Journal of Insect Biotechnology and Sericology 76, 113-120 (2007)

Microtubule Dynamics and Distribution of γ-Tubulin in Male Germ Cells of *Bombyx mori* (Lepidoptera)

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(Received February 19, 2007; Accepted May 11, 2007)

Male germ cells of *Bombyx mori* (Lepidoptera) have three cell kinds such as spermatogonia, eupyrene spermatocytes and apyrene spermatocytes. We observed spindle structure and the distribution of γ-tubulin in spermatogenesis. The microtubules (MTs) of mitotic apparatus in *Bombyx* spermatogonia were similar to that of general mammalian cells. In eupyrene spermatocytes, the asters were separated from the spindle pole, and then the spindle was transformed into a barrel shape at metaphase. In apyrene spermatocytes, the feeble-looking spindle body was formed, and asters were not separated at metaphase. The barrel-shaped spindle was not formed in apyrene metaphase cells. The chromosome arrangements on the equatorial plate were quite rough at metaphase and the movements toward the poles during anaphase were not synchronous. In all the three kinds of cells, spots of γ-tubulin were observed at centrosomes during cell division. Only in eupyrene spermatocytes, however, γ-tubulin was also disclosed in the spindle region from metaphase to anaphase. Many newly formed non-kinetochore-MTs appeared in the spindle interzonal region when the kinetochore-MTs disappeared and chromosomes reached the flat edge of spindle pole. It is indicated that non-kinetochore-MTs are built up by the action of spindle γ-tubulin. On the other hand, the distribution of γ-tubulin in apyrene spermatocytes appeared similar to the spermatogonia that performed regular chromosome separation. Therefore, we consider that the feeble spindle and the missegregation of chromosomes in apyrene meiosis are not caused by the γ-tubulin in the centrosomes.

Key words: *Bombyx mori*, male germ cells, spermatogenesis, γ-tubulin, microtubules

INTRODUCTION

Meves (1903) have found for the first time that males of Lepidoptera produce two types of sperm, eupyrene and apyrene. Eupyrene sperm with a nucleus are usual sperm that fertilize eggs, while apyrene sperm eliminate their nucleus by peristaltic squeezing at the end of spermatogenesis (Kawamura *et al.*, 2000). In lepidopteran species, the spermatogonia repeat mitosis for six times before becoming the spermatocytes (Phillips, 1970) and spermatogonial proliferation continues uninterruptedly throughout the insect's life (Friedländer *et al.*, 2005). Eupyrene spermatocytes are produced during the larval stage, while apyrene spermatocytes appear after pupation. The shift in commitment of the spermatocytes from eupyrene to apyrene spermatogenesis is assumed to relate with an apyrene spermatogenesis-inducing factor (ASIF) becoming active after pupation (Friedländer and Benz, 1982; Jans *et al.*, 1984).

The spermatogonial cells in *Ephestia kuehniella* (Lepidoptera) are structurally similar to those of mammalia (Wolf, 1990). In the proliferation stage, spermatogonia are not able to be discriminated which are to become eupyrene or apyrene sperm. The stark difference between eupyrene and apyrene cells is observed since the beginning of meiosis. In the primary and the secondary spermatocytes of the eupyrene, the spindle poles are separated from the asters at prometaphase and a unique barrel-shaped spindle body is produced at metaphase, though the chromosome separation during anaphase proceeds normally (Wolf and Bastmeyer, 1991; Wolf and Joshi, 1996; Yamashiki and Kawamura, 1998). On the other hand, an apyrene spermatocyte spindle that is not separated from the asters dose not become barrel-shaped (Reinholdt *et al.*, 2002). In addition, it is known that the apyrene spermatocytes never form a real equatorial plate at metaphase and the subsequent chromosome movements from anaphase to telophase is highly irregular (Wolf and Bastmeyer, 1991; Wolf, 1994; Wolf and Joshi, 1996).

