Determination and Formation of the Basic Body Pattern in Embryo of the Domesticated Silkmoth, *Bombyx mori* (Lepidoptera, Bombycidae)

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Synopsis

The determination and establishment of basic body pattern of the *Bombyx* germband are discussed chiefly from the morphological point of view. The anterior-posterior and ventral-dorsal polarities are distinguished easily in the newly laid egg by the shape and ultrastructures, but any genes concerned with the anterior-posterior polarity have not been found and experiments for modification of long axis of the egg have not succeeded. The first step of establishment of anterior-posterior organization of germband is performed by the protrusion of cytoplasmic masses from the germband at the lateral extremities of anterior and posterior parts, resulting in the pyriform germband. Then these cytoplasmic masses are subdivided into the numerous cytoplasmic granules and incorporated by the yolk cells.

From the pyriform stage, the gastrulation begins at the central region of the presumptive head lobe as an invagination and this process extends posteriorly. At the same time, several degenerating cytoplasmic masses appear among the ectodermal cells and/or between the ectoderm and the mesoderm and these masses also are protruded into the yolk. This phenomenon spreads posteriorly with the progress of gastrulation and seems to indicate the first step of establishment of ventral-dorsal organization of germband.

Introduction

The first experimental studies on embryogenesis of the domesticated silkmoth, *Bombyx mori*, were performed by Takami with cauterization (1942—1946). In the series of studies he revealed that the rough range of embryonic and extraembryonic regions, and the presumptive mesodermal and neural regions were predetermined in the newly laid egg. Then Miya (1950, 1952) carried out several experiments on the differentiation of primordial germ cells and the gonad formation using the similar technique and made clear the predetermination of germinal region and the relation between the segmental genital ridges and the primordial germ cells. From these results, Kuwana and Takami (1957) made a fate map of

Bombyx egg at first, and recently this fate map was reconfirmed by Katsuki *et al.* (1980) with the analysis of mosaics induced by super-cooling method.

Furthermore, several genes related to the morphogenesis of embryo were found in *Bombyx* and their genetical, morphological and biochemical studies have been carried out (see Tazima, 1964). These genes are divided into two categories; the one is maternal-effect mutant and the eggs laid by the homozygous female represent all this phenotype, while the other affects the embryogenesis only in the homozygote after fertilization. "Kidney-shaped egg" (*ki*) belongs to the first category and all *ki/ki* female moths lay kidney-shaped eggs, in which all embryos develop no mesodermal tissues either in *ki/ki* or *ki/+* combination, and "*E*-alleles" belong to the second and are all concerned not only with extra markings and supernumerary legs, but also with abnormalities of gonads and genital organs. Further, most of them are lethal in homozygous condition. Although *ki* gene is being re-examined recently from the morphological and biochemical points of view (Sakaguchi *et al.*, 1982; Kawaguchi, unpublished data; Miya, in press), *E*-alleles are not studied from the recent new viewpoint.

Since Yajima's epoch-making experiment (1960), induction of double-cephalon and double-abdomen by centrifugation in embryo of *Chironomus*, problems on formation of the embryonic body pattern have been studies in dipteran insects with centrifugation and UV-irradiation, for example, in *Smittia* (Kalthoff *et al.*, 1982) and *Sciara* (see Sander, 1983).

On the other hand, many genes relating to the morphogenesis of embryo are found in *Drosophila* and their analysis are being performed vigorously (see Lewis, 1978; Sander, 1982, 1983). Comparing with the results obtained in *Drosophila* and other dipteran insects, the studies on such phenomena in *Bombyx* seem to be far behind in this field, and hence in the present review the author intends to re-examine the results obtained in *Bombyx* until now and to make a basis for the future study.

Structure of Mature Egg and Determination of Basic Body Pattern

1. The structure of mature eggs

As the details of structure of the mature *Bombyx* eggs were described in the recent reviews (Miya, 1984a, b), only the brief description will be done in this paragraph from the viewpoint of determination of embryonic body pattern.

The mature egg is a flat spheroid, approximately 1.3 mm long, 1.0 mm wide, and 0.6 mm thick. At the anterior end, there is a micropylar apparatus, and hence the anterior-posterior polarity of the egg is easily distinguishable. One side of the egg is more convex than the other, and the convex side is the ventral side of the egg, at which the germ anlage appears at first. Thus ventral-dorsal polarity also seems to be determined during oogenesis (Fig. 1).

