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A *Bombyx* homolog of *ovo* is a segmentation gene that acts downstream of *Bm-wnt1*(*Bombyx wnt1* homolog)

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ABSTRACT

Insect embryogenesis is divided into long and short/intermediate germ types. The long germ type may exhibit *Drosophila*-like hierarchical segmentation mechanisms, whereas the short/intermediate type assumes some repeating mechanisms that are considered to be ancestral. Embryogenesis in *Bombyx mori* possesses both characteristics. Here, *Bombyx ovo* homolog (*Bm-ovo*) was identified as a gene involved in segmentation. *Ovo* is a *Drosophila* gene that encodes a zinc finger transcription factor and studies on its homolog functions in other systems have suggested that it acts as a switch to enable the initiation of differentiation from a progenitor cell state. This is the first description for *ovo* homologs being involved in insect segmentation. *Bm-ovo* is expressed dynamically during embryogenesis in a pattern that resembles that of gap and pair-rule genes. In *Bm-ovo* RNAi knockdown embryos, posterior segmentation does not proceed. In addition, defects in anterior segments are observed. In *Bm-wnt1* knockdown embryos, the *Bm-ovo* expression pattern was changed, suggesting that *Bm-wnt1* is an upstream regulator of *Bm-ovo*. The involvement of *Bm-ovo* may represent a novel ancestral step under the control of *wnt* genes in insect segmentation: this step may resemble those operating in cell differentiation processes.

1. Introduction

In insect embryos, maternal, gap, and pair-rule genes are successively expressed to produce segments along the anterior-posterior (AP) axis. Maternal genes dictate the AP axis. Gap genes subdivide embryos into broad domains according to their expression profiles. The expression of seven to eight stripes of pair-rule genes serves as a template for future segments. Insect embryogenesis has been largely categorized into long and intermediate/short germ types (Davis and Patel, 2002; Liu and Kaufman, 2005; Peel et al., 2005). In the long germ type, as represented by *Drosophila*, the combinatorial input of maternal and gap genes directs the expression of the individual stripes of pair-rule genes, which leads to the nearly simultaneous expression of pair-rule stripes, i.e., segment formation (Pankratz and Jäckle, 1993). In contrast, in the short/intermediate type, posterior segments are sequentially added, in which some repeating segmentation mechanisms are likely to operate. In the short germ-type insect *Tribolium*, the wave-like expression of pair-rule genes *even-skipped* (*eve*) and *odd-skipped* (*odd*) has been observed in the pre-segmental area (Sarrazin et al., 2012; El-Sherif et al., 2012). The pair-rule genes, *eve*, *odd*, and *runt* (*run*) interact positively and negatively to form a circuit that appears to be responsible for the striped expression of these genes. Thus, the wave-like expression of a subset of pair-rule genes may be passed onto the pair-rule gene circuit, leading to the production of segments (pair-rule gene stripes) in a clock-like manner (Choe et al., 2006; Lynch et al., 2012).

It is commonly acknowledged that the latter *Tribolium*-like segmentation mode is ancestral, while the former *Drosophila*-like hierarchical segmentation mode is derived. Recent evidence suggests that both modes may operate in some species (Rosenberg et al., 2014), such as *Bombyx mori*.

B. mori is a lepidopteran insect, the segmentation mechanisms of which have remained elusive. Its embryogenesis has long and short/intermediate-like features. The egg has a large embryonic primordium, within which individual segments are fate mapped without a growth zone early in embryogenesis similar to long germ insects (Myohara, 1994). However, when pair-rule gene interactions were examined, *B. mori* exhibited *Drosophila*- and *Tribolium*-like characteristics (Nakao, 2015). Furthermore, the roles of *Bombyx wnt1/wingless* and the *Krüppel* homolog resemble those in short/intermediate insects: embryos show truncated phenotypes in the posterior region when these gene (family) activities are perturbed (Beermann et al., 2011; Bolognesi et al., 2008, 2009; Miyawaki et al., 2004; Nakao, 2010, 2015; Yamaguchi et al., 2011; Cerny et al., 2005; Liu and Kaufman, 2004; Mito et al., 2006). Embryonic *Bm-wnt1* is considered to have two functions: its organizing

