Evolution of developmental systems underlying segmented body plans of bilaterian animals: insights from studies of segmentation in a cricket

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Abstract. Remarkable advances in developmental genetics in the past two decades allow us to approach the evolution of animal design by elucidating the molecular mechanisms underlying divergent body plans. The ancestry and evolution of segmented body plans in the bilaterians has been an active area of investigation in this field of study. Although segmentation mechanisms have been extensively studied for the fruit fly Drosophila melanogaster, Drosophila exhibits an evolutionarily derived mode of development, and molecular mechanisms underlying Drosophila segmentation may be unrepresentative for arthropods, even for insects. We have been studying the developmental system of the cricket, Gryllus bimaculatus, to understand more ancestral and general segmentation mechanisms for insects than those of Drosophila. In Gryllus, anterior segments are specified almost simultaneously, whereas posterior segments are specified sequentially in the extending posterior region. This mode of segmentation is general and probably ancestral for arthropods. Our RNA interference-based analyses of the functions and regulatory interactions of Gryllus orthologues of Drosophila segmentation genes have revealed surprisingly divergent aspects of the segmentation system in Gryllus in comparison with that of Drosophila. For example, the anteroposterior patterning in Gryllus is principally controlled by the caudal (cad) gene, probably without bicoid, unlike Drosophila. Comparisons of regulatory networks of segmentation genes between Gryllus and Drosophila suggest that regulatory interactions between the genes vary among insects, despite conservation of the network component genes. This implies that the molecular mechanisms of segmentation have changed dynamically during insect evolution, whereas the segmented body plan itself has been conserved. We also discuss evolution of developmental systems generating segment patterns in non-arthropod bilaterian animals.

Key words: body plan, developmental system, insect, segmentation

Introduction

The evolution of bilaterally symmetric animals, the bilaterians, and the explosive radiation of body plans at the phylum level in Cambrian time are central issues in paleontology. Remarkable advances in developmental genetics in the past two decades have enabled researchers to approach these issues by elucidating the molecular mechanisms underlying divergent body plans.

Segmented animals have been highly successful in the animal kingdom, thriving in a broad range of terrestrial and marine habitats. It is unclear, however, whether the common ancestor of all bilaterian animals was segmented. A major focus in trying to resolve this issue in recent years has been on elucidating the developmental systems underlying segmented body plans at the molecular level and comparing them among the three obviously segmented phyla: Annelida, Arthropoda and Chordata.

Molecular mechanisms of segmentation in arthropods have been extensively studied for the fruit fly Drosophila melanogaster. However, Drosophila exhibits an evolutionarily derived mode of development, and its segmentation mechanism may not be general for arthropods, even for insects. Comparing the developmental mechanism of Drosophila with those of non-derived insects is essential for understanding the evolutionary process of developmental systems in insects, and comparing the developmental system of arthropods with those of other phyla ought to clarify the ancestral condition in arthropods. We have employed a cricket, Gryllus bimaculatus, for this purpose. Our study of the molecular mechanisms of cricket develop-
ment has provided insights into the evolution of genetic regulatory networks underlying segmentation during insect evolution. It also implies that a feature shared between the segmentation mechanisms of arthropods and vertebrates may be conserved.

In this article, we review our findings of molecular mechanisms of segmentation in the cricket, and discuss the evolution of developmental systems generating segment patterns.

**Segmentation mechanisms in model organisms**

First we briefly review what is known about molecular mechanisms of segmentation in representative model systems. The most detailed understanding of the molecular basis of segmentation has been provided from studies of *Drosophila*. In *Drosophila* embryogenesis, the prolonged syncytial stage allows transcription factors to diffuse freely between the adjacent nuclei and exert their functions by forming diffusion-controlled gradients. Maternal factors, such as the transcription factor Bicoid (Bcd), regulate expression of gap genes, such as *hunchback* (*hb*) and *Krüppel* (*Kr*), in the early *Drosophila* embryo via their morphogenetic gradients. Each of the gap genes regulates the formation of a contiguous set of segments, and mutations in gap genes cause gaps in segmentation. The gap-gene products also diffuse to produce overlapping short-range gradients in the syncytial environment (Hülskamp and Tautz, 1991; Rivera-Pomar and Jäckle, 1996). These short-range gradients define the striped patterns of the primary pair-rule genes (Small and Levine, 1991). The pair-rule genes pattern segments in a double-segment periodicity and their mutants display defects in part of each pair of segments. The pair-rule genes in turn regulate expression of segment-polarity genes in a striped pattern of single-segment periodicity, and a segment pattern is established. In this process, all segments are specified almost simultaneously.