In spermatogenesis of Lepidoptera, each kind of germ cells such as spermatogonia, eupyrene spermatocytes and apyrene spermatocytes has a characteristic style of mitotic apparatus (Wolf, 1994). Microtubules (MTs) need to associate with a microtubule organizing center (MTOC) for stabilization. The polarity and positioning of MTs are generally determined by MTOC. The centrosomes in animal cells have been considered to be the major MTOC (Joshi *et al.*, 1992; Stearns *et al.*, 1991), with which the minus end of the MT is associated (Bergen *et al.*, 1980). γ-Tubulin, one of the tubulin superfamily members (Oakley and Oakley, 1989), is an important component of pericentriolar matrix that consists of centrosomal protein and locates at the centrosome (Stearns *et al.*, 1991; Zheng *et al.*, 1991, Joshi *et al.*, 1992; Julian *et al.*, 1993). γ-Tubulin

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plays a major role in the nucleation of MT assembly, because most of soluble γ-tubulin is composed of γ-tubulin ring complexes (γ-TuRCs) that binds to α/β-tubulin to promote MT growth, and that also caps the minus end of MTs to avoid depolymerization (Moritz *et al.*, 1995; Zheng *et al.*, 1995; Oegema *et al.*, 1999; Wiese and Zheng, 2000). Therefore, the centrosomes are evidently important for mitotic spindle formation in many cell types in most animal cells. In lepidopteran eupyrene spermatocytes, the spindle is separated from the centrosomes at metaphase. Wolf and Joshi (1996) demonstrated the distribution of γ-tubulin in *Phragmatobia fuliginosa* and *E. kuehniella* (Lepidoptera), though their interests were not focused on the distribution of γ-tubulin in the spermatogonia as well as the apyrene spermatocytes.

Our previous study (Matsuda *et al.*, 2007) provided an immunofluoresence method for γ-tubulin in *Bombyx mori* by using a polyclonal antibody for human γ-tubulin. In this study, we investigated the distribution of γ-tubulin and the difference in characteristic features of MTs between eupyrene and apyrene spermatocytes during meiosis. We also observed spermatogonia that form a usual spindle in order to compare with the eupyrene and apyrene spermatocytes with a particular shaped-spindle.

MATERIALS AND METHODS

Insects

Bombyx mori used for immunostain was the F_1 generation (rw2n) obtained by the cross between re9 (p^S/p^S, *re*/*re*) females and Tw1 (*p*/*p*, *w*-2/*w*-2) males (for details see Kawamura, 1978). The larvae were fed on the artificial diet, Silkmate 2M (Nosan Corporation, Tokyo, Japan) under the conditions at 26°C and 70% moisture. The testes of the forth instar larvae were utilized for observing mitosis in the spermatogonia and those of the fifth instar larvae or pupae for examining meiosis in the spermatocytes.

Indirect immunofluoresence staining

The contents of the testes were smeared on the coverslip (22 × 22 mm) coated with 1% 3-aminopropyl-triethoxy-silane in acetone. The specimens were fixed in 4% parafornaldehyde solution in PIPES buffer (10 mM PIPES, 1 mM $MgCl_2$, 2 mM EGTA, 100 mM NaCl, pH 7.0) for 30 min, and then rinsed with phosphate buffer (PBS, containing 0.1% bovine serum albumin, 0.1% NaN_3, and 0.05% Tween 20, pH 7.0). For additional fixation, the coverslip was soaked in cold methanol for 10 min. and lightly dried.

1. Double staining for α-tubulin or γ-tubulin and chromosomes

The primary antibody used was anti-human α-tubulin monoclonal antibody (CLT9002, Cedarlane Laboratories Ltd., Hornsby, Canada) or polyclonal antibody against human γ-tubulin peptide (T5192, Sigma-Aldrich, Inc., St. Louis, USA). The secondary antibodies used were FITC-conjugated anti-mouse antibody (H+L chain, Medical and Biological Laboratories Co., Ltd., Nagoya, Japan) for the anti-α-tubulin antibody, or FITC-conjugated anti-rabbit antibody (MP Biomedicals, Inc., Aurora, USA) for the anti-γ-tubulin antibody. In order to stain chromosomes, propidium iodide (Cambio Ltd., Cambridge, UK) was used before mounting on a slide.

2. Triple staining for α-tubulin, γ-tubulin and chromosomes

The primary antibody used was anti-human α-tubulin monoclonal antibody (CLT9002, Cedarlane Laboratories Ltd., Hornsby, Canada) and polyclonal antibody against human γ-tubulin peptide (T5192, Sigma-Aldrich, Inc., St. Louis, USA). And then the secondary antibody, TRITC-conjugate anti-mouse IgG (H+L) goat antibody (81-6154, Zymed Laboratories, Inc., California, USA) was applied for the anti-α-tubulin monoclonal antibody, and FITC-conjugated anti-rabbit antibody (MP Biomedicals, Inc., Aurora, USA) was applied for the anti-γ-tubulin polyclonal antibody. In order to stain chromosomes, Hoechst33258 (Sigma-Aldrich, Inc., St. Louis, USA) was used before mounting on a slide.

