Temporal and Spatial Expression Pattern of Segment Polarity Genes reveals an Intermediate Germ Segmentation in Lepidpteran *Bombyx mori*

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Abstract

To investigate how general the mechanisms of spatial patterning in early insect embryos, we have analyzed the expression patterns of the orthologues of *Wnt-1 (Bm Wnt-1)*, *engrailed (Bm en)* and *invected (Bm in)* during the segmentation in the silkworm *Bombyx mori* embryo which morphologically shows an intermediate germ type development. The *Bm Wnt-1* mRNA expression terminates just prior to the initiation of the segmentation process, the stripes of Bm Wnt-1 protein are progressively expressed in an anteroposterior order in the developing embryo, and the striped pattern expression of Bm Wnt-1 protein is preceded by that of the *Bm en* RNA. In conclusion, haed and thorax segments of *Bombyx* embryo have been already determined before morphological segmentation, and abdominal segments progressively durig morphological segmentation. These observations suggest that the difference of morphological segment patterning between short, intermediate and long germ band type insects depends on the difference of the expression pattern of the difference of the expression pattern of the segment polarity genes, especially *Wnt-1* expression during the segmentation.

Key Words : segment polarity gene, intermediate germ segmentation

Introduction

Animal bodies, especially insect bodies, are composed of a series of repeated units, and each of these is uniquely specified. The process of segmentation has been exclusively studied in *Drosophila* using a combination of genetic and molecular approaches[1), 2)]. How are segmental fates established in other types of insects? The most striking variation in insect embryogenesis is seen in the relative size of the initial anlage to the size of the egg[3)]. Insects with a very small anlage have been termed short germ insects, those with an anlage that occupies almost a half length of the egg are termed intermediate germ, and insects with an anlage that extends over the entire length of the egg are termed long germ. The process of early specification of segments takes place very rapidly in the *Drosophila* embryo, although not morphologically visible until late in gastrulation, and morphological classification places *Drosophila* into long-germ band insect[3)]. During *Drosophila* embryogenesis position within each segment along the anteroposterior axis is specified by the products if the segment-polarity genes which include *Drosophila Wnt-1(wingless)* and *engrailed*[4)]. These genes are assumed to control the anteroposterior patterning process as cellularization of the embryo is completed and gastrulation commences. Cross-regulatory interactions among the segment polarity gene products ultimately determined their patterns of expression in the embryo, and their expression in restricted domains provides instruction to specify and pattern the cuticular structures along the anteroposterior axis of the first instar larval cuticle[4)]. On the other hand it has been argued that earlier stages must differ dramatically between a long germ band embryo like *Drosophila* and a short germ embryo like *Schistocerca*, because short-germ embryo occur in a cellular epithelium, and therefore the diffusion gradients of large transcription factors like gap proteins could not form[5)]. Short germ band insect embryos show an early determination of only the anterior head segments whereas the more posterior thoracic and abdominal segments are sequentially added after formation of a primary germ anlage[3)]. This difference is discernible at the molecular level in the pattern of initiation of the segment polarity gene *engrailed*[6)].

Early embryogenesis of Lepidoptera is different from that of the other insects, and it has been difficult to categorize as short, intermediate, or long germ insects[7)]. Homologues to same of the segmentation and homeotic genes have already been isolated from both *Bombyx mori*[10), 11)] and

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Manduca sexta[8), 9)]. In *Manduca sexta*, the blastderm expression patterns of segmentation genes were found to show a molecular prepatterning typical of *Drosophila*[9)].

Experimental procedures

Cloning of *Bm Wnt-1*

A fragment of *Wnt-1* from *Bombyx* was amplified by PCR using the following primers GGGGAA TTCCA(AG)GA(AG)TG(TC)AA(AG)TG(TC)CA T and AAAATCTAGA(AG)CA(AG)CAC CA(AG) T(TG)(AG)AA (corresponding to positions 691-705 and 1129-1143 in the *Bombyx* sequence) and cloned into pBluescriptII KS(-) vector. The fragment was then used to screen a cDNA library of *Bombyx* embryo and cDNA which encodes whole open reading frame was obtained. cDNA of *engrailed* from *Bombyx* embryo was obtained by screening cDNA library from embryo with probe specific for *Bm en*[11)].

