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Evolution of Developmental Control Mechanisms

The gap gene *giant* of *Rhodnius prolixus* is maternally expressed and required for proper head and abdomen formation

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ABSTRACT

The segmentation process in insects depends on a hierarchical cascade of gene activity. The first effectors downstream of the maternal activation are the gap genes, which divide the embryo in broad fields. We discovered a sequence corresponding to the leucine-zipper domain of the orthologue of the gene *giant* (*Rp-gt*) in traces from the genome of *Rhodnius prolixus*, a hemipteran with intermediate germ-band development. We cloned the *Rp-gt* gene from a normalized cDNA library and characterized its expression and function. Bioinformatic analysis of 12.5 kbp of genomic sequence containing the *Rp-gt* transcriptional unit shows a cluster of *bona fide* regulatory binding sites, which is similar in location and structure to the predicted posterior expression domain of the *Drosophila* orthologue. *Rp-gt* is expressed in ovaries and maternally supplied in the early embryo. The maternal contribution forms a gradient of scattered patches of mRNA in the preblastoderm embryo. Zygotic *Rp-gt* is expressed in two domains that after germ band extension are restricted to the head and the posterior growth zone. Parental RNAi shows that *Rp-gt* is required for proper head and abdomen formation. The head lacks mandibulary and maxillary appendages and shows reduced clypeus-labrum, while the abdomen lacks anterior segments. We conclude that *Rp-gt* is a gap gene on the head and abdomen and, in addition, has a function in patterning the anterior head capsule suggesting that the function of *gt* in hemipterans is more similar to dipterans than expected.

Introduction

Insect embryogenesis displays a variety of mechanisms to generate similar segmented organisms. There are three major forms of embryonic development, depending on the formation of the germ band, from long to short germ-band embryos. Short germ-band embryogenesis is the most common and most primitive developmental way (reviewed in Davis and Patel, 2002; Liu and Kaufman, 2005). However, the main body of knowledge on the genetic and molecular developmental mechanisms of insect segmentation derives from studies on *Drosophila melanogaster*, a highly evolved insect with long germ-band mode of segmentation. In recent years, the development of genomics and parental RNAi allowed comparative studies in other insects such as the beetle *Tribolium castaneum* and the wasp *Nasonia vitripennis* – with sequenced genomes – as well as *Oncopeltus fasciatus* and the cricket *Gryllus bimaculatus* (reviewed by Peel et al., 2005).

In *Drosophila*, the segmentation cascade is initiated by the activity of maternal genes, which set the basic axis. Two transcription factors: bicoid and caudal form complementary gradients that activate gene expression throughout the blastoderm (Rivera-Pomar et al., 1995). Downstream of the maternal genes, the gap genes are expressed in overlapping domains that set broad regions of the embryo. Later on, the blastoderm becomes molecularly segmented by the setting of pair-rule and segment polarity. They interplay one with each other and with gap and maternal genes to determine the limits of their own expression and its precise position. This hierarchical model has been extensively studied in *Drosophila* (reviewed in Rivera-Pomar and Jackle, 1996). The advent of new developmental models such as *Tribolium castaneum*, *Oncopeltus fasciatus* and, more recently, *Nasonia vitripennis* – also a long germ insect – showed differences with the *Drosophila* paradigm. The most striking distinction is the use of different anterior determinants; *bicoid* in *Drosophila*, *orthodenticle* and *hunchback* in *Tribolium* (Schroder, 2003) and *orthodenticle* in *Nasonia* (Brent et al., 2007; Lynch et al., 2006). In the ancestral short and intermediate germ-band embryogenesis the segmentation process differs from the long germ-band segmentation. In long germ-band embryos, the segments are formed simultaneously throughout the embryo and are already determined in the blastoderm stage. In the short and intermediate germ-band embryos, the anterior segments – head and thoracic region – develop early and the posterior segments appear later in a sequential manner from a cell population in the posterior pole of the embryonic anlagen, called “growth zone”. Thus, only the anterior domain is defined in a syncytial environment while

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the most posterior structures are formed in a cellularized embryo from the growth zone, where diffusion of molecules is restricted (reviewed in Liu and Kaufman, 2005; Peel et al., 2005). In this context, the function of the genes is not necessarily to set broad domains in the entire blastoderm. Therefore, a real gap activity is unclear.

The gap gene *giant* encodes a basic leucine-zipper transcription factor conserved among different species (Brent et al., 2007; Bucher and Klingler, 2004; Capovilla et al., 1992; Liu and Patel, 2010; Wilson et al., 2010). It regulates other gap and pair rule genes by repression (Arnosti et al., 1996; Eldon and Pirrotta, 1991; Kraut and Levine, 1991; Small et al., 1992). In *D. melanogaster* the mutation of *gt* affects head formation and abdominal segmentation (Mohler et al., 1989; Petschek et al., 1987). In other long germ-band insects such as *N. vitripennis* and *Apis melifera* the lack of *gt* results in deletion of head and thoracic segments and the fusion of abdominal segments (Brent et al., 2007; Wilson et al., 2010). In short germ-band insects, *gt* expression is zygotic but maternal contribution has been proposed for *Tribolium* (Bucher and Klingler, 2004). In *T. castaneum* the lack of *gt* results in homeotic transformation of maxillary and labial segments to thoracic identity and alteration of thoracic and abdominal segments (Bucher and Klingler, 2004). In *O. fasciatus* it has been recently reported that the phenotype of *gt* is closer to *Drosophila* than other short germ-band insects. However, the diversity of insects and the different evolutionary pathways may represent a particular case rather than a rule.

The renaissance of the studies of the embryology of insects with intermediate and short germ-band combined with genomics and forward and reverse genetics allows the better understanding of the evolutionary processes leading to the different modes of segmentation and the generation of insect biodiversity. Progress in the genomics of *R. prolixus*, a vector of Chagas disease, led us to study it as a model to compare early development through both genomic analysis and parental RNAi. *R. prolixus*, a classical model for insect physiology, belongs to the intermediate germ-band insects; its embryology has already been studied (Kelly and Huebner, 1989; Mellanby, 1935, 1934). However, developmental genetic studies have not been carried out yet. In this context, we take advantage of the advances in genomics bringing back this model to study the segmentation process. Here we show that *Rp-gt* expression is both maternal and zygotic, and the lack of *Rp-gt* results in an anterior and posterior gap phenotype. Moreover, we compare the putative regulatory regions of *Rp-gt* with those described in *Drosophila* and show a similar distribution of transcription factors clusters.

Material and methods

Insect rearing

A colony of *R. prolixus* was maintained in our laboratory in a 12 h light/dark schedule at 28 °C and 80% humidity. In these conditions the embryogenesis takes 14 ± 1 days. Insects were regularly fed *ad libitum* on chickens once or twice before molting. When necessary, V larval instar were sexed before molting until adulthood and then mated.

