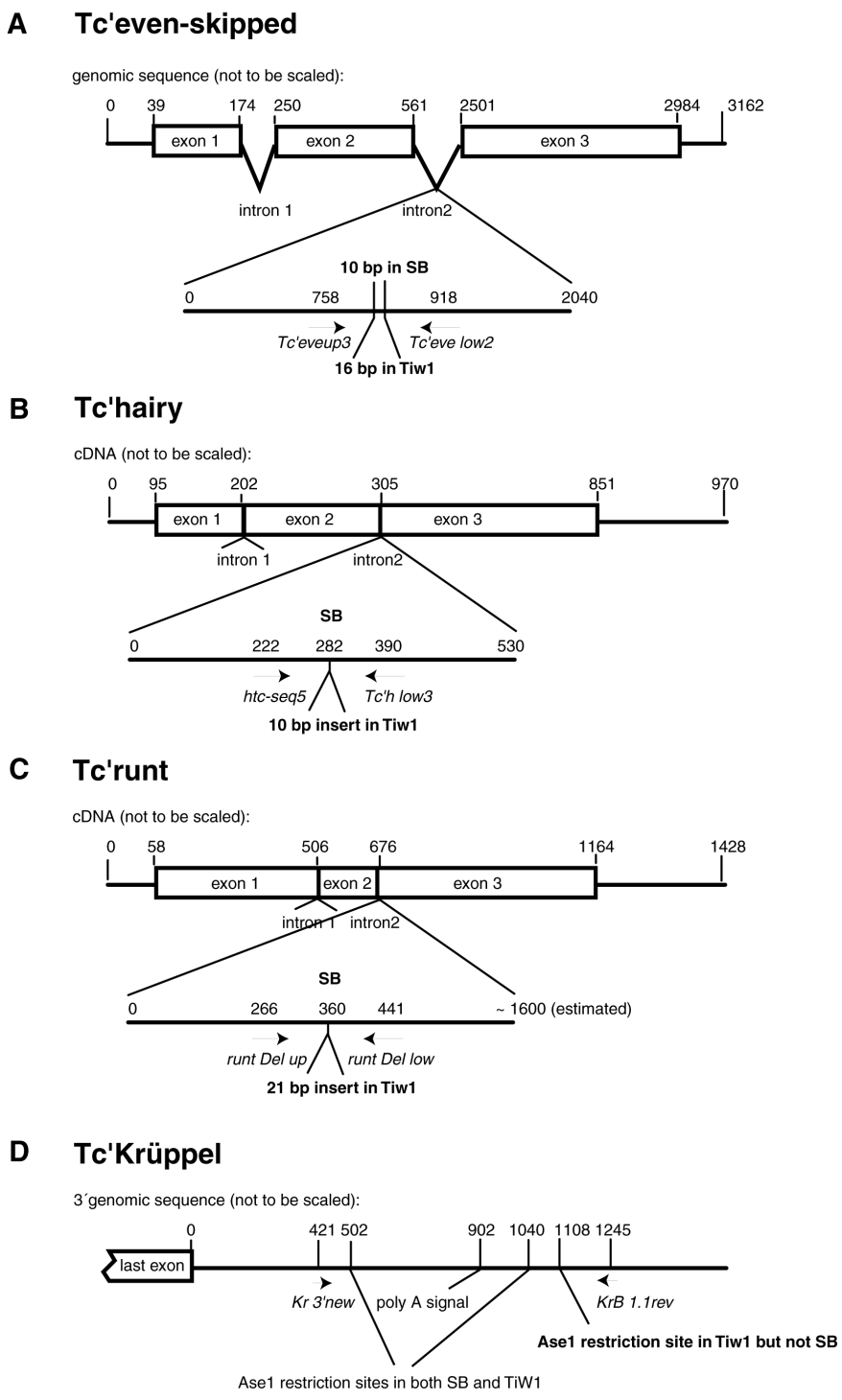


Figure 3
The position and nature of the polymorphisms that were used for the mapping experiment. The drawings are schematic and not to scale. The numbers indicate the position in base pairs relative to the indicated reference point (putative 5´ transcription start site in A through C; stop codon in D). For identification of polymorphisms, partial genomic sequences of Tc'eve *and* Tc'Krüppel *were available (Sue Brown, Reinhard Schröder, Diethard Tautz, Barbara Wigand, Christian Wolff; personal communications). In the case of* Tc'hairy *and* Tc'runt *only cDNA sequences were known (Sue Brown, Ralf Sommer; personal communications). In the latter cases, the position of potential introns were first guessed based on* Drosophila *gene structure and were then amplified by PCR using genomic DNA. The second intron of* Tc'runt *was not sequenced entirely, the length is therefore determined only approximately by gel electrophoresis of the genomic PCR fragment (C). In A through C, the polymorphisms consist of small deletions that were amplified by PCR using the primers (indicated in italics) and then separated by 3-3.5% agarose gel electrophoresis. For* Tc'Krüppel, *an* Ase1 *restriction length polymorphism was identified from the sequence. Two* Ase1 *sites are present in both strains, while a third site is only present in* Tiw1.

	males	females	sum	
icy	34	58	92	
scy^{tig}	32	56	88	186
scy	47	51	98	
god	36	50	86	
jaws	30	56	86	
bol	23	46	69	
kry	36	43	79	
rum	34	48	82	

Figure 4
The number of identified mutation carriers in the F2 generation of the mapping experiments. In order to identify mutation carriers, one F2 male was mated to three female siblings and the offspring was analyzed for the mutant phenotypes. The females of thus identified males were singled out and their offspring analyzed similarly. As scy^{tig} *has been shown to be allelic with* scy*, the numbers add to 186.*

The krusty *mutation lies close to, but not in the* Tc'runt *locus*

In the case of *krusty,* the portion of heterozygotes in the initial detection reaction for the *Tc'runt* locus was much higher then 50%. Therefore, additional animals were scored to a total of 50. Of these, 42 heterozygotes were found while only 8 animals were homozygous for the *Tiw1* allele (see figure 6 and boxed value in figure 7). The portion of 42/50 = 84% heterozygotes indicates that the mutation in *krusty* is linked to *Tc'runt*. The occurance of 8 recombinants shows, however, that the mutation lies in a certain distance to the locus and therefore does not affect the *Tc'runt* gene itself. The genetic distance is defined as the portion of recombinants in a crossing, that is 8/50 = 0.16 Morgan = 16 cMorgan for the *krusty*/*runt* experiment. *Tc'runt* has been mapped to linkage group *LG A* of the *Tribolium* map (Beeman, personal communication). By testing the available markers for linkage group *LG A* in the F2, the chromosomal region could be pinned down further. To positionally clone *krusty*, more markers would have to be developed and additional crossings would have to be performed. However, we decided not to pursue a positional cloning experiment at this point.

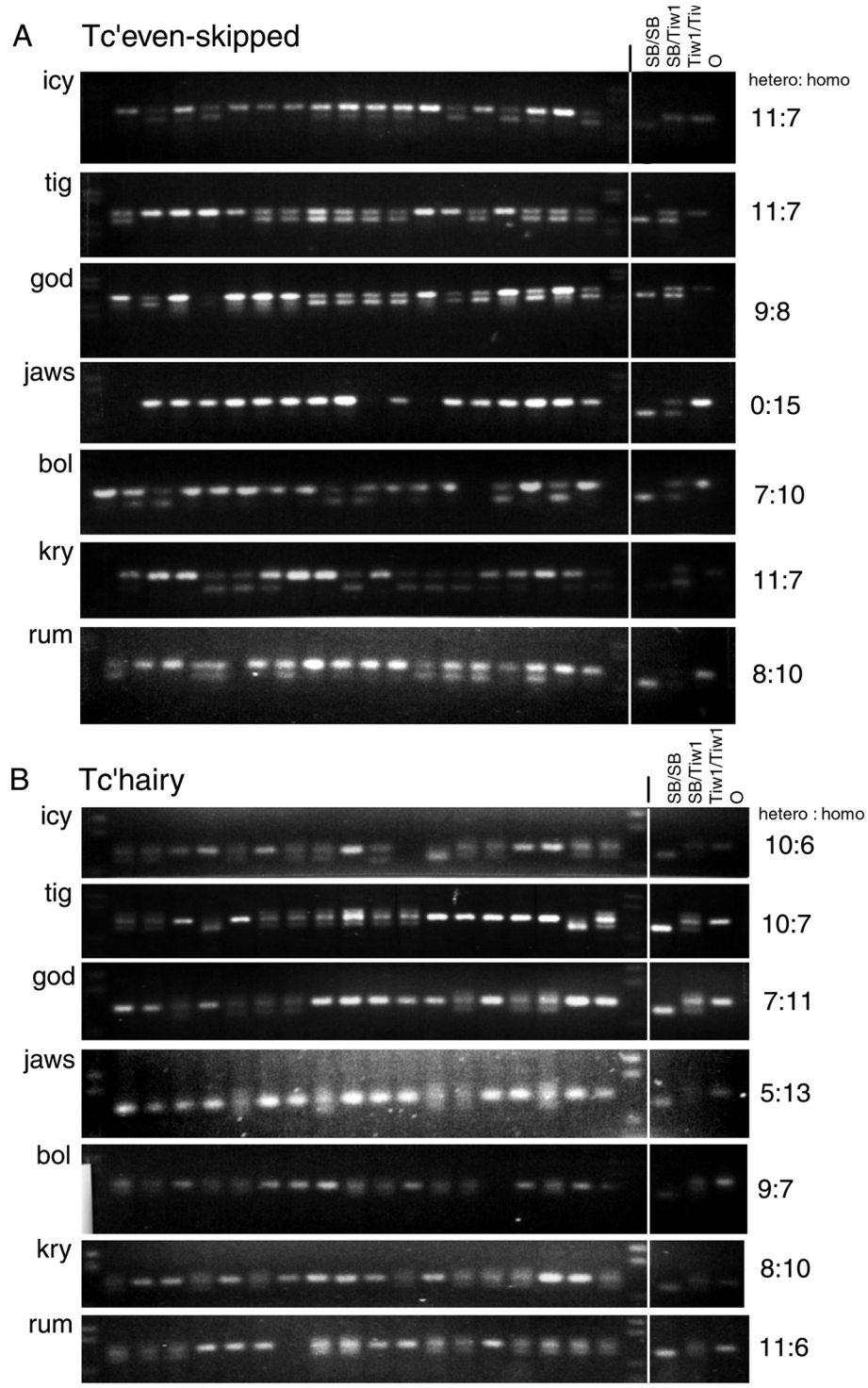


Figure 5
Gel electrophoresis to detect the alleles present in animals for the Tc'eve *(A) and* Tc'hairy *(B) polymorphisms. Both consist of small fragments of different length in the two strains, which were directly detected by PCR. The different alleles were separated by high percentage agarose gel electrophoresis. Right to the vertical bar, the controls are shown. Animals with defined genetic descendance were included (SB/SB,* SB/Tiw1 *and* Tiw1/Tiw1*) as well as a negative control without template (0). To the right to the gel photos, the numbers of hetero- and homozygous animals are given (summarized in figure 7).*