Expression of *Bm Wnt-1* and *Bm en* during the development of *Bombyx* embryo

The silkworm *Bombyx mori* was obtained from a laboratory breeding colony of a commercial hybrid, kin-Shu x Sho-wa, purchased from Kanebo Silk Co. (Kasugai City, Aichi-ken, Japan). Eggs were collected and grown at 25℃. For northern blot, total RNA was isolated from whole eggs by the acid guanidinium thiocyanate/phenol/chloroform method, and oligo(dT)-selected RNA was size-fractionated on a 1.1% agarose-formaldehyde gel and transferred to a nylon filter by a standard method. Blots were hybridized with a probe specific for *Bm en* at 65℃ in 5x SSC, 5x Denhardt's solution, 0.2% SDS, 50 mM phosphate buffer, pH6.5, and 100 μg/ml denatured salmon testes DNA. Following hybridization, filters were rinsed three times in 0.25x SSC/0.1% SDS for 30 min each at 54℃. For western blot, eggs were homogenized in SDS-sample buffer (2% (w/v) SDS, 4% (v/v) 2-mercaptoethanol, 30% (v/v) glycerol, 0.04% (w/v) Bromphenol blue). Western transfer and visualization of antigen were performed asa described before. To produce antibodies against Bm en, 15 mer peptide (AAELGLAEAQIKIWF) corresponding to residue no. 314-328 were synthesized[11)], conjugated with BSA and immunized to rats. To produce antibodies against Bm Wnt-1, cDNA fragment (aa. 125-191) was subcloned into pGEX expression vector (Pharmacia) to generate a glutathione S-transferase fusion protein. The fusion protein was injected into rats at two-week intervals. After four injections, the resulting antiserum was purified on a column of Protein G-sepharose. Embryonic protein samples from *Bombyx mori* at all stages were extracted by homogenization in SDS-PAGE sample buffer (2%(w/v) SDS, 4% (v/v) 2-mercaptoethanol, 30% (v/v) glycerol, 0.04% (w/v) Bromphenol blue). Samples were boiled for 5 min then stored at -20℃ until use for SDS-PAGE and Westernblot.

For antibody staining and *in situ* hybridization, eggs were collected, dechorionated in 10% Antiformin and rinsed twice in H_2O. After the rinsing, the eggs were fixed, dechorionated in ethanol series and embedded in paraplast. For in situ hybridization of BM en, riboprobes were prepared using the digoxigenin-labeling kit from Boehringer Mannheim according to the manufacturer's instructions. Hybridization and visualization for hybrid were performed as described before. For immunohistochemical study of Bm Wnt-1, eggs were embedded in paraplast, sectioned at 6 μm, and attached to gelatin-coated microscope slides. Avidin-biotin-peroxidase (ABC) complex staining was performed by the method of Hsu *et al*. (1981) with Vectastain ABC kit (Vector Labratories, Inc., Burlingame).

Results

To investigate how general the segmentation process in these insects, we have cloned the orthologue of *Wnt-1 (Bm Wnt-1)*, *engrailed (Bm en)* and *invected (Bm in)* from Bombyx embryo. In *Drosophila*, *wingless* will specify naked cuticle and *engrailed* the first denticle row in thoracic and abdominal segments of first instar larvae[4)]. *Wnt-1* encodes a cysteine-rich secreted glycoprotein, allowing the design of PCR primers within conserved portion of cysteine-rich domains[12)]. An orthologous *wingless* fragment was obtained in this way and used to clone the cDNA which encodes the whole open reading frame. Fig. 1 shows a comparison of the proteins encoded by the *Drosophila wingless* gene, mouse *Wnt-1* gene and the putative *Bombyx* orthologue. The fact that a cysteine residue conserved in *Drosophila wingless* and mouse *Wnt-1* is also present in the *Bombyx* sequence indicates that this *Bombyx* gene belongs to *Wnt-1* class of *Wnt* family genes[13)]. The *engrailed* gene encodes a homeo domain-containing nuclear protein which probably acts as a transcription factor to regulate the expression of other genes[14),15)]. *Bobmyx* orthologues of *engrailed* and *invected* have been cloned previously from the larval silk gland[11)]. In the present study, we have cloned *Bm en* cDNA from a cDNA library from *Bombyx* embryos. Two