Identification and cloning of the Rp-gt gene

Total RNA was isolated from mixed stages of *R. prolixus* embryos and adults using gaunidinium tiocyanate/acid phenol technique (Chomczynski and Sacchi, 1987). cDNA was synthesized using the Smart cDNA Library Construction Kit (Clontech). To enrich the library it was subsequently normalized with the Trimmer–Direct normalization kit (EVROGEN).

Gene discovery proceeded as described for other *R. prolixus* genes (Ons et al., 2011). Traces of *R. prolixus* whole genome sequence (WGS), EST and WGS assembly databases were used for homology search by local TBLASTN. *R. prolixus* genomic data was produced by the Washington University School of Medicine in St. Louis as part of the *R. prolixus* Genome Project (http://genome.wustl.edu/genomes/view/rhodnius_prolixus). From the genome traces a set of specific primers was designed spanning the entire basic leucine-zipper domain either containing or not T7 promoter sequence at the 5′ end for further use in *in vitro* transcription.

GTFwT7: CGACTCACTATAGGGAACCACCGTGAAGAAGAGAC,
GTRvT7: CGACTCACTATAGGGAAGAAAAGCCGCTCGTATAGC,
GTFw: GACCATTTAAAGCGTATCCAAAAG,
GTRv: CTTTCCC AATAGGCGGCATC.

The expression of *Rp-gt* was confirmed by PCR on standard and normalized embryonic cDNA libraries. The amplicons were cloned into the vector pGEM-T easy (Promega) and several independent clones were sequenced.

Sequence analysis

Prediction of the gene structure and open reading frame on the genomic sequence was done with Lasergene (DNASTAR) and by manual curation. *Rp-gt* sequence was aligned with the *gt* orthologues from different insects using Clustal W. This alignment was used for phylogenetic analysis with the centipede *Strigmia* sp. as outgroup. Phylogenetic analysis was performed using the Bayesian algorithm Mr. Bayes Online http://www.phylogeny.fr; (Dereeper et al., 2008; Huelsenbeck et al., 2001). The parameters used for the analysis were: Number of substitution types: 6 (GTR), Substitution model: Blosum62, Number of generations: 100,000 and Sample a tree every 10 generations. A larger genomic fragment was assembled using traces containing the partial *Rp-gt* ORF. For putative regulatory regions a fragment of 5.5 kbp upstream of the AUG was analyzed. We used the Position Weight Matrices (PWMs) described by Berman et al. (2002) for the Hb, Cad, Bcd, Kr, and Kni binding sites, adding our own matrices generated for Otd and Gt binding sites (see supplementary data). The software PATSER (http://rsat.ulb.ac.be/rsat/patser_form.cgi) was used for the search of PWMs that matches the genomic sequence. The parameters used were: Lower threshold estimation — weight store: 5; alphabet: a:t 0.297 c:g 0.203 (Berman et al., 2002). As validation methods we analyzed the presence of clustered binding sites using the software STUBB (http://stubb.rockefeller.edu/).

Embryonic techniques and RNA in situ hybridization

Embryos were collected at different time after egg laying (AEL) (24, 36 and 48 h AEL). For the embryo dissection two different strategies of fixation and dechorionation were tested. A group of embryos was dechorionated by hand after three cycles of heating at 60 °C and freezing in liquid nitrogen, fixation in 4% parafomaldeide in PBS (4% PFA) for 1 h, dechorionated manually, and stored in 100% methanol. Using this method, the embryo and the yolk are separated of the chorion, facilitating manual dechorionation without damage of the embryonic tissue. Other group of embryos was dechorionated and devitellinizated by hand, then fixed in 4% PFA for 1 h, and stored in 100% methanol. Early embryos were directly fixed in 4% PFA for 1 h after the removal of the egg operculum and then directly used for *in situ* hybridization. The dechorionation proceeded manually after probe hybridization. A detailed protocol can be obtained under request. Females were dissected after feeding and in reproductive activity to collect ovaries. The ovaries' fixation and *in-situ* hybridization was performed as described (Osborne and Dearden, 2005). Embryo *in situ* hybridization technique will be described elsewhere (Esponda-Behrens et al., unpublished results). After staining, the embryos were counterstained with DAPI for staging. Images were acquired with either a fluorescence microscope or binocular stereoscope (Leica DM 1000) and a CCD camera, Cool SNAP-Pro$_{cf}$ color (Media Cybernetics).

RNAi

dsRNA was produced by simultaneous T7 transcription on PCR products containing T7 promoter at both sides. dsRNA was quantified and injected in virgin females (2.0 μg in no more than 5 μl volume). In some experiments antisense ssRNA was used. The intra-abdominal injection proceeded between the third and fourth abdominal segments using a 5 μl Hamilton syringe. The females were let to recover for 2–3 days, and then fed to induce oogenesis. After mating, eggs were collected from individual females at different times of development. Embryos were immediately fixed or left to fully develop to account for lethality and/or cuticle preparation. Cuticles were prepared for optic and confocal microscopy in 10% potassium hydroxyde (clarification) for 16 h at room temperature, and then dehydrated in ethanol and xylol and mounted in Canada balsam. Internal cuticle morphology was performed taking advantage of the autofluorescence of the insect. Cuticular autofluorescence in the 520 to 660 nm range was detected using Argon laser at 488 nm on a Zeiss 510-META confocal microscope. A maximum projection images were generated from 15 image stacks. To quantify the head shortening in the interfered insects, we measured the distance between the most anterior part of the labrum and the anterior eyes border, the length of the clypeus, the labrum and the "clypeus-labrum". These parameters were measured in wild-type and *Rp-gt* interfered nymphs. As a negative control in every independent experiment we used dsRNA corresponding to the beta-lactamase gene of *Escherichia coli*, which did not show any detectable phenotype (not shown).

Results

Identification of the giant orthologue in R. prolixus

In order to identify the *giant* orthologue in *R. prolixus* we first performed a homology search on *R. prolixus* whole genome trace archives. This strategy resulted in the identification of several incomplete scaffolds that were assembled into a 12.5 kpb genomic region. The region shows an intronless 248 amino acid open reading frame similar to known *gt* orthologues (GenBank HQ853222). To validate the transcriptional activity of the putative *Rp-gt* gene, we designed specific primers from the ORF to perform PCR on cDNA derived from *R. prolixus* embryos. A PCR product of 332 bp was sequenced and showed the complete basic leucine-zipper domain (GenBank GU724146.1). Sequence comparison revealed high similarity to other insects *gt* orthologues, including the BLZ and CtBP domains and an additional putative phosphorylation domain shared with *Oncopeltus* (Fig. 1A). No evidence of duplication or paralog sequences was detected in the available genomic information, supporting the notion that this is the only *gt* orthologue in *Rhodnius*. Evolutionary analysis of the sequence revealed that *Rp-gt* fits within the hexapoda phylogenetic tree, grouping the hemimetabola and holometabola insects in two groups (Fig. 1B). This indicates that the evolution of the coding sequence of the gene followed the evolution of the group.

