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Evolution of Developmental Control Mechanisms

Gene expression suggests conserved mechanisms patterning the heads of insects and myriapods

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ABSTRACT

Segmentation, i.e. the subdivision of the body into serially homologous units, is one of the hallmarks of the arthropods. Arthropod segmentation is best understood in the fly *Drosophila melanogaster*. But different from the situation in most arthropods in this species all segments are formed from the early blastoderm (so called long-germ developmental mode). In most other arthropods only the anterior segments are formed in a similar way (so called short-germ developmental mode). Posterior segments are added one at a time or in pairs of two from a posterior segment addition zone. The segmentation mechanisms are not universally conserved among arthropods and only little is known about the genetic patterning of the anterior segments. Here we present the expression patterns of the insect head patterning gene orthologs *hunchback* (*hb*), *orthodenticle* (*otd*), *buttonhead-like* (*btdl*), *collier* (*col*), *cap-n-collar* (*cnc*) and *crocodile* (*croc*), and the trunk gap gene *Krüppel* (*Kr*) in the myriapod *Glomeris marginata*. Conserved expression of these genes in insects and a myriapod suggests that the anterior segmentation system may be conserved in at least these two classes of arthropods. This finding implies that the anterior patterning mechanism already existed in the last common ancestor of insects and myriapods.

Introduction

Axial patterning is a fundamental process in early development and provides the basis for subsequent patterning. One example is the formation of the segments, the metameric units of the arthropod body. A paradigm for arthropod segmentation is segmentation in the fly *Drosophila melanogaster* (e.g. Damen, 2007; Pankratz and Jäckle, 1993; Peel et al., 2005). However, the mode of segmentation in *Drosophila*, where all segments become specified at the blastoderm stage, is derived. Most arthropods only specify a small number of anterior segments from the blastoderm, and add their remaining posterior segments sequentially from the posterior (Davis and Patel, 2002).

Nonetheless, the knowledge on the molecular mechanisms underlying patterning and segmentation during *Drosophila* embryogenesis provided an excellent framework for studying the evolution of these processes among arthropods (e.g. Damen, 2007; Peel et al., 2005). A key feature of *Drosophila* segmentation is the hierarchic trunk segmentation gene cascade that controls the progressive subdivision of the embryo (Akam, 1987; Pankratz and Jäckle, 1993; St. Johnston and Nüsslein-Volhard, 1992). But the underlying molecular mechanisms appear far from being universally conserved, especially with respect to the terminal addition of the posterior segments (Peel et al., 2005). The sequential generation of the posterior segments in several other arthropods seems to depend on mechanisms that are not used during segmentation of the *Drosophila* embryo, but on mechanisms that are also used by vertebrate embryos to sequentially generate their somites (McGregor et al., 2009). While the posterior segments are generated sequentially in most arthropods, the anterior segments are specified more or less simultaneously from the early blastoderm with only a minimal delay in time, which is at least superficially more similar to what is seen in *Drosophila*. However, also knowledge on the mechanisms underlying patterning of the anterior segments in non-insect arthropods and how these mechanisms evolved is scarce. Data from the emerging model organism *Tribolium castaneum* however imply that key components of anterior patterning may be conserved in at least insects.

In *Drosophila* and other cyclorrhaphan flies maternally provided gene products, such as the anterior determinant bicoid (Bcd), provide the initial instructions of anterior–posterior (AP) patterning (Fröhnhofer and Nüsslein-Volhard, 1986; McGregor, 2005; Lemke et al., 2008). In more basal holometabolous insects, like the beetle *Tribolium* and the wasp *Nasonia vitripennis*, *orthodenticle* (*otd*) and *hunchback* (*hb*), two of the *bcd* target genes in *Drosophila* (Finkelstein and Perrimon, 1990; Gao and Finkelstein, 1998), were proposed to substitute for *bcd* (Lynch et al., 2006; Schröder, 2003). While in *Tribolium* the function of *hb* was later

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suggested to be rather that of a homeotic/regulatory gene (Marques-Souza et al., 2008), the function of *otd* was suggested to be more generally involved in early anterior–posterior and dorsal–ventral patterning (Kotkamp et al., 2010).

