# Non-canonical functions of *hunchback* in segment patterning of the intermediate germ cricket *Gryllus bimaculatus*

**Taro Mito, Isao Sarashina*, Hongjie Zhang†, Akihiro Iwahashi, Haruko Okamoto, Katsuyuki Miyawaki, Yohei Shinmyo, Hideyo Ohuchi and Sumihare Noji‡**

Department of Biological Science and Technology, Faculty of Engineering, The University of Tokushima, 2-1 Minami-Jyosanjima-cho, Tokushima City 770-8506, Japan

*Present address: Institute of Geoscience, University of Tsukuba, Tsukuba, 305-8572, Japan
†Present address: Department of Molecular Genetics, Albert Einstein College of Medicine, 1300 Morris Park Avenue, Bronx, NY 10461, USA
‡Author for correspondence (e-mail: noji@bio.tokushima-u.ac.jp)

*Accepted 14 February 2005*

Development 132, 2069-2079
Published by The Company of Biologists 2005
doi:10.1242/dev.01784

## Summary

**In short and intermediate germ insects, only the anterior segments are specified during the blastoderm stage, leaving the posterior segments to be specified later, during embryogenesis, which differs from the segmentation process in *Drosophila,* a long germ insect. To elucidate the segmentation mechanisms of short and intermediate germ insects, we have investigated the orthologs of the *Drosophila* segmentation genes in a phylogenetically basal, intermediate germ insect, *Gryllus bimaculatus* (*Gb*). Here, we have focused on its *hunchback* ortholog (*Gb'hb*), because *Drosophila hb* functions as a gap gene during anterior segmentation, referred as a canonical function. *Gb'hb* is expressed in a gap pattern during the early stages of embryogenesis, and later in the posterior growth zone. By means of embryonic and parental RNA interference for *Gb'hb*, we found the following: (1) *Gb'hb* regulates Hox gene expression to specify regional identity in the anterior region, as observed in *Drosophila* and *Oncopeltus*; (2) *Gb'hb* controls germband morphogenesis and segmentation of the anterior region, probably through the pair-rule gene, *even-skipped* at least; (3) *Gb'hb* may act as a gap gene in a limited region between the posterior of the prothoracic segment and the anterior of the mesothoracic segment; and (4) *Gb'hb* is involved in the formation of at least seven abdominal segments, probably through its expression in the posterior growth zone, which is not conserved in *Drosophila*. These findings suggest that *Gb'hb* functions in a non-canonical manner in segment patterning. A comparison of our results with the results for other derived species revealed that the canonical *hb* function may have evolved from the non-canonical *hb* functions during evolution.**

Key word: *Gryllus bimaculatus*, Orthoptera, Intermediate germ insect, Gap gene, *hunchback*, RNAi, Segmentation

## Introduction

The major features of the body plan of insects include segmentation and the organization of segments into three distinct regions: head, thorax and abdomen. Although almost all insects have segmented bodies in common, their patterning processes vary substantially from insect to insect. In long germ insects, such as *Drosophila*, the pattern of all segments is laid out simultaneously in a syncytial environment. In the cellularized embryos of short and intermediate germ insects, the anterior segments form almost simultaneously, and the remaining posterior segmentation occurs sequentially from a posterior growth (proliferative) zone after anterior segmentation is complete (Dearden and Akam, 2001). This mode of segmentation is believed to be ancestral (Davis and Patel, 2002; Tautz et al., 1994).

To understand the similarities and differences in the molecular mechanisms of short/intermediate and long germ segmentation, we need to compare the roles of the developmental genes involved in early embryonic patterning. In *Drosophila*, gap genes play a central role in the early subdivision of the blastoderm into broad regions. Maternal gradients lead to the activation of gap genes a broad domain, and the syncytial environment allows the gap-gene products to diffuse and produce overlapping short-range gradients (Hülskamp and Tautz, 1991; Rivera-Pomar and Jäckle, 1996). These short-range gradients define the stripe patterns of the primary pair-rule genes (Small and Levine, 1991). Gap genes are also responsible for providing positional information to regulate the expression of Hox genes, which assign identities to each segment (McGinnis and Krumlauf, 1992). Although gap genes act in a syncytial environment in *Drosophila*, the orthologs of gap genes in short and intermediate germ insects are expressed in a cellularized environment. The orthologs of the gap genes of these insects cannot act in the same manner as those in *Drosophila*. Thus, elucidating the functions of the gap genes in short and intermediate germ insects would provide crucial clues to clarify the molecular segmentation mechanisms.

The gap gene *hunchback* (*hb*), which codes for a zinc-finger type transcription factor, is crucial for anteroposterior patterning in various insects (Liu and Kaufman, 2004; Lehmann and Nüsslein-Volhard, 1987; Patel et al., 2001;

Schröder, 2003; Tautz et al., 1987). For example, in *Drosophila*, the loss-of-function alleles for *hb* show a canonical gap defect: i.e. deletion of the labial through the metathoracic segments. The *hb* RNAi depletion in *Tribolium*, a short germ insect, results in deletion of the gnathal and thoracic segments, suggesting that the canonical gap function of *hb* is conserved (Schröder, 2003). On the other hand, in the intermediate germ insect *Oncopeltus*, the *hb* (*Of'hb*) RNAi depletion results in a non-canonical, gap-like phenotype, i.e. a combination of the transformation of the gnathal and thoracic regions into an abdominal identity and defects in posterior elongation and segmentation (Liu and Kaufman, 2004). This indicates that *Of'hb* is required to suppress the abdominal identity and for proper germband growth and segmentation (Liu and Kaufman, 2004). These results suggest that *hb* function differs among insects.

To deepen our understanding of the mechanisms of insect segmentation by obtaining data from more phylogenetically basal species, we have focused on the intermediate germ cricket, *Gryllus bimaculatus* (*Gb*, Orthoptera), which is more basal than *Oncopeltus* (Hemiptera). In our study, we isolated *Gryllus hb* (*Gb'hb*) and analyzed its functions, using embryonic and parental RNA interference, and found the following: (1) *Gb'hb* regulates the Hox gene expression to specify a regional identity in the anterior region, as observed in *Drosophila* and *Oncopeltus*; (2) *Gb'hb* controls germband morphogenesis and segmentation of the anterior region probably through the pair-rule gene, *even-skipped* at least; (3) *Gb'hb* may act as a gap gene in a limited region between the posterior of the prothoracic segment and the anterior of the mesothoracic segment; and (4) *Gb'hb* is involved in forming at least seven abdominal segments, probably through its expression in the posterior growth zone, which is not conserved in *Drosophila*. These findings suggest that *Gb'hb* functions in a non-canonical manner in segment patterning. We will discuss both conserved and divergent aspects of *Gb'hb* functions from an evolutionary point of view.

## Materials and methods

### Cloning of *Gryllus* orthologs of *hunchback* and *Krüppel*

Total RNA was extracted from embryos within 24-72 hours after egg-laying (hAEL) using Isogen (Nippon-Gene). To clone *Gb'hb*, we first applied a degenerate polymerase chain reaction (PCR). cDNA was synthesized using the Superscript First Strand Synthesis Kit (Invitrogen) with random hexamers. For the PCR, we used *hb* degenerate primers that targeted GFVAVTK (forward) and GTRRGPK (reverse) motifs in the region coding the central zinc-finger domains. The nucleotide sequences of the primers were 5′-GGC TTC GTB GCN GTN ACN AA-3′ and 5′-TTA GGA CCA CKN CKN GTN CC-3′. To isolate of the *Gryllus Krüppel* fragment, we used the degenerate primers described by Sommer et al. (Sommer et al., 1992). Using the short-fragments sequences obtained from the degenerate PCRs, we designed gene-specific primers and performed 5′ and 3′ rapid amplification of cDNA ends (RACE) using the SMART RACE cDNA amplification kit (Clontech). The *Gb'hb* cDNA sequence was deposited in the DNA Data Bank of Japan (DDBJ) (Accession Number, AB120735).

### Whole-mount in situ hybridization

Standard protocols were used for whole-mount in situ hybridization with a digoxigenin (DIG)-labeled antisense RNA probe, as previously described (Niwa et al., 2000). In situ hybridization for double staining was carried out as follows. The antisense RNA probe was labeled with DIG or fluorescein. Hybridization was carried out following the standard protocol. After hybridization, anti-fluorescein-AP Fab fragments (Roche) were added and a color reaction for the fluorescein-labeled probe was performed using NBT/BCIP as the substrate. The samples were then washed in a TNT buffer, before re-fixing using 4% paraformaldehyde in PBS at 4°C overnight. After subsequent washing in TNT, anti-digoxigenin-POD Fab fragments (Roche) were added and then the samples were washed in TNT again. Treatment for signal enhancement was then carried out using the TSA Biotin System (PerkinElmer Life Sciences) following the manufacturer's instructions. The color reaction for the DIG-labeled probe was performed using the Vector Nova Red substrate kit (Vector).

### RNAi

We synthesized double-stranded RNA (dsRNA) using the MEGA-script Kit (Ambion), and used PCR fragments as the template for in vitro transcription. The PCR fragments were amplified using upstream and downstream primers that contained the T7 promoter sequence. The synthesized RNA was extracted using phenol/chloroform and ethanol precipitated. The RNA was denatured in boiled water after being suspended in a Tris-EDTA buffer and annealed at room temperature overnight. The resulting dsRNA was suspended in an appropriate volume of water after ethanol precipitation. The final concentration of dsRNA was adjusted to 20 μM for the *Gb'hb* dsRNA (395 bp, spanning the four central zinc fingers) and the *DsRed2* dsRNA [660 bp, derived from the pDsRed2-N1 (Clontech)]. The *DsRed2* dsRNA was used for negative control experiments. For embryonic RNAi, the cricket eggs were collected for 2 hours and used within 1 hour of collection. We microinjected the dsRNA in the posterior end of the egg, as previously described (Zhang et al., 2002). For parental RNAi, we injected adult females with a 0.6 μl dsRNA solution in a ventrolateral position, between segments T3 and A1 (details to be published elsewhere). Fifteen injected females were mated with untreated males, and the eggs were collected from 5 to 10 days after injection.