Clustering of regulators in the Rp-gt gene promoter

We then analyzed the genomic region upstream of the *Rp-gt* transcriptional unit, looking for the presence of clustered binding sites

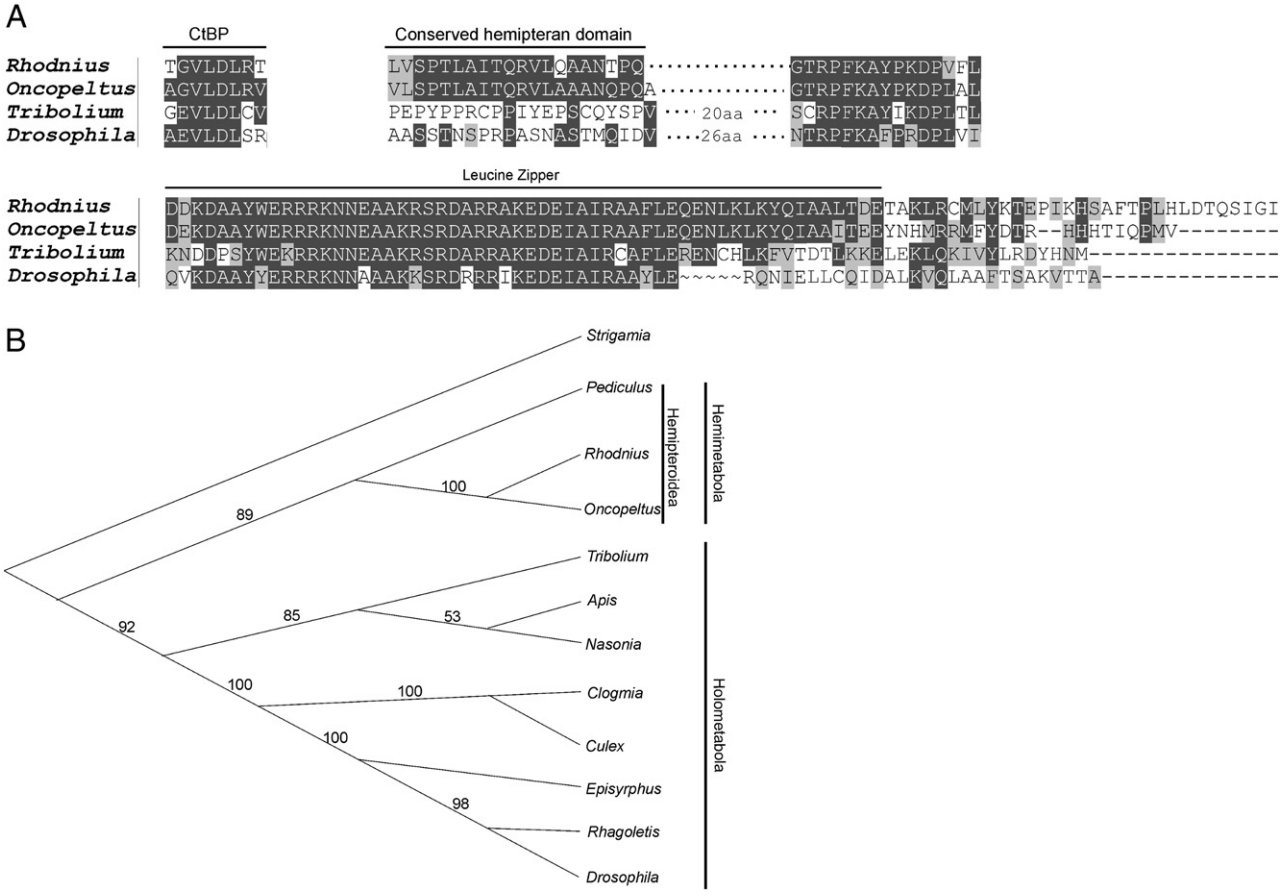

Fig. 1. Analysis of the *R. prolixus giant* gene. A. *Rp-gt* protein sequence alignment to other insect sequences. Black boxes indicate complete identity; three blocks are evident, the CtBP box, the leucine zipper domain and a conserved hemipteran domain. B. Phylogenetic analysis of *Rp-gt*. The tree was generated by Bayesian phylogenetic inference; the node values indicate the clade credibility values.

for putative regulators. A bioinformatic analysis using the PATSER software showed numerous binding sites for transcription factors which are putative regulators of gap gene activity (Hb: 30, Bcd/Otd: 4, Cad: 16, Kr: 3, Kni: 9, and Gt; 11). As the presence of binding sites could occur at random, a second parameter was analyzed: clustering. Using the software STUFF we can predict, with high confidence, a cluster of binding sites of 0.8 kbp at 1.5 kbp from the transcription start including the following transcription binding sites: Bcd/Otd: 1, Hb: 7, Kr: 1, Kni: 1, Gt: 3 and Cad: 3.(Fig. 2A). This analysis has been previously done in *Drosophila* and allowed to discover the abdominal activation enhancer of *gt* performed (Berman et al., 2002). To evaluate the reliability of our analysis, we used the same parameters to analyze the clustering in *Drosophila gt* as a control, and we also studied the *Tc-gt* upstream region. Using the same parameters for all three sequences, we could determine a 0.7 cluster at 1.7 kbp upstream of the transcriptional start in *Tribolium* and, as expected, a 1 kbp at 1.8 kbp from the +1 nucleotide in *Drosophila* (Figs. 2B and C). We noted that a similar cluster of regulators at equivalent relative position can be determined for the three species. Therefore we have defined a putative conserved regulatory sequence for *Rp-gt*, *Tc-gt* and *Dm-gt*.