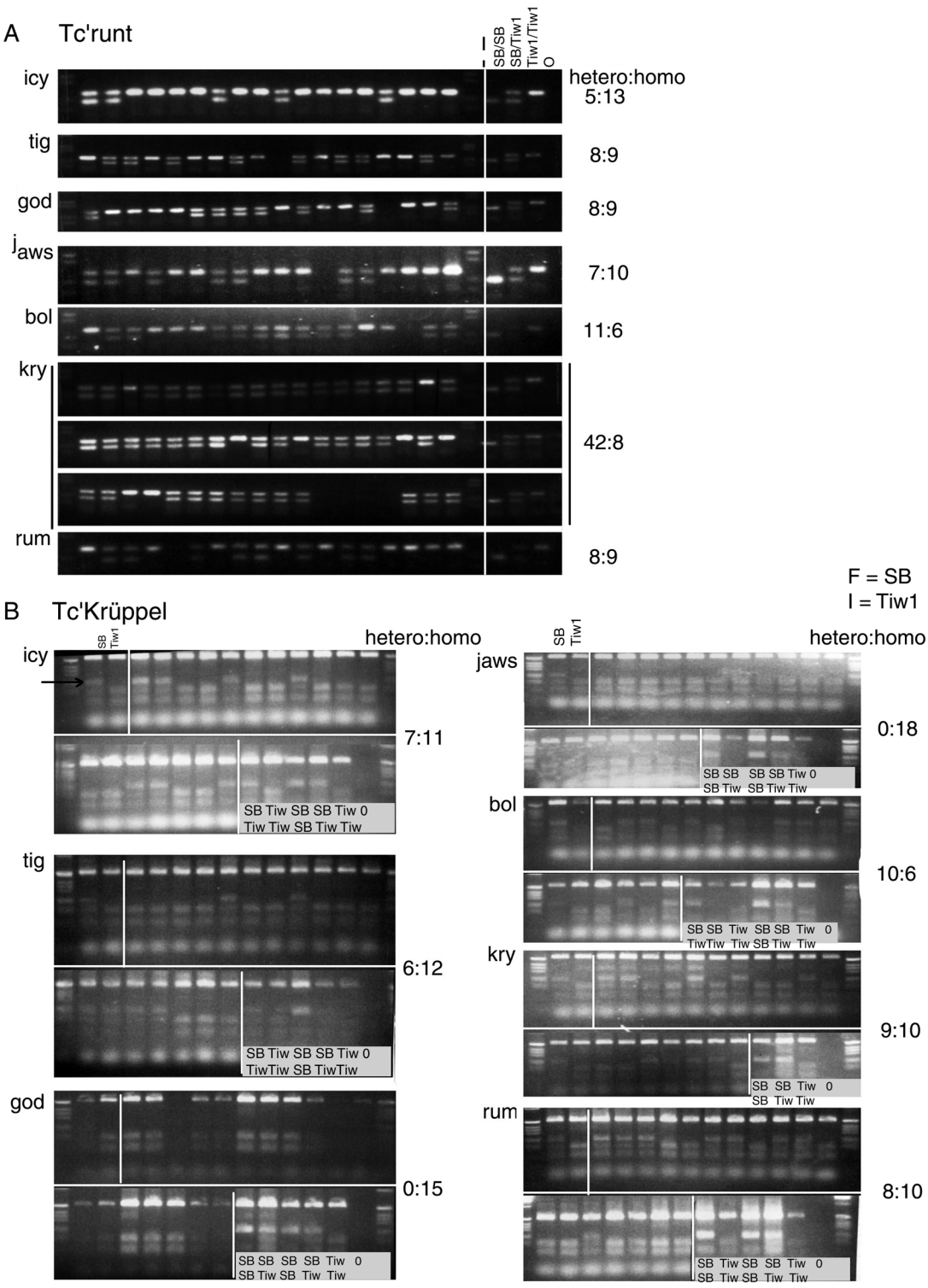


Figure 6

Gel electrophoresis to detect the alleles present in animals for the Tc'runt *(A) and* Tc'Krüppel *(B) polymorphisms. As with* Tc'eve *and* Tc'hairy, *fragments of different length were amplified by PCR and analyzed by high percentage agarose gel electrophoresis. The controls are as in figure 5. For* krusty, *more individuals were tested, because the portion of heterozygotes deviated from the 50% expected for the unlinked situation. The* Krüppel *polymorphism is a restriction site present in* Tiw1 *but not* SB *(see arrow in the icy result: one fragment present in* SB *is cut in* Tiw1*). Two gels are shown for each mutant. Controls are included in the first two lanes (top left) and in the last lanes (bottom right). As in fig 5, the numbers of hetero- and homozygotes for the indian allele are given (these values are summarized in fig 7).*

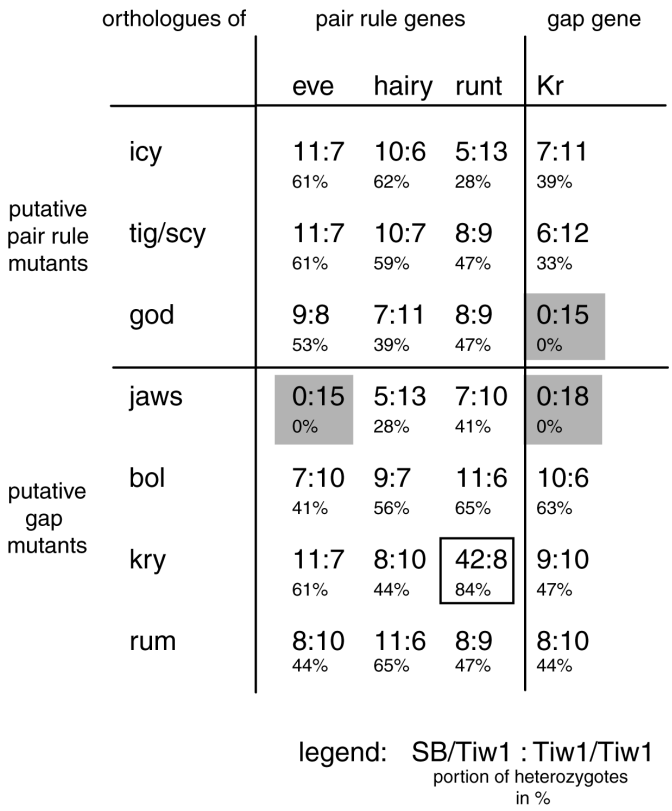


Figure 7
Summary of the results of the mapping experiments. Given are the absolute numbers of heterozygous and homozygous indian animals (same numbers as in figures 5 and 6). The portion of heterozygotes is given below (in %). Expected are 50% heterozygotes for a non-linked situation and 100% if the mutation lies very close to the polymorphism. Values significantly higher than 50% but not reaching the 100% indicate that the mutation lies at some distance to the polymorphism but in the same genomic region. All values not highlighted were interpreted as not deviating from the 50% value. The 84% portion in the krusty/Tc'runt *combination (boxed value) suggests that the* kry *mutation is located in a moderate distance to the* Tc'runt *gene. The values highlighted in grey show results that are unexpected for the crossing scheme: all F2 mutation carriers were homozygous for the* Tiw1 *allele. A likely explanation is, that the founder male carried the* Tiw1 *allele in spite of the expected* SB *allele (see text for evidence and more detailed discussion).*

The parental male of the jaws *crossing unexpectedly carried a* Tiw1 *allele*

In the *jaws* mapping experiment, all resulting F2 animals appeared to be homozygous for the *Tiw1* allele for both the *Tc'Krüppel* and *Tc'eve* polymorphism. This was also observed for the *Tc'Krüppel* polymorphism in the *godzilla* crossing (see values highlighted in grey in figure 7). This result is unexpected, because at least 50% of the animals should carry one *SB* allele. For the *jaws* / *eve* crossing this problem was further analyzed.

It is unlikely that by chance only homozygous individuals were scored despite a 50% portion of heterozygotes in the F2: As 18 individuals were analyzed, the probability for this is $p = 0{,}5^{18} =$

3,8 $^{-6}$. Another possibility is that *jaws* is a deletion mutant where the whole *Tc'eve* locus is missing or that it carries any other mutation or polymorphism that impedes amplification of the *SB* allele. As the *Tiw1* allele would nevertheless amplify, also heterozygous F2 animals would appear to be homozygous *Tiw1*. To test this hypothesis, homozygous mutant *jaws embryos* were collected from the stocks and scored for the *Tc'eve* polymorphism. These animals would be homozygous for the putative deletion and should therefore not amplify any allele. As a positive control, the *Tc'runt* polymorphism was tested in the same animals and Null controls (no DNA) were included. In all 6 larvae, the *SB* allele of the *Tc'eve* polymorphism was amplified, ruling out the above presented hypothesis (not shown). Another possibility is that the founder male used for the first crossing had carried the *Tiw1* allele in spite of the expected *SB* allele. In this case, the crossing would result in all F2 animals being homozygous for *Tiw1* irrespective of linkage. To test this, the parental and the F1 male were analyzed for the *Tc'eve* polymorphism. The parental male indeed amplified both alleles (in contrast to the expectation of being homozygous *SB,* not shown). After outcrossing this male with *Tiw1* females, half of the F1 offspring should be homozygous for *Tiw1*. And indeed the F1 male used in the crossing was homozygous for *Tiw1* (not shown). Outcrossing this male with *Tiw1* females necessarily resulted in all F2 animals being homozygous for *Tiw1* as observed in the *jaws/ eve* mapping. This result does therefore not provide any information about linkage of *Tc'eve* with *jaws* genes.

The reason for the presence of *Tiw1* alleles in the parental generation could be the genetic heterogeneity of the strains. Some polymorphisms were found to be present with both alleles in one or the other strain (see results and discussion below). Therefore, rare *Tiw1*-like alleles for *Tc'eve* could have been present in the *SB* strain, but were not detected in the controls, because of low frequency. By chance, such a beetle could have been chosen for the crossing. Some evidence suggests, however, that the mutations in *jaws* and *godzilla* were elicited in a strain with *Tiw1* like genetic background and that this is the reason for unexpected *Tiw1* alleles in the crossing. *jaws* and *godzilla* were isolated by Sulston et al (1996) in an independent screen in the *GA-1* strain. The genetic relationship of this strain with *Tiw1* or *SB* is unknown. Since the Sulston mutants have been integrated into our stock keeping system, they had been outcrossed at least two times with *SB* before they were used for the experiment. Thus, irrespective of their initial genetic background, at least 75% of the loci were replaced with *SB* alleles. Selecting one male for the crossing, 75% of the polymorphisms would then be expected to work. For 25% of the loci, however, the male would still carry a *Tiw1* allele (as shown above for *Tc'eve* in *jaws*). Mapping analysis with such loci would result in 100% offspring being homozygous for *Tiw1/Tiw1*. Regarding *god* and *jaws*, two out of seven polymorphisms tested turned out to have only *Tiw1/Tiw1* offspring: *Kr* in *godzilla* and *Tc'eve* in *jaws*. (*Kr* in *jaws* is excluded here, because it is probably linked, see below). The portion 2/7 =

28,5% is close to the expected value of 25% and thus compatible with that hypothesis. An additional and more direct evidence comes from our result that the *jaws* mutation is indeed linked to a*Tiw1*-like allele of the *Tc'Krüppel* polymorphism (see below). The mutation was therefore most likely elicited in a *Tiw1* like background (or the *GA-1* strain carried *Tiw1*-like alleles at least at the *Tc'eve* and *Tc'Krüppel* loci).

Alternative strategy to detect Tc'Krüppel *linkage in* jaws

Evidence presented above suggests that the Sulston mutants were induced in *Tiw1* like genetic background. Under this assumption, introduction of the mutants into our stock keeping procedure equals an unintentional outcrossing experiment. This allows an alternative strategy to investigate linkage of *Tc'Krüppel* with *jaws*: During stock keeping the mutants are outcrossed with *SB* wildtype about every 6 months. As this experiment was performed two years after the initial crossings, the putatively *Tiw1* like *jaws* strain had been outcrossed at least 6 times with *SB*. Therefore, most of the genome was expected to have been replaced by *SB* DNA. The genomic region close to the mutation, however, will remain *Tiw1*, because the mutation was elicited in *Tiw1*-like *GA-1* background. Mutation carriers isolated from the stocks can therefore be scored directly for the polymorphism: In the case of linkage, all individuals will be heterozygous SB/*Tiw1*: they all carry the *Tiw1* allele, because it is close to the mutation and the second allele must be derived from the wildtype partner of the crossing, i.e. the *SB* strain. In the case of non-linkage, also SB/SB individuals will appear. Because it is not exactly known how often the strains have been outcrossed, it is not defined, to what extent the genome has been replaced by *SB* DNA. It is thus impossible to give the expected ratio for homo- and heterozygous animals in the case of non-linkage. It is, however, expected to be much higher than 50%, because outcrossing during stock keeping was performed at least 6 times (versus two times in the mapping experiment).

