Histological Studies on the Yolk Granule Formation in the Egg Character Mutant, vit, of Bombyx mori

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The formation of protein yolk spheres during oogenesis in the egg character mutation, vit, of Bombyx mori, was studied by light microscopy and transmission electron microscopy of sections of the resin-embedded follicles. The vit ovary exhibited the normal polytrophic meristic form, in which the follicle has an oocyte, nurse cells and follicular epithelial cells. Also the accumulations of lipid droplets, glycogen granules and small yolk spheres took place normally in the vit oocytes during oogenesis. However, abnormality of the mutant appeared because large protein yolk spheres did not form. Instead, many small spheres were accumulated, which were not observed in the normal. Since the vit egg has been reported to have the egg specific protein but lack the two major yolk proteins, vitellin and the 30 kDa proteins, we infer that the vit mutation affects the incorporation of the precursors for these proteins from hemolymph to oocyte.

Key words: Bombyx mori, egg character mutation, vit, vitellogenesis, protein yolk spheres

INTRODUCTION

In the polytrophic meristic ovary of insects, a developing egg chamber or a follicle consists of an oocyte and plural nurse cells surrounded by a layer of follicular epithelial cells (FCs). The nurse cells and the FCs support the oocyte growth, and the ovarian follicle is considered to be a functional unit in this type of ovarian structure (King and Aggarwal, 1965; King, 1970; Mahowald, 1972; Telfer, 1975; Berry, 1982; Dobens and Raftery, 2000). The ovaries of Bombyx mori, which are also of this type, are comprised of eight ovarioles. In each of these, a series of follicles develop into matured ovarioles, initially utilizing a syncytium connecting the oocyte with seven nurse cells (Ozawa, 1959; Miya and Kurihara, 1966; Miya et al., 1970a,b, Yamauchi et al., 1981, 1984).

One of the most dynamic aspects of oogenesis is an accumulation of yolk proteins in the oocyte. Yolk proteins in B. mori can be classified into two categories according to its origin. The first category is vitellin and 30 kDa proteins synthesized in the fat bodies and the second is the egg-specific protein (ESP) synthesized by the ovaries per se. The hemolymph precursor of vitellin is a papal female protein, vitellogenin (Kawaguchi and Doira, 1973; Wyatt and Pan, 1978). The 30 kDa proteins are larval and pupal hemolymph components and sex non-specific (Izumi et al., 1980; Izumi et al., 1981; Zhu et al., 1986). The synthesis and secretion of ESP are contributed by the FCs (Ono et al., 1975; Irie and Yamashita, 1983).

Many mutations exist in B. mori with respect to the egg characteristics such as the size, shape, color and apparent yolk content (Doira, 1983, 1986). These include a remarkable recessive mutation, which was generated by treating normal eggs with N-methyl-N-nitrosourea. The eggs homogeneous to this mutation are sterile, less in weight than normal and are pure white in color, instead of the pale yellow of normal eggs shortly after oviposition. Thus the mutation was named "Shirotae-ran" (pure white egg) in Japanese and "scanty vitellin" in English, regulated by the gene symbol vit, which was localized at 23.0 centimorgans on the 20th linkage group (Fujikawa et al., 1993b, 1996). These traits of the vit mutation have been ascribed to some defects in the yolk components. This was confirmed to be true by our previous studies (Fujikawa et al., 1993a, 1995), indicating that the mutant eggs scarcely contain vitellin and the 30 kDa proteins, but with a normal amount of ESP. The present article deals with the morphological aspects of the mutant oocytes compared with the normal oocytes, as observed by light microscopy and transmission electron microscopy. We found that the large yolk spheres are not formed in the vit oocytes.

MATERIALS AND METHODS

Insects

The silkworm strain Yd501, maintained in the Faculty of Agriculture, Kyushu University Graduate School, was used. This strain possesses the vit gene. It also includes, as a marker, the recessive gene oh (hoarfrost translucent) belonging to the same linkage group as vit, as well as the dominant sex-limited yellow blood gene Y translocated to the W chromosome {T(W;2)Y}. The mating between Z/(T(W;2)Y); + / oh vit (females) and oh vit / oh vit (males) produces as offspring a mixture of oh-heterozy-

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gotes having the normal skin and oh-homozygotes having the oily skin in the same batch. Despite the skin characteristics, the females can be discriminated at the larval stages by the possession of yellow blood from males with white blood. These offspring were allowed to develop and the resulting animals were raised on mulberry leaves at 25°C during larval instars. The females with the oily skin produced vit eggs, whereas those with the normal skin produced normal eggs. Both types of females were sacrificed for ovarioles during the pupal period or shortly after emergence (unmated). The normal specimens were served as controls.