3. Observation

The double color stained specimens were observed by using a laser scanning confocal microscope (Fluoview, Olympus Co. Ltd., Tokyo, Japan) and captured images were processed by means of Adobe Photoshop (Mountainview, CA, USA). The triple color stained specimens were observed and captured images by using a fluorescence microscope (BX50, Olympus Co. Ltd., Tokyo, Japan) equipped with digital microscopy software slidebook (Intelligent Imaging Innovations, Inc., Denver, CO, USA).

RESULTS

Microtubule dynamics and γ-tubulin distribution in mitosis of spermatogonia

The size of spermatogonia at metaphase was much smaller than that of the primary spermatocytes at the meiotic metaphase. By immunostaining for α-tubulin, two asters that consisted of microtubules (MTs) were observed on the two opposite poles of the nucleus at prophase (Fig. 1a). By metaphase, spindle MTs developed between the two asters, assuming a usual mitotic spindle shape (Fig. 1b). When the sister chromosomes began to separate at anaphase, the kinetochore-MTs were still rich in the half spindle (Fig. 1c). The kinetochore-MTs almost disap-

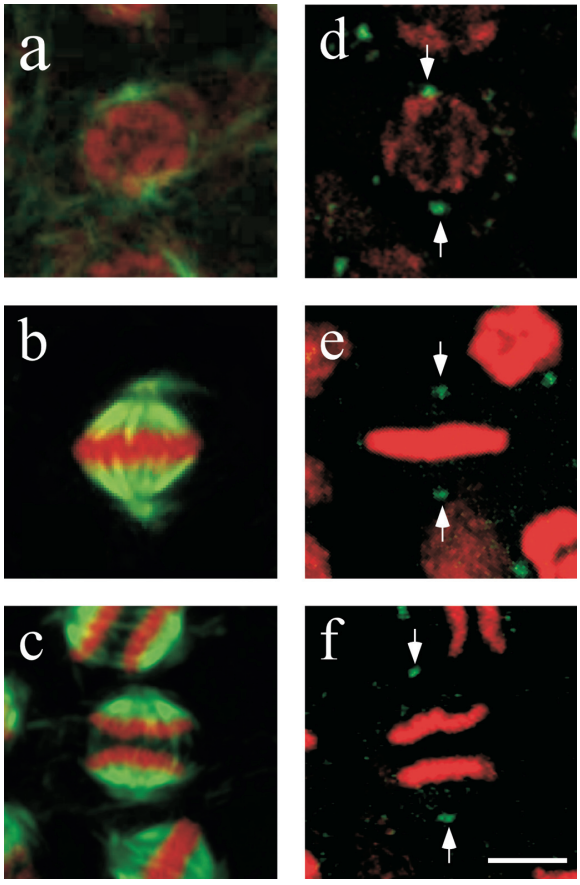

Fig. 1. Formation of mitotic apparatus in spermatogonia of *Bombyx*. Red indicates chromatin or chromosomes. The green color denotes α-tubulin in figures a, b and c, and γ-tubulin in figures d, e and f. (a, d) Prometaphase. (b, e) Metaphase. (c, f) Anaphase. Arrows in d, e and f show the γ-tubulin spots. Bar: 5 μm.

peared at late anaphase as the chromosomes reached the spindle pole.

To examine the distribution of the microtubule organizing center (MTOC), spermatogonia were stained by using the antibody for γ-tubulin. At prophase, two spots of γ-tubulin were located on the opposite sides of the nucleus (Fig. 1d), and then at the spindle poles during metaphase and anaphase (Fig. 1e, f).

Microtubule dynamics in eupyrene meiosis

During early prophase, no MT structures were found in the primary spermatocytes. At pachytene, four flagellar axonemes that were divided in two groups appeared side by side near the nuclear envelope. The small asters developed at the base of an each group of axonemes at diplotene. Diakinesis was the stage when the two asters with two axonemes each separated toward the opposite poles of the nucleus and the asters developed larger (Fig. 2a). After the breakdown of the nuclear envelope, MTs started to catch the bivalents (Fig. 2b). When all the bivalents made contact with MTs from both poles, the asters with a pair of axonemes were separated from the spindle poles. The further separation occurred at metaphase I, and at the same time, the spindle was transformed into a barrel-shaped that was the flat on the both poles (Fig. 2c). The spindle maintained the barrel shape till middle anaphase I; the homologous chromosomes reached the flat poles of the spindle and the kinetochore-MTs became shorter and then disappeared. At this stage, the asters were still separated from the spindle poles (Fig. 2d). Abundant non-kinetochore-MTs, which were not present before, appeared in the interzonal region of the now elongated spindle (Fig. 2e). The chromosomes moved toward the asters and at last reached the astral center at telophase I (Fig. 2f).