Rp-gt expression in the embryo

We used an antisense RNA probe targeting the transcribed genomic regions corresponding to the *Rp-gt* ORF and performed *in situ* hybridization in ovaries and embryos. In ovaries, *Rp-gt* mRNA is detected in the thopharium, as well as in early oocytes (Fig. 3A). The transcripts are located in the zone where the earliest oocytes are placed (comparable with the oocytes T-3 accordingly to Bjornsson and Huebner, 2004), and in advanced vitellogenesis oocytes — AvO (Fig. 3A). *Rp-gt* mRNA is also detected at low level at very early stages of embryogenesis. 6–12 h after egg laying (AEL), *Rp-gt* mRNA occurs in patches in the posterior half of the egg (Fig. 3B) and is slightly ventralized (Fig. A — supplementary data). At this stage, the nuclei have not migrated to the periphery of the egg and the blastoderm is not formed yet. Thus, the *Rp-gt* mRNA patches are not associated to any detectable cellular structure, as revealed by DAPI counterstaining (Figs. 3C and D). At 12 h AEL, the nuclei have reached the egg surface, the transcript distribution becomes more homogeneous and now is restricted to smaller perinuclear patches (Figs. 3E–G). In Figs. 3F and G we show the *Rp-gt* perinuclear distribution at blastoderm stage. Throughout the blastoderm stage the gradient is still visible with a maximum in the posterior region of the egg, which will correspond to the anterior region the germ band before katatrepsis. It is important to note that the anterior pole in the egg (operculum) corresponds to the posterior pole of the germ band. During the gastrulation (24–36 h AEL) the expression domain retracts to the posterior end of the egg (anterior-most part of the embryo) accompanying the migration of cells to form the germ band (Figs. 4A, B and C). In the ventral side, two regions of *Rp-gt* expression are detected: one anterior – head prospective (Fig. 4A; white arrowhead) – and one posterior with respect to the forming germ band — middle of the egg (Fig. 4A; black arrowhead). At the same time, at the dorsal side, the expression is split into two domains at each side of the gastrulation center (Figs. 4B and C). The embryos were counterstained with DAPI to determine the morphology of the germ band and establish the expression domains. At the germ band stage (36–48 h AEL), *Rp-gt* expression is placed in three regions: an anterior-most, a middle expression domain and a posterior domain (Figs. 4D, E and F). The anterior-most expression domain is in the presumptive region corresponding to pregnathal segments and shows four subdomains, two at each side (Fig. 4F). The middle expression domain coincides with the mandibulary and maxillary segments (see below). The posterior domain corresponds to the growth zone (Fig. 4E). After germ band extension (48–60 h AEL), the head expression occurs in the mandibulary and maxillary segments (Figs. 4G and H; white arrowhead) while the posterior expression domain is restricted to the so called the growth zone (Figs. 4H, black arrowhead and 4I). The gnathal and abdominal *gt* expression domains are conserved in other species while a pregnathal expression has only been described in *Drosophila*.

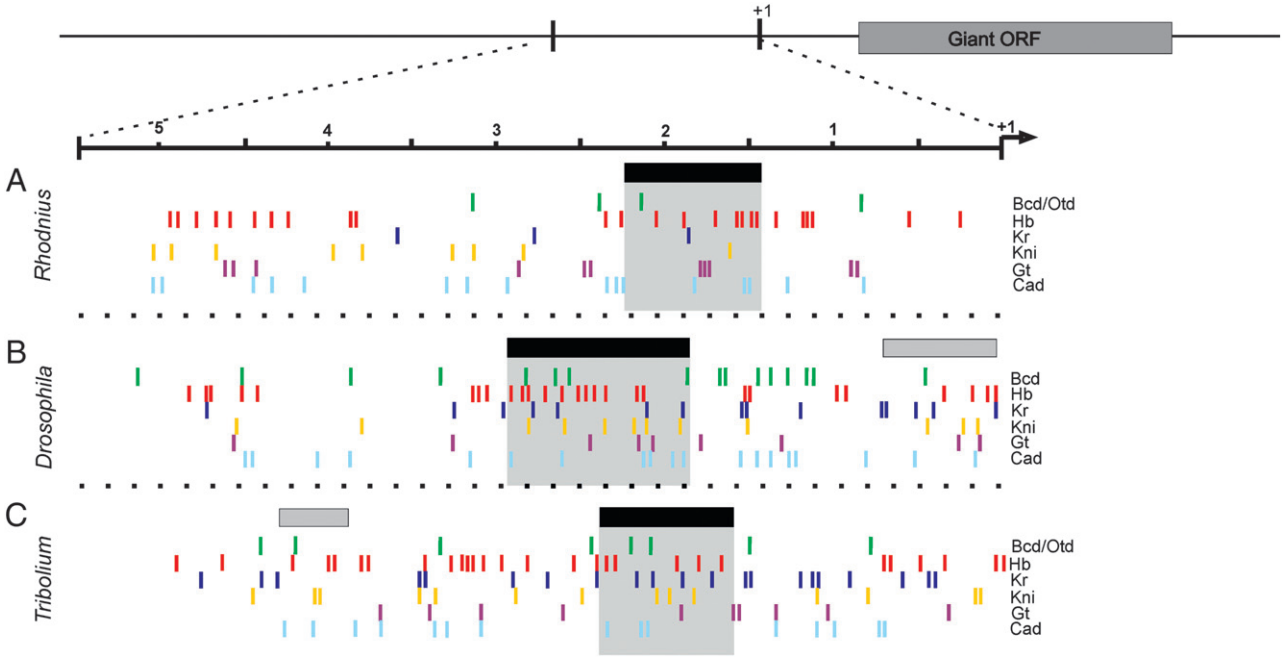

Fig. 2. Analysis of the putative *Rp-gt* promoter sequence compared to the different predicted regulatory clusters of *Drosophila* and *Tribolium*. The analysis was applied both to the *gt* promoter sequence of *R. prolixus* (A), *D. melanogaster* (B) and *T. castaneum* (C). On the right side are indicated different regulators. The color stripes represent the different binding sites for the transcription factors as predicted by the PATSER software. The black (highest probability) and gray boxes indicate predicted regulatory clusters by STUBB. The scale represents 1 kbp.