Sample preparation and histological observations

Freshly dissected ovarioles were washed in saline solution and cut to isolate follicles at the basal positions. These obtained follicles were fixed with 3% glutaraldehyde in 0.2 M sodium cacodylate buffer, pH 7.4, at 4°C, washed, postfixed in 1.3% osmium tetroxide, dehydrated in an ethanol series and embedded in epoxy resin. Then thin sections of 2.0 and 0.1 μm were cut with a glass knife. The former were stained with 0.5% toluidine blue and observed under a light microscope (Carl-Zeiss, Axioplan), while the latter were mounted on copper grids, stained with uranyl and lead acetates and examined under an electron microscope (Hitachi, H-300) at 75 kV.

RESULTS

The ovaries were taken out of the developing pupae, as well as the young adults, having the normal and vit phenotypes that were segregated in the same batches, and they were subjected to histological observations for follicles. Each follicle was allotted to any of the 12 oogenesis stages previously described by Yamauchi and Yoshitake (1984).

Follicles at Stage 5: In the pupae on Day 1 after the larval-pupal ecdysis, the four ovarioles are emerged from each of the paired ovarian capsules and gradually elongated into the body cavity. The follicle specimens, taken from the Day 1 ovarioles in both the normal and vit, seemed to be at Stage 5 (Figs. 1 and 2, respectively). There was no difference was detected between them, both showing what is called the typical form of polytrophic meroistic ovarioles, whose egg chambers are each composed of an oocyte, nurse cells and FCs. The oocyte pattern suggesting the inflow of cytoplasmic components from the nurse cells were seen in either normal or vit. The nurse cells were like enlarged hemispheres, and the oocyte occupied about half of the whole sectional area. An accumulation of densely stained materials was observed in the peripheral zone of the normal and vit oocytes (Figs. 3 and 4, respectively). These materials are considered to contain glycogen granules, lipid droplets and small, proteinaceous yolk spheres.

Follicles at Stage 6: In normal and vit pupae on Day 5, many follicles were at Stage 6 (Figs. 5 and 6, respectively). The inflow pattern was more significant than before. Each oocyte occupied about three-fourth of the whole sectional area, indicating its rapid growth. The intercellular spaces between the FCs were expanded. The central zone of the oocyte again seemed to contain glycogen granules and lipid droplets. These features exhibited no difference between the normal and the mutant. The large granules, that could be called the protein yolk spheres, were observed in the peripheral zone of the normal oocyte (Fig. 7, arrows). In contrast, such large spheres were not detected and the smaller ones were abundant in the vit oocyte (Fig. 8).

Follicles at Stage 9: In the follicle at Stage 9, taken out of a Day-7 pupae, the degenerating nurse cells were enclosed by the extension of the FCs (figures not shown). As to these basic features, a difference was not detected between the normal and vit. However, while the large yolk spheres were crowded in the peripheral zone of the normal oocyte (Fig. 9, arrows), the accumulation of large yolk spheres was not observed in the vit oocyte. In place

Figs. 1 to 8. Light micrographs of normal and vit follicles. Odd numbers, normal; even numbers, vit. 1 to 4, Stage 5; 5 to 8, Stage 6. 1, 2, 5 and 6, whole view; 3, 4, 7 and 8, ventral region. Arrows indicate large protein yolk spheres. FC, follicular epithelial cell; NC, nurse cell; OC, oocyte. Scale bars = 50 μm.
of these, there were weakly stained granules (Fig. 10, arrows), which were smaller than the normal yolk spheres.

**Follicles at Stage 11:** At Stage 11, the penultimate step of follicle development, the FCs decreased in height, especially in the ventral part of the follicle. They were no more columnar and reduce the intercellular spaces reduced. These features, observed in follicle specimens from Day 8 pupae, were similar between the normal and *vit* (Figs. 11 and 12; compare with the preceding figures for FC). Both exhibited the deposition of chorion (large arrows in Figs. 11 and 12). In the normal oocyte, the large yolk spheres in the peripheral zone became uncrowded, indicating their movement toward the central zone (Fig. 11, arrows). The large yolk spheres were not observable in the *vit* oocyte, in which weakly stained granules, similar to those found at Stage 9, became rich and seemed to be migrating toward the central zone (Fig. 12, arrows). There were electron-lucent materials in the normal oocyte at this stage, which may be the granules previously called the refractile bodies (Fig. 11, RB; see Discussion).