At prometaphase II, the spindle MTs developed (Fig. 2g) and caught chromosomes (Fig. 2h). The secondary spermatocytes also formed a barrel-shaped spindle and the asters were separated from the spindle just like the primary spermatocytes at metaphase (Fig. 2i). The chromatid separation at anaphase II followed the same procedure as anaphase I. The sister chromosomes finally reached the astral center and formed daughter nuclei at telophase II (Fig. 2j).

Microtubule dynamics in apyrene meiosis

The size of apyrene spermatocytes was much smaller than that of eupyrene ones. They furnished four axonemes as early as prophase I and small asters developed at the base of axonemes. At the diakinesis stage, the two asters with a pair of axonemes were located on the opposite position (Fig. 3a). The feeble-looking spindle body was formed between the asters (Fig. 3b) and, unlike eupyrene spermatocytes, spindle poles and asters were not separated at metaphase I. The chromosome arrangements on the equatorial plate were quite rough at metaphase I in apyrene spermatocytes (Fig. 3c). The chromosome movements toward the poles at anaphase I was not synchronous (Fig. 3d). Some of the chromosomes formed a chromosome bridge, while some remained near the equatorial plate (Fig. 3e). Finally, all the chromosomes moved to the pole at telophase I, though the daughter nuclei could not contain a few of them (Fig. 3f). The spindle of the secondary spermatocytes was much more meager and smaller than that of the primary ones during meiosis II though asters developed (Fig. 3g). At metaphase II, chromosome clumps were located at halfway between the spindle poles (Fig. 3h). With the smaller amount of spindle MTs, each chromosome migrated in splinters toward the spindle poles at anaphase II (Fig. 3i). The asynchronous chromosome movements prevented some of the chromosomes to be involved in the daughter nuclei at telophase II (Fig. 3j).