The head patterning genes encode transcription factors that typically are expressed in broad domains along the AP-axis and are under direct control of the anterior patterning system in *Drosophila* (e.g. Lemke et al., 2008, 2010; Rivera-Pomar and Jäckle, 1996). One can distinguish the anterior so-called procephalic head segments (ocular/labral, antennal and intercalary (premandibulary in myriapods)) from the gnathal head segments (mandibular, maxillary and labial (postmaxillary in myriapods)). The latter are under control of the trunk segmentation gene cascade, and the former are not (reviewed in Posnien et al., 2010). The general expression patterns of the anterior patterning genes appear to be widely conserved in insects, although some of these genes are expressed variably (e.g. Birkan et al., in press; Cohen and Jürgens, 1990; Economou and Telford, 2009; Liu and Kaufman, 2004a; Mito et al., 2006; Rogers et al., 2002; Schetelig et al., 2008; Schinko et al., 2008). Their function and interaction was also suggested to be more variable than what the mere expression patterns implied (e.g. Ben-David and Chipman, 2010; García-Solache et al., 2010; Schinko et al., 2008; reviewed in Jaeger, 2011). Although most data on anterior patterning gene orthologs in arthropods come from insects, recent data from the spider *Achaearanea tepidariorum* demonstrate that also here *otd* and *hb* are involved in anterior patterning, and that at least *hb* acts as a gap gene (Pechmann et al., 2009; Schwager et al., 2009). These data suggest that at least the anterior patterning system in arthropods is conserved, contradicting the emerging idea that the role of gap genes in segmentation may be an innovation of insects and therefore non-existent in other arthropods (Chipman and Stollewerk, 2006; Kontarakis et al., 2005; Peel et al., 2005).

To get further insights into the evolution of anterior axial patterning, and to elucidate to what extent the anterior patterning system is conserved among arthropods, we studied the expression of anterior patterning genes, i.e. the head gap gene orthologs *orthodenticle (otd)*, *hunchback (hb)* and *buttonhead-like* (*btd-l*), and secondary anterior patterning genes, i.e. *collier* (*col*), *cap-n-collar* (*cnc*) and *crocodile* (*croc*) in the millipede *Glomeris marginata* (Myriapoda: Diplopoda). Conserved expression of these anterior patterning genes suggests that a conserved set of anterior factors is involved in anterior patterning in insects and myriapods.

Materials and methods

Glomeris marginata husbandry, embryo treatment, and in situ hybridization

The general handling of adults and embryos of *Glomeris* has been described in Janssen et al. (2004) and Prpic et al. (2005). Oocytes were obtained by dissecting the ovaria from mature females. In situ hybridization was essentially done as described by Janssen et al. (2004). The embryos were fixed in a mix of 1 ml 37% formaldehyde and heptane for four hours at room temperature. The embryos were not treated with protein-K or acetic anhydride, and were not post-fixed prior to hybridization. Cell nuclei were visualized via incubation of the embryos in 1 μg/ml of the fluorescent dye 4-6-Diamidin-2-phenylindol (DAPI) in phosphate buffered saline with 0.1% Tween-20 (PBST) for 30 min followed by extensive washes in PBST (overnight).