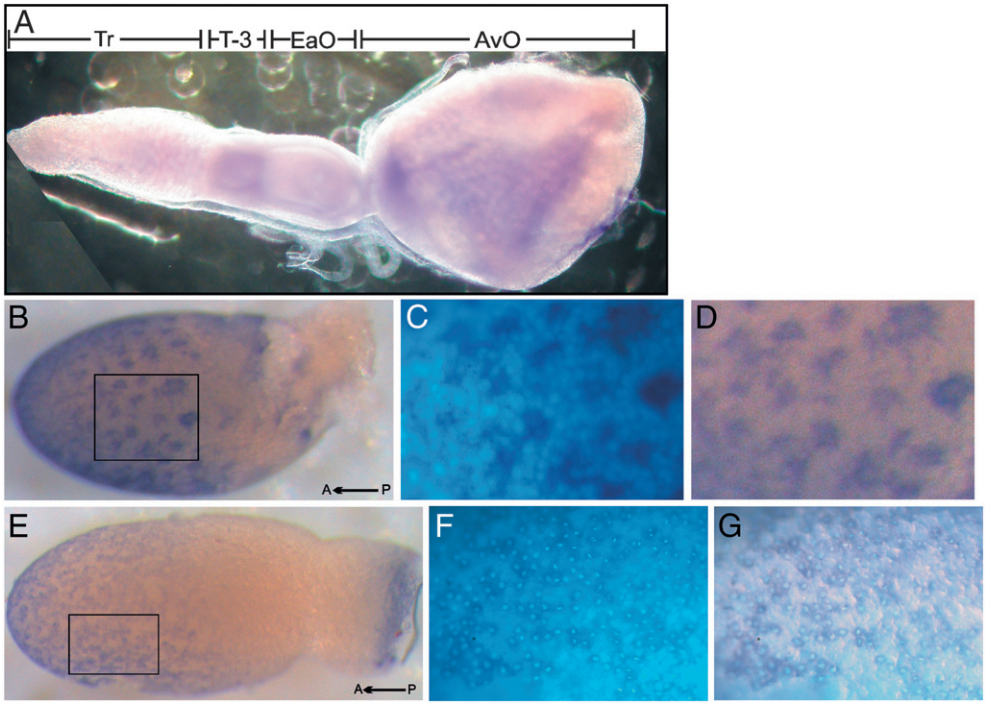

Fig. 3. Maternal expression of *Rp-gt*. A. *Rp*-gt mRNA distribution in ovaries. The tropharium (Tr), the early oocytes (T-3) and in advanced vitellogenesis oocyte (AvO) show expression, while that early oocytes without vitellogenesis (EaO) does not show expression. The *in-situ* hybridization of the sense probe is not shown. B–G. Distribution of *Rp*-gt mRNA in early embryos. B. Embryo at 4 h AEL, before nuclei migration shows the *Rp-gt* transcript distributed in irregular patches in the anterior half of the egg. The rectangle indicates the magnification, shown in C and D. The arrow indicates the anterior pole of the embryo (head), which coincides to the posterior pole of the egg (opposite to the operculum) before katatrepsis. C. Image of the embryo staining with DAPI (the nuclei are not evident in the egg surface). D. Same image that in C with epiillumination, showing the *in situ* hybridization pattern. E. Embryo at 24 h AEL. *Rp-gt* expression becomes more homogeneous compare to B. F. Detail of the embryo surface showing the nuclei stain with DAPI. G. Same image in F with epiillumination, where the transcript localization becomes more dispersed and with peri-nuclear distribution. This image was acquired by double exposure by epiillumination with visible and UV epifluorescence.

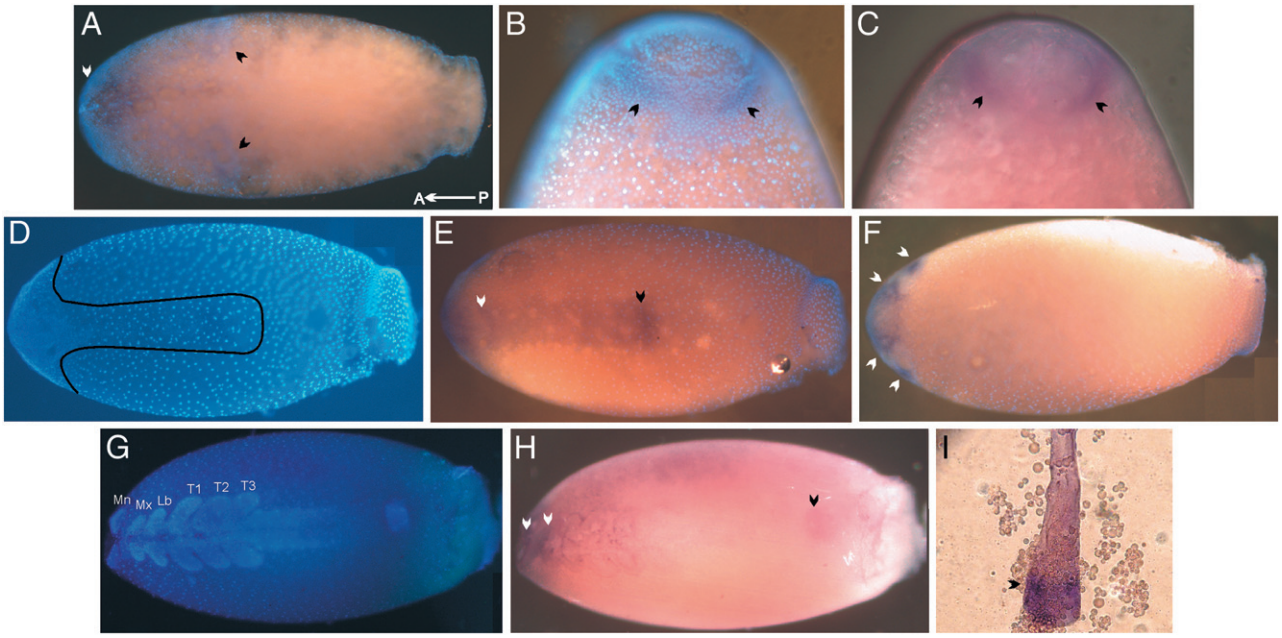

Fig. 4. Zygotic expression of *Rp-gt*. A–C. Embryo in invagination state (36 h AEL). A. Ventral view showing the anterior expression domain, and the early posterior domain. B–C. Dorsal view of the invaginating region shows the distribution of the *Rp-gt* transcript in the invagination border. B. *In situ* hybridization and DAPI staining, C, same embryo, showing only the in-situ hybridization. D–E. Embryos during germ band extension in ventral view. D. DAPI staining. The black line marks the position of the embryo in the egg. Note, again, that anterior of the embryo is posterior of the egg before katatrepsis. E. *In situ* hybridization showing the same embryo as D. The black arrowhead indicates the posterior expression domain in the growth zone. F. Dorsal view of the same embryo is shown in E. The white arrowheads indicate the gnathal expression domains. G–I. Embryos at full germ band elongation. G. DAPI image showing a ventral view of the embryo indicating the head and thoracic segments (Mn, mandible; Mx, maxillae; Lb, labium; T1–T3, thoracic segments 1 to 3). H. *In situ* hybridization of the same embryo is shown in G. The arrowhead marks the different expression domains in the embryos: mandibular and maxillary (white arrowheads) and abdominal domain (black arrowhead). I. Detail of *Rp-gt* posterior domain expression in the "growth zone" in a dissected germ band.

Table 1
Results of the parental RNAi.