distinct classes of cDNAs were obtained. We have determined the sequences of several cDNA clones, and confirmed that each class of cDNAs has the same sequence in their protein coding region and the difference from *Bm en* of the silk gland[11] is only the length of 3' untranslated region (data not shown). We also found that the sequence of embryonic *Bm in* was identical with that of the *Bm in* from the silkgland.

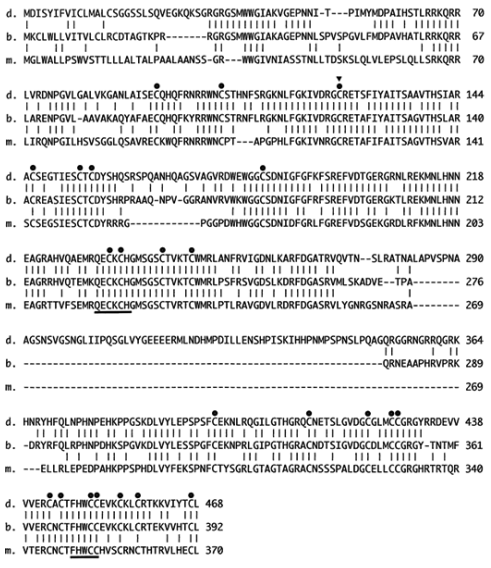

Fig. 1 Protein sequence comparison (single letter amino-acid code) between the *Wnt-1* orthologues from *Drosophila*, *Bombyx* and *mouse*.

Bars indicate complete conservation of amino acid in the *Wnt-1* genes. Closed circle indicates cysteine residue presents specifically in *Drosophila wingless*, *Bm Wnt-1* and *mouse Wnt-1*.

Bombyx embryogenesis starts with the formation of a syncytial blastderm followed by a cellular blastderm, similar to *Drosophila*. While the *Drosophila* embryos proceed directly to the formation of segments, the *Bombyx* embryos form first the germband and serosa. A stage 6 (36 hr) embryo reveals a non-segmented morphology at the presumed head, thorax regions and growth zone which makes abdominal region later. By stage 16 (48 hr) the embryo is very much elongated by growth and segmented from head to tail (see Fig. 2(1)). Based on these observations and the morphological appearance we concluded that the *Bombyx* embryo is clearly different from the long germ band type and belongs to either the intermediate or short germ band type. As shown in Fig. 2(2)(a), a single band of 3.7 kb transcripts of *Bm Wnt-1* was detected just after the fertilization. This is not due to maternal transcripts in ovary and unfertilized eggs. Bm Wnt-1 transcripts were most strongly expressed during the cellular blastderm stage and before the beginning of the segmentation process. The signals decreased precipitously after the beginning of segmentation and were not detectable during the segmentation process. After the segmentation, *Bm Wnt-1* transcripts were expressed again during the blastokinesis stage and not detectable during the rest of embryonic stages. Transcripts of *Bm en* gene were also detected shortly after the fertilization. As shown in Fig. 2(2)(b), during embryogenesis three kind of *Bm en* transcripts of 5.1, 2.8 and 2.6 kb were detected. Two kinds of transcripts of about 2.6 and 2.8 kb were expressed at almost constant level throughout early embryogenesis and larval tissue development. Transcripts of 5.1 kb were expressed during segmentation and larval tissue development, but not in the early and late embryos. In our previous study we examined the expression of *Bm en* gene in the silk gland and detected only the *Bm en* transcripts of 5.1 kb[11]. On the other hand, transcripts of *Bm in* gene were not detected until the beginning of the segmentation process. Only the transcripts of 6.2 kb were expressed constantly during the segmentation process and most strongly at the blastokinesisi stage. The signals of *Bm in* transcripts were not detectable during the rest of embryonic stages (Fig. 2(2)(c)). Both in *Drosophila* and *Bombyx*, the expression of *invected* gene takes place later than that of *engrailed* during the early embryogenesis[16].