We identified 31 mutation carriers of *jaws* from the stocks. 20 animals were tested for *Tc'eve* and all were homozygous for the *SB* allele. This shows that *Tc'eve* is not linked to the mutation in *jaws* (figure 8 B). All 30 animals, however, were heterozygous for the *Tc'Kr* polymorphism (figure 8 A). The Tiw1 allele has obviously not been replaced despite extensive outcrossing - this suggests that the mutation in *jaws* lies very close to the *Tc'Krüppel* gene. Possibly (but not necessarily) the mutation affects the *Tc'Krüppel* locus. Intriguingly, the phenotype of *jaws* differs strongly from *Drosophila Krüppel* (see discussion).

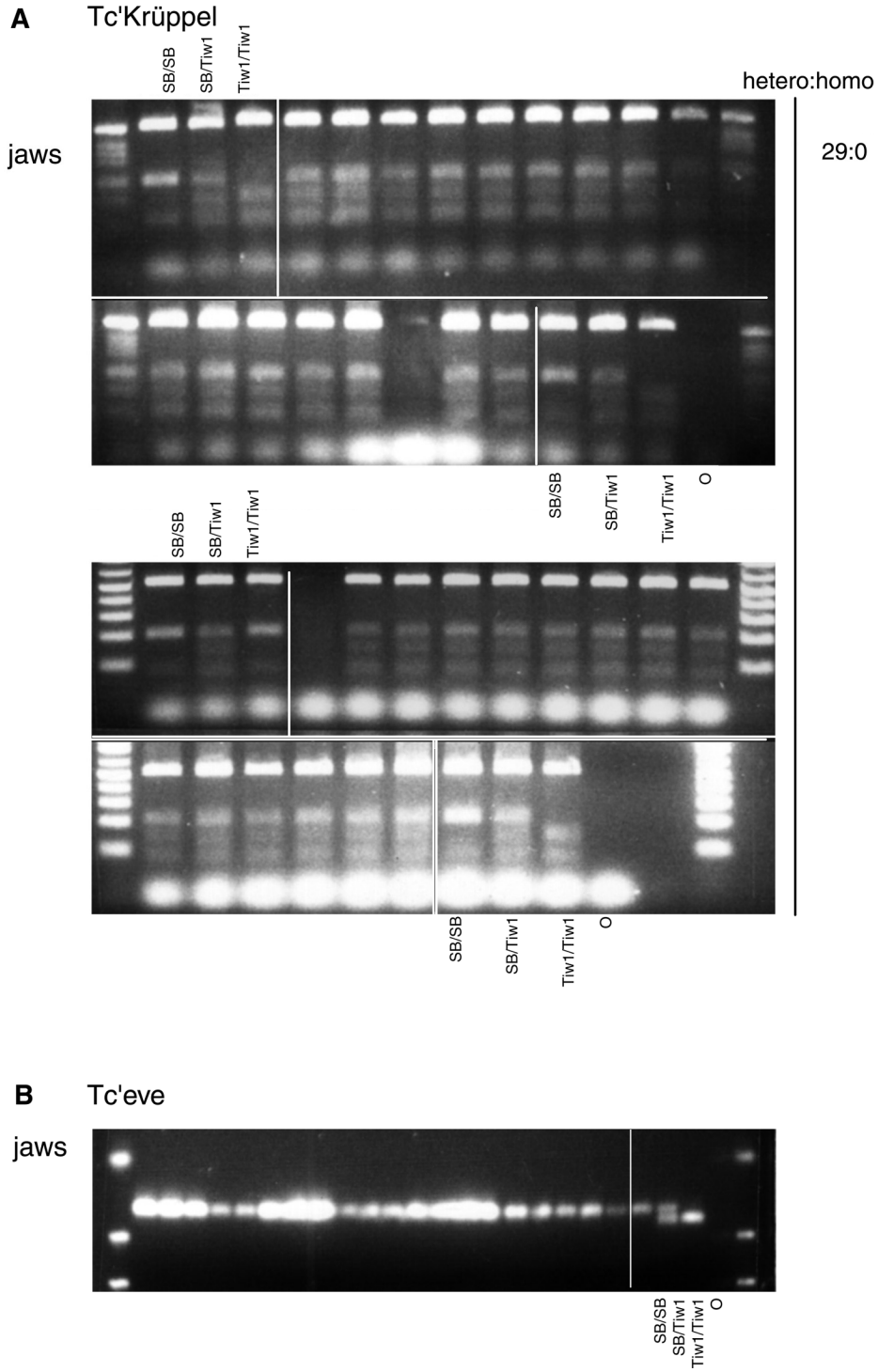


Figure 8
Alternative mapping of jaws *using mutation carriers from the stock collection. (A) The* jaws *mutant with putative* Tiw1 *like genetic background (see text for evidence) was crossed with our wildtype strain* SB *for several generations during stock keeping. Therefore, in the case of linkage, all mutation carriers from the stocks are expected to be heterozygous for the polymorphism. Shown are the results for 30 mutation carriers isolated from the stocks. All are heterozygous for the* Tc'Krüppel *polymorphism. This result shows that the mutation lies close to the* Tc'Krüppel *gene and it suggests that it might affect the gene itself. (B) As a control, the same animals were also tested for the* Tc'eve *locus. In contrast to* Tc'Krüppel*, all 20 animals checked were homozygous for the* SB *strain. This is expected for an unlinked situation, where repeated outcrossing should lead to replacement of most of the genome with* SB.

Complications encountered during the mapping experiment

Some polymorphisms are present with both alleles in one strain

It was not clear, whether the strains would be genetically sufficiently separate to identify polymorphisms. It turned out, that enough polymorphic sites are present with fixed alleles in both strains. The strains are, however, not totally isogenic. This means that some polymorphisms are also present within one population with both alleles. For instance two small deletions in the hunchback gene were present with both alleles in one or both strains. As the mapping analysis relies on defined genetic traits, such polymorphisms could not be used. Others observed similar problems in other mapping experiments (Sue Brown, personal communication).

New mutations were isolated

As the strains are not totally isogenic, they can also contain embryonic lethal mutations. During the selfings performed to identify mutation carriers in the F1 and F2 generation, such new mutations were indeed isolated unintentionally. In order to distinguish them from mutants that change their phenotype in different genetic backgrounds (see below), they were backcrossed to SB. Two mutants were integrated into our stocks. A subsequent screen for embryonic lethal mutations in the wildtype strains revealed, that the mutations described below were indeed present in high frequency in the *Tiw1* wildtype strain.

Kegelfuß: This leg mutation was independently identified in crossings of scy^{tig}, *bol* and *rum*. It shows reduced legs where trochanter, femur and tibiotarsus are fused.

Klootzack: This segmentation mutant was identified in a crossing with *kry*. Cuticles of *klootzack* have severe disturbances along the whole body axes. It appears as if the segments were established initially but not maintained correctly. This suggests that klootzack could be a mutation in a segment polarity or another gene that is required for maintenance of segment borders.

Some phenotypes change in different genetic backgrounds

When *bollig* and *hintenrum* offspring were selfed in order to identify mutation carriers, novel phenotypes appeared in the offspring while the *bollig* and *hintenrum* specific cuticle pattern was not observed any more. In both cases, the "new" phenotype was stronger and extended to additional body regions. Two effects can account for "new" phenotypes: embryonic lethal mutations are present in wildtype populations (see above). Alternatively, the phenotype of the mutants could be

modified when combined with a different genetic background. To distinguish between these possibilities, the respective new/modified mutations were backcrossed with *SB*. When the "new" phenotype was also observed in the offspring of the backcrossing, it was interpreted as a newly isolated mutation. When the new/modified phenotype switched back to the "old" one, it was regarded as being the original mutation whose effect is modified by different genetic backgrounds.

Both *bollig* and *hintenrum* consistently changed their phenotype in these test crossings (not shown). Their "new" phenotype was interpreted as a modified "old" one and was used to identify mutation carriers in the F2 generation. The mapping therefore should not have been adversely affected.

Discussion

jaws *is probably a* Tc'Krüppel *allele*

I have shown that all analyzed mutation carriers of *jaws* (n=31) from our stock collection are heterozygous for the *SB* and *Tiw1* allele of the *Tc'Krüppel* polymorphism. This suggests that *jaws* is closely linked to the *Tc'Krüppel* gene or may affect the gene itself. Alternatively, the animals could have carried the *Tiw1* allele by chance. The probability for this is, however, very low: *jaws* has been outcrossed at least 6 times with *SB* before mutation carriers were identified for the experiment. SB DNA had therefore replaced most of their genome. Some parts of the genome were, however, still *Tiw1*, namely: $0.5^6 = 0.016 = 1.6\ \%$. Under this assumption, the probability of choosing by chance 31 animals heterozygous at one locus is extremely low. Also, if the mutation in *jaws* involves a genomic rearrangement that interferes with proper recombination, our result could be misleading. There is, however, independent evidence that corroborates the hypothesis that *jaws* is *Tc'Krüppel: Tc'Krüppel* RNAi elicits variable phenotypes that include transformations of thorax to gnathal segments and segmentation defects similar to *jaws* (Schröder, personal communication) and in the meantime, a student in the lab has identified a mutation in an essential amino acid of the *Tc'Krüppel* zinc finger in *jaws* mutant embryos (Alex Cern y, personal communication).

The finding that *jaws* is indeed a *Tc'Krüppel* allele was unexpected, because the phenotype of *Drosophila Krüppel* is quite different: it is expressed in T2 through A3 (Preiss, Rosenberg et al. 1985) and lack of *Krüppel* function leads to a gap phenotype from T1 through A5 (Wieschaus, Nüsslein-Volhard et al. 1984). *Drosophila Krüppel* functions by providing short range gradients (Rivera-Pomar and Jäckle 1996) and strong mutual repressive interactions with the gap gene *giant* has been shown as well as interactions with other gap genes (Kraut and Levine 1991). Finally, *Drosophila Krüppel* is crucial for the regulation of primary pair rule genes and is also involved in Hox gene regulation (Riley, Carroll et al. 1987).

During abdominal segmentation, *Tc'Krüppel* is transcribed in the thorax only, as shown in this work by double stainings with *Tc'giant* (see figure 3 in the *giant* chapter). Therefore, *Tribolium Krüppel* expression is about three segments more anterior than in *Drosophila*. *jaws* embryos display a homeotic transformation of thoracic segments to alternating maxilla and labium, while segmentation is not disturbed in gnathocephalon and thorax. This suggests that also *Tc'Krüppel* is involved in the regulation of Hox genes. In contrast to the *Drosophila* situation, however, it is not required for the formation of segments that abut and overlap its anterior expression border. Intriguingly, these segments are specified during the blastoderm stage, where a more conserved segmentation mechanism could have been expected. *jaws* nevertheless affects segmentation: all

segments posterior to A1 are disturbed. Again this is surprising, because *Tc'Krüppel* is not expressed in abdominal segments. Nevertheless, the defects extend even into body regions that are patterned long after *Tc'Krüppel* expression has ceased, and at locations where it is not expressed at all during segmentation. Interestingly, a similar combination of far reaching posterior segmentation defects and anterior homeotic transformations has also been observed in *Tc'giant* RNAi embryos (see chapter 2). The spatial separation of these two functions appear to be common in *Tribolium*, but have not been observed in *Drosophila* apart from rare *hunchback* alleles (Bender, Turner et al. 1987). It is also conspicuous that the transformation of thoracic segments to maxilla and labium is exactly the opposite of the effects in *Tc'giant* RNAi, where gnathal segments are transformed to thorax. This suggests that both genes negatively interact to regulate Hox genes. The implications of these results on the segmentation machinery, on probable interactions of *Krüppel* and *giant* in *Tribolium* homeosis, and the evolution of gap gene orthologues are discussed in the general discussion.

