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Hunchback knockdown induces supernumerary segment formation in Bombyx

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ABSTRACT

Insect segment number within species appears to be fixed irrespective of germ types: long vs. short/intermediate. The present study showed induction of supernumerary segment formation by the knockdown of *Bombyx hunchback* (*Bm-hb*), presumably by terminal segment addition, a short/intermediate-like-segmentation mode that is not observed in normal *Bombyx* embryogenesis. This suggests that *Bm-hb* suppresses segmentation. The results obtained also suggest that the gap gene *Bm-Kr* (*Bombyx Krüppel*) provides a permissive environment for the progression of segmentation by suppressing the expression *Bm-hb*, which terminates segmentation. This indicates a novel mechanism by which the gap gene is involved in segmentation. It appears that *Bm-Kr* and *Bm-hb* are involved in segment counting and their interplay contributes to the correct number of segments being formed in *Bombyx*. Similar mechanisms may be operating in insects that employ the non-*Drosophilan* mode of segmentation such as in short/intermediate-germ insects.

1. Introduction

Insect embryogenesis has been classified into long- and short/intermediate-germ types (Davis and Patel, 2002; Liu and Kaufman, 2005; Peel et al., 2005). An example of the segmentation mechanisms occurring in the long-germ type is well-known from *Drosophila* studies and involves hierarchical gene interactions that occur in the syncytium (Nüsslein-Volhard and Wieschaus, 1980; Akam, 1987; Ingham, 1988; Pankratz and Jäckle, 1993). In brief, maternal genes provide positional information along the anterior–posterior axis of embryos, which is sensed by gap genes, resulting in subdivisions into several gap gene expression domains. The combinatorial code of these maternal and gap genes is utilized to produce seven stripes of pair-rule gene expression, which forms the basis of segment formation. Thus, there are no repeating mechanisms for the generation of the 'repeating' segmental structure. This mechanism is referred to as the long-germ mode in the present study. On the other hand, posterior segments in the short/intermediate-germ type are added sequentially from the growth zone, which is situated at the posterior terminus of the embryo, in a cellular environment (short-germ mode), whereas anterior segments may be generated by the long-germ mode. The mechanisms underlying the short-germ mode remain elusive. However, some repeating mechanisms, i.e. a segmentation clock were previously suggested to be involved (Choe et al., 2006; El-Sherif et al., 2012; Sarrazin et al., 2012).

The body segment number appears to be invariant within insect species, although slight variations in the abdominal segment number have been observed between species. In long-germ insects, the mechanisms responsible for segmentation suggest the generation of a fixed number of segments, in which gap genes contribute to this process. Although gap gene mutants produce individuals with a reduced number of segments, supernumerary segment formation by the mechanism described above is not expected. In spite of extensive mutagenic screens, the supernumerary phenotype has not been identified in *Drosophila*. In contrast, the supernumerary phenotype may occur in short-germ insects. A segmentation clock that continues to function without perturbation may produce this phenotype. The pair-rule gene circuit model proposed in *Tribolium* (an insect that possesses short-germ mode) appears to represent such a clock-like mechanism; the pair-rule gene circuit is assumed to operate by itself and does not require other inputs such as provided by gap genes for its progression (Choe et al., 2006; El-Sherif et al., 2012; Lynch et al., 2012; Sarrazin et al., 2012). However, gap mutants have been reported in *Tribolium* (Maderspacher et al., 1998). Their relationship to a putative segmentation clock remains to be established. The existence of such mutants suggests that gap gene homologs are also involved in segmentation or in segment counting in short/intermediate-germ insects.

The expression patterns of some of the gap genes, such as *hb* or *Kr* are well-conserved across insects irrespective of the germ types

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(Pankratz and Jäckle, 1993; Wolff et al., 1995; Patel et al., 2001; Liu and Kaufman, 2004a, 2004b; Cerny et al., 2005; Mito et al., 2005, 2006; Pultz et al., 2005; He et al., 2006; Wilson et al., 2009; Huang et al., 2010; Wilson and Dearden, 2011; Nakao, 2012). Thus, a gap system may be involved in the generation of insect specific characteristics. The formation of fixed number of segments is a good candidate. The *Tribolium* homologs of *hb* (*Tc-hb*), *giant* (*Tc-gt*) and *Kr* (*Tc-Kr*) have been suggested to function in the initiation, maintenance and termination of the segmentation clock (Bucher and Klingler, 2004; Cerny et al., 2005). Therefore, these genes may be involved in segment number determination through such functions.

The lepidopteran insect *Bombyx mori* possesses long- and short-germ characteristics. Its embryonic primordium occupies a large surface area of the egg, a classical criterion of long germ insects, with all segments being fate mapped without a growth zone within the primordium, another characteristic of long germ insects (Nagy et al., 1994; Myohara, 1994). However, a *Bombyx* homolog of *wnt1*/*wingless* is expressed from the early embryonic stage and its knockdown embryo exhibits a large posterior truncation (Nakao, 2010; Yamaguchi et al., 2011). reminiscent of the case in *wnt* family gene knockdown in short germ insects; *wnt* family genes have been implicated in growth zone functions in short germ insects (Miyawaki et al., 2004; Bolognesi et al., 2008, 2009; Beermann et al., 2011). Additionally, each knockdown embryo of the pair-rule genes, *Bombyx even skipped* (*Bm-eve*), *runt* (*Bm-run*), and *odd-skipped* (*Bm-odd*), show asegmental phenotypes (Nakao, 2015) similar to knockdown embryos of *Tribolium* counterparts, although the interactions of these genes appear to differ between these species (Choe et al., 2006). Knockdown embryos of *Bombyx Kr* (*Bm-Kr*) also exhibit a large posterior deletion similar to short-germ insects (Liu and Kaufman, 2004b; Cerny et al., 2005; Mito et al., 2006; Nakao, 2015), instead of the gap in the middle region as observed in long-germ insects. Interestingly, some forms of hierarchical gene interactions appear to exist between these pair-rule genes and *Bm-Kr*.