Gene cloning

RNA isolation and cDNA synthesis were done as described in Janssen et al. (2004). We used the following primers (all primers in 5′ to 3′ direction) in RT-PCR experiments to amplify gene fragments of 1) *orthodenticle otd-fw1* (AAR CAR MGN MGN GAR MGN ACN AC) and *otd-bw1* (TTN GCN CKN CKR TTY TTR AAC CA) in an initial PCR and the oligonucleotides *otd-fw2* (TTY ACN MGN GCN CAR YTN GAY GT) and *otd-bw2* (WSN CCN TAY GAR TGG ATN AA) in a nested PCR, 2) *hunchback hb-fw1* (AAR CAY CAY YTN GAR TAY CA) and *hb-bw1* (RTG RCA RTA YTT NGT NGC RTA) in an initial PCR and *hb-fw2* (AAY CAY TTY GGN WSN AAR CC) and *hb-bw1* in a nested PCR, 3) *buttonhead-like btdl-fw1* (TGY ACN TGY CCN AAY TG) and *btdl-bw* (TGN CKY TGN ARY TCR TC) in an initial and *btdl-fw2* (AAR CAR CAY ATH TGY CAY AT) and *btdl-bw* in a nested PCR, 4) *Krüppel Kr-fw1* (GGN TAY AAR CAY GTN YTN CA) and *Kr-bw* (GCY TTN ARY TGR TTN SWR TC) for the initial PCR and *Kr-fw2* (CAR AAY CAY GAR MGN ACN CA) and *Kr-bw* in a semi-nested PCR, 5) *cap-n-collar cnc-fw1* (THA THA AYY TNC CNR TNG ANG A) and *cnc-bw1* (TTN CKR CAR TTY TGN GCN GC) for the initial PCR and *cnc-fw2* (CCN RTN GAN GAR TTY AAY GA) and *cnc-bw2* (GCN GCN ACY TTR TTY TTN CC) for the nested PCR, 6) *crocodile croc-fw* (GTN AAR CCN CCN TAY WSN TAY AT) and *croc-bw1* (AAC ATR TTR TAN SWR TCN GGR TC) for an initial PCR and *croc-fw* and *croc-bw2* (GTN CAR TAN SWN CCY TTN CC) for a nested PCR. The annealing temperature was calculated with the 2 + 4 method; successful PCRs were run at the calculated minimum annealing temperature minus 2 °C. Isolation of a *collier* ortholog is described in Janssen et al. (2011a). We obtained larger fragments of the genes by RACE-PCR using the MARATHON RACE Kit (Clontech, Heidelberg, Germany). Fragments were cloned into a plasmid vector (pBlueScript KS) and sequences were determined from both strands on an ABI-3100 automated sequencer (Applied Biosystems, Foster City, CA, USA), using Big Dye dye-terminators (Version. 3.1 Applied Biosystems). Sequences were submitted to similarity analysis using BLAST (Altschul et al., 1997) and sequence alignments were made using Clustal X (Thompson et al., 1997). Gene sequences are available under accession numbers AM279691 (*Gm-otd*), AM279689 (*Gm-hb* TypeA), AM279690 (*Gm-hb* TypeB), AM279683 (*Gm-btdl*), AM279684 (*Gm-cnc*), AM279686 (*Gm-croc*) and FR716827 (*Gm-Kr*).

RT-PCR

RT-PCR was performed on timed embryos (zero to seven days). mRNA was isolated using the PolyATract mRNA Isolation System (Promega, Madison, WI, USA). The PCR reactions were carried out using the Titan One Tube RT-PCR System (Roche, Indianapolis, IN, USA); control reactions were performed as suggested by the manufacturers. The oligonucleotide primers amplified a *Gm-otd* fragment of 749 bp, and a *Gm-hb* fragment of 502 bp. In the same way we tested the presence of the two variants of the *Gm-hb* transcripts that differ in their 5′UTR using specific primers that result in fragments of 230 bp for variant I and 190 bp for variant II.

Documentation techniques

Embryos were analyzed as whole mounts under a Leica (Heerbrugg, Switzerland) dissection microscope and pictures were taken with an Axiocam camera (Zeiss, Jena, Germany). Brightness, contrast, and color values were adjusted in all images using the image processing software Adobe Photoshop CS2 (Version 9.0.1 for Apple Macintosh (Adobe Systems Inc. San Jose, CA, USA)).

Results

The millipede hunchback and orthodenticle genes

Using RT-PCR and RACE-PCR we recovered cDNAs of the millipede *hb* and *otd* genes. Two variants of *Gm-hb* were found. They only differ in their 5′UTR (Fig. S1A), which may be the result of alternative promoters as it is the case in the fly *Drosophila* and the beetle *Tribolium* (Schröder et al., 1988; Wolff et al., 1995). The deduced *Gm*-Hb protein contains nine zinc fingers (Fig. S1B), which is similar to other metazoans, but is different from insect Hb proteins that lack