As shown diagrammatically in Fig. 1, the egg is covered with a thick, tough, and elastic chorion, which is formed by the follicular epithelium at the last period of oogenesis. Beneath the chorion there is a complex vitelline membrane, which consists of a very thin outer membrane, an electron-dense outer layer, and a thick inner layer containing the numerous electron-dense irregular granules. As in the most insect eggs, the ooplasm of the *Bombyx* egg is divided into a superficial layer, the periplasm, and inner endoplasm, which occupies the
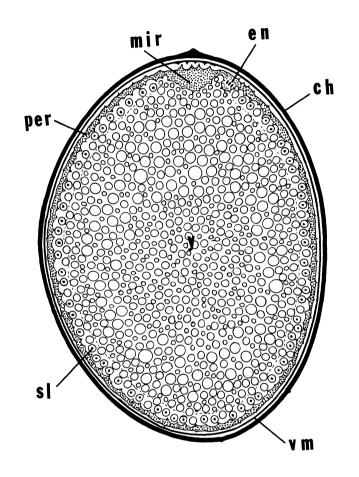
Fig. 1. Diagrammatic figure of the mature *Bombyx* egg showing anterior-posterior polarity (top − bottom) and dorsal-ventral polarity (right − left).
ch chorion, en egg nucleus, mir micropylar region, per periplasm, sl subcortical layer, vm vitelline membrane, y yolk system.
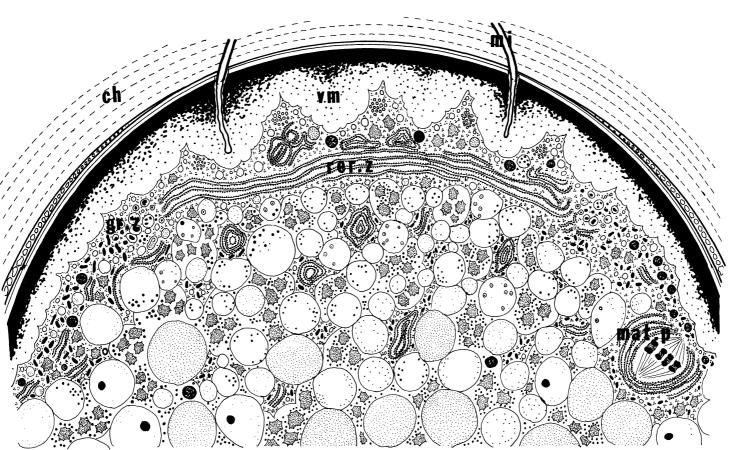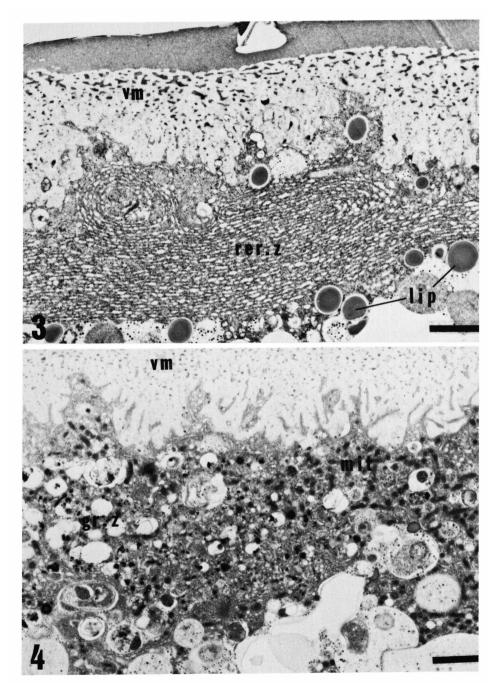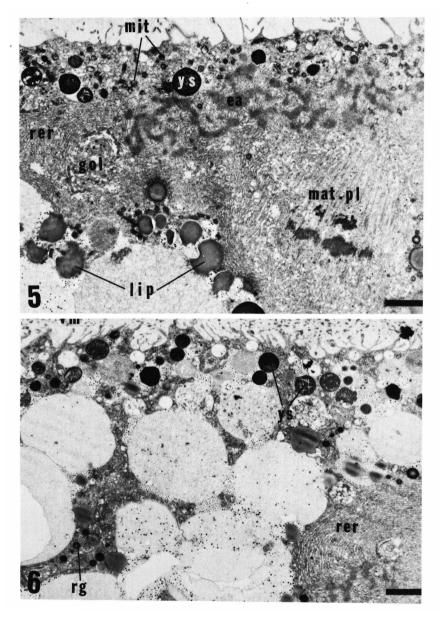
Fig. 2. Diagrammatic figure of the anterior part of mature egg.
ch chorion, gr.z zone of granules, mat.p maturation plasm, mi micropyle, rer.z
zone of rough-surfaced endoplasmic reticulum, vm: vitelline membrane.