**Follicles at Stage 12:** On Day 10 after the larval-pupal ecdysis, shortly before or after the emergence, a large fraction of follicles were at Stage 12, the final step. The mature eggs were completed in the chorion deposition in the normal and mutant (Figs. 13 and 15, CH), and the degenerating FC layer was disposed. In the normal eggs, the large yolk spheres accumulated all over the ooplasm except at the narrow periplasm region, although they significantly lowered the stainability (Fig. 13, asterisks). This type of yolk spheres seemed to be composed of somewhat homogeneous elements as far as observed by electron microscopy (Fig. 14). The small particles, probably lipid droplets, were stained more strongly than the yolk spheres (Figs. 13 and 14). Such features were in agreement with the previous observations of the matured silkworm eggs (e.g., by Takesue et al., 1976). In the mature eggs of *vit*, however, the large yolk spheres were not observed. Instead, the weakly stained/electron-lucent granules were accumulated (Fig. 15, arrows; Fig. 16, C), featuring the strong stainability of putative lipid droplets (L). With respect to this feature changes between Stage 11 and 12 in the mutant did not occur (cf. Figs. 12 and 15).

**DISCUSSION**

The development of the follicles in *B. mori* was first subdivided into seven stages (Ozawa, 1959; Miya and Kurihara, 1966; Miya et al., 1970a), then into ten stages excluding the choriogenesis steps (Yamauchi et al., 1981) and finally into 12 stages, which covered all of the steps of oogenesis including the formation of the oocyte-nurse cell complex and the maturation of the ovarian eggs (Yamauchi and Yoshitake, 1984). This staging was done by introducing various morphological criteria such as those published for *Drosophila melanogaster* (in which 16 stages from 1 to 14B have been depicted (King, 1970). The subdivision into 12 stages was also reported in *Hyalaphora cecropia* (King and Aggarwal, 1965). Here, we applied the staging by Yamauchi and Yoshitake (1984) as a measure, and found it to be convenient that their description about the relationships between the age in days of pu-

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**Figs. 9 to 12.** Light micrographs of normal and *vit* follicles (continuation). Odd numbers, normal; even numbers, *vit*. 9 and 10. Stage 9: 11 and 12. Stage 11. Ventral region only. Arrows in 9 and 11 indicate large protein yolk spheres, while those in 10 and 12 show weakly stained "core" structures for protein yolk spheres proposed in the current study (see text). CH, chorion; FC, follicular epithelial cell; OC, oocyte; RB, refractile body. Scale bars = 50 µm.

**Figs. 13 to 16.** Light micrographs (13 and 15) and electron micrographs (14 and 16) of normal and *vit* follicles (continuation). 13 and 14, normal; 15 and 16, *vit*. All at Stage 12. Asterisks indicate large protein yolk spheres, whose ultrastructural feature is seen in 14. Arrows in 15 and "C" in 16 indicate the weakly stained "core" structures for protein yolk spheres proposed in the current study (see Discussion), whose ultrastructural feature is seen in 16. Scale bars in 13 and 15 = 50 µm; those in 14 and 16 = 1 µm.
pal development and the oogenesis stage of the follicles at
the basal positions was roughly reproducible, although a
slight difference existed; e.g., the typical follicles in Day-
1 pupae in Yamauchi and Yoshitake (1984) were at Stage
4, instead of Stage 5 in the current case. This is probably
attributed to the difference in the silkworm strains em-
ployed.