The segmentation process of vertebrate model systems differs largely from that of *Drosophila*. In vertebrates, a metameric pattern is established by the segmentation of the embryonic paraxial mesoderm into transient structures known as somites, which later mature and give rise to the reiterated structures of the trunk such as vertebrae and associated muscles (Rida et al., 2004). As the presomitic mesoderm (PSM) extends at its posterior, somites arise at its anterior. In the process of vertebrate segmentation, morphogenetic gradients and a molecular clock are known to play central roles (reviewed by Aulehla and Herrman, 2004). A fibroblast growth factor (FGF) and Wnt signaling gradient control stepwise setting of the segment boundary position (Dubrulle and Pourquie, 2004; Aulehla et al., 2003), while vertebrate *hairy* homologues, members of the Hairy/Enhancer of the split related (HER) gene family, may be part of the clock mechanism, showing cyclic patterns of expression in the PSM (Palmeirim et al., 1997). In the vertebrate model systems (i.e., mouse, chick, zebrafish, and *Xenopus*), HER genes are expressed in dynamic stripes in the PSM (Rida et al., 2004), which oscillate with the same periodicity as that of the somite formation. The Notch/Delta cell-cell signaling pathway is an additional component of the clock mechanism and linked to HER genes. HER genes are thought to be both the target of Notch signaling and the repressors of Notch pathway genes (Rida et al., 2004).

**Mode of embryogenesis in the cricket**

Since the known segmentation mechanism of *Drosophila* does not indicate clear commonality with that of vertebrates, segmentation seems to have evolved independently in the lineages leading to arthropods and vertebrates. For example, in *Drosophila*, Notch/Delta signaling does not appear to play a role in establishing segmentation of the trunk. However, such a conclusion appears hasty when one considers that the *Drosophila* developmental system may not be representative of insects, as explained below.

Modes of embryogenesis of insects are classified into three groups: short, intermediate, and long germ embryogenesis. *Drosophila* is classified as a long germ insect, in which entire segments are specified almost simultaneously. In short and intermediate germ embryogenesis, only anterior segments are specified at the blastoderm stage and the remaining segments arise later during elongation of the posterior region of the embryo. Since short and intermediate germ modes are widely found across the insect orders, whereas the long germ mode is restricted to the holometabolous insects, it is likely that the mode of short or intermediate germ segmentation is ancestral (reviewed by Davis and Patel, 2002). Thus, we use the cricket *Gryllus bimaculatus*, a phylogenetically basal, intermediate germ insect, as a model system to understand segmentation mechanisms which are more likely to be ancestral and generally representative of insects than *Drosophila*.

During embryogenesis of *Gryllus*, the embryonic rudiment is formed in the posterior region of the egg at about 30 hours after egg laying (Sarashina et al., 2005). Gnathal and thoracic segments are specified in the embryonic rudiment, while abdominal segments are
Evolution of segmentation mechanisms

Figure 1. Comparison of segmentation modes between *Drosophila* and *Gryllus*. In *Drosophila*, a long germ insect, simultaneous segment patterning takes place through the genetic cascade of maternal, gap, pair-rule, and segment polarity genes. Early steps of segment patterning progress in a syncytial condition, so that products of the maternal and gap genes can diffuse in the egg. Bicoid plays an especially important role in early steps of the anterior-posterior pattern formation via its morphogenetic gradient. The developmental process of the intermediate germ insect *Gryllus* is different from *Drosophila*. In a *Gryllus* egg, a germ band is formed in its posterior region. The germ band elongates as development proceeds. Anterior segmentation occurs almost simultaneously, while posterior segmentation takes place sequentially during elongation of the posterior portion of the germ band. Most of the segments are specified in a cellular condition, in contrast to *Drosophila*.