	Strong (%)	Weak (%)	WT (%)	Total
ssRNA antisens	0 (0%)	8 (17%)	39 (83%)	47
dsRNA antisens	143 (94.1%)	5 (3.3%)	4 (2.6%)	152
	143 (71.9%)	13 (6.5%)	43 (21.6%)	199 (100%)

Rp-gt phenotype

To determine the lack-of-function phenotype of *Rp-gt*, we generated phenocopies by parental RNAi. Injected virgin females produced a total of 199 eggs from two independent experiments. The embryos were studied at different time points to determine the phenotype (Table 1). 78% of the interfered embryos showed a phenotype different to the wild-type. Two groups were distinguished out of them: namely weak (8%) and strong (92%) phenotypes. The weak phenocopies completed embryogenesis but they did not hatch. They corresponded to the eggs laid during the first days of the experiment.

The wild-type abdomen is formed by 10 segments (Fig. 5A). The head consists of the pregnathal segments – formed by the clypeus, the labrum and the antennal segment – and the gnathal segments — the mandibulary, maxillary and labial segments associated to form the proboscis (Figs. 5B and C). The head structure derives from the anterior segments ventrally folded. The clypeus and the labrum are in the most anterior part of the hemipteran head capsule. The labrum has two segments and is anterior to the clypeus. In the wild-type first instar larva (nymph) the length of the head from the anterior border of the eyes to the anterior tip is 630 μm; the length of the "clypeus–labrum" is 360–210 μm, which corresponds to the clypeus and 150 μm to the labrum. The proboscis is formed by the labium, which wraps the stylets. The stylets are formed by modified mandibles and maxillae. The mandibles have a serrated tip and the maxillae a spatle-like shape (Fig. 5D).

The interfered embryos and hatchlings showed an unequivocal phenotype in the regions that would correspond to the *Rp-gt* mRNA expression: modifications in the head, lack of gnathal segments and lack or fusion of abdominal segments (Fig. 6). All of them show a normal development of the labial segment and a normal proboscis (Figs. 6A–D). Cuticle analysis of clarified nymphs and/or fluorescent confocal optical sections of the cuticle show a normal development of the proboscis formed by the labium, but lacking the mandibulary and maxillary appendages, which correspond to the stylets (Fig. 6A). In addition, the head shows shortening of the clypeus and the labrum, compared to the wild-type (Figs. 6C–E). These two structures are fused and result is impossible to distinguish from each other — we refer to this structure as "clypeus–labrum". In these mutants the length of the "clypeus–labrum" is 250 μm and the length of the head before the anterior eye border is 390 μm. After comparing these parameters to the wild-type we calculated that there was a 30% reduction in the length of the "clypeus–labrum", while there was a 40% reduction in the anterior part of the head. This phenotype correlates with the anterior-most expression domain in the pregnathal region. The reduction or deletion of these structures has been also described for the *Drosophila gt*-mutant, providing evidence of the functional similarity of *Rp-gt* to *Dm-gt*, rather than to other insects analyzed up to date. In some cases – when the effect of the parental RNAi technique was weak – a reduced mandibulary appendage can be also observed (red arrow in Fig. 6D). This also correlates with the expression domains described in the head. In addition, the interfered embryos show defective abdomen, either fusion or lack of abdominal segments (Figs. 6E and H). Weak phenotypes in first instar larvae consistently show fusion of abdominal segments 4 and 5 (Fig. 6F). Embryos with stronger phenotype (N = 143) are highly affected compared to the wild-type (Figs. 6G and H). They consistently arrest development at 72 h AEL. The head phenotype is similar to the weak phenotype, they lack mandibulary and maxillary segments, but the labium is always present. On the other hand, the posterior phenotype is more dramatic, being the number of abdominal segments persistently reduced to four (Figs. 6G, compared to H, wild-type). In these embryos we observed additional defects such as incomplete dorsal closure; however, we attribute it to the arrest of development before dorsal closure occurs rather than to an effect of *Rp-gt* in that process. In all cases there is an effect in the thoracic appendages, such as the deformation of the legs, particularly femur and tibia (data not shown). This could be an indirect effect of the lack of *Rp-gt* in other gap and hox genes required for leg formation.

Discussion

During the segmentation process, the gap genes are the first zygotic genes that are expressed in the embryo. Within the gap genes, *giant* has been widely studied in short and long germ-band insects. In this work, we studied the *Rp-gt* expression pattern and its phenotype. At the same time, we analyzed the promoter sequence and found a putative regulatory cluster. As a result of this analysis, we have seen that the position and extent of the *gt* regulatory cluster are conserved in *Rhodnius*, *Drosophila* and *Tribolium*. We also show that *Rp-gt* is maternally expressed, being the first hemimetabolous insect where the maternal expression of this gap gene is observed. The zygotic expression is distributed in three expression domains: two anterior domains, and the posterior domain. RNAi against the *Rp-gt* transcript results in an embryo which is defective in the formation of the pregnathal segment, the mandibulary and maxillary segments and the anterior abdominal segments.

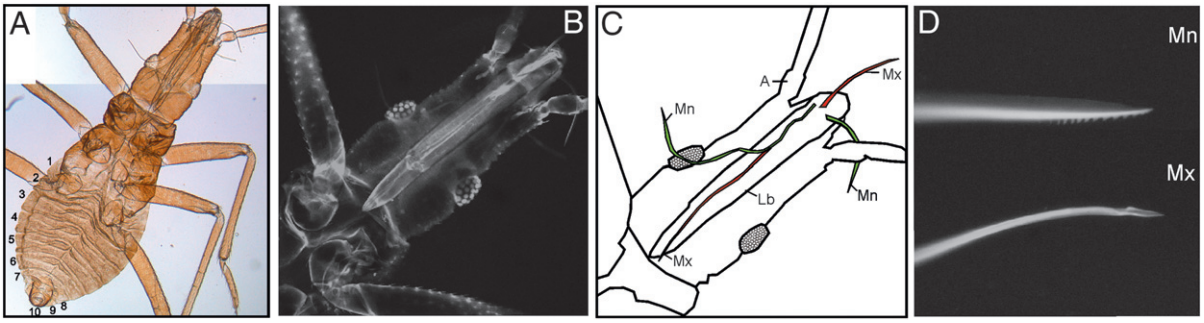

Fig. 5. Wild-type *R. prolixus* first instar larvae morphology. A. Cuticle preparation in dorsal view, showing the ten abdominal segments. B. Projection of 10 confocal images of the head. C. Scheme of picture B, indicating the different cephalic appendages (A, antenna; CL, clypeus–labrum; L, labium; Mn, mandible and Mx, maxillae). D. Structure of mandibular (serrated tip) and maxillary (spatle tip) stylets.