Distribution of γ-tubulin in eupyrene spermatocytes

The meiotic spindle of eupyrene cells showed the

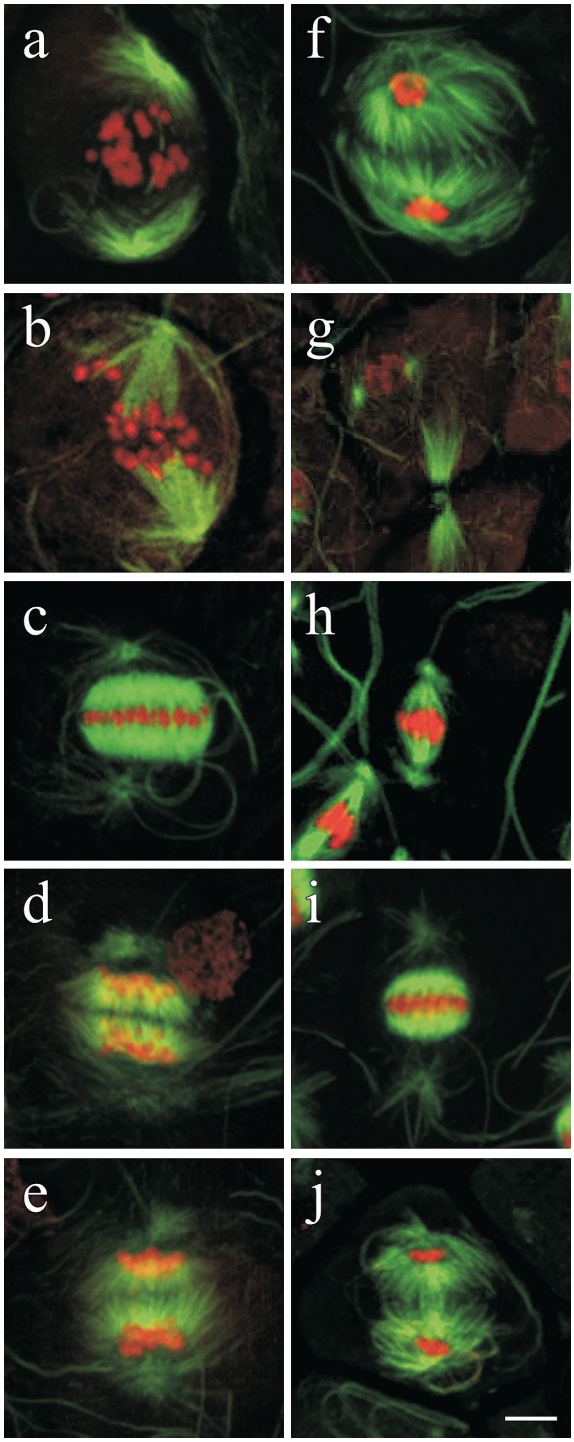

Fig. 2. Formation of meiotic apparatus in eupyrene spermatocytes. Red indicates chromosomes. The green color denotes α-tubulin. (a~f) Meiosis I. (g~j) Meiosis II. Note the separation of asters with axonemes and the spindle body at metaphase I and II (c, i). Chromosomes reach the flat spindle pole at middle anaphase I, but asters are still separated from the spindle poles (d). Daughter nuclei reach the asters at telophase I and II (f, j). Bar: 5 μm.

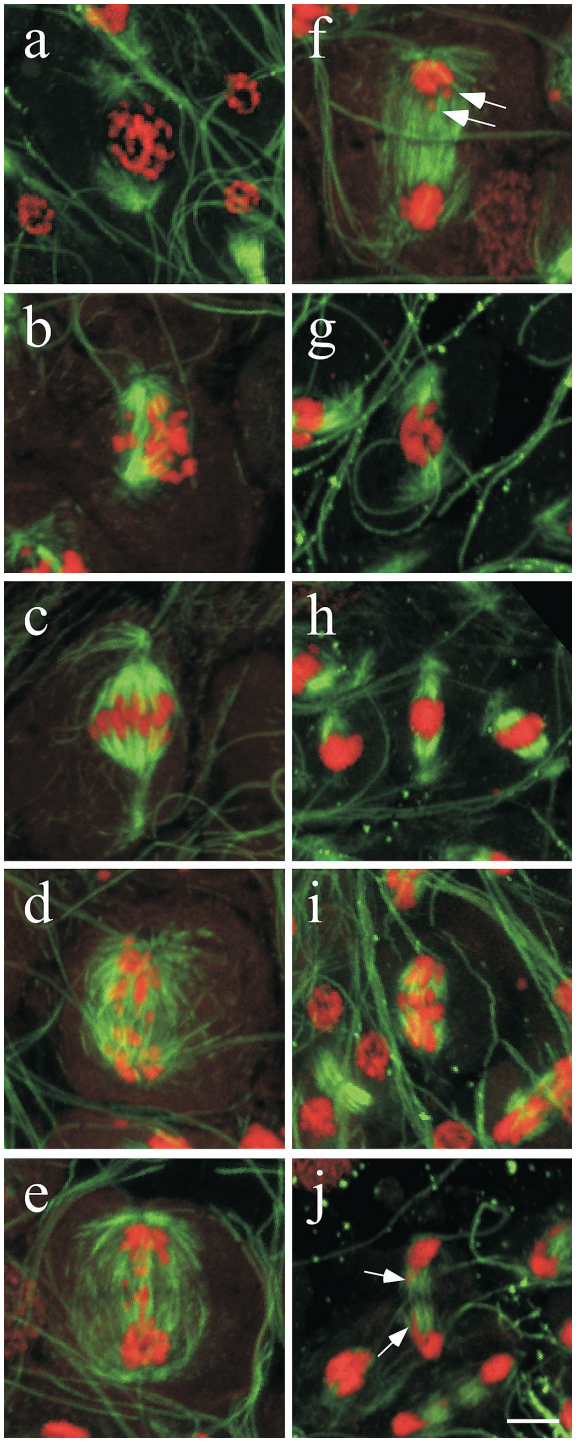

Fig. 3. Formation of meiotic apparatus in apyrene spermatocytes. Red indicates chromosomes. The green color denotes α-tubulin. (a~f) Meiosis I. (g~j) Meiosis II. Note the rough chromosome arrangement at metaphase (c, h), asynchronized chromosome movements at anaphase (d, i), and chromosome bridges at late anaphase (e). Some chromosomes shown by arrows are left in the interzonal region at telophase (f, j). Bar: 5 μm.

unique barrel shape and asters were separated from the spindle pole during metaphase and anaphase. In the eupyrene spermatocytes, MTOC shown by the distribution of γ-tubulin was examined to confirm the relation between the astral centers and the spindle poles.