## Results

### Sequence analysis of the *hunchback* ortholog in *Gryllus*

We isolated cDNA clones of *Gb'hb* from embryos at 24-72 hAEL using the degenerate PCR and 5′ and 3′ RACE strategy. Nucleotide sequences of three independent clones were analyzed in each RACE experiment and they showed identical structure each other. The *Gb'hb* cDNA is predicted to encode 572 amino acids. The alignments of conserved domains of *Gb'*Hb compared with those of other insects are shown in Fig. 1A. *Gb'*Hb contains six zinc-finger domains, four in the middle region (MF 1-4) and two in the C-terminal region (CF 1-2), which correspond to the MF 1-4 and the CF 1-2 of *Drosophila* Hb, respectively. In addition, sequences similar to the A-box and C-box of *Drosophila* Hb (Tautz et al., 1987; Hülskamp et al., 1994) are conserved in *Gb'*Hb. The overall structure of the *Gb'*Hb protein is shown in Fig. 1B. In our PCR clone, we did not find the basic box (Hülskamp et al., 1994) or the two N-terminal finger domains found in *C. elegans*, *Oncopeltus* and grasshopper Hb (Liu and Kaufman, 2004; Patel et al., 2001). From a phylogenetic perspective, there could be an isoform containing these domains in *Gryllus*.

### Expression patterns of *Gb'hb* during embryogenesis

The embryogenesis of *Gryllus* begins with mitoses, without cellular divisions. The nuclei then migrate to the egg periphery,

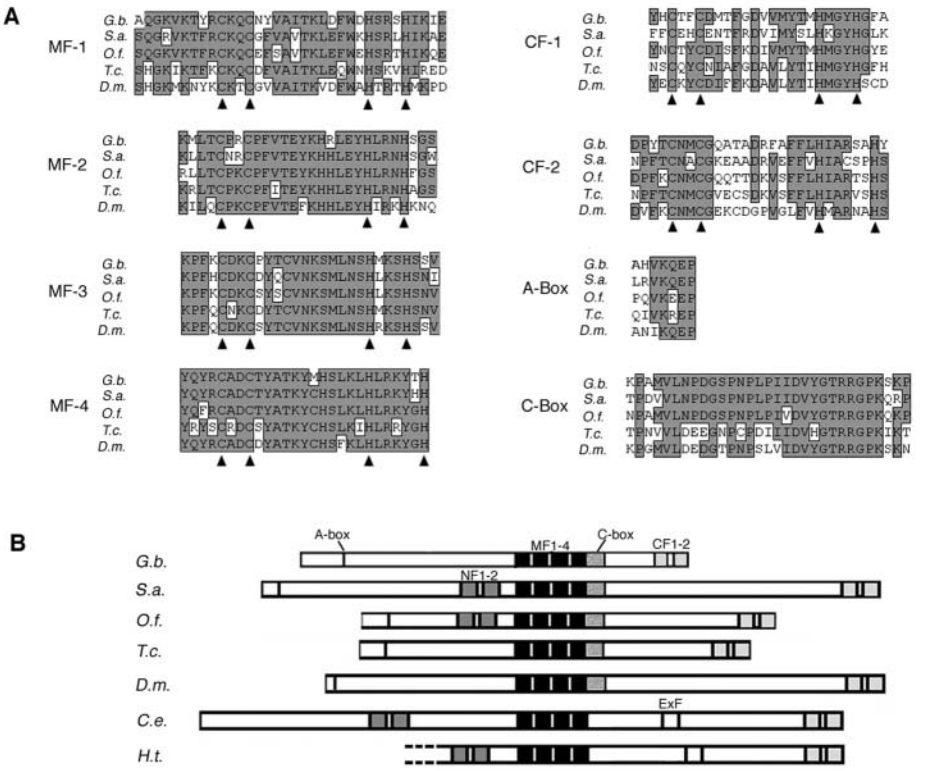


**Fig. 1.** Alignments of conserved domains of *Gb'hb* compared with orthologous sequences from other insects. (A) Four central fingers (MF-1 to 4), two C terminal fingers (CF-1 and 2), and A-Box and C-Box of *Gryllus bimaculatus* (*G.b.*) *hunchback* aligned with those of *Schistocerca americana* (S.a.), *Oncopeltus fasciatus* (*O.f.*), *Tribolium castaneum* (*T.c.*) and *Drosophila melanogaster* (*D.m.*). Arrowheads indicate the structural residues of the putative metal-binding fingers. (B) Schematic structures of various Hunchback proteins, comparing the organization of zinc fingers. Each box shows a zinc finger or C-box. Corresponding zinc fingers of different species are shown in the same shade. Four central fingers (MF-1 to 4) and two C terminal fingers (CF-1 and 2) are conserved in all insects in this figure. The structure of *H.t.* Hb was predicted from the genomic sequence (Patel et al., 2001; Savage and Shankland, 1996). *C.e.*, *Caenorhabditis elegans*; *H.t.*, *Helobdella triserialis*.

followed by cellularization (Sarashina et al., 2005). During the blastoderm stage, the germ anlage forms in the posteroventral region of the egg around 30 hAEL. At 30 hAEL, *Gb'hb* was expressed weakly as a band in the middle of the embryo and also expressed in a stripe along the midline, probably in the invaginating mesoderm (Fig. 2A). As *Gryllus wingless* (*Gb'wg*) is expressed in each segment (Miyawaki et al., 2004), we used it as a segment-marker gene. The middle *Gb'hb* domain was located between the anterior and posterior expression domains of *Gb'wg* (Fig. 2B). The middle *Gb'hb* domain, corresponding to the prospective gnathal region, then incompletely resolved into two bands at 32 hAEL (Fig. 2C), and the anterior band was further resolved into two stripes at 36 hAEL (Fig. 2D). Double staining for *Gb'hb* and *Gb'wg* at 40 hAEL indicated that the gap-like middle domain of *Gb'hb* expression spanned from the prospective mandibular to labial segments (Fig. 2E). By 42 hAEL, the expression domain in the gnathal region became faint and eventually faded out (Fig. 2F).

In the later stages, *Gb'hb* was expressed as spots in the head region (Fig. 2I), and then in the trunk (Fig. 2H-J). Because we know that *hb* is expressed in neuroblasts as spots in *Drosophila* (Isshiki et al., 2001), and is probably expressed the same in *Tribolium* (Wolff et al., 1995) and in the grasshopper (Patel et al., 2001), each spot may correspond to a neuroblast. As in *Drosophila, Gb'hb* may be also involved in neural patterning.

An additional *Gb'hb* expression domain appeared near the posterior end of the abdomen (Fig. 2G) and became more intense as the posterior region elongated (Fig. 2H). The posterior band then split into two stripes, and a new band appeared in the posterior region (Fig. 2J). Double staining for *Gb'hb* and *Gb'wg* indicated that the two intense stripes were located between the A7- and A9-*Gb'wg* stripes (Fig. 2K). In *Drosophila*, the posterior *hb* expression domain is located in the region of parasegment (PS) 13/14 and is required to form the abdominal segments A7 and A8 (Tautz et al., 1987; Lehmann and Nüsslein-Volhard, 1987; Bender et al., 1987). In the grasshopper, the *hb* posterior expression domain spans a region from the posterior compartment of A7 to the anterior compartment of A9 (Patel et al., 2001).

We detected *Gb'hb* transcripts in the ovary and early eggs using RT-PCR (data not shown), which indicated the presence of maternal transcripts. It is unclear, though, when the maternal transcripts are replaced by the zygotic ones.

## RNAi analysis of *Gb'hb*

To investigate the *Gb'hb* function, we applied both embryonic RNAi (eRNAi) and parental RNAi (pRNAi) to deplete the transcript (Bucher et al., 2002; Miyawaki et al., 2004). We confirmed that no qualitative phenotype differences were produced by either the eRNAi or the pRNAi. We used pRNAi for further analyses using in situ hybridization, because it does not produce injection artifacts and all of the developed embryos showed effects of RNAi by our pRNAi for *Gb'hb* (Table 1). We were able to categorize the resulting embryos into three phenotypic classes, based on the severity, as shown in the embryos just before hatching and in the embryos stained with a segment marker gene *Gb'wg* at stage 9 (Fig. 3). The most severe class I embryos (Fig. 3J-M) showed a gap-like phenotype, in which the head with the mandible was followed by several segments without appendages. In the class I embryos, the antenna, mandible and cercus formed almost normally. In milder phenotypes, the class II (Fig. 3G-I) and III (Fig. 3D-F), a part of the appendages was suppressed. Of the affected appendages, the T3 leg was the most resistant to *Gb'hb* depletion. In the class II embryos, all gnathal and thoracic appendages were suppressed, except for vestigial legs in the T3 segment, and the embryos were shortened as a result of the reduced number of segments (Fig. 3G). Segmentation