By the mapping experiment only *jaws* could be shown to be closely linked to a candidate gene. The other mapping results are also important in that they exclude some candidate genes from being mutated in our strains. The depth of conclusions by such negative results are naturally limited, because they exclude only a few but leave open many other possibilities. Nevertheless, for some mutations the negative results are worth to be considered in some detail.

Bollig *and* krusty *are not* Tc'Krüppel *alleles*

In *Drosophila*, *Krüppel* elicits deletions in a domain from T2 to A5. *bollig* has a gap like phenotype from T3 to A2 and *krusty* has a deletion domain spanning from T3 into the abdomen (Maderspacher, Bucher et al. 1998). In addition, the labium is deleted as well as the urogomphi (A9). Thus, both *bollig* and *krusty* were considered candidates for *Tc'Krüppel*. We have shown for both mutants that this is not the case. Other candidate gap genes are *hunchback* and *knirps*. Our results with *giant* and *jaws* show, however, that regarding gap genes, phenotypes look quite distinct in *Drosophila* and *Tribolium* and that determining candidate genes by analogy to *Drosophila* may often be missleading. Another possibility is that the *bollig* or *krusty* mutations might affect a stripe specific element of a primary pair rule gene. In *Drosophila*, four pair rule genes are regulated by stripe specific elements and are therefore included in the class of primary pair rule genes (*eve*, *hairy*, *runt* and *ftz*). We have excluded *Tc'eve*, *Tc'hairy* and *Tc'runt*. To hold up the hypothesis of a regulatory mutation of a stripe specific element, a novel primary pair rule gene had to be assumed to be mutated in *bollig* or *krusty,* because *Tc'ftz* has no segmentation phenotype at all.

kry lies in approximately 16 cMorgan genetic distance to *Tc'runt*. This gene has been mapped to linkage group *LG A* (Beeman, Brown, personal communication). While the distance is still too

far for a direct positional cloning approach, the DNAs isolated from the F2 generation could be used to determine the genomic location more precisely by using markers that have already been developed for that linkage group. As *Tc'Krüppel* as an important candidate gene is not mutated in *krusty*, a positional cloning approach could be worth the effort in the future. Similarly, the mapping DNAs from the other mutants could be used to roughly determine their positions on the genomic map. As more and more segmentation genes are being mapped, this information could eventually reveal concordance of the positions of a mutation with that of a segmentation gene. That gene would then be a good candidate that could be molecularly analyzed subsequently.

Itchy, scratchy *and* godzilla *are not primary pair rule genes*

icy, *scy* and *god* have been described as pair rule genes. While this interpretation is unambiguous for *icy* and *scy*, the *god* mutation has no clear double segmental deletion pattern. It was assumed to be a pair rule gene, because segmentation is disturbed throughout the body. We have shown that none of these mutants is affecting an orthologue of one of the primary pair rule genes. Under the assumption that the set of primary pair rule genes (e.g. those that establish the repetitive pattern) is conserved between both insects, these mutants appear to be orthologues of secondary pair rule genes (e.g. those that refine the already established repetitive pattern and transmit it to the segment polarity genes). In this case, the mutants would be less revealing for the question of how a short germ embryo segments its body because the crucial question here is how the repetitive pattern is established and not how it is transmitted and refined afterwards. Further studies will have to show, whether the set of pair rule genes that initiate the repetitive pattern is conserved or if it includes additional members.

Lessons for future mapping experiments

This was the first molecular mapping experiment involving embryonic lethal mutations in *Tribolium*. Some experiences made during the procedure could help future experiments of the same type. One problem encountered was that the *Tribolium* strains available today are not totally isogenic. Some polymorphisms were present with both alleles in at least one strain and could thus not be used. In addition, we even isolated embryonic lethal mutations with embryonic phenotypes from the "wildtype" strains. While the newly isolated mutations did not adversely interfere with the mapping experiment, the heterogeneity regarding polymorphisms is a problem. It could be circumvented in part by further inbreeding both strains before using them for mutation screens and mapping experiments. For highly inbred strains, however, many generations are necessary. Moreover, inbred strains loose vitality and fecundity and are therefore less apt for experiments where single

crossings should produce a large number of offspring. The problem can thus not be completely eliminated - the strains have to be inbred as much as possible, but without affecting vitality and fecundity too much. A further possibility lies in setting up several crossings in parallel and to check the parental individuals for the presence of the expected alleles of a polymorphism before proceeding. Only those founder males would then be used, that contain the expected alleles. This procedure will, however, not help for polymorphisms of genes that will be identified and tested in the future. As the strains have been shown to carry plenty of polymorphisms, it should be possible in most cases to test several polymorphisms for one locus until one is found to be suitable for the experiment.

Materials and methods

Molecular methods

Unless indicated otherwise, standard molecular procedures were performed according to standard protocols and standard buffer receipts (Sambrook et al 1989).

Cloning of Tc'gt

Based on alignments of *Dm'gt* (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 3 redundant primers. 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° Celsius, containing all segmentation stages) following standard procedures (Sambrook, Fritsch et al. 1989). cDNA was prepared with the SuperScriptTM Preamplification System (GibcoBRL) using polyT primers. 3 ul 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 sec at 94°; annealing for 1 min in all cycles, at 53° in first 5 cycles, 51° during the next 5 cycles, and 47° in the remaining 20 cycles; elongation was 15 sec at 72° for all cycles. Of this reaction, 0.5 ul were used as template for a nested PCR with primer gt-5' and gt-3'b (same PCR conditions; Perkin Elmer AmpliTaq and the provided standard buffer was used in both reactions). 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). 21 independent inserts were sequenced, all of which turned out to represent the same sequence.

To obtain a complete transcript, this fragment was radioactively labeled (alpha 32p dCTP) using the Random Primer DNA Labeling System (GibcoBRL), with random primers supplemented by the primers gt-5' and gt-3'b. Using this probe, a cDNA lamda library (Lamda ZAP) was screened employing HighBond-XL filters (Amersham). Five independent cDNA clones with identical sequence were isolated. In addition, we utilized RACE (rapid amplification of cDNA ends) in order to search for additional transcripts. 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'gt* and *Tc'gt* was done using Clustal W (1.5) (Higgins, Bleasby et al. 1991) 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, Madden et al. 1997) search with the leucin zipper domain of *Tc'gt* 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, again using default settings. Bootstrap analysis was also done 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 leucin zipper.

DNA extraction from single beetles

Process 18 beetles in parallel:

(read also instructions of manufacturer of microcon device)

5 ml homogenization buffer, add proteinase K to a final concentration of 100ug/ml
Add 200 ul homogenization buffer (incl PK) to a single beetle in a 1,5 ml Eppendorf tube
Homogenize with a tightly fitting pestle
Digest at least 1h at 55° Celsius (mix all 20 min)
Add 200 ul 5M NaCl, mix by inverting 3X
Add 300 ul chloroform
Centrifuge for 10 min at maximum speed
Bring upper phase into Microcon devices (Millipore) and add 150 ul H2O
Centrifuge 15 min at 2400 U/min
Discard flowthrough, add 400 ul H2O
Centrifuge 15 min at 2400 U/min
Add another 400 H2O and centrifuge 15 min at 2400 U/min

Add 200 ul TE and dissolve DNA by moving device
Put Microcon filter device upside down onto marked Eppendorf tubes
Centrifuge 3 min for 3400 U/min to bring DNA with TE into eppi
Test 5ul on a 1% Gel,
DNA should appear as a bright band of high molecular weight, two short bands represent ribosomal RNA

Preparing high percentage gels

Put100 ml cold buffer in erlenmeyer
Add the agarose slowly while stirring (adding buffer to the agarose or adding agarose too rapidly will result in clumping of the agarose)
Let incubate for 5 min at RT
Boil carefully in microwave until dissolved completely

Embryonic and parental RNAi

For embryo injections, sense and antisense RNAs were synthesized from a full length *Tc'gt* cDNA plasmid using the T7 Megascript Kit (Ambion), utilizing T7 RNA polymerase (Ambion) and T3 RNA polymerase (LaRoche). Annealing was performed in injection buffer (potassium phosphate 20mM, sodium citrate 3mM pH7.5; (Fire, Xu et al. 1998)). Different concentrations of resulting dsRNA (*Tc'gt*: 750 ng/ul, 75 ng/ul and 7.5 ng/ul, *Tc'dll*: 2 ug/ul) were supplemented with 1/10 volume phenol red to 0.05% (Sigma) and filtered (Ultrafree 0.45 um, Millipore) prior to injection. *Tribolium* eggs were collected for one hour at 25°C and kept for another hour at 33°C to improve injection survival. The embryos were then dechorionized two times for 1,5 minutes using 1% "Klorix" bleach, washed carefully in water and mounted on microscope slides without applying glue or oil. They were injected in air at an intermediate anterior-posterior position to minimize damage to egg poles where maternal morphogens may be localized, and were the growth zone will develop at later stages. After injection, embryos on the slide were placed onto an agar plate, that was closed and put into a closed TupperWare box, which contained humid paper. The 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. 32% of the injected eggs differentiated cuticles, and of these, 56% displayed *Tc'gt* phenotypes. Both higher dsRNA concentrations resulted in similar frequencies of RNAi phenotypes, while the lowest concentration produced mostly wild type cuticles.

For parental RNAi, mature female pupae (i.e. pupae with well pigmented eyes, sclerotized mandibles and claws, grayish wing discs) were affixed to a microscope slide, ventral side up, using drops of rubber cement ("Fixogum", Marabu). In order not to interfere with eclosion, only the posteriormost portion of the abdomen was allowed to contact the rubber cement. Approximately 0.15 ul of dsRNA (750 ng/ul) was injected between abdominal segments three and four, at a ventro-laterally position, in order not to damage the CNS. About 30 eclosed females were mated to untreated males, and eggs were collected beginning one week after injection. All embryos in the first egglay displayed *Tc'gt* phenotypes in cuticle preparations. During the following two weeks, eggs were fixed for histochemistry using standard procedures. Three weeks after injection, the portion of embryos displaying *Tc'gt* phenotypes dropped to 40% and egg collection was discontinued. Therefore, at least 40% of embryos used for histochemistry were expected to display *Tc'gt* phenotypes.

Morpholino oligonucleotide analysis

A Morpholino oligo against the giant gene (Gene-Tools) was designed to cover both possible starting ATGs (oligo sequence: 5'CCATCGCAAATTCTGCTTTTTCCAT-3'). Injection of 1mM and 0.66 mM concentrations (in injection buffer) resulted in premature termination of development in all embryos. With lower concentrations (0.4 and 0.2 mM) the portion of fully differentiated embryos (32%) and cuticles displaying phenotypes (42% of differentiated embryos) was similar as in our embryonic RNAi experiments.

Subcloning

Cloning, DNA restriction and plasmid preparations were performed according to Sambrook et al. (1989) and to manufacturers advices.