When four axonemes appeared at pacytene, signal of

γ-tubulin was not observed. The spots of γ-tubulin appeared at the astral center as the asters developed at diplotene. Just before nuclear envelope broke down, two spots of γ-tubulin were observed on the two opposite sides of the nucleus and the stainability of the two spots increased (Fig. 4a, a'). As asters were separated from the meiotic spindle at metaphase I, two spots of γ-tubulin were also separated from the spindle pole (Fig. 4b, b'). γ-Tubulin spots on the astral center were observed until the end of meiosis. At metaphase I, however, the barrel-shaped spindle became rich with γ-tubulin signal (Fig. 4b, b'). The signals of γ-tubulin on the spindle remained until kinetochore-MTs disappeared at anaphase I. Many non-kinetochore-MTs appeared at middle anaphase, and the signals of γ-tubulin were observed close to chromosome groups in the spindle interzonal region (Fig. 4c, c'). At telophase I, γ-tubulin signals were not observed on the spindle region. The secondary spermatocytes showed the same features of γ-tubulin as the primary ones (data not shown).

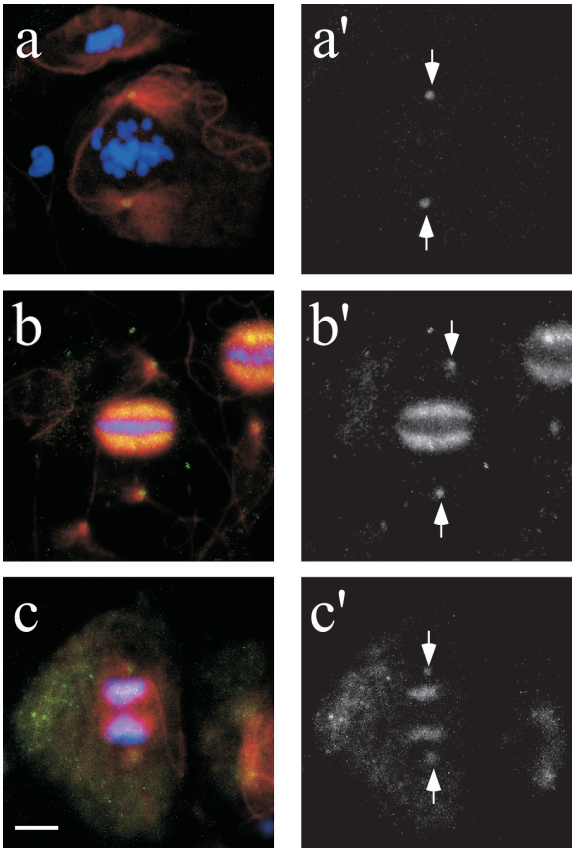

Fig. 4. Color photographs show triple staining for chromosomes (blue), microtubules (red) and γ-tubulin (green) in eupyrene spermatocytes (a, b and c). Gray photographs show only distribution of γ-tubulin (a', b' and c'). (a, a') Prophase I. The spots of γ-tubulin are located at the astral centers (arrows). (b, b') Metaphase I. γ-Tubulin is located at the astral centers that separated from the spindle body. Note the appearance of γ-tubulin on the half spindle. (c, c') Anaphase I. The γ-tubulin spots are still observed at astral centers. Note the signals of γ-tubulin on the spindle inte zonal region. Bar: 5 μm.

Distribution of γ-tubulin in apyrene spermatocytes

Two γ-tubulin spots were located at the astral center side by side near the nucleus at prophase I. Before the nuclear envelope broke down, the spots were separated toward the opposite side (Fig. 5a, a'). At metaphase I and anaphase I, γ-tubulin spots located at the spindle poles that coincided with the astral centers (Fig. 5b, b', c, c'). On the contrary to the eupyrene meiosis, the signals of γ-tubulin were not recognizable on the spindle region at metaphase I and anaphase I. At telophase I, stainability of γ-tubulin in the spindle poles decreased.

γ-Tubulin spots were also located on the spindle poles at prometaphase II and metaphase II, though they were not observed at anaphase II and telophase II (data not shown). The stainability of γ-tubulin in meiosis II was less than that in meiosis I.