Figs. 3, 4. Electron micrographs showing zones of rough-surfaced endoplasmic reticulum (Fig. 3, rer.z) and granules (Fig. 4, gr.z).
lip lipid droplet, mit mitochondria, vm vitelline membrane. Scale: 2 μm.

Fig. 5. Electron micrograph of the anterior polar plasm, in which the egg nucleus shows
        metaphase of the 1st maturation division.
        ea   dense organelles connecting with endoplasmic reticulum, gol: Golgi body, lip
        lipid droplet, mit   mitochondria, rer   rough-surfaced endoplasmic reticulum, ys
        small yolk sphere. Scale: 2 μm.
Fig. 6. Electron micrograph of the ventral periplasm to which a large cytoplasmic mass
        containing rough-surfaced endoplasmic reticulum (rer) attaches.
        rg   round granule with thick cortex, ys   small yolk sphere, vm   vitelline
        membrane. Scale: 2 μm.

central part of the egg and contains yolk organelles in the meshwork. Another special layer, the subcortical layer, lies adjacent to the periplasm. This layer seems to react sensitively to the fixing conditions, and it appears to be thicker and more distinct in the eggs fixed after removing the chorion.

Fig. 2 represents diagrammatically fine structures of the anterior part of the egg. The surface of the micropylar region shows a petal-like pattern, and at its center the micropylar canals open (Kanda et al., 1974; Ohtsuki, 1979). These pattern and canals are formed by three types of cells: the micropylar channel-forming cells, the micropylar orifice-forming cells, and the micropylar rosette-forming cells (Yamauchi and Yoshitake, 1984). In the micropylar region the vitelline membrane becomes thicker than in the other regions, and the periplasm lying under the micropylar canals indicates the peculiar structure, comparing with the other egg regions. This periplasm consists of a relatively thick layer of ooplasm which terminates in a series of small cone-like processes, from which short microprojections protrude into the inner layer of the vitelline membrane. Stacks of endoplasmic reticulum (ER) develop well parallel to the surface of egg (rer. z). Fig. 3 shows the ultrastructure of this zone. Another ooplasmic zone encloses the ER zone, and in this zone many vesicles containing electron-dense granules are observed and slender mitochondria are present numerously(gr. z). Fig. 4 shows the ultrastructure of this granule zone. At the short distance dorsally from the micropylar region, there is the maturation plasm (mat. p), in which the egg nucleus is in metaphase of the first maturation division. Fig. 5 shows the ultrastructure of this plasm, in which the chromosomes and spindle fibers are enclosed by concentric circle-like stacks of rER.

In the periplasm of the other egg regions, there are no remarkable differences except the configuration of rER. In the newly laid egg many pyronin-positive granules were observed attaching to the periplasm (Suzuki, 1969). These structures are constituted of large whorls of rER (Fig. 6, rer; Miya 1984b) and their distribution is limited to the ventral and lateral sides of the egg, the presumptive embryonic region (Kobayashi and Miya, unpublished data).

2. Source and supply of cytoplasmic components in the mature egg

The ovary of Bombyx belongs to the meroistic-polytrophic type and large amount of cytoplasmic components of the egg cells, except for the yolk substance, are produced in the nurse cells and supplied from them (Miya et al., 1969; Yamauchi et al., 1981). Table 1 shows changes of the cytoplasmic components in the nurse cells with the progress of oogenesis. Not all, but almost all of them are supplied to the oocyte corresponding to the developmental stages (Yamauchi et al., 1981).