later specified sequentially in the extending posterior region. Figure 1 shows a comparison between segmentation processes in *Drosophila* and *Gryllus*. As mentioned in the last section, in *Drosophila* the segmental pattern is formed through the action of maternal gradients and zygotic gap, pair-rule, and segment polarity genes. All segments are specified almost simultaneously in the syncytial blastoderm embryo of *Drosophila*. In *Gryllus*, on the other hand, the anterior segments are specified almost simultaneously in a pre-existing field of cells, whereas the remaining more posterior segments form sequentially from a posterior growth zone following gastrulation. Since *Gryllus* embryos are cellularized throughout the segmentation process, it is inferred that a cell-cell signaling system would be necessary in lieu of the protein diffusion system seen in *Drosophila*. Additionally, *Gryllus* probably does not use the Bcd system for anterior-posterior patterning, since the *bcd* gene was acquired in the line-

eage of higher dipterans and thus the determination of the anterior-posterior polarity by Bcd is a derived developmental mechanism, limited to a derived group of flies (cycorrhaphans) (McGregor, 2005). Thus, since the mode of segmentation in *Gryllus* largely differs from that in *Drosophila*, the mechanism underlying it could be divergent as well.

Molecular mechanisms of segmentation in the cricket

The cricket, *Gryllus bimaculatus*, is amenable to functional analyses of genes via RNA interference (RNAi), either by introducing double-stranded RNA (dsRNA) into an egg (embryonic RNAi) or an individual (Figure 2). Application of parental RNAi, a phenomenon in which female adult crickets injected with dsRNA produce progeny showing an RNAi knockdown phenotype (Figures 2.3 and 2.4), has drastically improved the efficiency of functional analysis of
Gryllus genes. In this section, we describe the molecular mechanisms of segmentation in Gryllus, which have been revealed by RNAi approaches.

How is the anterior-posterior axis determined in Gryllus, which is thought not to possess a bcd gene? To address this question, Shinmyo et al. (2005) focused on the caulal (cad) gene, which is involved in the formation of abdominal structures in Drosophila, by analyzing the function of its orthologue in Gryllus using RNAi. We found that reduction of the Gb'cad expression by RNAi results in deletion of the gnathal, thorax, and abdomen in embryos, leaving only the anterior head (Figures 2.1 and 2.2), suggesting that, in Gryllus, cad is required for formation of a surprisingly larger part of the body than in Drosophila. Such cad function may be conserved in short and intermediate germ insects, because a similar cad phenotype was reported in the short germ beetle, Tribolium castaneum (Copf et al., 2004). For the formation of the head region, Gryllus orthodentine (otd) also appears to be essential (Mito et al., unpublished data).

As for gap genes, we have examined the roles of the gap gene orthologues, Gryllus hb (Gb'hb) and Gryllus Kr (Gb'Kr). We found that RNAi knockdown of Gb'hb results in a gap-like phenotype, which lacks gnathal and thoracic segments. Detailed analyses of the Gb'hb phenotype revealed that the phenotype results from both transformation of gnathal and thoracic regions into abdominal identity and reduction of the number of abdominal segments (Mito et al., 2005) (Figure 2.5). This differs from the typical gap phenotypes caused by hb depletion in Drosophila and Tribolium, which exhibit deletion of contiguous segments in the gnathal and thoracic regions (Lehmann and
Nüsslein-Volhard, 1987; Schröder, 2003), but resembles the phenotype of the intermediate germ insect, Oncopeltus fasciatus (Liu and Kaufman, 2004). On the other hand, Gb’Kr apparently acts as a gap gene in a manner similar to Drosophila, since Gb’Kr RNAi results in several contiguous segments in the thorax and abdomen (Mito et al., 2006). These results suggest that the gap patterning system, a system of patterning several contiguous segments, operates in Gryllus. However, it is unclear how the Gryllus gap genes act in the cellular environment, where transcription factors cannot diffuse to produce morphogenetic gradients, as in Drosophila.

We further examined conservation of pair-rule patterning, which is downstream of gap patterning in the Drosophila segmentation hierarchy. We found that applying RNAi for the pair-rule gene orthologue, Gryllus even-skipped (Gb’eve), results in segment fusion in alternative segmental units in the anterior region of the embryo (Mito et al., in press). This suggests that Gb’eve acts as a pair-rule gene at least during anterior segmentation.

In later stages of Gryllus segmentation, abdominal segments are sequentially determined as the posterior region of the embryo elongates. Miyawaki et al. (2004) demonstrated using RNAi that the Wingless (Wg)/Armadillo (Arm) signaling pathway (a canonical Wnt signaling pathway) is required for posterior segmentation. Since a Wnt signaling pathway is involved also in vertebrate segmentation, a common mechanism of sequential segmentation involving Wnt/Wg could exist in Gryllus and vertebrates. In addition, Gb’eve may be also involved in posterior elongation, because its RNAi depletion results in severe shortening of the abdomen (Mito et al., in press).