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function in posterior development, as reflected in the knockdown phenotype as described above, and its function as a segment polarity gene, similar to that in *Drosophila*. During normal embryogenesis, *Bm-wnt1* is expressed in the large posterior domain in the early stages, the expression domain then recedes posteriorly, and segmental stripes begin to appear in an anterior to posterior sequence (Nakao, 2010). Posterior expression appears to be responsible for posterior organization functions, while segmental expression appears to exhibit a segment polarity function that has not yet been experimentally validated in *Bombyx*. Furthermore, the addition of supernumerary posterior segments is induced at the posterior terminus in a manner that resembles that of short/intermediate insects by the knockdown of the *hunchback* homolog (*Bm-hb*) (Nakao, 2016). These findings strongly suggest that the short/intermediate type mode of segmentation is operating in normal *Bombyx* embryogenesis.

An *ovo* homolog from *B. mori* (*Bm-ovo*) was described herein. *Ovo* is a *Drosophila* gene that encodes an evolutionarily conserved zinc finger transcription factor with various biological functions. It is required in the female germ line for proper oogenesis: the mutants display egg chambers filled with excess undifferentiated germ cells, an ovarian tumor phenotype (Oliver et al., 1987). *Ovo* is also involved in early germ line development: the transcript is deposited in the germ plasm as a maternal factor, regulating the expression of *vasa* (Yatsu et al., 2008). In the zygote, often referred to as *shavenbaby* (*svb*) at this stage, it is necessary and sufficient to cell-autonomously direct the formation of denticles (trichome-cytoplasmic extrusion of epidermal cells) in the ventral epidermis of the embryo abdomen, in which there is a segmentally repeating denticle pattern separated by naked cuticle, thereby contributing to the binary choice to produce either a naked cuticle or denticles. The upstream regulation of *svb* has been investigated. The high mobility group (HMG)-domain protein SoxNeuro (SoxN) is necessary and sufficient to cell-autonomously direct the expression of *svb*. SoxN, in turn, receives positive and negative inputs from the epidermal growth factor receptor (Egfr) ligand Spitz (Spi) and Wingless (Wg), respectively. These mechanisms result in the restriction of trichome-producing cells. The closely related protein of Spi, Dichaete is co-regulated with Spi and has a partially redundant function in the activation of *svb*, albeit to a lesser extent. However, these regulatory relationships are not strictly hierarchical, but complex feedback mechanisms are involved (Overton et al., 2007).

Apart from *Drosophila*, *ovo* homologs are isolated from diverse animals. The mouse homolog also controls germline and epidermis differentiation. In *C. elegans*, the *ovo* homolog *lin-48* is required for the development of hindgut. These findings point to the role of *ovo* homologs in the differentiation and maintenance of specific cell types (Wieschaus et al., 1984; Oliver et al., 1987; Dai et al., 1998; Johnson et al., 2001). More recently, human homolog of *ovo* (*OVOL1*) was shown to regulate the transition of progenitor to differentiated trophoblast cells (Renaud et al., 2015).

In the present study, the embryonic functions of *Bm-ovo* were examined. *Bm-ovo* expression during the embryonic stage resembled that of gap and pair-rule genes. Embryonic *Bm-ovo* RNAi embryos showed segmentation defects in the gnathal/thoracic region and posterior abdomen. In the posterior body part, the abnormal expression of some pair-rule genes that are crucial for establishing pair-rule gene expression patterns was observed: the posterior part did not produce pair-rule stripes, but a broad band of expression was observed in the affected region for the pair-rule genes examined. In *Bm-wnt1* knockdown embryos, alternation in *Bm-ovo* expression pattern was observed, suggesting that *Bm-wnt1* is an upstream regulator of *Bm-ovo*. This interaction may be related to those operating in *Drosophila* embryonic epidermal patterning as described above.

The involvement of *Bm-ovo* may represent a novel ancestral step under the control of *wnt* genes in insect segmentation, and this step may resemble those operating in cell differentiation processes.

2. Materials and methods

2.1. Silkworm strains, rearing, and development

The *B. mori* strain *pnd-2* was used in this study. Silkworms were reared on an artificial diet (Nippon Nosanko). Refer to Nagy et al. (1994) for a general description of early *Bombyx* development.