It was confirmed that vit females did not loose the basic
developmental programs for the polytrophic meristic
eriovaries, since the nurse cells and the FCs were normal
in terms of morphology. Moreover, the vit oocytes became
rich in the putative lipid droplets and glycogen granules,
indicating that the flow of cytoplasmic components via
the syncytial bridges was also ordinary. A direct analysis
using extracts of matured eggs has not indicated any dif-
fERENCE in the glycogen and lipid contents between the nor-
mal and vit (Fujikawa et al., 1993). The transportation of
cytoplasmic components, such as r-ER, Golgi bodies and
ribosomes, as well as different kinds of RNAs (Kawa-
guchi and Fujii, 1983), from nurse cells to oocyte takes
place during Stages 1-8 (Yamauchi et al., 1981). Moreo-
VER, the lipid droplets and glycogen granules accumulated
during the previtellogenic period (Stages 1-3) and the
large protein yolk spheres accumulated during the vitel-
logenetic period (Stages 4-10) (Yamauchi and Yoshitake,
1984). In the present article, these aspects were partly con-
firmed, except that the large protein yolk spheres did not
appear in the vit oocytes.

The total protein content in the vit egg is about one-
third of the normal (Fujikawa et al., 1993). As described
in the Introduction, the vit eggs lack vitellin and the 30
kDa proteins, i.e., two kinds of major yolk proteins,
whereas normal with respect to the ESP content. Vitel-
genin, the precursor of vitellin, and the 30 kDa proteins
must normally be incorporated from hemolymph into ma-
turing oocytes as discussed previously (Fujikawa et al.,
1995). It is worth noting that vitellin and the 30 kDa pro-
teins are present in the maternal hemolymph of the vit
mutant. Taken together, these facts strongly indicate that
the mutant females can synthesize vitellogenin and the 30
kDa proteins but do not utilize these for yolk sphere con-
struction. It is highly likely that the vit alleles are related
to some of the incorporation steps of the precursor pro-
teins from hemolymph to oocyte. This inference is sup-
pported by the present indication that the mutant oocyte
lacks the large proteinaceous yolk spheres. One of our pre-
vious supposition that the vit mutation brings about abnor-
mal interfollicular spaces (Fujikawa et al., 1995), which
provide the hemolymph-oocyte migration rout, could be
ruled out, since the above observation indicated that the
columnar arrangement of the mutant FCs were morpho-
logically normal. Thus, the plausible explanation may be
that certain receptor-mediated endocytosis steps under the
control of FCs and/or peripheral ooplasm are affected by
the vit gene.

The present mutation differs from the D. melanogaster
mutation in yolk protein 3, YPS3I (Butterworth et al.,
1999), wherein the leader sequence is not cleaved from
the protein and this causes defects in the secretion machin-
ery. It is difficult to assume that, in our case, vitellogenin
and all of the three 30 kDa protein components have re-
ceived their respective leader sequence mutations at once.
In D. melanogaster, females homozygous for YPS3I pro-
duce abnormal yolk spheres and lay less viable eggs, indi-
cating that the yolk protein 3 is not completely essential
for viability but is required for normal yolk sphere mor-
phogenesis. In contrast, the normal phenotypes of vit
seemed to be necessary for the completion of embryos,
since all vit eggs die before hatching.

The vit mutant oocyte was found to have unusual gran-
ules, which were weak in stainability for toluidine blue
(Fig. 15), and very translucent to the electron beam (Fig.
16). There is the possibility that these granules, which are
smaller than the normal protein yolk spheres, are each
functioning as a “core” to construct the final structure of
the yolk sphere, but incomplete without vitellin and the 30
kDa proteins. It is tempting to suppose that the hypotheti-
cal “core” is constructed by ESP, which is synthesized in,
and secreted from the FCs without disturbance by the vit
mutation.

On the whole, the present findings support the idea that
the vit mutation brings about a shortage of yolk proteins
which are of the extra-ovarian origin, and that the trans-
portation of the yolk precursor proteins is supported by
the normal-type vit allele. In this connection, the so-called
refractile bodies (RSs) are of interest (see Figs. 11 and
13). The RBs are electron-lucent, limited to the peripheral
ooplasm and apparently distinguishable from the protein
yolk spheres (Yamauchi and Yoshitake, 1984). The corre-
sponding structures have previously been observed in B.
mori and other insect species (Cruckshank, 1972; Ruben-
stein, 1979; Takesue et al., 1980; Yamauchi and
Yoshitake, 1984). We found that there were only a few
RBs in the vit mutation (Figs. 12 and 15). This fact sug-
gests that the formation of RB is under the control of the
FCs and/or the peripheral ooplasm and is suppressed by
the vit mutation. Further studies on the vit gene expres-
sion in relation to the RB formation will provide a clue to a
deeper understanding of oogenesis including the construc-
tion processes of the protein yolk spheres.

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REFERENCES