In order to investigate their protein accumulation, we have made specific antibodies against Bm Wnt-1 and Bn en. Western blot analysis revealed that Bm Wnt-1 and Bm en proteins are expressed just after the fertilization (Fig. 2(3)). The Bm Wnt-1 protein accumulated gradually, reached the maximum level at 36 hr after fertilization just before the beginning of the segmentation process, and maintained the same level during the segmentation process. On the other hand the Bm en protein maintained almost constant level throughout the early embryogenesis. From these results, it appears that *Bm Wnt-1* transcription finishes before the beginning of the morphological segmentation and its protein maintained during the rest of segmentation process.

The expression patterns of *Bm Wnt-1* and *Bm en* were analyzed during the segmentation process by in situ hybridization and antibody staining. In *Drosophila*, *wg* and *en* become dependent upon each other for their continued and stable expression,

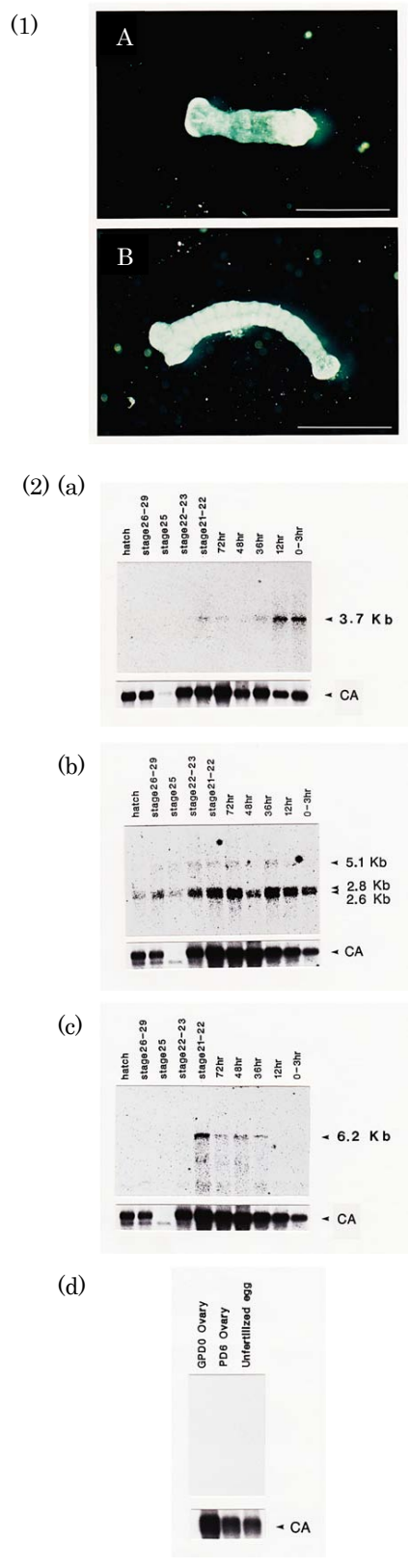

Figure 2. Expression of *Bm Wnt-1* and *Bm en* during the development of *Bombyx* embryo. (1) Early development of *Bombyx* embryos. (A), early germ anlage just before the beginning of the segmentation (stage 6, 36 hr). (B), embryo after the completion of segmentation process (stage 16, 48 hr). (2) Northern blot analysis of the expression of *Bm Wnt-1* (a), *Bm en* (b) and *Bm in* (c) during embryogenesis. Northern blot analysis