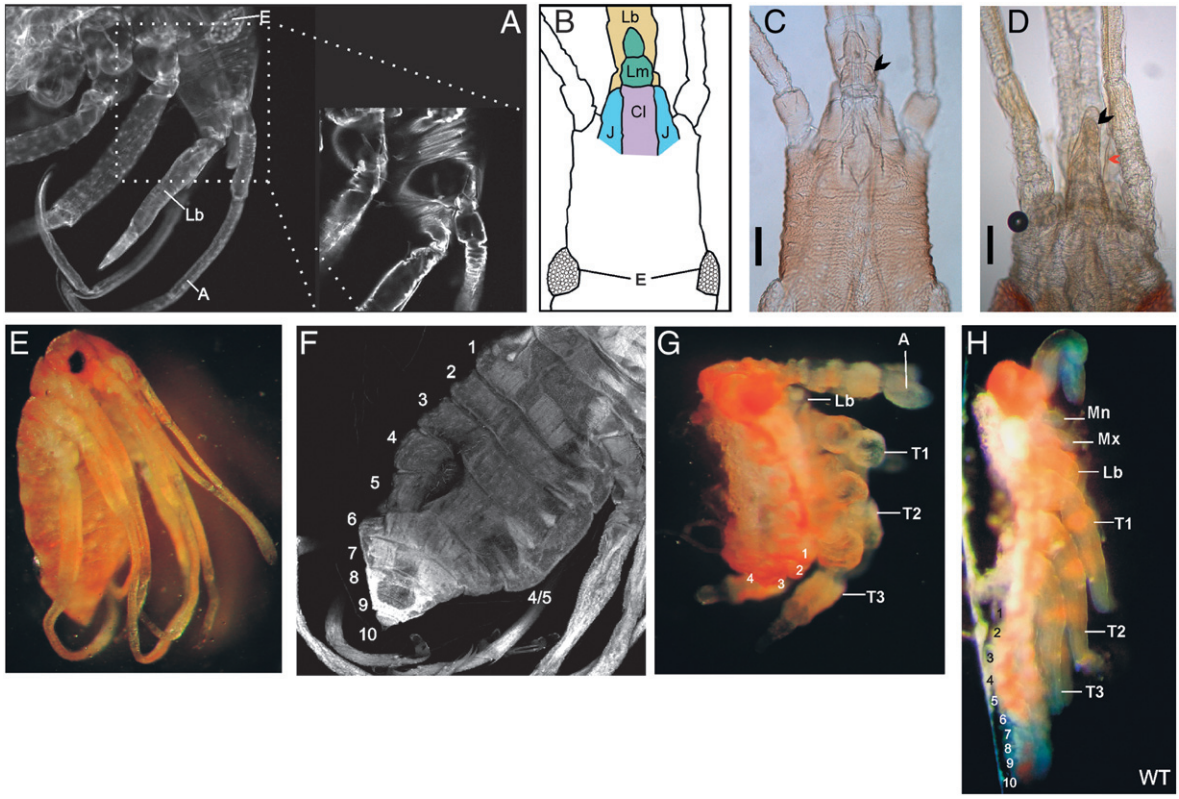

Fig. 6. RNAi phenotypes. A–D. Head phenotype. A. Confocal image of a first instar larva. Inset shows a magnified view of the proboscis to evidence the lack of stylets. B. Scheme of the wild-type head in dorsal view. Here the structures that form the pre-gnathal head are marked. C–D. Cuticle preparation of wild-type (C) and *Gt*-RNAi (D) heads showing the "clypeus–labrum" morphology. The black arrowhead points the "clypeus–labrum", which is smaller in the interfered animal compared to the wild-type. The red arrowhead marks a smaller mandible in *Rp-gt* interfered first instar larva. E–H. Abdominal phenotype. E. Lateral view of a hatchling showing abdominal segment fusions. F. Projection of 10 confocal images of the same embryo in E where the segments 4th and 5th are fused. G–H. Strong phenotypes. G. Image of 72 h AEL embryo in lateral view, where the labium is the only gnathal appendage and the abdominal segments is reduced to four. H. Lateral view of a wild-type embryo at the same developmental stage as that embryo in G. Abbreviations: A, antenna; E, eye; Cl, clypeus; Lb, labium; Lm, labrum; Mn, mandibles; Mx, Maxilae, J, jugum. Scale bar = 100 μm.

Conserved features of the gt genes

The gene *gt* is highly conserved in insects, but our results presented here suggest that *Rp-gt* is closer in function to the *Drosophila* gene rather than other known *gt* orthologues in insects. The similarities extend to the putative promoter region.

Rp-gt shows the characteristic domains present in other *gt* orthologues, namely the CtBP repression domain (Strunk et al., 2001) and the BLZ domain (Capovilla et al., 1992). In addition, *Rhodnius* and *Oncopeltus* protein sequences share a conserved domain, with a putative phosphorylation site. The similarity of sequence is reflected in the phylogenetic analysis, where *Oncopeltus* and *Rhodnius* form a monophyletic group, the order Hemiptera, and along to *Pediculus*, the Hemimetabola. The grouping of the species in the tree and the supporting branch value generate a robust phylogenetic tree that indicates that the evolution of the *gt* coding sequence followed the evolution of the insects.

The sequence relationship within insects seems to extend also to the regulatory sequences. *gt* expression regulation in the blastoderm has been studied in *Drosophila*, where activators and repressor transcription factors have been identified (Capovilla et al., 1992; Kraut and Levine, 1991). However, the enhancers involved, different to other gap and segmentation genes were not precisely defined until recently. Berman et al. (2002) have predicted and validated a cluster that regulates the posterior expression domain of *Dm-gt*. Using the same approach and parameters we define a putative regulatory cluster in the *Rp-gt* upstream sequence. The cluster is in quality and position similar to the one in *Drosophila*. We have also performed the same analysis for *Tc-gt* upstream sequence, and found a cluster with the same characteristics as in *Rhodnius* and *Drosophila*. Thus, it is likely that *gt* is not only similar at the protein sequence level, but also equivalent – sequence similarity does not count at this level – in non coding regions, at least for the putative abdominal domain.

The transcriptional regulatory elements of gaps genes have been studied in minute detail in *D. melanogaster*. However, information regarding to other insects is still scarce. The transcriptional regulatory elements of *hunchback* and *caudal* have been analyzed in *T. castaneum* and *D. melanogaster* and among different dipteran species (Shaw et al., 2002; Wolff et al., 1998). We present here, up to our knowledge, the first comparative analysis of *giant* genomic upstream region in different insect species. Our analysis, based on the *gt* abdominal domain element in *Drosophila* (Berman et al., 2002), suggests a putative conserved regulatory region in *Tribolium* and *Rhodnius*. The anterior expression control element is still unknown in insects, including *Drosophila*. Further experiments are currently under way to demonstrate the occupancy of the predicted binding sites *in vitro*.