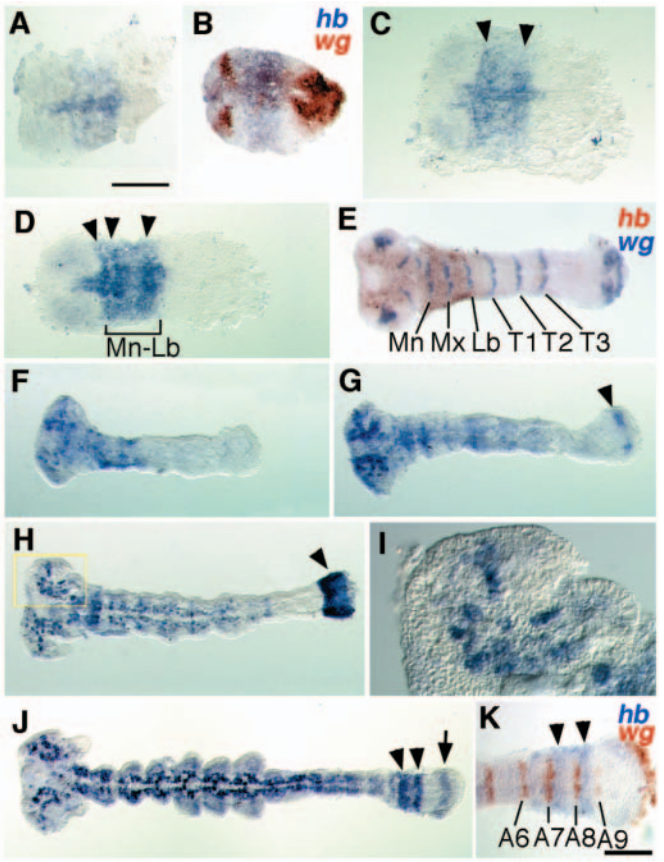


**Fig. 2.** Expression of *Gb'hb* during embryogenesis. Expression patterns of *Gb'hb* at 30 (A,B), 32 (C), 36 (D), 40 (E), 42 (F), 48 (G), 52 (H,I) and 72 (J,K) hAEL. The embryo was double-stained with *Gb'wg* in B (*Gb'hb*, blue; *Gb'wg*, brown), E (*Gb'hb*, brown; *Gb'wg*, blue) and K (*Gb'hb*, blue; *Gb'wg*, brown). (C,D) Stripes of *Gb'hb* expression in the middle gap domain are indicated by arrowheads. The posterior expression domain (arrowheads in G,H,J) appeared at 48 hAEL (G) and split into two stripes (J). (K) Double staining for *Gb'hb* (blue) and *Gb'wg* (brown) indicates that the splitting intense domain of the *Gb'hb* expression near the posterior end spanned from the A7 stripe to the A9 stripe of *Gb'wg*. (I) High-magnification view of the boxed area in H, showing spotted expressions. Mn, mandible; Mx, maxilla; Lb, labium; T1-3, thoracic segments 1 to 3; A7-9, abdominal segments 7 to 9. Scale bars: 250 μm in A-H,J; 100 μm in K.

disturbances were frequently observed in all of the phenotypic classes (Fig. 3F,H,I). Such phenotypes are reminiscent of those resulting from *hb* depletion in another intermediate germ insect, *Oncopeltus*, in which the gnathal and thoracic regions are transformed into an abdominal identity, forming a small abdomen with defective segments (Liu and Kaufman, 2004). Even in the most severe class I embryos, vestiges of T3 legs were observed in 70% of the RNAi embryos ($n$=28 out of 40, Fig. 3L). Most of these embryos had only three or four segments posterior to the leg vestiges, indicating that seven abdominal segments had been deleted in the severe case. This number may not be maximal because it was often difficult to ascertain the precise number of deleted segments because of segmentation disturbances. The class I embryos without leg vestiges showed no crucial differences from embryos with leg vestiges in their external morphology, indicating that all of the class I embryos were resulted from suppressing the gnathal and thoracic identities and growth and segmentation defects in the posterior region.

We observed the internal structure of the *Gb'hb* RNAi embryos using sagittal sections. The digestive tract of the wild-type embryos consisted of the fore-, mid- and hindgut with characteristic constriction patterns (Fig. 3N). The midgut contained yolk granules. In the class II embryos, the fore-, mid- and hindgut were formed, although the fore- and hindgut were compressed in the shortened *Gb'hb* RNAi embryos (Fig. 3O). The class I embryos had a sac-like midgut, and the fore- and hindgut were further compressed (Fig. 3P). Our observation of the segmental musculature revealed that anterior segments of the *Gb'hb* RNAi embryos were associated with the segmental musculature of the abdominal character, even in severe phenotypes (Fig. 3R,S). However, these segments were wider than the abdominal segments of the wild type and were not arranged in an orderly reiterated pattern, suggesting incomplete transformation to the abdomen.

## Effects of *Gb'hb* RNAi on regional identities

To further investigate the effect of *Gb'hb* depletion on segment identity, we examined the expression patterns of the Hox genes in the *Gb'hb* RNAi embryos. In the wild-type embryo at stage 9 (Niwa et al., 1997), *Gryllus abdominal-A* (*Gb'abdA*) is expressed in the posterior compartment of A1 and the remaining abdominal segments (Fig. 4A) (Miyawaki et al., 2004; Zhang et al., 2005), thus making it an abdominal-marker gene. We observed ectopic expression of *Gb'abdA* in the gnathal and thoracic regions in the *Gb'hb* RNAi embryos (Fig. 4B,C). This supports our interpretation based on the morphological observation that suppression of appendages in the *Gb'hb* RNAi embryos is a result of the homeosis of the gnathal and thoracic regions towards an abdominal identity, even in the most severe phenotype (Fig. 4D). This is essentially consistent with the results of an RNAi analysis of *Oncopeltus hb* (Liu and Kaufman, 2004).

Transformation of the gnathal and thoracic regions should be accompanied by a change in the expression patterns of the Hox genes, other than *Gb'abdA*. *Gryllus Sex combs reduced*

**Table 1. Effect of parental and embryonic RNAi for *Gryllus hunchback***

| | Total ($n$) | Class I | Class II | Class III | Wild type | Nonspecific |
|---|---|---|---|---|---|---|
| *Gb'hb* pRNAi | 257 | 40 (15.6) | 154 (59.9) | 39 (15.2) | 0 (0) | 24 (9.3) |
| *DsRed2* pRNAi | 188 | 0 (0) | 0 (0) | 0 (0) | 176 (93.6) | 12 (6.4) |
| *Gb'hb* eRNAi | 71 | 26 (36.6) | 17 (23.9) | 3 (4.2) | 18 (25.3) | 7 (9.9) |
| *DsRed2* eRNAi | 131 | 0 (0) | 0 (0) | 0 (0) | 115 (87.8) | 16 (12.2) |

Number of embryos of given phenotypic class with percentages in parentheses is shown.
*DsRed2* dsRNA was used for negative control experiments.