Table 2 summarizes the source and route of supply of the cytoplasmic components in the mature egg. Among them, the rER seems to have the closest relation to the maternal informations for the embryonic body pattern, at least their expression. As indicated in Table 2, the rER lying under the micropylar region originates from the anterior polar plasm (Yamauchi, unpublished data) and that in the periplasm of the other egg regions is formed from the annulate lamellae derived from the nuclear membrane of nurse cells at the last stage of vitellogenesis.

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Table 1. Stage-dependent changes of cytoplasmic components in nurse cells
(Yamauchi et al., 1981)

Organelles	Previtellogenesis				Vitellogenesis			
Stage	I	II	III	IV	V	VI	VII	VIII
Mitochondria*	++	++	++	+	+	+	++	++
Ribosome*	++	++	++	++	++	++	++	++
r-ER*	+	+	+	+	+	++	++	++
Dense ground granules*	+	+	+	+	+	+	+	+
Golgi bodies*	+	+	+	+	+	+	+	+
Annulate lamellae*	+	+	+	+	+	+	++	+
Lipid droplets*	++	++	++	++	+	+	+	+
Glycogen granules*						++	+	+
Electron dense masses		+	+	++	++	++	++	++
DP masses					+	+	+	+
Microtubules*		+	+	+	+	+	++	++
Pinocytotic vesicles		+	+	+	+	++	++	+
Vacuoles					++	++	+	+

* : inflow into oocyte, ++ : presence of larger amount

Table 2. Source and route of supply of cytoplasmic components in mature egg cell

EGG CELL	FOLLICLE CELLS	NURSE CELLS	HEMOLYMPH

Yolk spheres   a ← .... ← (vitellogenin[1,2]
(proteid yolk)   (b)   (other proteins[2]
                    c ← egg-specific[2]
                          protein
Small yolk sphere ← ..... ?
Lipid droplets ← .... ← diglycerides[3]
(lipid yolk)
Glycogen granules ← .... ← glycogen granules[4]
) ← .... ← trehalose[7]
Mitochondria ← .... ← mitochondria[4,5]
Ribosomes ← .... ← ribosomes[4,5]
Rough-surfaced
   endoplasmic reticulum
   (micropylar region) ← (anterior polar plasm of oocyte)[6]
                                                        nuclear membrane[4]
   (other egg regions) ← .... ← annulate lamellae ←
Golgi bodies ← ..... ?
Round granules with
   thick cortes ← ..... ?

*1   Izumi et al. (1980),   *2   Irie and Yamashita (1983),   *3   Ichimasa (1977),
*4   Yamauchi et al. (1981),   *5   Miya et al. (1969),   *6   Yamauchi (unpublished data),
*7   Yamashita and Hasegawa (1974).

3. Determination of basic body pattern of embryo

Fig. 7 represents the fate map of *Bombyx* egg (Kuwana and Takami, 1957). Because of thick and tough chorion, it is impossible in the *Bombyx* egg that the detailed fate map is made by the defect method with point irradiation of UV-light or others. Hence, Katsuki *et al.* (1980) analyzed the mosaic individuals induced by the super-cooling method introduced by Tamazawa (1977) and made a more detailed fate map. This fate map coincides roughly with the Takami's original one.

As to determination of the anterior-posterior polarity, there are a series of studies on maternal-effect mutants in *Drosophila*, such as *dicephalic* (*dic*) and *bicaudal* (*bic*). *Dic* females produce eggs with anterior markers at both poles and these eggs produce mostly "double anterior" embryos and occasionally a perfect double cephalon. Some egg follicles from homo-zygous *dic* females carry nurse cells at the both poles of the oocyte instead of only the anterior pole and this anomaly results from aberrant arrangement of the normal number of nurse cells (Lohs-Schardin,1982). On the other hand, *bicaudal* homo-zygous females produce eggs which develop double abdomen instead of normal larvae (Nüsslein-Volhard, 1979).

In the *Bombyx* egg, no maternal-effect mutant for the anterior-posterior polarity has been found and experiments for modification of long axis of the egg have not succeeded. The most characteristic structure in the mature *Bombyx* egg is the structure of the anterior part, that is, micropylar apparatus of chorion, ultra-structure of vitelline membrane and ooplasm. These structures seem to relate with sperm entry into the egg and activation of the egg, considering their changes at sperm entry (Miya, 1984b). Though origin and function of the granule zone existing in this region remain unsolved, this zone might have any relation to anterior determinants.

