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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).