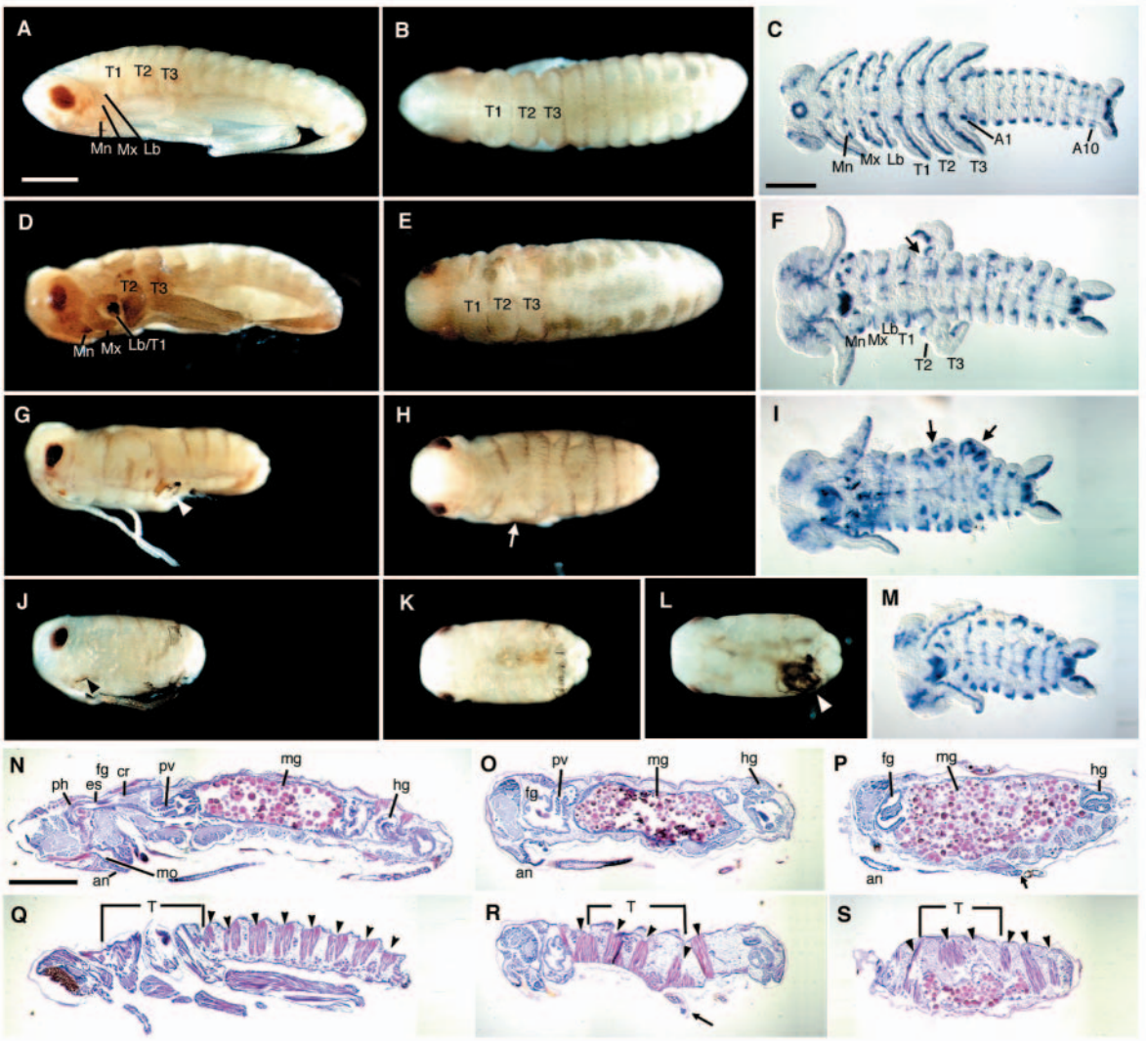


**Fig. 3.** Effect of *Gb'hb* RNAi on *G. bimaculatus* embryos. Anterior towards the left. (A,B) Wild-type embryo in the stage just before hatching. (A) Lateral view. (B) Dorsal view. (C) Wild-type embryo stained for *Gb'wg*. (D,E) Class III embryo. (D) Lateral view. (E) Dorsal view. (F) Putative class III embryo stained for *Gb'wg*. In this embryo, segmentation of the T1 to T3 was partially disturbed (arrow). Segmentation defects were frequently observed in *Gb'hb* RNAi embryos (also shown in H and I). (G,H) Class II embryo. (G) Lateral view. Arrowhead indicates a defective T3 leg. (H) Dorsal view. (I) Putative class II embryo stained for *Gb'wg*. Arrows in H and I indicate segmentation disturbances. (J-L) Class I embryo. (J) Lateral view. Arrowhead indicates the mandible. (K) Dorsal view. (L) Ventral view. Arrowhead indicates a vestige of a T3 leg. (M) Putative class I embryo stained for *Gb'wg*. (N-S) Sagittal sections through wild-type and *Gb'hb* RNAi embryos. (N,Q) Wild type. (O,R) Class II embryo. (P,S) Class I embryo. Each of these pairs was derived from the same specimen. The digestive tract consists of a fore-, mid- and hindgut. (N) In wild type, the pharynx (ph), esophagus (es), crop (cr) and proventriculus (pv) are observed in the foregut. The midgut contains yolk granules. (O) In class II embryos, the fore- and hindgut were compressed and the midgut was relatively shifted towards the anterior. (P) In more severe cases, a sac-like midgut was observed, and the fore- and hindgut were further compressed. (Q) The musculature in the abdominal segments shows a reiterated pattern (arrowheads). (R,S) In *Gb'hb* RNAi embryos, musculature was observed in each segment, although the reiterated pattern was not orderly. Arrows in P and R indicate a vestige of the T3 leg. an, antenna; cr, crop; es, esophagus; fg, foregut; ph, pharynx; mo, mouth; pv, proventriculus; mg, midgut; hg, hindgut; T, thorax; Mn, mandible; Mx, maxilla; Lb, labium; T1-3, thoracic segments 1 to 3. Scale bars: in A, 400 μm for A,B,D,E,G,H,J,K,L; in N, 400 μm for N-S; 200 μm in C,F,I,M.

(*Gb'Scr*) is intensely expressed in the labial and T1 segments in the wild-type embryos (Fig. 4E) (Miyawaki et al., 2004; Zhang et al., 2005). In the *Gb'hb* RNAi embryos, the *Gb'Scr* expression in the labial and T1 segments was greatly reduced (Fig. 4F,G) and disappeared in the most severe case (Fig. 4H). This is concordant with suppression of the gnathal and thoracic identity in the *Gb'hb* RNAi embryos. *Gryllus Antennapedia* (*Gb'Antp*) is expressed in all of the thoracic and abdominal segments in the wild-type embryos (Fig. 4I) (Zhang et al., 2005). In the abdominal region, strong expression of *Gb'Antp* is observed along the midline. In the *Gb'hb* RNAi embryos, the anterior expression boundary was shifted to the labial segment in all phenotype classes (Fig. 4J-L). The *Gb'Antp* expression in the anterior region of the *Gb'hb* RNAi embryos was strong in midline, suggesting that *Gb'Antp* expression in abdomen-like patterns was ectopically induced in the anterior

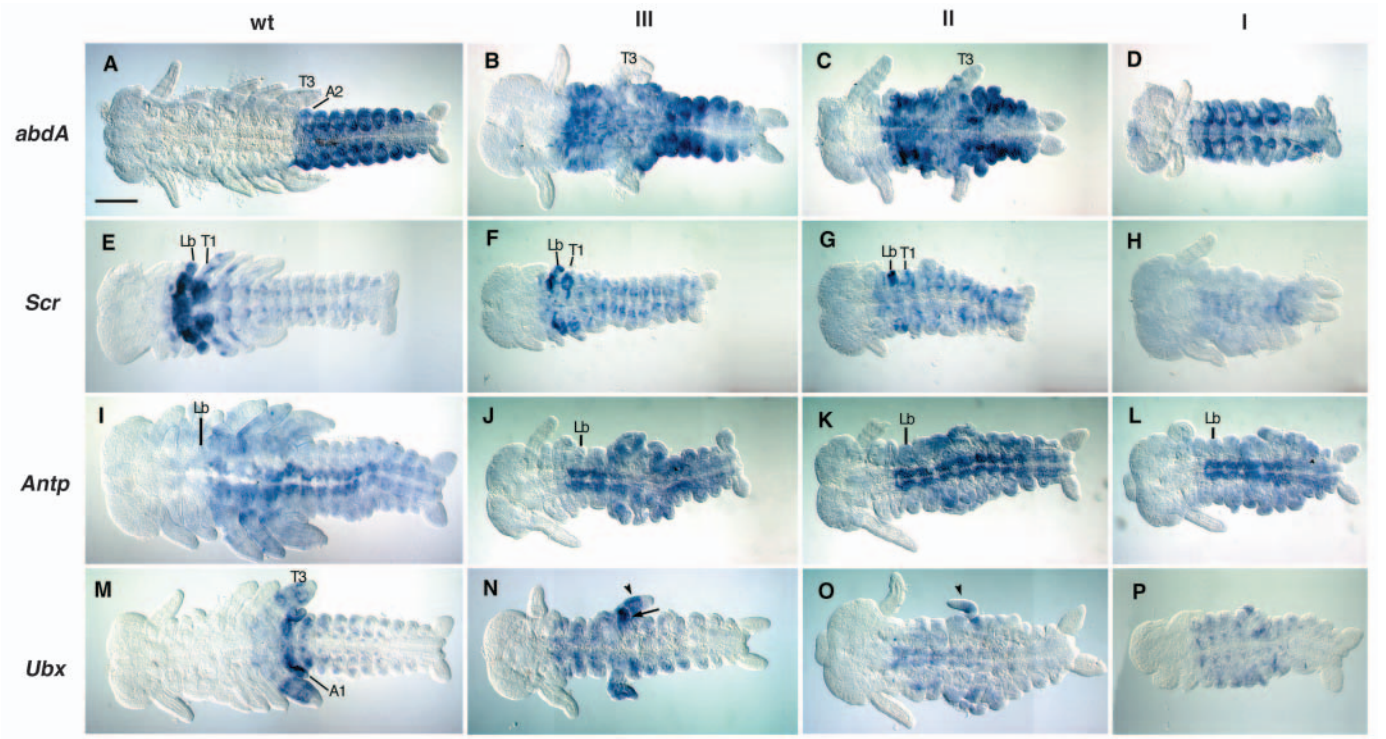


**Fig. 4.** Expression patterns of Hox genes in embryos depleted of *Gb'hb* by RNAi. (A) Wild-type embryo stained for *Gb'abdA*. *Gb'abdA* is expressed in the posterior compartment of A1 and the remaining abdominal segments. (B-D) Putative class III-I embryos stained for *Gb'abdA*. Ectopic expression of *Gb'abdA* in gnathal and thoracic regions was observed in B and C, suggesting that the strong phenotype (D) consists of transformed gnathal and thoracic segments and a shortened abdomen. (E) Wild-type embryo stained for *Gb'Scr*. *Gb'Scr* is strongly expressed in the labial and T1 segments. Expression in the labial segment is more intense than in the T1 segment. (F-H) Putative class III-I embryos stained for *Gb'Scr*. *Gb'Scr* expression in the labial and T1 segments was gradually reduced as the phenotype became more severe. (I) Wild-type embryo stained for *Gb'Antp*. The anterior boundary of the *Gb'Antp* expression is the T1 segment. (J-L) Putative class III-I embryos stained for *Gb'Antp*. The anterior boundary of the *Gb'Antp* expression was shifted to the labial segment in all phenotype classes. The strong midline expression in the anterior region is an abdomen-like pattern and probably ectopically induced. (M) Wild-type embryo stained for *Gb'Ubx*. *Gb'Ubx* is strongly expressed in the T3 legs and A1 prolegs, and weakly expressed in the abdominal segments. In the abdomen, the intensity of expression follows a gradient from anterior to posterior. (N-P) Putative class III-I embryos stained for *Gb'Ubx*. (N) Expression patterns of *Gb'Ubx* indicates that the leg most resistant to RNAi (arrowhead) was T3. Strong expression of *Gb'Ubx* in the A1 prolegs was also detected in this class of embryos (arrow). Scale bar: 250 μm.

region. *Gryllus Ultrabithorax* (*Gb'Ubx*) is strongly expressed in T3 legs and A1 prolegs, and weakly in the abdominal segments (Fig. 4M) (Zhang et al., 2005). We used *Gb'Ubx* as a marker gene for the T3 and A1 segments in the RNAi embryos. In the *Gb'hb* RNAi embryos, *Gb'Ubx* was ectopically expressed in the anterior region, in the maxillary to T2 segments (Fig. 4N,O). In weak phenotypes, the *Gb'Ubx* expression in the T3 leg bud was retained (Fig. 4N), indicating that the T3 segment was the most resistant to *Gb'hb* RNAi depletion. In more severe cases, it was reduced (Fig. 4O). In the most severe case, *Gb'Ubx* was expressed from the maxillary segments towards the posterior end in a similar pattern to the abdominal segments of wild type (Fig. 4P). Thus, expression patterns of the Hox genes suggest that in the *Gb'hb* RNAi embryos, gnathal and thoracic identities were suppressed and transformed towards an abdominal identity.