As to determination of the ventral-dorsal polarity, several interesting results are reported in *Drosophila*. A maternal-effect mutant, *dorsal* (*dl*), affects determination of the dorsoventral coordinate and the ventral cells formed no mesoderm, but ventral hypodermis (Nüsslein-Volhard *et al.* 1980). Recently, Anderson and Nüsslein-Volhard (1984) reported the experimental results on "dorsalized" embryos in *Drosophila*. Ten loci have been found which were required for the production of lateral and ventral pattern elements. The lack of each gene function in the mother resulted in an embryonic phenotype identical to the maternal-effect mutation *dorsal*. Injection of RNA isolated from wild-type embryos into the mutant at six loci partially restored ventral-dorsal polarity. For the mutant *snake*, especially, injection of poly $(A)^+$ RNA isolated from wild-type embryos restored a complete ventral-dorsal pattern.

In the *Bombyx* egg, a similar maternal-effect mutant "kidney-shaped egg" (*ki*), was found by Suzuki (1932) and has been studied from genetical, embryological, and biochemical points of view. The homozygous female moths lay eggs, the ventral side of which is slightly concave showing kidney-like shape. The egg died both in homozygous or heterozygous state of *ki* gene without any mesodermal organs, namely the *ki* embryo was constructed with only ectodermal organs, such as epidermis, setae, mouthparts, and so on. Furthermore, the nervous system could not be formed except the brain (Suzuki and Ichimaru, 1955; Miya, in press).

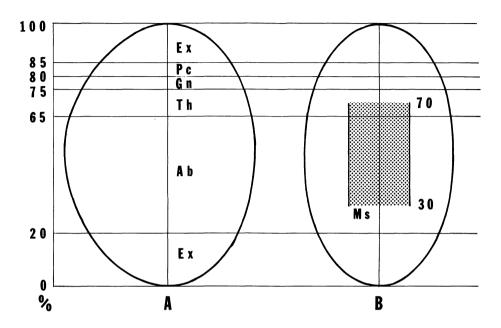Sakaguchi *et al.*, (1982) observed oogenesis of the *ki* egg and reported that the *ki* follicle

Fig. 7. The fate map of *Bombyx* egg (Reproduced from Kuwana and Takami, 1957).
A: lateral view, B: ventral view, Ab abdomen, Ex extraembryonic region, Gn gnathocephalon, Ms mesodermal region, Pc protocephalon, Th thorax.
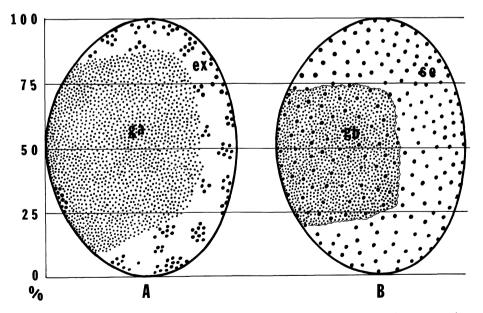

Fig. 8. Diagrammatic figures showing segregation of germ anlage from the extraembryonic blastoderm (A) and completion of the germband and the serosa (B).
ex extraembryonic blastoderm, ga germ anlage, gb germband, se serosa.

cells at the ventral side were narrower and longer than those at the other sides. They suggested that the *ki* gene affected the size and shape of follicle cells at the ventral side, the presumptive embryonic region. However, the comparative studies of ultrastructure between the normal and the *ki* eggs revealed that there were no remarkable differences in the egg cell during oogenesis. Further, no differences could be detected between the normal and the *ki* egg in the qualitative and quantitative changes of yolk proteins or their synthetic capacity during oogenesis (Kawaguchi, unpublished data). Analysis of *ki* gene function might contribute to the solution of ventral-dorsal polarity as a useful clue.