Maternal expression of Rp-giant

In *D. melanogaster*, gap genes are zygotic and become activated after cycle 13 — with the exception of *hunchback* (*hb*). In insects such as *Nasonia* and *Tribolium*, some gap gene orthologues also show maternal expression where they regulate the initiation of the segmentation processes (Brent et al., 2007; Lynch et al., 2006; Pultz et al., 2005; Schroder, 2003). We have shown that *Rp-gt* is accumulated in the oocyte during oogenesis. Maternal expression and localization of *gt* mRNA have been shown in *A. melifera* and *Nasonia* (Olesnicky and Desplan, 2007; Wilson et al., 2010). In *Nasonia* the instructive role of *gt* in setting the anterior patterning by the

indirect effect on *hunchback* expression has been also demonstrated (Brent et al., 2007). In short and intermediate germ-band insects maternal *gt* expression has not been described, although Bucher and Klingler (2004) have inferred a maternal contribution for *gt* in *Tribolium*. *Rp-gt* mRNA is expressed in the tropharium and early oocytes as well as in freshly laid eggs. The structure, cytology and physiology of the telotrophic ovariole of *Rhodnius* have been studied in detail (Bjornsson and Huebner, 2004; Huebner, 1981; Huebner and Anderson, 1972a, 1972b). In the ovariole of *R. prolixus*, RNA and protein synthesis as well as its transfer to the oocyte is governed by the trophic cell in the tropharium and the follicular cells that surround the oocyte (Vanderberg, 1963). Nucleic acids and proteins are transported to the oocytes through the trophic cord. For each different stages of oocyte the charge of DNA, RNA and proteins is different, and it is proportional to the diameter of the trophic cord (Vanderberg, 1963). These data are consistent with the expression of *Rp-gt* transcript. In the ovarioles *Rp-gt* mRNA is restricted to the tropharium, to early oocytes and to oocytes in advanced vitellogenesis. *Rp-gt* mRNA is likely synthesized in the tropharium and loaded into the oocytes. We have detected *Rp-gt* mRNA as early as in T-3 oocytes (nomenclature according to Bjornsson and Huebner, 2004). We attribute the higher level of expression in T-3 oocytes to the tight packing of several oocytes. Oocytes in advanced vitellogenesis (AvO), but not early oocytes in the final stages of development (EaO), also show *Rp-gt* mRNA expression. We attribute this difference to the vectorial transfer of yolk and nucleic acids through the trophic cord directly to the oocyte in advanced vitellogenesis. In-situ hybridization on very early embryos shows that a few hours after egg laying, before the nuclei have migrated to the surface of the blastoderm, *Rp-gt* display a patched and disperse pattern. These patches of mRNA accumulate on the egg cortex in cytoplasmatic islands between the yolk granules. Twelve hours AEL, when the nuclei have migrated to the periphery, the localization of the transcript becomes perinuclear. The perinuclear localization of maternal *gt* has been also observed in *Nasonia*, although only during oogenesis (Olesnicky and Desplan, 2007). We hypothesize that the perinuclear mRNA localization in the cortex results from the redistribution of *Rp-gt* when the syncytial blastoderm is formed. However, we cannot establish if the co-localization of *Rp-gt* mRNA along with some of the nuclei is a cause or a consequence of the nuclear migration process. We conjecture that a stochastic process occurs causing some nuclei to fall into the patches and, in consequence, it might trigger different gene expression programs that will influence the embryonic patterning. We observed a similar effect on other early genes such as *Krüppel* (*Kr*), *caudal* (*cad*) and *decapentaplegic* (*dpp*) (unpublished data). Whether this is a general effect or consequence of loading of mRNAs in a large yolky egg remains to be established.

Zygotic Rp-gt expression and the giant gap phenotype

In *Drosophila gt* is expressed after cell cycle 13 in three domains: the anterior-most domain (91% to 97% egg length), the head domain (75% to 83%) and the abdominal domain (25% to 33% egg length; Mohler et al., 1989). They correlate to the phenotype: the clypeus–labrum – defects of the anterior-most domain – the cephalopharingeal skeleton and labium – defects of head domain – and the abdominal segments (A5–A7) — defects of the posterior expression domain. In the intermediate germ-band insects *Tribolium* and *Oncopeltus*, two embryonic *gt* expression domains have been described: one anterior – at the gnathal segments – and another posterior — at the growth zone. In *Tribolium* parental RNAi resulted in a classical abdominal gap phenotype and in homeotic transformations of maxillary and labial segments, not being required for head formation (Bucher and Klingler, 2004). In *Oncopeltus*, interfered embryos show the classical gap phenotype as described in *Drosophila*, in which both gnathal segments – maxillary and labial – and abdominal segments are deleted (Liu and Patel, 2010). In addition to this, some homeotics changes have been described – comb in T3 leg – as a consequence of ectopic expression of *Scr* in the third thoracic appendage. Our data indicates a similar gap phenotype; however, we have not observed homeotic changes in *Rp-gt* interfered embryos.

We have determined two embryonic expression domains in *R. prolixus*: the anterior and the posterior domain — abdominal segments and the growth zone. The anterior domain of expression is complex and two regions can be defined: an anterior-most and a middle domain, which resemble the expression in *Drosophila*. The results of parental RNAi experiments show the requirement of *Rp-gt* for the proper formation of the most anterior region of the head, maxillae, mandibles and the abdomen. Previous research has also described the effect of *gt* in the most anterior part of the embryo (Brent et al., 2007; Lynch et al., 2006) where *gt* contributes to the proper formation of the anterior half of the embryo. In *Rhodnius*, *Rp-gt* does not have this function but it is involved in the formation of the anterior part of the head capsule, mandibles and maxillae in a gap-like fashion. Therefore we have shown that *Rp-gt* is a gap gene that affects cephalic and abdominal segments and, as in *Drosophila*, it is required for the development of the "clypeus–labrum", the most distal part of the head capsule in hemipterans.

Conclusions

R. prolixus is an intermediate germ-band insect with a genome just sequenced and a wide potential for developmental studies. We show a novel maternal expression pattern and provide new clues on the function of *gt* in intermediate germ-band insects. We show a close relationship to *Drosophila* from the zygotic expression pattern, gap phenotype in head and abdomen and the conservation of an upstream putative regulatory sequence. In *Nasonia*, *Nv-gt* is also maternal and contributes to the anterior patterning, however, *Nv-gt* seems to act as an instructive factor rather than an anterior gap gene as in *Drosophila*. Being *Nasonia* and *Drosophila* long germ-band insects, a comprehensive study of the maternal contribution in intermediate and short germ insects and a closer analysis of head phenotypes in these and other insects will provide valuable information on *gt* gene function and its role in head formation as well as in the evolution of the patterning in insect embryos.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.ydbio.2011.06.038.

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