### Effects of *Gb'hb* RNAi on early embryonic patterning

The *Gb'hb* RNAi effects extend outside the expression domain of the gap pattern in the early stages. Effects outside of this expression domain may be caused through regulation of other segmentation genes and/or Hox genes during early embryogenesis. To investigate the effects of *Gb'hb* depletion on early embryogenesis, we examined the expression patterns of the *Gryllus* orthologs of *Drosophila* segmentation genes (*Krüppel*, *even-skipped* and *wingless*) and Hox genes (*Antennapedia* and *abdominal-A*).

We isolated a fragment of the gap-gene ortholog *Gryllus Krüppel* (*Gb'Kr*) from the degenerate PCR and 5′ RACE, using cDNA from embryos within 24-72 hAEL. This fragment contained the Type 1 to 3 zinc fingers and part of the Type 4 zinc finger of the five zinc fingers (Type 1-5) in *Drosophila Kr* (Fig. 5) (Rosenberg et al., 1986). In the wild-type embryos at 40-42 hAEL, *Gb'Kr* is expressed in a broad domain in the thoracic region and spot-like domains in the gnathal region (Fig. 6A, details to be published elsewhere). In *Gb'hb* RNAi embryos, *Gb'Kr* expression was severely affected. The *Gb'Kr* expression domain in the thoracic region was reduced in the RNAi embryos (Fig. 6B,C). In severe cases, the thoracic broad domain was suppressed, except in the peripheral region (Fig. 6C). This suggests that *Gb'hb* is involved in transcriptional regulation of *Gb'Kr* in the thoracic region directly or indirectly.


**Fig. 5.** Alignment of *Gb'Kr* with orthologous sequences of *Drosophila melanogaster* (Dm) and *Tribolium castaneum* (Tc). The labeled brackets indicate zinc fingers (type 1 to 4).

The reduction of the *Gb'Kr* expression domain could be partially due to the defect in embryonic growth, because the *Gb'hb* RNAi embryos in the early stages were shorter and wider than wild-type embryos at the same time after egg-laying. Such a morphological change in the *Gb'hb* RNAi embryos suggests that *Gb'hb* is required for proper development of the germband.

Next, we observed the expression patterns of pair-rule and segment polarity gene orthologs in *Gb'hb* RNAi embryos. In wild-type embryos at 38-40 hAEL, *Gryllus even-skipped* (*Gb'eve*) is expressed in the five clearly resolved stripes in the gnathal and thoracic regions, and an additional stripe or two are resolved from the broad expression domain in the posterior growth zone (Fig. 6D). The five anterior stripes first appear almost simultaneously, as the most anterior segmental stripe and two pair-rule-like broad stripes, each of which resolves into two segmental stripes (data not shown). The *Gb'hb* expression domain covers the gnathal region (Fig. 2E). However, we found that the first to third *Gb'eve* stripes in the gnathal region were not affected in the *Gb'hb* RNAi embryos, even though the total number of stripes was reduced at this stage (Fig. 6E,F). In the most severe case, only three stripes were observed (Fig. 6F). This suggests retardation in the timing of the sequential formation of the stripes, or suppression of the formation of segmental stripes 4 and 5 (derived from the secondary pair-rule-like stripe). *Gb'wg* is expressed in seven segmental stripes in the gnathal/thoracic and abdominal regions in the wild-type embryos at 45-47 hAEL (Fig. 6G). In the *Gb'hb* RNAi embryos, the number of stripes was reduced to five, and the shapes of thoracic stripes became irregular (Fig. 6H,I). The T1 stripe of *Gb'wg* was the most severely affected, indicating fusion with the T2 stripe (Fig. 6H). In severe cases, the labial stripe was also affected, while the first and second gnathal stripes appeared normal (Fig. 6I). In addition to defects in the anterior region, the shape of the elongating posterior region in the *Gb'hb* RNAi embryos was abnormal at this stage (Fig. 6H,I).

To see the effects of *Gb'hb* depletion in the homeotic segment specification in early embryos, we examined the expression patterns of the homeotic genes in the *Gb'hb* RNAi embryos. *Gb'Antp* is strongly expressed in the thoracic region of the wild-type embryos at 38-40 hAEL (Fig. 6J) (Zhang et al., 2005). The *Gb'Antp* domain was reduced in the *Gb'hb* RNAi embryos (Fig. 6K). The expression of *Gb'abdA* begins at the boundary between the thoracic region and the abdominal region in the wild-type embryos by 45-47 hAEL (Fig. 6L)

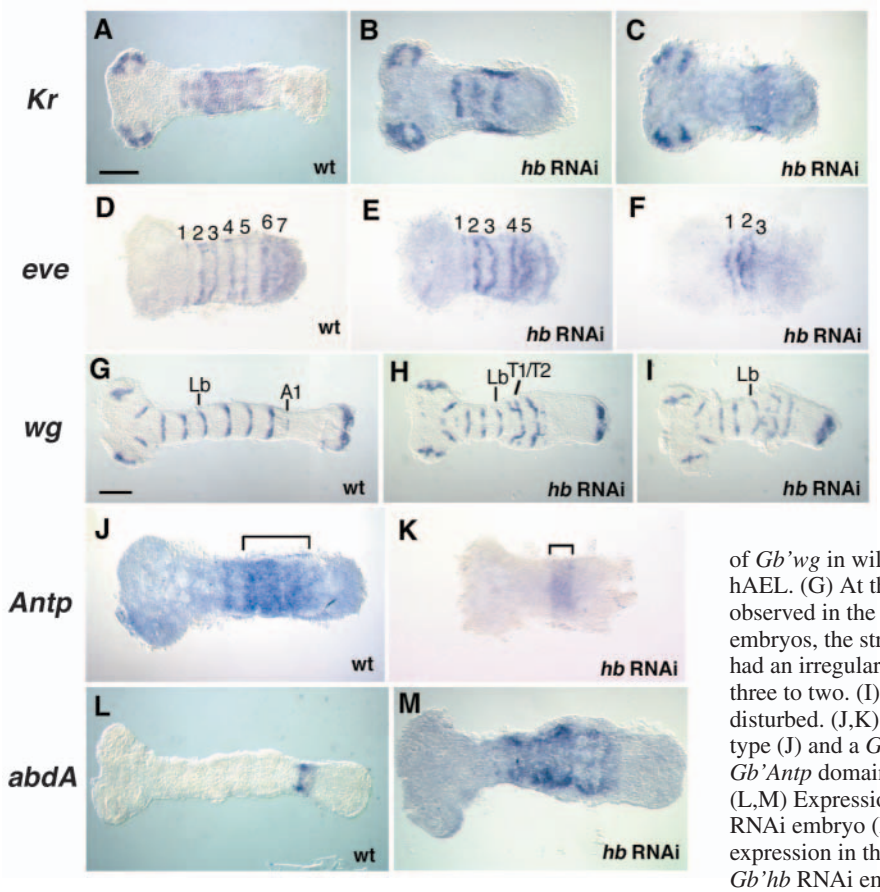


**Fig. 6.** In situ detection of segmentation and Hox genes in early-stage embryos depleted of *Gb'hb* by RNAi. (A-C) Expression patterns of *Gb'Kr* in wild-type (A) and *Gb'hb* RNAi embryos (B,C) at 40-42 hAEL. *Gb'Kr* is expressed in the gnathal and thoracic regions at this stage in wild-type embryos. Expression in this domain was reduced by *Gb'hb* depletion and, in severe cases, almost eliminated. The embryo shown in C was affected more severely than that shown in B. *Gb'hb* RNAi embryos are shorter and flatter than the wild type. (D-F) Expression patterns of *Gb'eve* in wild-type (D) and *Gb'hb* RNAi embryos (E,F) at 38-40 hAEL. In wild-type embryos at this stage, *Gb'eve* is expressed as five clearly resolved stripes and an additional one or two stripes are resolving from the posterior broad domain. (E) In *Gb'hb* RNAi embryos, the number of stripes was reduced to less than five. (F) In more severe cases, only three stripes were observed. (G-I) Expression patterns of *Gb'wg* in wild-type (G) and *Gb'hb* RNAi embryos (H,I) at 45-47 hAEL. (G) At this stage of wild type, seven *Gb'wg* stripes are observed in the gnathal and thoracic regions. (H) In *Gb'hb* RNAi embryos, the stripes in the thoracic region were affected in that they had an irregular shape and the number of stripes was reduced from three to two. (I) In more severe cases, the labial stripe was also disturbed. (J,K) Expression patterns of *Gb'Antp* (brackets) in wild type (J) and a *Gb'hb* RNAi embryo (K) at 38-40 hAEL. (K) The *Gb'Antp* domain was severely reduced in *Gb'hb* RNAi embryos. (L,M) Expression patterns of *Gb'abdA* in wild type (L) and a *Gb'hb* RNAi embryo (M) at 45-47 hAEL. (L) In wild type, the *Gb'abdA* expression in the abdominal region begins close to this stage. (M) In *Gb'hb* RNAi embryos, the ectopic *Gb'abdA* expression was observed in the gnathal and thoracic regions. Scale bar: 250 μm.