In the present study, supernumerary segment formation that occurs in *Bombyx hunchback* (*Bm-hb*) knockdown embryos, a hitherto unknown phenotype in insects, was introduced. Supernumerary segment formation appears to occur through short-germ-like terminal segment addition, a mode of segmentation that is not observed in normal *Bombyx* development. The results obtained suggest that *Bm-hb* is involved in the termination of segmentation as well as segment counting. This *Bm-hb* function has also been suggested by comparing the phenotypes of *Bm-Kr* single and *Bm-hb*/*Bm-Kr* double knockdown embryos: the knockdown of *Bm-Kr* induced the premature termination of segmentation and *Bm-hb* overexpression, whereas the simultaneous knockdown of *Bm-hb* largely rescued segmentation defects. These results suggested that, in normal *Bombyx* development, *Bm-Kr* provides a permissive environment for the progression of posterior segmentation by suppressing or spatially delaying the expression of *Bm-hb*. This is a novel mechanisms by which the gap gene is involved in segmentation and highlights the importance of the interaction between the gap gene and *hb* homolog in fixed number segment formation or segment counting in *Bombyx*. Similar mechanisms may be operating in insects that employ the non-*Drosophilan* mode of segmentation such as short/intermediate-germ insects.

2. Materials and methods

2.1. Silkworm strains, rearing and development

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

2.2. In situ hybridization

Fixation and *in situ* hybridization were performed as previously described (Nakao et al., 2008; Nakao, 2010). In order to obtain probes for *Bm-hb*, about 0.6kbp HindIII fragment encompassing 313-906 base positions of the full-length cDNA clone (e10004F06; DDBJ/GenBank/EMBL accession number AK385224) were first cloned into the HindIII site of pBluescript. For *Bm-Kr* and the HOX genes, corresponding PCR fragments were first cloned into pBluescript vectors. Primer pairs used for the amplification of these PCR fragments were summarized in Supplementary Table 1. 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. The DIG RNA labeling kit (Roche) was used for the synthesis. The procedures for *Bm-en*, *Bm-eve*, *Bm-run* and *Bm-odd* were previously described (Nakao, 2010, 2015).

2.3. RNAi

The RNAi procedure used was described previously (Nakao, 2012). 3 μg/μl or 6 μg/μl dsRNA was injected for single RNAi experiments. The results were essentially the same. A total of 6 μg/μl dsRNA consisting of 3 μg/μl of each RNA species was injected for double RNAi experiments. The templates used for the *in vitro* transcription of double-stranded RNAs were PCR fragments of the corresponding genes, flanked by T7 promoter sequences. The primers used for the amplification of *Bm-Kr* fragments were the same as those described previously (Nakao, 2015). The following primers were used for *Bm-hb* amplification: *Bm-hb* RNAi1F, 5′-taatacgactcactatagggagacgagttcatccgtgagatgt-3′, *Bm-hb* RNAi1R, 5′-taatacgactcactatagggagagttctgggcgagtaagactc-3′; *Bm-hb* RNAi 2F, 5′-taatacgactcactatagggagatcaccacaaccatcactgct-3′, *Bm-hb* RNAi 2R, 5′-taatacgactcactatagggagagatgaagaaagccactcggt-3′. Two independent double-stranded RNAs were used in analyses of each gene. The results obtained were essentially the same.

3. Results

3.1. Bm-hb expression follows a conserved expression pattern among insects

In addition to the broad anterior expression domain observed from earlier stages (Nakao, 2012), a posterior band of expression of *Bm-hb* was observed at the beginning of germ band elongation (see Supplementary Figs. 1A and Fig. 4B). As the elongation progressed, the posterior band split into two: one situated at approximately the 7th abdominal segment and the other at the posterior end (Fig. 1A and B). Segmentally reiterated blocks of expression subsequently became evident (Fig. 1B), as observed in other insects. The expression pattern was later replaced by neural expression in two rows of cells along the midline (Fig. 1C). These results demonstrated that the *Bm-hb* expression largely follows a conserved expression pattern among insects (Wolff et al., 1995; Patel et al., 2001; Liu and Kaufman, 2004a; Mito et al., 2005; Pultz et al., 2005; He et al., 2006; Huang et al., 2010; Wilson and Dearden, 2011).

3.2. Bm-hb RNAi knockdown phenotypes

Embryonic RNAi was used to examine the functions of *Bm-hb*. The effectiveness of the RNAi is shown in Supplementary Fig. 1. Defects in RNAi-treated embryos were identified anteriorly and

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