of *Bm Wnt-1* expression during oogenesis and unfertilized eggs (d). (3) Western blot analysis of Bm Wnt-1 (a) and Bm en (b). Embryonic stages were determined according to the stage given by Toyama (1909). 0-3 hr (stage 1-2), fertilization and debision of yolk nuclei; 12 hr (stage 3), cellular blastderm; 24 hr (stage 4-4), early germ anlage; 36 hr (stage 6), beginning of the segmentation process; 48 hr (stage 16), end of the segmentation process; 72 hr (stage 17-18), maximum elongation of the germ band; stage 21-22, blastkinesis and dorsal closure; stage 22-23, bristle formation; stage 25, completion of larval tissue development; stage 26-29, pigmentation; stage 30, hatch. Note the amount of actin mRNA in lane stage 25 is seen much reduced in comparison with those in other lanes probably by the failure of applying poly $(A)^+$ RNA in this lane. (4) *In situ* hybridization for *Bm en*. (a) and (b), early germ anlage (stage 4-5), earliest *Bm en* striped expression in the thoracic region. (c) and (d), the germ anlage at the beginning of the segmental growth process (stage 6). (e), the germ anlage at the end of the segmental growth process (stage 16). (f), just after the end of segmentation. (5) Antibody staining for *Bm Wnt-1*. (a) and (b), early germ anlage (stage 4-5). Striped pattern expression of *Bm Wnt-1* proteins detected only in the ectodermal cells. (c), the germ anlage at the beginning of the segmental growth process (stage 6). (d), the germ anlage at the end of the segmental growth process (stage 16). The embryos are oriented with the anterior to the left. Each scale bar represents 100 μ m.

and both the *wg* and *en* signaling pathways involve several other segment polarity genes. In *Bombyx*, *Bm en* expression was first observed at early gern anlage stage and the final *Bm en* stripe appears at the end of the segmentation process (Fig. 2(4)). The first *Bm en* stripe appears shortly after the formation of early germ anlage in a region that presumably corresponds to the second head segment, and other two stripes of the head and thoracic segment appear almost simultaneously. At the beginning of the segmentation process, stripes appear in the thorax segments and subesophagal segments. Upon careful examination of the transition from the three stripes at 24 hr to the eight stripes at 36 hr, it became clear that the second and fourth *Bm en* stripes at 36 hr occurs between the three stripes of 24 hr embryos. It suggests that the control of segmentation in subesophagal and thorax segments is the same as that of *Drosophila*. Starting at stage 4 of development (24 hr), the abdominal anlagen undergoes a major phase of growth, and the embryo rapidly elongates caudally and is segmented. In these developmental processes the abdominal *Bm en* stripes appear segmentally. At stage 16 of development (48 hr), the abdomen stop segmentation, and *Bm en* stripes appear in every segments including the telson. At stage 17-18 of development (72 hr), germ band exhibits maximum elongation, and *Bm en* stripes disappear at this time. Antibody staining also confirm the same striped expression of pattern of *Bm en* proteins. It also revealed that the expression of *Bm en* in the central nervous system at the blastokinesis stage of *Bombyx* embryo (data not shown). The difference of the way in which the *engrailed* pattern is generated reflects the difference in how these insects generate their metameres. These differences call into question what role homologous of Drosophila *wingless* gene might play in these insects that generate metameres sequentially in abdominal segments. The Bm Wnt-1 antibody was used to determine the spatial and temporal patterns of the Bm Wnt-1 protein during segmentation process. In cellular blastderm stage (stage 3) Bm Wnt-1 proteins are expressed evenly in mesodermal cells (data not shown). Shortly after the formation of early germ anlage (stage 4-5, 24 hr), a striped pattern of Bm Wnt-1 protein expression becomes visible in endodermal cells in the region correspond to the thoracic segments (Fig. 2(5)). In this stage, Bm Wnt-1 is also expressed broadly in the posterior part of germ band, that presumably represent the cellularized growth growth zone which proliferate and generate the posterior segments. At the beginning of the segmentation process, when the *Bm en* stripes appear in the abdominal segments, Bm Wnt-1 stripes also appear in the abdominal segments and disappear in the thoracic segments. Bm Wnt-1 expression in the cellularized growth zone is continued during this stage. At the end of the segmentation process, some of Bm Wnt-1 stripes have already disappeared in the anterior abdominal segments and remained mainly in the posterior abdominal segments.