(Zhang et al., 2005). The *Gb'hb* RNAi resulted in ectopic expression of *Gb'abdA* in the gnathal and thoracic regions (Fig. 6M). These results indicate that *Gb'hb* is involved in regulating the Hox genes in the gnathal and thoracic regions during early embryogenesis.

## Discussion

In this study, we examined the expression patterns and functions of an ortholog of the *Drosophila* gap gene *hb* in an orthopteran *Gryllus bimaculatus*, which phylogenetically is the most basal species of insects ever examined for *hb* functions. So far, the functions of insect *hb* genes have been studied in a dipteran *Drosophila*, coleopteran *Tribolium* and hemipteran *Oncopeltus* (*Of'hb*). Interestingly, the *Of'hb* functions were found to differ from the canonical functions of *Drosophila* or *Tribolium hb* (Liu and Kaufman, 2004). Furthermore, in this study we found that the non-canonical functions of *Gb'hb* partly differ from those of *Of'hb*. Here, in comparison with the functions of *hb* in other species, we discuss the *Gb'hb* functions in the anterior region at first (Fig. 7A-D), the regulatory network among *Gryllus* segmentation genes (Fig. 7E) and the evolution of *hb* functions.

### *Gb'hb* is involved in the morphogenesis of the germband

In the early stages of *Gryllus* embryos, we observed a large morphological change, in which the germ anlage becomes thinner and longer to form the germband (Miyawaki et al., 2004), probably caused by cell rearrangement. This morphological change is reminiscent of the germband extension observed in *Drosophila* embryogenesis. In the early stages of *Gb'hb* RNAi embryos, the germband was shorter and wider than in the wild type, and the *Gb'wg* stripes corresponding to the prospective thoracic segments became irregular (Fig. 7B), suggesting that the cell rearrangement defects occurred during the morphogenesis of the germband (Fig. 7A). In *Drosophila*, *hb* is required for proper cell rearrangement in the germband extension through regulation of expression of pair-rule genes (Irvine and Wieschaus, 1994). Therefore, this fact and our results suggest that *Gb'hb* may be involved in the cell rearrangement for morphogenesis in the germband.

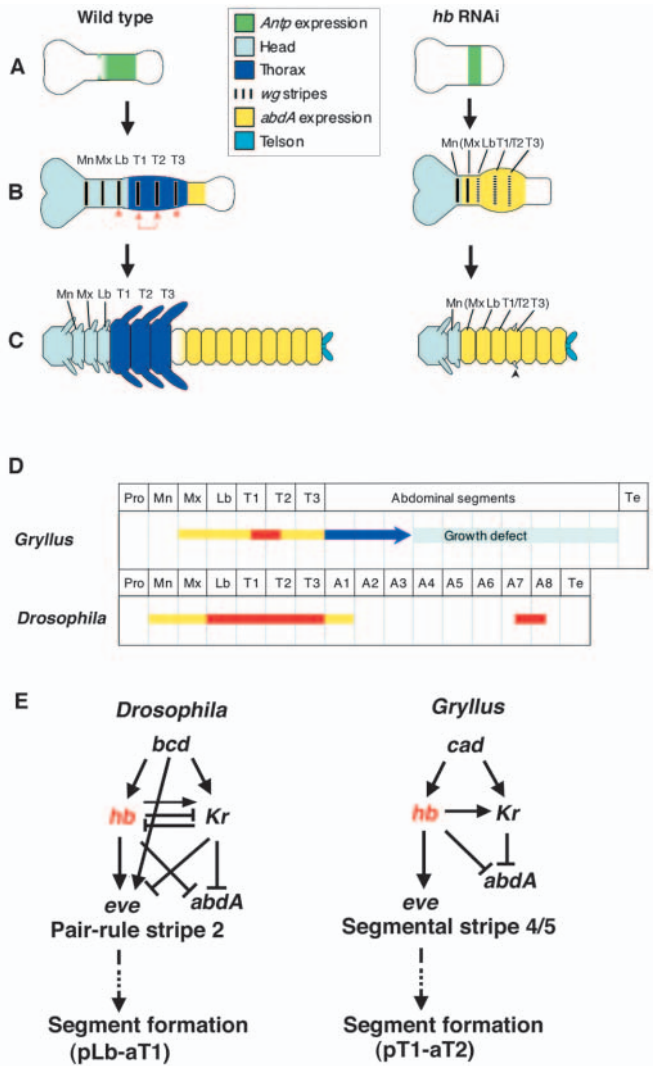


**Fig. 7.** Schematic drawing of the effects of *Gb'hb* RNAi during embryogenesis. All embryos are shown from a ventral view. (A) Wild-type and *Gb'hb* RNAi embryos at early germband stage (38-42 hAEL). In *Gb'hb* RNAi, early germbands are shortened and flattened. Reduction of *Gb'Antp* expression domain indicates that the prospective thoracic region is reduced in *Gb'hb* RNAi embryos. (B) Wild-type and *Gb'hb* RNAi embryos at the beginning of germband elongation. Vertical lines on the embryos represent the anterior segmental stripes of *Gb'wg*. The stripes affected by *Gb'hb* RNAi segments are indicated by red arrowheads in the wild-type embryo, with the deleted region (posterior T1 to anterior T2) shown by a red bracket. In *Gb'hb* RNAi, the *Gb'wg* stripes indicated by dotted lines are not eliminated but irregularly shaped. *Gb'abdA* is expressed in a limited domain in the posterior region of the wild-type embryo, while its ectopic expression is induced in the anterior region of the *Gb'hb* RNAi embryos. Accordingly, the segment identities of *Gb'hb* RNAi embryos change in the prospective mandible to T3 segments (labeled by parenthesized abbreviations). (C) Wild-type and *Gb'hb* RNAi embryos at stage 9. The *Gb'hb* RNAi phenotype is a combination of the transformation of the gnathal and thoracic regions (the maxillary to T3 segments) towards the abdominal identity and the reduction of the number of segments in the abdomen. The vestiges of T3 legs (arrowhead) are observed in most RNAi embryos. The phenotype would also represent the deletion of one segment, as inferred from the modification of *Gb'wg* stripe pattern resulting from the fusion of the T1 and T2 segments, though it was not obvious from the inspection of embryos in later stages because of segmentation disturbances. (D) Comparison of the *hb* functions in *Gryllus* and *Drosophila*. Red bars indicate deletions in the affected regions, while yellow bars indicate transformation into abdominal identity (or more posterior identity in the A1 segment of *Drosophila*). For *Drosophila*, the case of the class V alleles (Lehmann and Nüsslein-Volhard, 1987) is shown. In *Gryllus*, only three abdominal segments (dark blue arrow) are formed in severe cases of *Gb'hb* RNAi depletion, probably owing to the defect in the posterior growth accompanied by segmentation. (E) Models for regulatory networks of *hb* and other segment-patterning genes in the anterior region of *Drosophila* and *Gryllus*. In *Drosophila*, *bcd* activates *hb* and *Kr* in the anterior region of the embryo. *hb* and *bcd* activate *eve* (stripe 2), while *Kr* represses it. In *Gryllus*, *hb* is activated by *cad* and regulates the *Kr* and *eve* expression in the thoracic region, directly or indirectly. *Gryllus hb* and probably *Kr* repress *abdA* as in *Drosophila* (see text).

### *Gb'hb* functions as a gap gene in the anterior region

In *Drosophila hb* mutants, the region between the labial and metathoracic segments is known to be deleted. In *Gb'hb* RNAi embryos, we were not able to find such a large deletion in the anterior region. However, precise analysis of the expression patterns of *Gb'wg* and *Gb'eve* stripes in *Gb'hb* RNAi embryos revealed that a narrow region was deleted. In wild-type *Gryllus* embryos, the *Gb'wg* stripes are located at the anteroposterior boundary of each segment (Miyawaki et al., 2004; Niwa et al., 2000), as shown in Fig. 7B; in the *Gb'hb* RNAi embryos the T1 and T2 stripes of *Gb'wg* were fused, indicating that the region from the posterior T1 to the anterior T2 was deleted (indicated by a bracket in Fig. 7B). This is supported by the fact that segmental stripes 4 and 5 of the *Gb'eve* were not observed in the *Gb'hb* RNAi embryos. These results indicated that *Gb'hb* functions as a gap gene in a limited region from the posterior T1 to the anterior T2 (Fig. 7D). We can not deny that more segments would be deleted in more severe *Gb'hb* phenotype, because in our RNAi experiments we are unable to confirm a complete knockdown of *Gb'*Hb activity. However, the most severe class of the *Gb'hb* phenotypes that we observed is likely to result from the strongest depletion of *Gb'hb,* because appearance frequency of the most severe phenotype increased with injected amount of dsRNA for *Gb'hb* without change in the phenotype itself (data not shown). This presumption is supported by the fact that the anterior deleted region was not extended in *Gb'hb* RNAi embryos, when more severe effects of RNAi on abdominal regions appeared.

It is very mysterious that the segmental deletion appears in the thoracic region where *Gb'hb* transcripts were not detected. Similar phenomena were reported for gap gene orthologs of other short/intermediate germ insects such as *Oncopeltus* (Liu and Kaufman, 2004) and *Tribolium* (Bucher and Klingler, 2004). The following two possibilities might explain the phenomenon: one is that *Gb'hb* indirectly regulates the expression of pair-rule genes, such as *Gb'eve* in the thoracic region through *Gb'Kr* and/or other downstream genes; the other is that very low levels of *Gb'hb* expression could directly regulate the expression of pair-rule genes in the thoracic region. The latter possibility may be supported by the fact that in grasshopper embryos, transcripts of *hb* and its proteins exhibit distinct stepped expression levels in the gnathal/thoracic region, i.e. high levels in the gnathal region and low levels in the T1 region (Patel et al., 2001).