Colony PCR

To identify clones that contain insert, colony PCR was performed. First, PCR-tubes were filled with a standard 10ul PCR Mix containing all components but DNA. The primers pBSA and pBSE were used, that prime close to M13 and M13rev, respectively. Colonies were picked from the plate with autoclaved toothpicks, choosing small, large and intermediate colonies. They were first dipped onto a plate marked with numbers and then inmersed into the PCR-tube with the corresponding number. After spinning the toothpick in order to set cells free, they were discarded. After 30 rounds of a standard PCR program (melting 30 seconds at 95°C, annealing 45sec at 60°C and elongation

1-2min at 72°C) the products were separated and those clones chosen for minipreps that displayed the expected length.

Single embryo PCR

Single embryos for PCR were carefully dechorionated (flour inhibits reactions) and washed extensively with PCR grade water to avoid contamination. Then they were placed into 10ul NTEK buffer containing PK (no SDS because this inhibits PCR), smashed with a 2ul tip and digested for 1 hour. By heating, the PK was denatured. After 30´ spinning, 4 ul of the supernatant was used as template for one PCR reaction. (NTEK buffer: NaCl: 25mM; Tris pH8: 10mM; EDTA 1mM; PK (added freshly) 200 ug/ml)

Mapping

The outcrossing procedure

As our mutants are not balanced, only 50% offspring in the stock collections are mutation carriers. To identify founder males from these stocks, 5 test crossings were set up per mutation: One male pupa was added to three female sibling pupae from the stocks. Their offspring was analyzed for the cuticle phenotype as described in (Berghammer, Bucher et al. 1999). Two males whose offspring showed the phenotype were chosen for each mutation.

Two such identified mutation carrier males were crossed to three wildtype pupae from the *Tiw1* strain. From the resulting F1 offspring, five single matings were made per mutation: Again, in each case, one male was crossed with three female sibling pupae, respectively. Their offspring was analyzed for the cuticle phenotype. Again, two positive males were selected per mutation for further crossings.

One of the two identified F1 mutation carrier males was used for the second crossing (the second one was kept as a backup in case of death of the first male). It was crossed to 5 to 8 wildtype females of the *Tiw1* strain in order to provide sufficient F2 offspring (regarding the genetic heterogeneity of the strains, it is, however, better to use only 2-3 females and collect offspring for a longer time).

In order to identify mutation carriers in the F2 generation, single matings were again set up, by crossing one male with three female pupae. For each mutation, 4-6 blocks á 24 crossings were prepared (appr. 120 crossings). Two subsequent 3-day egglays were collected and let develop at 33°. The eggs of both egglays were pooled in the block used for dechorionation and embedded together on microscope slides in Hoyers' medium. The identified male carriers were frozen (in 1.5 ml Eppendorf tubes without buffer) and their females singled out for additional egglays. As they still carry sperm from the mutation carrier, they keep on laying eggs including homozygous mutant offspring. Again, two egglays were collected, pooled and embedded. The females that proved to be mutation carriers because of mutant offspring were frozen. For each identified F2 beetle, the number of homozygous mutant offspring was documented and the microscopic preparation was kept for future reference. For the allele detection reactions, the individuals with most mutant offspring were chosen. Individuals with only one or two mutant offspring were kept but should be treated with care, as it is possible, that during egg collection and embedding, single embryos might have been transmitted to adjacent vials.

In case of ambiguous results it proved to be important to test the parental and F1 males and females for the alleles. They were therefore also frozen.

Polymorphism detection protocols

Tc'eve

1) PCR amplification of the fragment:

	per 11 ul reaction:	master mix for 25 reactions:
DNA	1	-
H2O	5,6	140
MgCl (25mM)	0,8	20
10X buffer (with 15mM Mg)	1,1	27,5
dNTP	1,1	27,5
primer: *Tc'eve up*	*3*0,55	13,75
Tc'eve low2	0,55	13,75
ExpandPolymerase (Boehringer)	0,3	7,5
	——-	
	11	

(primers: *Tc'eve up3*: 5´ATA ATT AGT TCT ATA CCT TAT3´; *Tc'eve low2*:5´CAA TTT TTT GGG ATA AAA CAC3´)
10 ul of the master mix was added to 1 ul DNA (in TE).
PCR program (MWG machine, name of the program: " Greg_DP5"):

1´	95°	
1´	95°	
1´	50°	30X
1´	72°	
2´	72°	

2) Gel electrophoresis:
2 ul loading buffer were added. The products were separated in a 3,5% NuSieve gel at 50 Volt for 3-4 hours.

<u>*Tc'hairy*</u>
1) PCR amplification of the fragment:

per 11 ul reaction:		master mix for 25 reactions:
DNA	1	-
H2O	6,1	152,5
MgCl (25mM)	0,4	10
10X buffer (with 15mM Mg)	1,1	27,5
dNTP	1,1	27,5
primer:*hTc-sq5*	0,55	13,75
Tc'h low3	0,55	13,75
AmpliTaq (Perkin Elmer)	0,2	5
	11	

(primers: *hTc-seq5*: 5´TTT CGG ATA TTT AGT TCT TGA3´; *Tc'h low3*: 5´GCA CCG TTT CAA AGT TAG A3´)

10 ul of master mix was added to 1 ul DNA (in TE).

PCR program (MWG machine, name of the program: " Greg DP_5"):

1´	95°	
1´	95°	
1´	50°	30X
1´	72°	
2´	72°	

2) Gel electrophoresis:

2 ul loading buffer were added. The products were separated in a 2,5% NuSieve gel at 50 Volt for 3-4 hours.

Tc'runt

1) PCR amplification of the fragment:

	per 11 ul reaction:	master mix for 25 reactions:
DNA	1	-
H2O	6,6	165
10X buffer (with 15mM Mg)	1,1	27,5
dNTP	1,1	27,5
primer:*runt Del up*	20,55	13,75
runt Del low	0,55	13,75
AmpliTaq (Perkin Elmer)	0,1	2,5
	——- 11	

(primers:*runt Del up2*: 5´GTG AGT TTG CTT ATT GTC TG3´, *runt Del low*: 5´AAA ATA ACA TAT TAC GAG GTA TTA 3´)

10 ul of the master mix was added to 1 ul DNA (in TE).

PCR program (biometra machine, name of the program: "*runt* DP"):

1´	95°	
30´´	95°	
1´	50°	30X
30´´	72°	
2´	72°	

2) Gel electrophoresis:
2 ul blue juice were added. The products were separated in a 2,5% NuSieve gel at 50 Volt for 3-4 hours.

Tc'Krüppel

1) PCR amplification of the fragment:

	per 20 ul reaction:	master mix for 25 reactions:
DNA	1	-
H2O	12,7	317,5
10Xbuffer (incl15mM $MgCl_2$)	2	50
dNTP	2	50
primer: *Kr B1.1rev*	1	25
Kr3´new	1	25
AmpliTaq (Perkin Elmer)	0,3	7,5
	20	

(primers: *Kr B1.1rev*: 5´TAC GAA AGT AGG CAC ACA AC3´; *Kr3´new*: 5´ACG ACT TGG CGG TTA ATG3´)
19 ul of the master mix were added to 1 ul DNA (in TE).

PCR program (MWG machine, name of the program: “Greg_*Kr*u”):

1´	94°	
30´´	94°	
30´´	60°	30X
1´	72°	
2´	72°	

2) Ase1 digest:

	per reaction:	master mix for 25 reactions:
PCR product	20	-
10X buffer	6	150
BSA	0,6	15
H20	33,15	828,75
Ase1 (2,5U)	0,25	6,25
(NEB)	———	
	60	

40 ul of the master mix were added to the 20 ul PCR and digested for 1 hour at 37° (longer digests lead to star activity of the enzyme)

3) Gel electrophoresis:
10 ul blue juice were added. The products were separated in a 2,5% NuSieve gel at 55 Volt for 1,5 hours.

Beetle handling and strains

Data on the life cycle and the biology of *Tribolium* castaneum (coleoptera) is published in Sokoloff (1974). The stock keeping and the procedure to identify mutation carriers are described in Berghammmer et al. (1999).

Two wildtype strains were used: *San Bernardino* (*SB*) was isolated in the USA and is the standard wildtype strain used for the screen in Munich. *Tiw1*is a strain that is derived from India.

Dick Beeman, Kansas State University provided both strains. The mutant strains *godzilla (god)* and *jaws* were isolated in a screen in the *GA-1* strain (Sulston et al 1996). *itchy (icy), scratchy (scy), krusty (kry)* and *bollig (bol)* were isolated in the Munich screen (Maderspacher et al 1998) as well as the mutants *hintenrum (rum)* and *tigerente (tig)* (unpublished). *tig* is allelic with *scy* and has been renamed to scy^{tig}.

Histology

Whole mount in situ hybridizations were done according to established protocols (Tautz, Pfeifle 1989). For double staining, fluorescein- and digoxigenin-labelled probes were detected using alkaline phosphatase and betagalaktosidase, the latter after signal enhancement via biotin deposition (Prpic, Wigand et al. 2001). Cuticle preparations were performed according to standard procedures (Berghammer et al. 1999)

General discussion

I already discussed the results of my three thesis projects (pRNAi, *Tc'giant* and mapping of segmentation mutants) in the previous chapters. In this general discussion I will first elaborate on implications that parental RNAi could have on the field of evolutionary development. In the second part, I will consider the likely possibility that *jaws* is indeed a *Tc'Krüppel* allele and will present a model for Hox gene regulation in gnathum and thorax including both genes. Then I will show that some common features of *Tc'giant* and *Tc'Krüppel* corroborate the idea of a fundamentally different segmentation mechanism in *Drosophila* and *Tribolium*. Finally, I will discuss the fact that several abdominal gap gene orthologues are shifted anteriorly on the *Tribolium* fate map. Based on this, a model for the evolution of abdominal gap gene othologues in short and long germ embryos will be presented.

The potential of parental RNAi for functional studies in evolutionary developmental biology

The evolution of developmental pathways was initially studied by comparing the expression patterns of orthologous genes in phylogenetically distant organisms. For a deeper understanding, however, the function of genes must be compared, and *Tribolium* was originally chosen as new insect model system because it is suitable for classical genetic approaches (Beeman, Stuart et al. 1989). In this thesis, I followed the genetic approach by mapping previously induced segmentation mutants relative to molecularly identified segmentation genes. While these experiments eventually revealed the function of *Tc'Krüppel* in *Tribolium* (see project 3), an alternative approach, RNAi, was successful in the investigation of *Tc'giant* function.

The RNAi technique allows a rapid answer for a crucial question in the field of developmental evolution: To which degree is the function of orthologous genes conserved in different species? The time and effort required for identifying a gene and uncovering its function is substantially reduced by using RNAi analysis in comparison to classical genetic approaches. The reason is that RNAi studies obviate the time and resource consuming genetic screens as well as the constant investment necessary for stock keeping. The parental RNAi technique presented in this work further broadens the experimental possibilities of RNAi: First, the eggs of some species do not easily survive microinjection - injecting pupae or adults will in most cases be less demanding. Therefore, through pRNAi additional species could become available for functional studies, which is of special

interest in the field of evolutionary development but also in the study of species of economic interest. Secondly, large numbers of eggs layed by injected pupae can be collected, fixed and stained following standard procedures. Thus, alterations of gene expression in RNAi embryos can be investigated rapidly obviating laborious embryonic injections and devitellinisation by hand. Therefore, functional analysis of gene interactions is now also feasible in species other than the well-developed genetic model systems. Third, parental RNAi offers the possibility to perform large scale screens for gene function: Injection of three pupae provides about 100 „mutant" cuticles. For a similar number of larvae, 300 embryonic injections are necessary because 60-70 % of the eggs do not survive the treatment. This ease of application allows for large-scale functional analysis of genes, that are identified by cDNA sequencing (EST screens) or genomic sequencing. In *Caenorhabditis elegans* for instance, large-scale RNAi screens uncovered the function of many genes that were predicted from the genomic sequence (Gonczy, Echeverri et al. 2000). In fact, the ease of pRNAi in *Tribolium* could be an important argument for promoting this species for genomic sequencing.