Establishment of Basic Body Pattern of Embryo

1. Anterior-posterior organization

Fig. 8 represents diagrammatically the eggs in which segregation of the germ anlage and the extraembryonic blastoderm (A) and completion of the germband and the serosa (B) occur. The blastoderm cells existing in the embryonic region continue parallel divisions to the surface of egg to increase cell population and to form the germ anlage, and those in the other region become the extraembryonic blastoderm. The area of germ anlage coincides roughly with the embryonic region represented in the Takami's fate map. As mentioned above, many cytoplasmic masses containing large whorls of rER, attach to the periplasm of presumptive embryonic region in the newly laid egg. These stacks of rER break down to form numerous tubular and/or vesicular structures, but the direct relation between such structural changes and the establishment of germ anlage does not yet confirmed.

Yajima's experiments in *Chironomus* suggested that the anterior-posterior polarity predetermined during oogenesis could be changed before the establishment of protocephalic-caudal organization. Afterwards, the similar results have been obtained in *Eucelis* (Sander, 1960), in Smittia (Kalthoff, 1979) and so on. Ripley and Kalthoff (1983) reported that the anterior determinants were cytoplasmic ribonucleoprotein particles existing in a yolk-free cytoplasmic cone observed behind the anterior pole of newly laid egg, diffused posteriorly with the progress of development, and then migrated into the periplasm of the anterior part of the egg.

In the *Bombyx* egg, such anterior determinants have not been found and any experiments have not succeeded in changing the anterior-posterior polarity. Morphologically, the anterior-posterior organization of germband is not yet established until the early germband stage, as shown in Fig. 8B. However, many cytoplasmic masses are protruded from the germband into the yolk at the lateral extremities of anterior part at this stage (Fig. 10A). This phenomenon seems to be the first process of anterior-posterior organization of germband. As shown in Fig. 11, these cytoplasmic masses contain many usual cell organelles, such as mitochondria, rER, and ribosomes. Then the protrusion of cytoplasmic masses occurs in the posteriormost region of germband, and consequently the anterior and posterior parts of germband become visible, resulting in the pyriform germband (Fig. 9A).

At the pyriform stage an invagination occurs in the center of presumptive head lobe of germband and it indicates the first sign of mesoderm differentiation (Figs. 9A, 10B). At this stage the protruded cytoplasmic masses are subdivided into numerous cytoplasmic granules, which are incorporated by phagocytotic activity of the yolk cells, as shown in Figs. 10B and
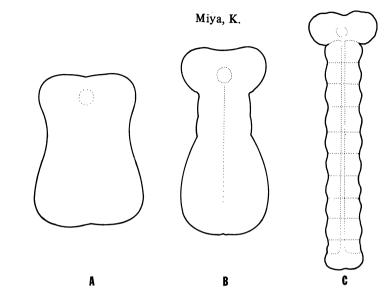
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Fig. 9. Diagrammatic figures of germband at the pyriform (A), the head lobe formation (B),
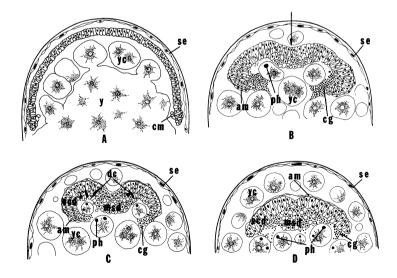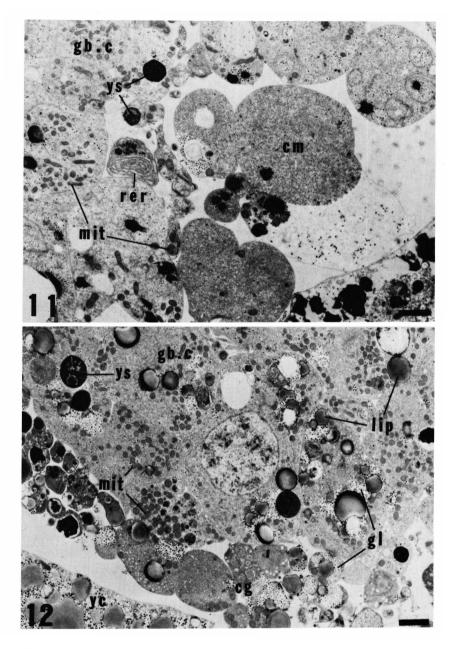and the early diapause (C) stages.