### Maternal functions of *Gb'hb* are unknown

We detected maternal transcripts in *Gryllus* eggs using RT-PCR. However, in *Schistocerca*, which belongs to Orthoptera along with *Gryllus*, the Hb protein rather than the *hb* transcript appears to be maternally provided (Patel et al., 2001). It remains unclear whether maternal transcripts or proteins of *Gb'hb* functions in the early stages. Because our embryonic and parental RNAi experiments exhibited no qualitative differences and the embryonic RNAi yielded the severest class of phenotypes at a higher rate than the parental RNAi, the *Gb'hb* RNAi phenotypes we obtained seem to be a result of the depletion of zygotic transcripts. Thus, we could not estimate the contribution of the maternal transcripts of *Gb'hb* or its proteins to the RNAi phenotypes.

### *Gb'hb* determines anterior identities through regulation of expression of the Hox genes

We found that the maxillary, labial and thoracic segments were homeotically transformed into abdominal identity in the late stages of *Gb'hb* RNAi embryos, while the anterior head and mandibular segment remained intact (Fig. 7C,D). A similar transformation has been reported in *Of'hb* RNAi embryos (Liu and Kaufman, 2004). As we observed that the ectopic expression of *Gb'abdA* in the maxillary, labial and thoracic segments is induced in early *Gb'hb* RNAi embryos not later than the stage when normal expression of *Gb'abdA* begins in the abdominal region (Fig. 7B), *Gb'hb* probably suppresses the abdominal identity in the maxillary, labial and thoracic segments by repressing the *Gb'abdA* expression during early embryogenesis, which is consistent with a report for *Of'hb*.

As the homeotic transformation occurs in a much larger region than the *hb* expression domain, it is also mysterious how *Gb'hb* regulates the expression of homeotic genes. In *Oncopeltus*, *Of'hb* was speculated to indirectly regulate the expression of *Of'abdA*, because ectopic expression of *Of'abdA* was not detected in the blastoderm stage (Liu and Kaufman, 2004). This may be true in the thoracic region of the *Gryllus* embryos, because the T3 segment is the most resistant to the homeotic transformation into abdominal identity caused by *Gb'hb* RNAi depletion. Because the expression of *Gb'Kr* was reduced in *Gb'hb* RNAi embryos, and we know that both *hb* and *Kr* act as *abdA* repressors in *Drosophila* (Casares and Sánchez-Herrero, 1995; Shimell et al., 2000), *Gb'Kr* appears to be a candidate of *Gb'abdA* repressor in the thoracic region, although the precise mechanism for induction of the *Gb'Kr* expression by *Gb'hb* remains to be clarified.

We also observed that *Gb'Antp* was expressed in the prospective labial and thoracic segments in the early stages (Fig. 7A). In the shortened and widened *Gb'hb* RNAi embryos, the *Gb'Antp* expression domain was severely reduced (Fig. 7A). Thus, *Gb'hb* regulates expression of *Gb'Antp,* directly or indirectly. The reduced *Gb'Antp* expression domain may correspond to the region most resistant to the homeotic transformation into abdominal identity caused by *Gb'hb* RNAi depletion, probably overlapping the *Gb'Ubx* expression and leading to T3 segment identity (Zhang et al., 2005). This should be closely related to the formation of the residual leg-like structures observed in *Gb'hb* RNAi embryos (Fig. 7C).

In the late stages, *Gb'hb* RNAi depletion also affected the expression patterns of *Gb'Scr*, *Gb'Antp* and *Gb'Ubx*. We found that *Gb'hb* is required for the expression of *Gb'Scr* in the labial and T1 segments, for the suppression of *Gb'Antp* expression in the maxillary segment, and for the suppression of *Gb'Ubx* expression in the region from the maxillary to T2 segments. Our results suggest that *Gb'hb* determines the anterior segment identities through regulation of expression of the Hox genes, as found in *Drosophila*.

### Regulatory network among *Gryllus* segmentation genes

Fig. 7E shows a regulatory network involving *hb* in *Drosophila* and a putative one in *Gryllus*. In *Drosophila*, the prolonged syncytial stage allows transcription factors to diffuse freely between the adjacent nuclei and exert their functions by forming diffusion-controlled gradients. Bicoid (Bcd) activates

*hb* and *Kr* in the anterior region of the early *Drosophila* embryo with its morphogenetic gradient (Driever and Nüsslein-Volhard, 1989; Hoch et al., 1991). In turn, the morphogenetic gradient of the Hb protein organizes the expression of the other gap genes *Kr* and *knirps* (*kni*) (Hülskamp et al., 1990). Hb acts as an activator of *Kr* and a repressor of *kni* at low levels, while high levels of Hb repress the *Kr* expression (Hülskamp et al., 1990). These gap genes define the expression domains of pair-rule genes, such as *eve*, to generate their periodic expression patterns. The functions of *hb* and *Kr* as an activator and a repressor, respectively, for the second pair-rule stripe (stripe 2) formation of *eve* have been extensively investigated (Small et al., 1991; Stanojevic et al., 1991). Additionally, *hb* regulates other pair-rule stripes of *eve* (stripe 3) and other pair-rule genes, e.g. *runt* and *paired* (Gutjahr et al., 1993; Klingler et al., 1996; Small et al., 1996).

In *Gryllus* (Fig. 7E), it was shown that *Gryllus caudal* (*Gb'cad*), instead of *bcd*, organizes the gap domains of *Gb'hb* and *Gb'Kr* (Shinmyo et al., 2005). Our *Gb'hb* RNAi analysis suggested that *Gb'hb* activates *Gb'Kr,* directly or indirectly, although more data are needed to establish this regulatory interaction. Furthermore, we found that *Gb'hb* directly or indirectly regulates the *Gb'eve* expression in the prospective thoracic region of the embryo, suggesting that the hierarchical relationship between *hb* and *eve* is conserved between *Gryllus* and *Drosophila*. Our results also suggested that the *Gb'abdA* expression is suppressed by *Gb'hb* and *Gb'Kr*, directly or indirectly, in the anterior region, as in *Drosophila*. Thus, although the maternal morphogenetic organizer in the regulatory network of segmentation may have switched from *cad* to *bcd* during insect evolution, its downstream relationship in the segmentation and Hox genes appears to have been mostly conserved between *Gryllus* and *Drosophila* (Fig. 7E).

### Evolution of *hb* functions in insect segmentation

In spite of these conserved aspects of the putative regulatory network, we found that *Gb'hb* functions differ considerably from those of *Drosophila hb* in segment formation (Fig. 7D). Although we cannot deny that more segments might be deleted in *Gryllus*, as observed in the *Drosophila hb* mutant, it is likely that *Gb'hb* plays the role of a gap gene that patterns fewer segments than in *Drosophila*. Interestingly, in the *hb* RNAi phenotypes of *Oncopeltus,* no segmentation gap was observed in the gnathal and thoracic regions, suggesting that the requirement of the *hb* gap function in this species is minimal, if at all present (Liu and Kaufman, 2004). On the contrary, *Tribolium hb* was reported to have the anterior gap function similar to *Drosophila* (Schröder, 2003). In *Tribolium*, (a short germ insect) some of the anterior segments are patterned under syncytial conditions (Sommer and Tautz, 1993). These lines of evidence suggest that the number of anterior segments regulated by *hb* increased during evolution from cellular to syncytial segmentation, or that the canonical function of *hb* may have evolved from the non-canonical functions of an ancestral *hb* during insect evolution. Although the anterior *hb*-expression domain appears to be fundamentally conserved in insects, the number of pair-rule stripes of the pair-rule genes regulated by *hb* in the anterior region may increase during evolution, probably owing to modification of the *cis*-regulatory elements of the pair-rule genes. Comparative analyses of the *cis*-regulatory elements of the pair-rule genes would shed some light on this issue.

In short/intermediate germ insects, the posterior segments form sequentially from the posterior growth zone during germband elongation. We found that *Gb'hb* is required in the formation of at least seven posterior segments. In addition to our finding, *Of'hb* was found to be expressed in the posterior growth zone and involved in growth and segmentation (Liu and Kaufman, 2004). On the other hand, in *Drosophila*, *hb* is reported to be required only for the formation of the A7 and A8 segments (Lehmann and Nüsslein-Volhard, 1987) (Fig. 7D). These facts suggest that the *hb* function in the posterior region may have been reduced during evolution from the short/intermediate to long germ embryogenesis. As *hb* is similarly expressed in the prospective A7 to A9 segments in *Gryllus* and *Drosophila*, the *hb* functions in these segments may have been conserved in both short/intermediate and long germ embryogenesis. Recently, Peel and Akam (Peel and Akam, 2003) proposed an assumption that the posterior sequential segmentation in short/intermediate germ insects is controlled by a Notch signaling-dependent segmentation clock. If it is the case, *Gb'hb* might be involved in regulating segmentation clock. It is also possible that *Gb'hb* controls posterior growth through regulation of the morphogenesis of the germband, because the shape of the elongating posterior region was affected by *Gb'hb* RNAi depletion (Fig. 7B). Further precise analyses of posterior segmentation in short/intermediate germ insects will be required to elucidate its mechanisms.

We thank K. Matsushima, A. Takeuchi, C. Kobayashi and W. Shinahara for their technical assistance. We also thank the anonymous reviewers whose comments were very helpful. This work was supported by a grant from the Ministry of Education, Culture, Sports, Science and Technology of Japan to T.M., I.S., O.H. and S.N.