Our present knowledge about the Gryllus segmentation process can be summarized as follows. First, the anterior-posterior axis is determined by Gb’cad and probably Gb’ott without the Bcd system. Next, in the process of anterior segmentation, gap and pair-rule patterning occur despite a cellularized environment. The remaining posterior segments are then formed sequentially under the control of the Wg/Arm signaling pathway and Gb’eve.

**Evolution of developmental systems in insects**

Information about functions of genes involved in segmentation of the cricket has been obtained by RNAi approaches as described above. In addition, RNAi reveals the regulatory interactions between these genes. For example, it has been shown that expression of Gb’hb and Gb’Kr is repressed in Gb’cad RNAi embryos, suggesting that Gb’cad activates these gap genes (Shinmyo et al., 2005). In some cases of Gb’cad RNAi, expression domains of Gb’hb and Gb’Kr shift to a more posterior region. This fact, together with the observation that the distribution of Gb’cad mRNA in wild-type embryos shows a posterior to anterior gradient, suggests that the expression domains of Gb’hb and Gb’Kr may be determined by a series of concentration thresholds of Gb’Cad in Gryllus embryos: The Gb’hb and Gb’Kr expression domains may be defined by low and middle concentrations of Gb’Cad, respectively (Shinmyo et al., 2005). In addition, analyses of Gb’hb and Gb’Kr RNAi embryos reveal that Gb’hb activates Gb’Kr and that Gb’Kr represses Gb’hb (Mito et al., 2005, 2006). Furthermore, since inhibition of the Wg/Arm signaling pathway by RNAi for Gb’arm results in elimination of Gb’cad expression, it is suggested that the Wg/Arm signaling pathway positively regulates expression of Gb’cad (Shinmyo et al., 2005).

Figure 3.1 shows regulatory networks among genes involved in segmentation in Gryllus, which have been revealed through our RNAi analyses. In Gryllus, Gb’cad regulates the gap genes, Gb’hb and Gb’Kr. Gb’hb and Gb’Kr mutually regulate their expression, positively or negatively. Gb’Kr also regulates Gb’eve. The Wg/Arm signaling pathway regulates Gb’cad expression in the posterior region of the embryo to control posterior elongation and segmentation. In comparison with the Drosophila networks (Figure 3.2), conserved and divergent aspects of the regulatory networks of segmentation genes in Gryllus are obviously shown. In Drosophila, bcd regulates hb and Kr expression, whereas in Gryllus, cad regulates them. There is a difference in regulation of eve expression by gap genes between Gryllus and Drosophila. The regulatory interaction between hb and Kr is fundamentally conserved in these species. Furthermore, the Wg/Cad system operating in abdominal segmentation in Gryllus has not been found in Drosophila. A counterpart of the Wg/Cad system exists in vertebrates. It is known that, prior to somite formation in vertebrates, the action of the Wnt signaling pathway is required for the correct patterning of the posterior embryo, mediated by the Drosophila cad-related gene, Cdx (reviewed in Lohnes, 2003). Thus, it is suggested that a common mechanism underlies sequential segmentation in Gryllus and vertebrates.

The evolutionary process of segmentation mechanisms in insects can now be inferred as follows. Fundamental gap and pair-rule patterning systems, which included at least some of the components of these systems known from Drosophila, evolved prior to the di-
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1

Gryllus
Cellularized embryo

hb  Wg/Arm
Kr

eve
posterior growth

head and thorax
abdomen

2

Drosophila
Syncytial embryo

bcd
hb

Kr

eve

head, thorax, and abdomen

Figure 3. Models for regulatory networks of segmentation genes in Gryllus and Drosophila. Relationship between genes does not necessarily imply direct regulation. 1. In Gryllus, Gb’cad activates Gb’Kr and Gb’hb. Gb’hb activates Gb’Kr expression, while Gb’Kr represses Gb’hb expression in the thoracic region. Gb’Kr is required for Gb’eve thoracic stripes corresponding to T1 and T2. Gb’Kr is also involved in segmentation in the posterior region, possibly via expression of other gap genes. 2. In Drosophila, bcd activates Kr and hb. Hb activates Kr at low levels and represses it at high levels (asterisk). Kr acts as a repressor of hb and eve in the thoracic region.

vergence of crickets and flies, since these systems operate in Gryllus. Furthermore, the anterior-posterior patterning system organized by cad is ancestral for insects. In the evolutionary process of higher dipterans, the bicoid gene evolved to organize the anterior-posterior axis, taking over the activation of hb by cad. On the other hand, the Wg/Cad system to regulate sequential segmentation was lost during the evolution of the long germ mode of segmentation exhibited by Drosophila.