Fig. 10. Diagrammatic figures showing the cross sections through the anterior part of the
germband at the early germband (A), the pyriform (B), the head lobe formation
(C), the early diapause (D) stages.
am amnion, cg cytoplasmic granule, cm cytoplasmic mass, dc degenerating
cytoplasmic mass, ecd ectoderm, msd mesoderm, ph phagosome se serosa, y
yolk system, yc yolk cell, arrow an invagination occurring at the central region
of the presumptive head lobe ÷ the first sign of gastrulation.

Fig. 11. Electron micrograph showing the cytoplasmic masses protruded from the germband

cm cytoplasmic mass, gb.c: germband cell, mit mitochondria, rer rough-surfaced endoplasmic reticulum. Scale. 2 μm.

Fig. 12. Electron micrograph showing the cytoplasmic granules.

cg cytoplasmic granule, gb.c germband cell, gl glycogen granule, lip lipid droplet, mit mitochondria, yc yolk cell, ys small yolk sphere. Scale. 2 μm.
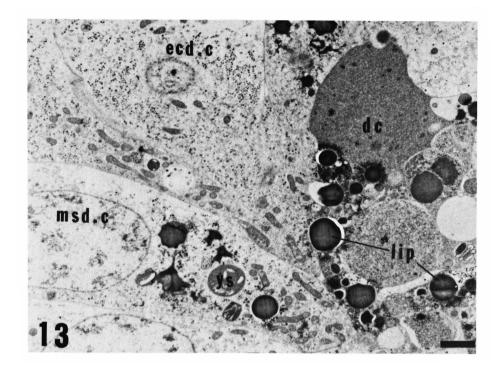
Fig. 13. Electron micrograph showing degenerating cytoplasmic mass inserted between the
ectoderm and the mesoderm.
dc degenerating cytoplasmic mass, ecd.c ectodermal cell, lip lipid droplet,
msd. c mesodermal cell, ys small yolk sphere. Scale: 2 μm.

12. The next stage is the head lobe formation stage, at which the anterior part of germband
develops into a wide head lobe (Fig. 9B). The invagination becomes deeper and extends
posteriorly, and the invaginated cells segregate from the outer layer of germband, the
ectoderm, to differentiate to mesodermal cells (Fig. 10C). With the differentiation of
mesoderm, several degenerating cytoplasmic masses appear, inserted among the ectodermal
cells and/or between the ectoderm and the mesoderm, and then these masses also are
protruded into the yolk and subdivided into the numerous cytoplasmic granules (Figs. 10C,
13, dc). This phenomenon spreads posteriorly with the progress of gastrulation.

From the above-mentioned results, the establishment of anterior-posterior organization in
the *Bombyx* germband seems to accompany the protrusion of cytoplasmic masses into the
yolk at the lateral extremities of anterior part of germband, the presumptive head lobe, and
the posterior part, the presumptive telson. The formation of head lobe results in the
subsequent occurrence of an invagination at its center. This invagination is a first sign of
formation of mesoderm, and then extends posteriorly (Figs. 9C, 10D). This process of
gastrulation seems to indicate the establishment of ventral-dorsal pattern elements.

2. Ventral-dorsal organization

As mentioned above, the ventral-dorsal organization of germband may be closely related to the establishment of anterior-posterior organization, because the formation of head lobe may trigger the initiation of gastrulation, which means the first step of establishment of the ventral-dorsal body pattern.

The maternal-effect mutant, "Kidney-shaped egg", serves as a clue for such a suggestion. In the *ki* germband, protrusion of cytoplasmic masses occurs at the lateral extremities of anterior part of germband as in the normal egg and the anterior-posterior organization is established. However, as development proceeds, such protrusion of cytoplasmic masses spreads not only to the middle part of anterior region, but also to the entire length of germband. Consequently, the normal head lobe is not formed in the anterior region of germband and the process of gastrulation beginning as an invagination in the center of head lobe is inhibited, resulting in no differentiation of mesoderm (Miya, in press).

The similar result was obtained by centrifugation. Some abnormal embryos consisting of only ectodermal tissues were induced in the eggs centrifuged at the early stages, because of the inhibition of gastrulation by the effect of centrifugation (Miya, 1967, 1973). The results on the *ki* germband and the experimental results suggest that the ventral-dorsal organization in the *Bombyx* egg might be established through the process of gastrulation.

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