## References

**Bender, M., Turner, F. R. and Kaufman, T. C.** (1987). A developmental genetic analysis of the gene *Regulator of postbithorax* in *Drosophila melanogaster. Dev. Biol.* **119**, 418-432.

**Bucher, G. and Klingler, M.** (2004). Divergent segmentation mechanism in the short germ insect *Tribolium* revealed by *giant* expression and function. *Development* **131**, 1729-1740.

**Bucher, G., Scholten, J. and Klingler, M.** (2002). Parental RNAi in *Tribolium* (Coleoptera). *Curr. Biol.* **12**, R85-R86.

**Casares, F. and Sanchez-Herrero, E.** (1995). Regulation of the infraabdominal regions of the bithorax complex of *Drosophila* by gap genes. *Development* **121**, 1855-1866.

**Davis, G. K. and Patel, N. H.** (2002). Short, long, and beyond: molecular and embryological approaches to insect segmentation. *Annu. Rev. Entomol.* **47**, 669-699.

**Dearden, P. K. and Akam, M.** (2001). Early embryo patterning in the grasshopper, *Schistocerca gregaria*: *wingless*, *decapentaplegic* and *caudal* expression. *Development* **128**, 3435-3444.

**Driever, W. and Nüsslein-Volhard, C.** (1989). The bicoid protein is a positive regulator of *hunchback* transcription in the early *Drosophila* embryo. *Nature* **337**, 138-143.

**Gutjahr, T., Frei, E. and Noll, M.** (1993). Complex regulation of early *paired* expression: initial activation by gap genes and pattern modulation by pair-rule genes. *Development* **117**, 609-623.

**Hoch, M., Seifert, E. and Jäckle, H.** (1991). Gene expression mediated by cis-acting sequences of the *Krüppel* gene in response to the *Drosophila* morphogens *bicoid* and *hunchback. EMBO J.* **10**, 2267-2278.

**Hülskamp, M. and Tautz, D.** (1991). Gap genes and gradients-the logic behind the gaps. *BioEssays* **13**, 261-268.

**Hülskamp, M., Pfeifle, C. and Tautz, D.** (1990). Morphogenetic gradient of

*hunchback* protein organizes the expression of the gap genes *Krüppel* and *knirps* in the early *Drosophila* embryo. *Nature* **346**, 577-580.

**Hülskamp, M., Lukowitz, W., Beermann, A., Glaser, G. and Tautz, D.** (1994). Differential regulation of target genes by different alleles of the segmentation gene *hunchback* in *Drosophila*. *Genetics* **138**, 125-134.

**Irvine, K. D. and Wieschaus, E.** (1994). Cell intercalation during *Drosophila* germband extension and its regulation by pair-rule segmentation genes. *Development* **120**, 827-841.

**Isshiki, T., Pearson, B., Holbrook, S. and Doe, C. Q. D.** (2001). *Drosophila* neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell* **106**, 511-521.

**Klingler, M., Soong, J., Butler, B. and Gergen, J. P.** (1996). Disperse versus compact elements for the regulation of *runt* stripes in *Drosophila*. *Dev. Biol.* **177**, 73-84.

**Lehmann, R. and Nüsslein-Volhard, C.** (1987). *hunchback*, a gene required for segmentation of an anterior and posterior region of the *Drosophila* embryo. *Dev. Biol.* **119**, 402-417.

**Liu, P. Z. and Kaufman, T. C.** (2004). *hunchback* is required for suppression of abdominal identity, and for proper germband growth and segmentation in the intermediate germband insect *Oncopeltus fasciatus*. *Development* **131**, 1515-1527.

**McGinnis, W. and Krumlauf, R.** (1992). Homeobox genes and axial patterning. *Cell* **68**, 283-302.

**Miyawaki, K., Mito, T., Sarashina, I., Zhang, H., Shinmyo, Y., Ohuchi, H. and Noji, S.** (2004). Involvement of Wingless/Armadillo signaling in the posterior sequential segmentation in the cricket, *Gryllus bimaculatus* (Orthoptera), as revealed byRNAi analysis. *Mech. Dev.* **121**, 119-130.

**Niwa, N., Saitoh, M., Ohuchi, H., Yoshioka, H. and Noji, S.** (1997). Correlation between *Distal-less* expression patterns and structures of appendages in development of the two-spotted cricket, *Gryllus bimaculatus*. *Zool. Sci.* **14**, 115-125.

**Niwa, N., Inoue, Y., Nozawa, A., Saitoh, M., Misumi, Y., Ohuchi, H., Yoshioka, H. and Noji, S.** (2000). Correlation of diversity of leg morphology in *Gryllus bimaculatus* (cricket) with divergence in *dpp* expression pattern during leg development. *Development* **127**, 4373-4381.

**Patel, N. H., Hayward, D. C., Lall, S., Pirkl, N. R., DiPietro, D. and Ball, E. E.** (2001). Grasshopper *hunchback* expression reveals conserved and novel aspects of axis formation and segmentation. *Development* **128**, 3459-3472.

**Peel, A. and Akam, M.** (2003). Evolution of segmentation: rolling back the clock. *Curr. Biol.* **13**, R708-R710.

**Rosenberg, U. B., Schröder, C., Preiss, A., Kienlin, A., Cote, S., Riede, I. and Jäckle, H.** (1986). Structural homology of the product of the *Drosophila Krüppel* gene with *Xenopus* transcription factor IIIA. *Nature* **319**, 336-339.

**Rivera-Pomar, R. and Jäckle, H.** (1996). From gradients to stripes in *Drosophila* embryogenesis: filling in the gaps. *Trends Genet.* **12**, 478-483.

**Sarashina, I., Mito, T., Saito, M., Uneme, H., Miyawaki, K., Shinmyo, Y., Ohuchi, H. and Noji, S.** (2005). Location of micropyles and early embryonic development of the two-spotted cricket *Gryllus binaculatus* (Insecta, Orthoptera). *Dev. Growth Differ.* **47**, 99-108.

**Savage, R. M. and Shankland, M.** (1996). Identification and characterization of a hunchback orthologue, Lzf2, and its expression during leech embryogenesis. *Dev. Biol.* **175**, 205-217.

**Schröder, R.** (2003). The genes *orthodenticle* and *hunchback* substitute for *bicoid* in the beetle *Tribolium*. *Nature* **422**, 621-625.

**Shimell, M. J., Peterson, A. J., Burr, J., Simon, J. A. and O'Connor, M. B.** (2000). Functional analysis of repressor binding sites in the *iab-2* regulatory region of the *abdominal-A* homeotic gene. *Dev. Biol.* **218**, 38-52.

**Shinmyo, Y., Mito, T., Matsushita, T., Sarashina, I., Miyawaki, K., Ohuchi, H. and Noji, S.** (2005). *caudal* is required for gnathal and thoracic patterning and for posterior elongation in the intermediate-germband cricket *Gryllus bimaculatus*. *Mech. Dev.* **122**, 231-239.

**Small, S. and Levine, M.** (1991). The initiation of pair-rule stripes in the *Drosophila* blastoderm. *Curr. Opin. Genet. Dev.* **1**, 255-260.

**Small, S., Kraut, R., Hoey, T., Warrior, R. and Levine, M.** (1991). Transcriptional regulation of a pair-rule stripe in *Drosophila*. *Genes Dev.* **5**, 827-839.

**Small, S., Blair, A. and Levine, M.** (1996). Regulation of two pair-rule stripes by a single enhancer in the *Drosophila* embryo. *Dev. Biol.* **175**, 314-324.

**Sommer, R. J. and Tautz, D.** (1993). Involvement of an orthologue of the *Drosophila* pair-rule gene *hairy* in segment formation of the short germ-band embryo of *Tribolium* (Coleoptera). *Nature* **361**, 448-450.

**Sommer, R. J., Retzlaff, M., Goerlich, K., Sander, K. and Tautz, D.** (1992). Evolutionary conservation pattern of zinc-finger domains of *Drosophila* segmentation genes. *Proc. Natl. Acad. Sci. USA* **89**, 10782-10786.

**Stanojevic, D., Small, S. and Levine, M.** (1991). Regulation of a segmentation stripe by overlapping activators and repressors in the *Drosophila* embryo. *Science* **254**, 1385-1387.

**Tautz, D., Friedrich, M. and Schöder, R.** (1994). Insect embryogenesis–what is ancestral and what is derived? *Development Suppl*. 193-199.

**Tautz, D., Lehmann, R., Schürch, H., Shuh, R., Seifert, E., Kienlin, A., Jones, K. and Jäckle, H.** (1987). Finger protein of novel structure encoded by *hunchback*, a second member of the gap class of *Drosophila* segmentation genes. *Nature* **327**, 383-389.

**Wolff, C., Sommer, R., Schröder, R., Glaser, G. and Tautz, D.** (1995). Conserved and divergent expression aspects of the *Drosophila* segmentation gene *hunchback* in the short germ band embryo of the flour beetle *Tribolium*. *Development* **121**, 4227-4236.

**Zhang, H., Shinmyo, Y., Hirose, A., Mito, T., Inoue, Y., Ohuchi, H., Loukeris, T. G., Eggleston, P. and Noji, S.** (2002). Extrachromosomal transposition of the transposable element *Minos* in embryos of the cricket *Gryllus bimaculatus*. *Dev. Growth Differ.* **44**, 409-417.

**Zhang, H., Shinmyo, Y., Mito, T., Miyawaki, K., Sarashina, I., Ohuchi, H. and Noji, S.** (2005). Expression patterns of the homeotic genes *Scr*, *Antp*, *Ubx*, and *abd-A* during embryogenesis of the cricket *Gryllus bimaculatus*. *Gene Expr. Patt.* **5**, 491-502.