2.2. Identification and isolation of Bm-ovo cDNA

The *Drosophila ovo* sequence was used to search the *Bombyx* cDNA database (Mita et al., 2003) for *Bombyx ovo* homologs and one wing-disk-derived cDNA was identified. cDNA was obtained and initial analyses were performed using this cDNA. The annotated genome database (KAIKObase; Mita et al., 2004) subsequently became available, which revealed that the open reading frame of *Bm-ovo* comprises four exons (E1, E2, E3 and E4; gene ID: BMgn000987). The cDNA clone described above was found to lack the second exon. To obtain cDNA species expressed during early embryogenesis, a PCR amplification procedure was employed. Total RNAs in embryos 14 and 20 h AEL were prepared using TRIZOL reagent (Invitrogen). Single-strand cDNAs were synthesized using total RNAs as templates by PrimeScript™II 1st strand cDNA Synthesis Kit (TAKARA) according to the manufacturer's instructions. With these cDNAs, PCR amplification was performed using PrimeSTAR GXL DNA Polymerase (TAKARA). A primer pair was set at the sequence near the start and stop codons, respectively, so as to amplify the full open reading frame. PCR conditions were 40 cycles at 98 °C for 10 s; at 55 °C for 15 s; at 68 °C for 3 min. The primer pair was the *Bm-ovoCp*, 5′-primer and *Bm-ovoE3-4p*, 3′-primer (see below). The amplified product was analyzed using 1% agarose gel electrophoresis. The results obtained revealed the predominant amplification of an approximately 2.6-kbp product from both templates (Supplementary Fig. 1). The amplified product was cloned and sequenced. The sequence analysis revealed that it had all four exons. Subsequent analyses were performed using this cDNA.

2.3. In situ hybridization

Fixation and *in situ* hybridization were performed as previously described (Nakao, 1999, 2010). Probes for *Bm-eve* and *Bm-wnt1* were previously described (Nakao, 2010). Probes for *Bm-ovo*, designated as *Bm-ovoCp*, *Bm-ovoE1p*, *Bm-ovoE2p* and *Bm-ovoE3-4p*, were synthesized using cloned cDNAs. cDNAs were PCR fragments amplified using the primers described below. The primer pairs used for the amplification of these fragments were as follows: *Bm-ovoCp*, 5′-primer: 5′- GGGGGATCCAGTCCTAACGAAGCGGCCAA-3′, 3′-primer: 5′- CCCAAGCTTTTTATACGGTCTGACTCCGG-3’; *Bm-ovoE1p*, 5′-primer: 5′- GGGGGATCCAGTCCTAACGAAGCGGCCAA -3′, 3′-primer 5′- CCCAAGCTTCAGTGCA TTCCTTTTCTTTATCC -3’; *Bm-ovoE2p*, 5′-primer: 5′- GGGGGATCCCGCACAAAAGAAACTAGACG -3′, 3′-primer 5′- CCCAAGCTTAGCACTAAAACAGGTCGTGC -3’; *Bm-ovoE3-4p*, 5′-primer: 5′- GGGGGATCCTAGGACTACCAGCAGAGCTT -3′, 3′-primer 5′- CCCAAGCTTAATTGTGT ACTGGCATGGGC -3’. The *Bm-ovoCp* primer pair was used for amplification from the cloned cDNA template (fufe-P20_F_P18), and the amplified product comprised approximately 0.3-kb exon 1 and 0.3-kb exon 3 (see the Results section). *Bm-ovoE1p*, *E2p* and *E3-4p* primer pairs were used for amplification from the cDNA prepared using PrimeScript™ II 1st strand cDNA Synthesis Kit (TAKARA) and total RNA at 20 h AEL. The amplified products were approximately 0.3, 0.5, and 0.6 kbp, which corresponded to parts of exons 1, 2, and 3–4, respectively. After amplification, cDNAs were cloned into pBluescript vectors. In order to obtain probes, plasmid DNA was cut with an appropriate enzyme and RNA probes were synthesized using either T3 or T7 polymerase, depending on the direction of the insert using DIG RNA Labeling Kit (SP6/T7) (Roche).