A model for homeosis in head and thorax of Tribolium

Tc'Krüppel (the gene most likely mutated in *jaws*) and *Tc'giant* have opposing effects on homeosis of gnathum and thorax: In *jaws*, the thorax and the first abdominal segment are transformed to alternating maxilla and labium. In *Tc'giant* RNAi embryos, in contrast, maxilla and labium are transformed to T1 and T2, respectively. These phenotypes suggest opposing action of *Tc'Krüppel* and *Tc'giant* on homeotic target genes. However, these opposing effects could also be due to direct negative interactions of both genes. The *Drosophila Krüppel* and *giant* genes repress each other thus that the expression domain of one expands in absence of the other (Kraut and Levine 1991). Similarily, the opposing homeotic effects of *Tc'Krüppel* and *Tc'giant* could reflect expansion of the anterior *giant* domain in *jaws,* and of *Tc'Krüppel* in *Tc'giant* RNAi embryos.

Based on that assumption, a model is proposed in figure 1. The anterior domain of *Tc'giant* and the anterior border of the *Tc'Krüppel* domain form opposing repressive gradients in the blastoderm. *Tc'giant* expression defines gnathal and *Tc'Krüppel* expression thoracic destiny by directly or indirectly activating Hox genes in their respective domains and/or repressing those of the opposing domain. As already proposed in the *Tc'giant* discussion, the different identities within the gnathum (maxilla versus labium) and the thorax (T1 versus T2) likely depend on additional information provided by pair rule genes. The double segmental transformation in *jaws* also argues for pair rule input in homeosis.

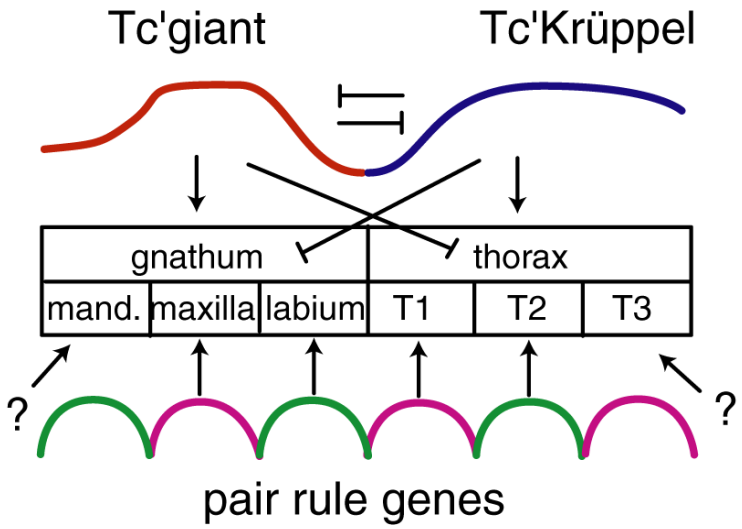


Figure 1

A model for homeosis in head and thorax of Tribolium. Tc'giant *and Tc'Krüppel define gnathum and thorax by their mutually exclusive expression (shown as opposing gradients above the fate map). They activate and/or repress the respective Hox genes. Further information is provided by the pair rule genes (below the fate map). In concert with* Tc'giant *and* Tc'Krüppel, *respectively, they define labium versus maxilla and T2 versus T3. Additional information is needed for specification of mandible and T3. Note that not all of the indicated interactions are required for the model to work.*

To date, none of the interactions suggested in figure 1 have been shown directly and not all indicated interactions are required for the model to function. Nevertheless, some interactions are reminiscent of the *Drosophila* situation and might well be conserved between both insects: Strong mutual repression between *Krüppel* and *giant* is described for the fly (Kraut and Levine 1991) and *Drosophila* pair rule genes are also involved in Hox gene regulation (Ingham and Martinez-Arias 1986; Irish, Martinez-Arias et al. 1989). It has also been shown that *giant* defines the anterior border of *Antennapedia* and is involved in the regulation of *sex-combs-reduced* in *Drosophila* (Reinitz and Levine 1990; Riley, Carroll and Scott 1987)

.

It should be noted, however, that the above presented model alone cannot account for all gnathal and thoracic identities, because T1 versus T3 and mandible versus labium have the same pair rule code within gnathal and thoracic domains, respectively. Additional information is therefore required for specification of the mandible, the third thoracic segment and the border between thorax and

abdomen (question marks in figure 1).

Common features of Tc'giant *and* Tc'Krüppel *that deviate from* Drosophila

Tc'giant and *Tc'Krüppel* (i.e. *jaws*) have some features in common that distinguish them from their *Drosophila* orthologues. Both genes function in homeosis as well as segmentation. But in contrast to the fly, they do so in separate domains in *Tribolium*: Anteriorly they regulate segment identity but are not required for segmentation of the respective segments. Only posteriorly both genes are required for segmentation. Also *Drosophila Krüppel* and *giant* provide input for both, homeosis and segmentation (Klingler and Tautz 1999). But in contrast to *Tribolium*, the same segments are affected by both processes (therefore, the homeotic function of *Drosophila* gap genes is usually not evident from the cuticle phenotype because the transformed segments are deleted by the gap function). One might have assumed that orthologous genes expressed in a similar cellular environment (the blastoderm) would function similarly in long and short germ embryos. Therefore it is surprising that anterior (blastodermal) aspects of the *Tribolium* orthologues do not function in segmentation. As *Tribolium* most likely reflects the ancestral situation, it is probable that the *Drosophila* orthologues acquired the anterior gap function after the split of the two lines (a model for the evolutionary change of abdominal gap gene function is given at the end of this discussion).

Another common feature of *Krüppel* and *giant* is that the expression of both, *Tc'Krüppel* and the posterior *Tc'giant* domain, are shifted anteriorly on the fate map by several segments (see figure 2). Therefore they hardly can regulate the same pair rule stripes as their *Drosophila* orthologues. The anterior domain of *Drosophila giant,* for instance, is directly required for defining the anterior border of the second *even-skipped* stripe (Small, Blair et al. 1992). The corresponding domain of *Tc'giant*, in contrast, appears to function only in homeosis and has no effect on *even-skipped* stripe 2 (not shown). Similarly, the anterior border of *Tc'Krüppel* (in the anterior thorax) appears not to be involved in segmentation, while its *Drosophila* orthologue regulates pair rule gene expression using both concentration slopes of its expression domain. Evidently, the regulative network activating pair rule stripes has undergone major changes in long and short germ embryogenesis.

This is further corroborated by the third common feature of *Tc'giant* and *Tc'Krüppel*: Apparently, both do not function by the „typical" gap gene mechanism. Gap genes were initially defined by their phenotype: lack or disturbance in a group of adjacent segments. As the *Drosophila* segmentation hierarchy was uncovered, this definition was modified to comprise their position within the segmentation gene network: gap genes are maternally activated, they interact with other gap genes, and they regulate primary pair rule and Hox genes (Kraut and Levine 1991). Their gene

products are believed to form short-range gradients at both slopes of their expression domains and provide concentration dependent positional information (Hülskamp and Tautz 1991; Rivera-Pomar and Jäckle 1996). In contrast to their *Drosophila* orthologues, lack of *Tc'giant* and *Tc'Krüppel* function interferes with segmentation of the whole abdomen. The defects include some posterior segments that are formed long after *Tc'Krüppel* and *Tc'giant* expression has ceased. Apparently, lack of their function leads to a breakdown of patterning in all posterior segments. A *Drosophila*-like short-range morphogenetic gradient can hardly explain these long-reaching effects.

Finally, the activation of both genes probably differs from *Drosophila*: the posterior *Tc'giant* domain, and the *Tc'Krüppel* arise *de novo* during late blastoderm. Therefore, maternal activation as in in the fly is unlikely.

I have shown that *Tc'giant* and *Tc'Krüppel have* several features in common that deviate from their *Drosophila* orthologues. If these differences were observed in one gene only, they might be assigned to evolutionary change restricted to that single gene in an otherwise conserved gene network. But the fact that two abdominal gap gene orthologues display similar deviations from the *Drosophila* situation strengthens another interpretation: The mechanism of segmentation deviates substantially in both insects. At this point, it is still open, how posterior pair rule stripes are generated in *Tribolium*. Because of the similarities outlined above, the *Tc'Krüppel* phenotype has similar implications on possible models of *Tribolium* segmentation as the *Tc'giant* results. Thus, the models presented in the *giant* chapter can also account for the *Tc'Krüppel* phenotype and will not be discussed here further. As both genes probably play a different role in segmentation, it is missleading to call *Tc'giant* and *Tc'Krüppel* "gap genes". They should rather be referred to as "gap gene orthologues". Analysis of other gap and pair-rule gene orthologues as well as functional dissection of regulatory regions of pair-rule genes are still required to finally unravel the principle of segmentation in *Tribolium*.

The *Tc'giant* and *Tc'Krüppel* results also shed new light on the interpretation of segmentation mutants. The *Tribolium* mutants *bollig* and *krusty* lack a group of adjacent segments (Maderspacher, Bucher et al. 1998). Therefore, by analogy to *Drosophila* phenotypes, they have been suggested to be gap gene mutants. The *Tc'giant* and *Tc'Krüppel* results alter the bases of this interpretation in two ways: First, two genes that were assumed to be among the best candidates, namely *Tc'giant* and *Tc'Krüppel*, have been shown not to be affected in *bollig* and *krusty*. Thus, the interpretation based on analogy to *Drosophila* has to be questioned such that the mutants should not be taken any more as evidence for conservation of gap gene function in *Tribolium*. Secondly, and in more general terms, phenotypic similarities of *Tribolium* and *Drosophila* mutants should not easily be interpreted

as mutations in homologous genes. In the context of this new interpretation, it may be worthwhile to positionally clone the *krusty* mutation starting from the information that it maps in moderate distance to the *Tc'runt* gene (see mapping project).