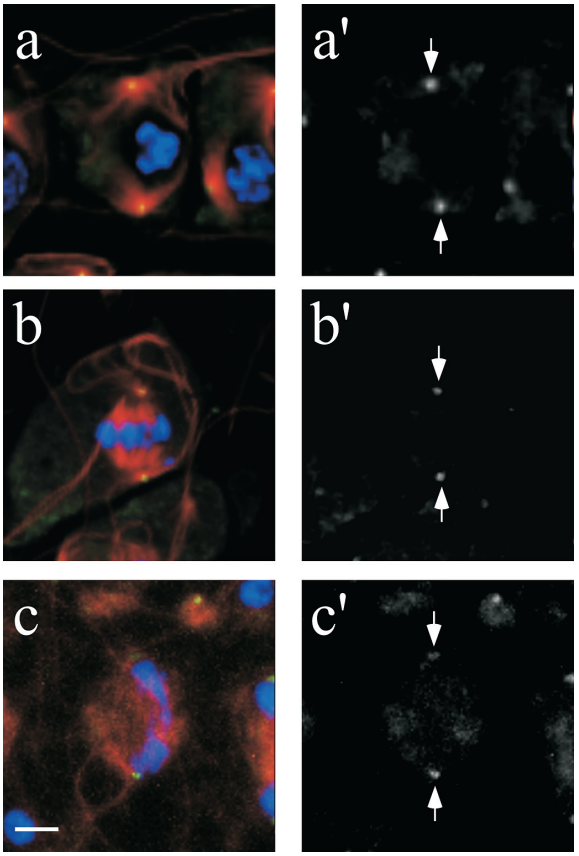

Fig. 5. Color photographs show triple staining for chromosomes (blue), microtubules (red) and γ-tubulin (green) in apyrene spermatocytes (a, b and c). Gray photographs show only distribution of γ-tubulin (a', b' and c'). (a, a') Prophase I. (b and b') Metaphase I. (c and c') Anaphase I. The signals of γ-tubulin appear only at the spindle pole in apyrene meiosis (arrows). Bar: 5 μm.

DISCUSSION

In *Bombyx* spermatogenesis, three kinds of germ cells, spermatogonia, eupyrene spermatocytes and apyrene spermatocytes are coexisted in the testis. We have shown the characteristic features of microtubules (MTs) and the distribution of γ-tubulin in each of the germ cells. The characteristic behaviors of the chromosomes, MTs and distribution of γ-tubulin in the three kinds of germ cells are schematically summarized in Fig. 6.

The mitotic apparatus of *Bombyx* spermatogonia appeared to be similar to that of general mammalian cells. Since two spots shown by γ-tubulin staining were located in the astral centers that coincided with spindle poles, the spots were considered to be the centrosomes (Fig. 6a-d).

In both of the eupyrene and apyrene spermatocytes, two spots of γ-tubulin became visible in the center of the asters that started to develop in the base of axonemes at diplotene and existed throughout meiosis (Fig. 6e-l). In eupyrene spermatocytes, however, the asters with a pair of axonemes were separated from the spindle pole at prometaphase, and then the spindle was transformed into the barrel-shaped at metaphase. Spindle structure without the centrosomes has been reported in the mutant embryonic cell line of *Drosophila melanogaster* and the mouse oocytes. In these cells, γ-tubulin was located at the spindle poles but not on the spindle itself in any stage (Debec *et al.*, 1995; Palacios *et al.*, 1993; Sanfins *et al.*, 2003). In *Bombyx* eupyrene spermatocytes, signals of γ-tubulin were disclosed in the spindle region as well as the astral centers. In male meiosis of lepidopteran species, *Phragmatobia fulinginosa*, Wolf and Joshi (1996) also demonstrated that γ-tubulin located on the spindle at metaphase of eupyrene spermatocytes. When they applied a cold-shock to the eupyrene spermatocytes, the astral-MTs of the metaphase cells were depolymerized and became small, while the spindle-MTs were not depolymerized and the spindle size was constant. They suggested, therefore, that γ-tubulin in the half spindle stabilized the spindle body separated from asters.

In the present study, we analyzed MT dynamics of the spindle and the change of γ-tubulin distribution during meiotic anaphase. In eupyrene spermatocytes, the chromosomes reached the flat poles of barrel-shaped spindle that was separated from the centrosomes and the kinetochore-MTs disappeared at early anaphase. When the chromo-