Ancestry of segmentation systems in bilaterians

Based on phylogenetic trees constructed with molecular data, bilaterian animals fall into three distinct lineages: the deuterostomes, ecdysozoans, and lophotrochozoans (Aguinaldo et al., 1997). Each clade contains both segmented (vertebrates in the deuterostomes, arthropods in the ecdysozoans, and annelids in the lophotrochozoans) and unsegmented animals. Therefore, the following three evolutionary patterns of segmentation during bilaterian evolution can be hypothesized (Davis and Patel, 1999): (1) A segmented bilaterian common ancestor lost the segmented body plan at least three times in each of the above lineages. (2) Segmentation arose twice, in the lineages leading to protostomes (ecdysozoans and lophotrochozoans) and vertebrates, respectively. (3) Segmentation arose independently three times in the lineages leading to vertebrates, arthropods, and annelids.

In the annelid Platynereis dumerilii, the orthologues of the Drosophila segment-polarity genes engrailed and wingless are expressed as segmental stripes during segment formation, similar to arthropods (Prud’homme et al., 2003). This result was interpreted by the authors as molecular evidence of a segmented ancestor of protostomes (Prud’homme et al., 2003). However, this claim is still controversial, because some of the segment-polarity gene orthologues in three other annelid species (Capitellida sp. I, Hydroídes elegans, and Chaetopterus sp.) are not expressed in a striped segmental pattern (Seaver et al., 2001; Seaver and Kaneshige, 2006).

Stollewerk et al. (2003) demonstrated using an RNAi approach that in the spider Cupiennius salei, the Notch/Delta pathway is utilized in posterior segmentation, in which segments form sequentially in an anterior to posterior progression. In addition, a previous study also showed a dynamic pattern of hairy expression in the posterior region of Cupiennius embryos, also resolving into a pattern of stripes (Damen et al., 2000). These facts suggest that similarity between somitogenesis in vertebrates and posterior segmentation in arthropods is not merely morphological in nature, and that segmentation may be ancestral to vertebrates and arthropods. Our finding of the Wg/Cad system in Gryllus possibly homologous to the Wnt/Cdx in vertebrates also supports this view. However, it should be noted that the use of Notch/Delta signaling in spider segmentation could have still evolved independently of vertebrates, namely repre-
senting an independent cooption, because the Notch/Delta signaling is used to make boundaries in structures other than trunk segments even in *Drosophila*.

Clearly, we need to obtain further information of molecular mechanisms of segmentation from segmented animals to examine which hypothesis is most likely. However, comparisons of the molecular mechanisms may be insufficient to clarify the evolutionary process of segmentation, because we cannot rule out the possibility of an independent cooption of homologous genes into analogous developmental systems. It would be required to integrate evolution of segmented morphologies and their underlying molecular mechanisms into the framework of the evolutionary history of bilaterians. Budd (2001) examined the segmentation of the arthropod epidermis on the basis of the fossil record and the extant animals of the phyla closely related to arthropods. From the point of view of evolution of functional morphology, the author suggested that the epidermal segmentation evolved gradually in the lineage leading to arthropods, and thus at least some aspects of segmentation may have been independently derived in arthropods. Such an approach based on morphological and paleontological data would define the framework of evolutionary changes of developmental systems in the ancestry of segmentation.

**Concluding remarks**

Most of the segmentation genes identified by studies of *Drosophila* development are likely to be found in other insects. However, the regulatory networks of those genes vary among insects, as shown by our study of the *Gryllus* segmentation system. In insects, the derived segmentation mechanism of *Drosophila* evolved from an ancestral segmentation mechanism with a commonality to the vertebrate one. This implies that molecular mechanisms of segmentation changed dynamically during insect evolution, whereas the segmented body plan itself remained substantially unchanged. Such evolutionary changes in the molecular mechanisms should be mainly attributed to alterations in cis-regulatory elements of segmentation genes (Carroll et al., 2001). In addition to further functional analyses of genes involving *Gryllus* embryonic patterning, analyses of cis-regulatory DNA of those genes are now underway.

**References**