Evolution of abdominal gap gene orthologues

Above, I mentioned the anterior shift of both, *Tc'giant* and *Tc'Krüppel.* Also *Tc'tailless* expression does not cover the same set of segments as in *Drosophila* (see figure 2). In *Drosophila*, *tailless* is expressed at the posterior tip of the blastoderm. There, it is responsible for terminal fates and for positioning of the posteriormost pair rule stripes (Pankratz, Seifert et al. 1990; Klingler, Erdelyi et al. 1988). Also *Tribolium tailless* is expressed at the posterior tip in the early blastoderm. In this stage, however, the abdomen is not patterned yet and *Tc'Tll* protein becomes undetectable before the first abdominal segments are formed. Therefore, *Tc'tailless* cannot regulate the posteriormost pair rule stripes (Schröder et al. 2000). Neither *Tc'giant*, *Tc'Krüppel* nor *Tc'tailless* are expressed at positions where they could give similar input on homologous pair rule stripes as

Figure 2

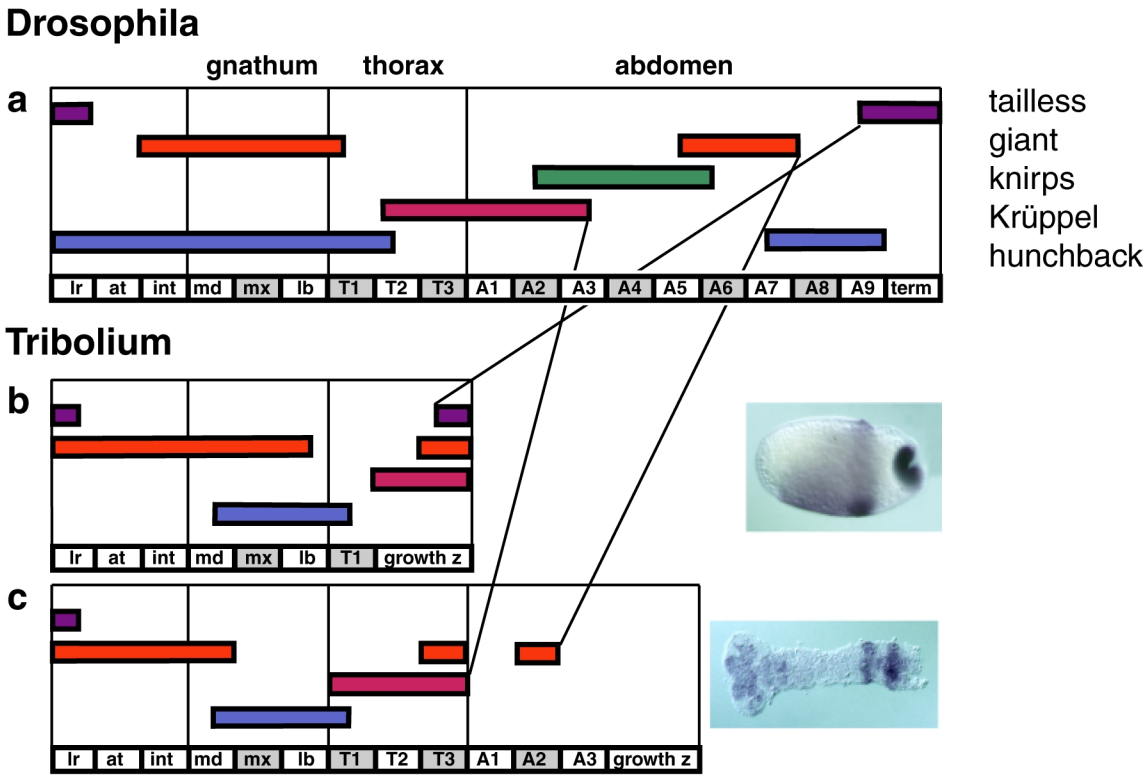
(A) The expression domains of abdominal gap genes in the Drosophila *blastoderm (a) are more posteriorly on the fate map than their* Tribolium *orthologues (b and c). The position of segment primordia is depicted below each panel; vertical bars separate cephalic, gnathal, thoracic and abdominal body parts. For Tribolium, two different stages are depicted, a late blastoderm (b; comparable to the embryo shown to the right) and a mid germ band (c; compare with germ band shown to the right). In the late* Tribolium *blastoderm, only head and anterior thoracic segments are specified (fate map in b). The posterior pole comprises the growth and patterning zone (growth z) and probably includes terminal fates (not shown). The anterior part of the* Tribolium *blastoderm consists of extraembryonic tissue but has been omitted for simplicity.*

While anterior expression domains are at corresponding positions in both species (compare anterior domains of tailless, giant *and* hunchback*), the posterior gap domains are shifted relative to the segments they will form:* Tc'tailless *expression at the posterior pole of the blastoderm ceases before gastrulation (expression in b but not in c).* Tc'Krüppel *arises during late blastoderm and does not extend beyond the 3rd thoracic segment.* Tc'giant*´s most posterior expression is in A2.*

(B) Conservation of relative positions: Despite different positions on the fate map, all three posterior gap gene domains are expressed in the same relative order in the blastoderm in Drosophila *(a) and* Tribolium *(b)(compare anterior borders of* tll, giant *and* Krüppel*). However, the domains overlap much more extensively in* Tribolium *than in* Drosophila*.*

lr:labrum, at: antenna, int: intercalary segment, md: mandible, mx: maxilla, lb: labium, T1-3: first to third thoracic segments, A1-8: abdominal segments, term: terminal structures, growthz: growth zone

A Expression domains on the fate map



B Expression domains in the blastoderm stage

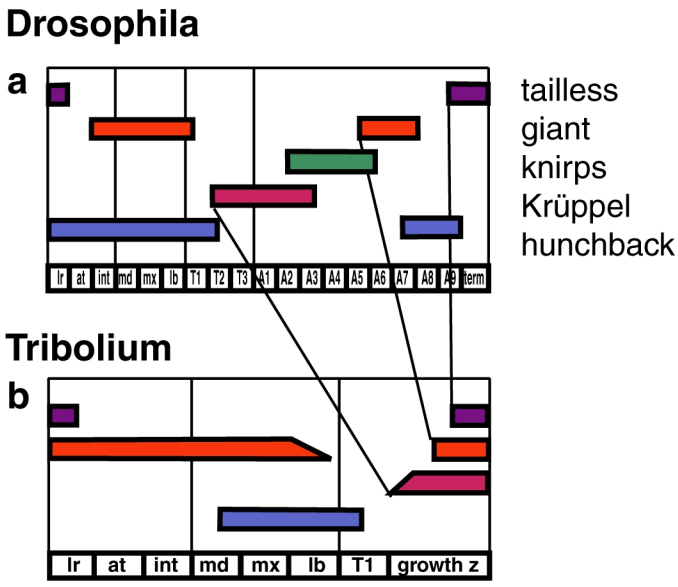


Figure 2 Comparison of the expression patterns of gap gene orthologues in *Drosophila* and *Tribolium* (see text on preceeding page)

known from *Drosophila*. In contrast to this notion, anterior expression domains of some gap gene orthologues are remarkably conserved regarding their position on the fate map. These include the head gap gene *Tc'otd-1* (Li et al. 1996) and anterior aspects of *Tc'hb, Tc'giant* and *Tc'tailless* (Wolff et al. 1995; Schröder et al. 2000). So far, no orthologue of the abdominal gap gene *knirps* has been identified in *Tribolium*. But even if its position on the fate map should be conserved, its position relative to the other gap gene orthologues would have changed fundamentally.

For speculations on the course of evolutionary change of abdominal gap gene orthologues, it is necessary to distinguish between ancestral and derived features. Such interpretations require the comparison of several species from different taxonomic groups. Based on classical comparative embryology the short germ insect *Tribolium* is believed to represent the ancestral mode of segmentation within insects (reviewed in Tautz, Friedrich et al. 1994). Therefore, features of the *Tribolium* genes likely reflect the ancestral function more closely than their *Drosophila* orthologues. However, also in the line from the last common ancestor to *Tribolium*, genes may have evolved different functions. Thus, only features, which are conserved in both taxa, can be regarded ancestral with high certainty.

Despite the marked differences in expression and function outlined above, there are indeed conserved aspects regarding abdominal gap gene orthologues in *Drosophila* and *Tribolium*: First, in the blastoderm, the anterior borders of *Tc'tll*, *Tc'giant* and *Tc'Krüppel* are expressed in the same relative position in both insects (although in overlapping instead of mutually exclusive domains and covering different segment anlagen; see figure 2 B). Thus, the relative order in a specific stage (the blastoderm) rather than the position on the fate map is conserved. Secondly, in both organisms, *giant* and *Krüppel* serve to subdivide the blastoderm into domains spanning several segment anlagen (used in the *Tribolium* blastoderm for homeosis of gnathum and thorax, in *Drosophila* for homeosis and segmentation). Finally, both genes are required for segmentation although their mode of action is probably different. It is not clear yet, whether *Tc'tailless* functions in segmentation and/or homeosis but is has been argued that it probably determines terminal fates (Schröder et al 2000).

Based on these conserved and divergent aspects, I suggest a (highly hypothetical) model for the evolution of abdominal gap gene orthologues: It assumes that *giant*, *Krüppel* and *tailless* orthologues of the last common ancestor had a function in subdividing the blastoderm. This information was used to position Hox genes (*giant*, *Krüppel*) and perhaps to define terminal cells at the posterior pole of the blastoderm (*tailless*). In addition, *Krüppel* and *giant* expression in the posterior blastoderm were required for segmentation. Possibly, they were involved in setting up or starting a segmentation machinery that subsequently patterned the abdomen by an autonomous

mechanism (see discussion in the *giant* chapter). During evolution of the dipteran clade, this mechanism was altered such that more and more posterior segments became specified already in the blastoderm stage. Subsequently, these blastodermal pair rule stripes acquired additional regulatory input by those genes that ancestrally regionalized the blastoderm for use in homeosis. In the course of dipteran evolution, direct input by gap genes became dominant over the autonomous segmentation machinery. When finally all pair rule stripes became specified by gap genes in the blastoderm, the ancestral segmentation machinery became dispensable and degenerated leaving the fly with the well-known hierarchical system.

From this study it becomes clear that the transition from short to long germ embryogenesis was accompagnied by marked changes in the orthologues of abdominal gap genes. This is in contrast to the conserved expression of pair rule and segment polarity genes between *Tribolium* and *Drosophila*. Future comparative studies should therefore focus on genes of the gap gene class. Especially, it will be interesting to investigate expression and function of the *knirps* orthologue, and to find out, whether anterior gap genes have also diverged functionally. Important insight is also likely to be derived from the analysis of pair rule gene upstream regions. Eventually, this will lead to an understanding of the genetic changes that were necessary for the evolution of long and short germ development in insects.

Altschul, S. F., T. L. Madden, et al. (1997). “Gapped BLAST and PSI-BLAST: a new generation of protein database search programs” Nucleic Acids Res. **22**: 3389-3402.

Beeman, R. W., J. J. Stuart, et al. (1989). ”Genetic analysis of the homeotic gene complex (HOM-C) in the beetle Tribolium castaneum.” Dev Biol **133**(1): 196-209.

Beermann, A., D. G. Jay, et al. (2001). ”The Short antennae gene of Tribolium is required for limb development and encodes the orthologue of the Drosophila Distal-less protein.” Development **128**(2): 287-97.

Bender, M., F. R. Turner, et al. (1987). ”A development genetic analysis of the gene regulator of postbithorax in Drosophila melanogaster.” Dev Biol **119**(2): 418-32.

Bennett, R. L., S. J. Brown, et al. (1999). ”Molecular and genetic analysis of the Tribolium Ultrabithorax ortholog, Ultrathorax.” Dev Genes Evol **209**(10): 608-19.

Berghammer, A., G. Bucher, et al. (1999). ”A system to efficiently maintain embryonic lethal mutations in the flour beetle Tribolium castaneum.” Dev Genes Evol **209**(6): 382-9.

Berghammer, A. J., M. Klingler, et al. (1999). ”A universal marker for transgenic insects.” Nature **402**(6760): 370-1.

Bernstein, E., A. A. Caudy, et al. (2001). ”Role for a bidentate ribonuclease in the initiation step of RNA interference.” Nature **409**(6818): 363-6.

Brown, S., J. Fellers, et al. (2001). ”A strategy for mapping bicoid on the phylogenetic tree.” Curr Biol **11**(2): R43-4.

Brown, S. J., R. B. Hilgenfeld, et al. (1994). ”The beetle Tribolium castaneum has a fushi tarazu homolog expressed in stripes during segmentation.” Proc Natl Acad Sci U S A **91**(26): 12922-6.

Brown, S. J., J. P. Mahaffey, et al. (1999). ”Using RNAi to investigate orthologous homeotic gene function during development of distantly related insects.” Evol Dev **1**(1): 11-5.

Brown, S. J., J. K. Parrish, et al. (1997). ”Molecular characterization and embryonic expression of the even-skipped ortholog of Tribolium castaneum.” Mech Dev **61**(1-2): 165-73.

Brown, S. J., J. K. Parrish, et al. (1994). ”Genetic control of early embryogenesis in the red flour beetle, Tribolium castaneum.” Am Zool **34**(3): 343-52.