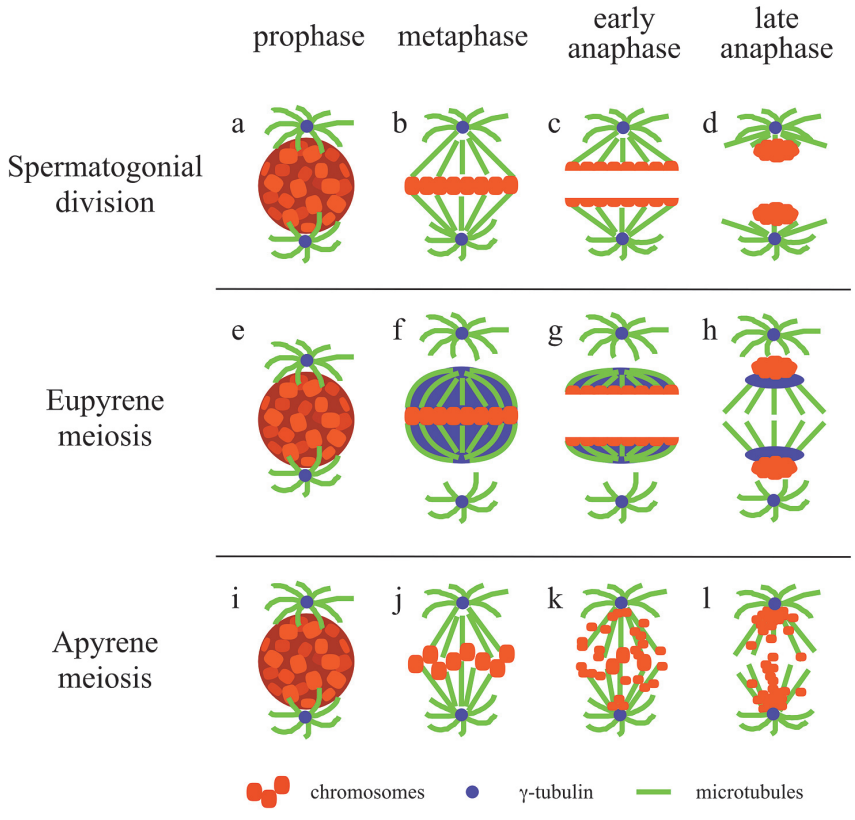

Fig. 6. Schematic overview showing the chromosome behavior (orange), microtubule dynamics (green) and γ-tubulin distribution (blue) in the three kinds of *Bombyx* male germ cells. (a-d) Spermatogonial cells. (e-h) Eupyrene spermatocytes. (i-l) Apyrene spermatocytes. Each stage of the cell division indicates prophase (a, e, i), metaphase (b, f, j), early anaphase (c, g, k) and late anaphase (d, h, l). Note that γ-tubulin on the spindle area in eupyrene cell (f, g) and distribution of γ-tubulin changes from the polar side to the equatorial side of the chromosomes (g, h). Flagellar axonemes developed from the astral center of the eupyrene and apyrene cells are omitted in the diagram.

somes moved further toward the centrosomes, many newly formed non-kinetochore-MTs appeared in the spindle interzonal region without any participation of centrosomes. γ-Tubulin distribution changes from the polar side to the equatorial side of the chromosomes at middle anaphase (Fig. 6g, h).

Generally, γ-tubulin molecules nucleate MTs and associate with the minus-end of MTs at the centrosome. Our findings in *Bombyx* eupyrene spermatocytes indicate that γ-tubulin of the spindle functions as MTOC to nucleate non-kinetochore-MTs in the spindle interzonal region. It seems that the non-kinetochore-MTs developing from the opposite sides are overlapped in the spindle interzonal region and may act to push the chromosomes toward the astral centers at late anaphase.

In apyrene spermatocytes, the arrangement of MTs at anaphase considerably differed from eupyrene spermatocytes. Kinetochore-MTs and non- kinetochore-MTs were not distinguishable because the chromosome segregation was asynchronous. Signals of γ-tubulin in apyrene spermatocytes located on the centrosomes and were not observed on the spindle that was not separated from the centrosomes at anaphase. The distribution of γ-tubulin in apyrene spermatocytes appeared similar to that in the spermatogonia that performed regular chromosome separation (Fig. 6). Therefore, we consider that the feeble spindle and the missegregation of chromosomes in apyrene meiosis are not caused by the γ-tubulin in the centrosomes.

ACKNOWLEDGMENTS

We thank Dr. Ken Sahara (Laboratory of Applied Molecular Entomology, Graduate School of Agriculture, Hokkaido University) for his generous supply of silkworm eggs and valuable comments to our study. Our thanks also go to Dr. Naoko Kawamura and Mr. Tsubasa Yamamoto (Laboratory of Developmental Biology, Rakuno Gakuen University) for helps and critical discussion. This study was partially supported by a Grant-in-Aid for Scientific Research no. 15380044 (N.Y.) from the Japan Society for the Promotion of Science (JSPS).

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