Discussion

Our results indicate that the relationship of the expression control of *engrailed* and *wingless* as well as their relative locations with respect to the developing segments are conserved between short, intermediate and long germ band insects (Fig. 3). The idea that insect segmentation mechanisms are diverse was supported by the demonstration that the locust expresses a homologue of *even-skipped* in the nervous system, but not in a pair-rule pattern during segmentation[6]. But it was also shown that

the short (intermediate) germ band flour beetle, *Tribolium*, express orthologues of the gap gene, *Kruppel*, and of the pair-rule gene, *hairy*, in patterns resembling those of *Drosophila*[17]. Examination of the expression of even-skipped in both short- and long-germ beetles reveales that germ-type designations do not necessarily predict the mechanistic details of development but those designations reflect the temporal aspects of segmentation[17]. We have already shown that the stripes of the *Bombyx even-skipped* are progressively expressed in an anteroposterior order and, later, these stripes disappear anteriorly at first. And we also shown that *even-skipped* expression proceed *engrailed* expression and the anterior margin of each *even-skipped* stripe coincide with an odd-numbered *engrailed* stripes[19]. The largest difference between long germ type and short germ type development is the difference in the generation of the posterior segments. In short germ and intermediate germ insects, the small early embryo appears to comprise only prospective head, thorac and a growth zone, the remaining segments being formed sequentially during posterior cell division, and visible segmentation is proceeded by the sequential appearance of stripes of *engrailed* expression. In our study, it was shown that the segmentation process in head and thoracic segmentation process in head and thoracic segments of *Bombyx mori* resemble the long germ band type insect *Drosophila* and finishes its determination before morphological segmentation, on the other hand abdominal segments were sequentially determined during morphological segmentation (Fig. 3, between 36 hr and 48 hr). These temporal differences of head, thorax and abdominal segmentation could be considered a reflection of the germ type difference. In conclusion *Bombyx mori* segmentation processes resemble the long germ insects like *Drosophila* in head and thorax regions, but in the abdominal region it resembles the short germ insects like *Tribolium*, so the *Bombyx* segmentation process should be considered as an intermediate germ type. Further study of the expression pattern of the orthologues of higher hierarchy of segmentation genes such as gap-genes and pair-rule genes of this system should give clues about the regulation of segmentation in earlier stages of development and to understand the general mechanisms of segmentation.

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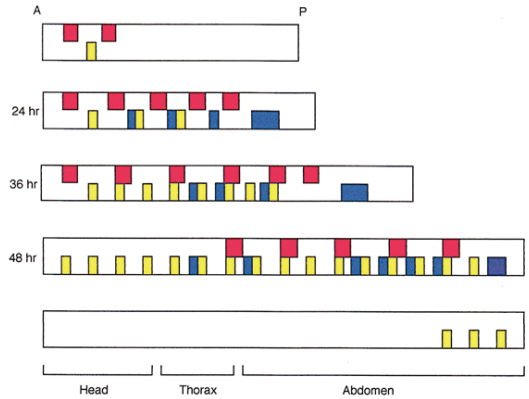

Figure 3. Schematic diagram of the special expression pattern of *even-skipped*, *Bm en* and *Bm Wnt-1* during segmentation process of *Bombyx mori*. Expression patterns of *even-skipped* red box), *Bm en* (yellow box) and *Bm Wnt-1* (blue box) are represented furing the segmentation process of *Bombyx mori* embryo. Before the early germ anlage formation, *Bm Wnt-1* protein expressed in mesodermal cells. During the visible metameric pattern formation (between 36 and 48 hr embryo), germ band elongation occurs only in the region of abdominal segments.

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