Brown, S. J., N. H. Patel, et al. (1994). ”Embryonic expression of the single Tribolium engrailed homolog.” Dev Genet **15**(1): 7-18.

Bucher, G., J. Scholten, M. Klingler (2002). ”Parental RNAi in Tribolium (Coleoptera).” Current biology **12**(3): R85-R86

Capovilla, M., E. D. Eldon, et al. (1992). ”The giant gene of Drosophila encodes a b-ZIP DNA-binding protein that regulates the expression of other segmentation gap genes.”

Development **114**(1): 99-112.

Carroll, S. B., R. A. Laymon, et al. (1986). "The localization and regulation of Antennapedia protein expression in Drosophila embryos." Cell **47**(1): 113-22.

Darwin, C. (1859). On the Origin of Species by Means of Natural Selection, or the preservation of favoured Races in the Struggle for Life. London, Murray J.

Eldon, E. D. and V. Pirrotta (1991). "Interactions of the Drosophila gap gene giant with maternal and zygotic pattern-forming genes." Development **111**(2): 367-78.

Fire, A., S. Xu, et al. (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-11.

Fraser, A. G., R. S. Kamath, et al. (2000). "Functional genomic analysis of C. elegans chromosome I by systematic RNA interference." Nature **408**(6810): 325-30.

Fujioka, M., Y. Emi-Sarker, et al. (1999). "Analysis of an even-skipped rescue transgene reveals both composite and discrete neuronal and early blastoderm enhancers, and multi-stripe positioning by gap gene repressor gradients." Development **126**(11): 2527-38.

Gonczy, P., G. Echeverri, et al. (2000). "Functional genomic analysis of cell division in C. elegans using RNAi of genes on chromosome III." Nature **408**(6810): 331-6.

Higgins, D. G., Thompson, J. D., et al. (1996). «Using CLUSTAL for multiple sequence alignments» Methods Enzymol **266**: 383-402.

Hülskamp, M. and D. Tautz (1991). "Gap genes and gradients—the logic behind the gaps." Bioessays **13**(6): 261-8.

Ingham, P. W. (1988). "The molecular genetics of embryonic pattern formation in *Drosophila*." Nature **335**: 25-34.

Ingham, P. W. and A. Martinez-Arias (1986). "The correct activation of Antennapedia and bithorax complex genes requires the fushi tarazu gene." Nature **324**(6097): 592-7.

Irish, V. F., A. Martinez-Arias, et al. (1989). "Spatial regulation of the Antennapedia and Ultrabithorax homeotic genes during Drosophila early development." Embo J **8**(5): 1527-37.

Kimura, M. (1979). "The neutral theory of molecular evolution." Sci Am **241**(5): 98-100, 102, 108 passim.

Klingler, M., M. Erdelyi, et al. (1988). "Function of torso in determining the terminal anlagen of the Drosophila embryo." Nature **335**(6187): 275-7.

Klingler, M. and J. P. Gergen (1993). "Regulation of *runt* transcription by *Drosophila* segmentation genes." Mech. Dev. **43**(1): 3-19.

Klingler, M. and D. Tautz (1999). Formation of embryonic axes and blastodermal pattern in Drosophila. Development; genetics, epigenetics and environmental regulation. V. E. A. Russo. Berlin, Springer**:** 311 pp.

Kraut, R. and M. Levine (1991). "Mutually repressive interactions between the gap genes giant and Kruppel define middle body regions of the Drosophila embryo." Development **111**(2): 611-21.

Kraut, R. and M. Levine (1991). "Spatial regulation of the gap gene giant during Drosophila development." Development **111**(2): 601-9.

Langeland, J. A., S. F. Attai, et al. (1994). "Positioning adjacent pair-rule stripes in the posterior Drosophila embryo." Development **120**(10): 2945-55.

Li, Y., S. J. Brown, et al. (1996). "Two orthodenticle-related genes in the short-germ beetle Tribolium castaneum." Dev Genes Evol **206**: 35-45.

Maderspacher, F., G. Bucher, et al. (1998). "Pair-rule and gap gene mutants in the flour beetle Tribolium castaneum." Dev Genes Evol **208**(10): 558-68.

Meinhardt, H. (1982). Models of Biological Pattern Formation, London, Academic Press.

Mendel, G. (1865). "Versuche über Pflanzen-Hybriden." Verhandl. Naturf. Ver. in Bruenn(4): 3-47.

Mohler, J., E. D. Eldon, et al. (1989). "A novel spatial transcription pattern associated with the segmentation gene, giant, of Drosophila." Embo J **8**(5): 1539-48.

Nagy, L. M. and S. Carroll (1994). "Conservation of wingless patterning functions in the short-germ embryos of Tribolium castaneum." Nature **367**(6462): 460-3.

Nibu, Y. and M. S. Levine (2001). "CtBP-dependent activities of the short-range Giant repressor in the Drosophila embryo." Proc Natl Acad Sci U S A **98**(11): 6204-8.

Oppenheimer, D. I., A. M. MacNicol, et al. (1999). "Functional conservation of the wingless-engrailed interaction as shown by a widely applicable baculovirus misexpression system." Curr Biol **9**(22): 1288-96.

Palmeirim, I., Henrique D., Ish-Horowicz D., Pourquie O. (1997). "Avian hairy gene expression identifies a molecular clock linked to vertebrate segmentation and somitogenesis." Cell **91**(5): 639-48

Pankratz, M. J. and H. Jäckle (1990). "Making stripes in the Drosophila embryo." Trends Genet **6**(9): 287-92.

Pankratz, M. J., E. Seifert, et al. (1990). "Gradients of Krüppel and knirps gene products direct pair-rule gene stripe patterning in the posterior region of the Drosophila embryo." Cell **61**(2): 309-17.

Patel, N. H., B. G. Condron, et al. (1994). "Pair-rule expression patterns of even-skipped are found in both short- and long-germ beetles." Nature **367**(6462): 429-34.

Petschek, J. P. and A. P. Mahowald (1990). "Different requirements for l(1) giant in two embryonic domains of Drosophila melanogaster." Dev Genet **11**(1): 88-96.

Petschek, J. P., N. Perrimon, et al. (1987). "Region-specific defects in l(1)giant embryos of

Drosophila melanogaster." Dev Biol **119**(1): 175-89.

Preiss, A., U. B. Rosenberg, et al. (1985). "Molecular genetics of Kruppel, a gene required for segmentation of the Drosophila embryo." Nature **313**(5997): 27-32.

Prpic, N. M., B. Wigand, et al. (2001). "Expression of dachshund in wild-type and Distal-less mutant Tribolium corroborates serial homologies in insect appendages." Dev Genes Evol **211**(10): 467-77.

Riley, P.D., S.B. Carroll , M.P. Scott (1987). „The expression and regulation of Sex combs reduced protein in Drosophila embryos." Genes Dev **1**(7):716-30

Reinitz, J. and M. Levine (1990). "Control of the initiation of homeotic gene expression by the gap genes giant and tailless in Drosophila." Dev Biol **140**(1): 57-72.

Rivera-Pomar, R. and H. Jackle (1996). "From gradients to stripes in Drosophila embryogenesis: filling in the gaps." Trends Genet **12**(11): 478-83.

Sambrook, J., E. F. Fritsch, et al. (1989). Molecular cloning, Cold Spring Harbor Laboratory Press.

Sander (1976). "Specification of the basic body pattern in insect embryogenesis." Advances in Insect Physiology **12**: 125-238.

Schmid, K. J. and D. Tautz (1999). "A comparison of homologous developmental genes from Drosophila and Tribolium reveals major differences in length and trinucleotide repeat content." J Mol Evol **49**(5): 558-66.

Schröder, R., C. Eckert, et al. (2000). "Conserved and divergent aspects of terminal patterning in the beetle Tribolium castaneum." Proc Natl Acad Sci U S A **97**(12): 6591-6.

Seidel (1960). "Körpergrundgestalt und Keimstruktur." Zool. Anz. **164**: 245-305.

Shippy, T. D., J. Guo, et al. (2000). "Analysis of maxillopedia expression pattern and larval cuticular phenotype in wild-type and mutant tribolium." Genetics **155**(2): 721-31.

Small, S., A. Blair, et al. (1992). "Regulation of even-skipped stripe 2 in the Drosophila embryo." Embo J **11**(11): 4047-57.

Small, S., R. Kraut, et al. (1991). "Transcriptional regulation of a pair-rule stripe in Drosophila." Genes Dev **5**(5): 827-39.

Small, S. and M. Levine (1991). "The initiation of pair-rule stripes in the Drosophila blastoderm." Curr Opin Genet Dev **1**(2): 255-60.

Sokoloff, A. (1974). The Biology of *Tribolium*, Oxford Univ. Press.

Sommer, R. J. and D. Tautz (1993). "Involvement of an orthologue of the Drosophila pair-rule gene hairy in segment formation of the short germ-band embryo of Tribolium (Coleoptera) [see comments]." Nature **361**(6411): 448-50.

St Johnston, D. and C. Nüsslein-Volhard (1992). "The origin of pattern and polarity in the Drosophila embryo." Cell **68**(2): 201-19.

Stauber, M., H. Jäckle, et al. (1999). "The anterior determinant bicoid of Drosophila is a derived Hox class 3 gene." Proc Natl Acad Sci U S A **96**(7): 3786-9.

Strimmer, K. and D. G. von Haeseler (1996). Mol. Biol. Evol. **13**: 964-969.

Stuart, J. J., S. J. Brown, et al. (1993). "The Tribolium homeotic gene Abdominal is homologous to abdominal-A of the Drosophila bithorax complex." Development **117**(1): 233-43.

Sulston, I. A. and K. V. Anderson (1996). "Embryonic patterning mutants of Tribolium castaneum." Development **122**(3): 805-14.

Sulston, I. A. and K. V. Anderson (1998). "Altered patterns of gene expression in Tribolium segmentation mutants." Dev Genet **23**(1): 56-64.

Swofford, D. L. (1998). PAUP, Phylogenetic Analysis Using Parsimony. Washington, DC, Smithsonian Institution.

Tautz, D. (1992). Genetic and molecular analysis of early pattern formation in Drosophila. Development; the molecular genetic approach. Berlin, Russo V.E.A.

Tautz, D., M. Friedrich, et al. (1994). "Insect embryogenesis - what is ancestral and what is derived?" Development (Suppl.): 193-199.

Timmons, L. and A. Fire (1998). "Specific interference by ingested dsRNA." Nature **395**(6705): 854.

Wieschaus, E., C. Nüsslein-Volhard, et al. (1984). "Krüppel, a gene whose activity is required early in the zygotic genome for normal embryonic segmentation." Dev Biol **104**(1): 172-86.

Wolff, C., R. Schröder, et al. (1998). "Regulation of the Tribolium homologues of caudal and hunchback in Drosophila: evidence for maternal gradient systems in a short germ embryo." Development **125**(18): 3645-54.

Wolff, C., R. Sommer, et al. (1995). "Conserved and divergent expression aspects of the Drosophila segmentation gene hunchback in the short germ band embryo of the flour beetle Tribolium." Development **121**(12): 4227-36.

Wu, X., R. Vakani, et al. (1998). "Two distinct mechanisms for differential positioning of gene expression borders involving the Drosophila gap protein giant." Development **125**(19): 3765-74.

Zhao, Z., Y. Cao, et al. (2001). "Double-stranded RNA injection produces nonspecific defects in zebrafish." Dev Biol **229**(1): 215-23.

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Hiermit erkläre ich, die vorliegende Arbeit eigenständig und nur mit den angegebenen Quellen und Hilfsmitteln angefertigt zu haben.

München im März 2002,

Gregor Bucher