Chromatin and microtubule organization during premeiotic, meiotic and early postmeiotic stages of Drosophila melanogaster spermatogenesis*

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*This paper is dedicated to the memory of Fritz Sobels, in recognition of his studies on Drosophila spermatogenesis

SUMMARY

Larval and pupal testes of Drosophila melanogaster were fixed with a methanol/acetone fixation procedure that results in good preservation of cell morphology; fixed cells viewed by phase-contrast optics exhibit most of the structural details that can be seen in live material. Fixed testis preparations were treated with anti-tubulin antibodies and Hoechst 33258 to selectively stain microtubules and DNA. The combined analysis of cell morphology, chromatin and microtubule organization allowed a fine cytological dissection of gonial cell multiplication, spermatocyte development, meiosis and the early stages of spermatid differentiation. We placed special emphasis on the spermatocyte growth phase and the meiotic divisions, providing a description of these processes that is much more detailed than those previously reported. In addition, by means of bromo-deoxyuridine incorporation experiments, we were able to demonstrate that premeiotic DNA synthesis occurs very early during spermatocyte growth.

Key words: Drosophila, spermatogenesis, meiosis

INTRODUCTION

The differentiation of sperm from the relatively undifferentiated gonial cells is one of the most complex and elaborate of developmental processes. This process encompasses two types of cell divisions: the mitotic divisions needed for the multiplication of gonial cells, and the meiotic divisions leading to the formation of haploid spermatids. In addition, both premeiotic and postmeiotic cells undergo a series of dramatic changes in cellular morphology that culminate in the differentiation of the sperm tail. All these events, collectively defined as spermatogenesis, are characterized by a remarkable degree of evolutionary conservation, so that insects and mammals exhibit only minor differences in the basic morphogenetic features involved in sperm development. Thus, an understanding of spermatogenesis in a model system should provide a conceptual framework of wide utility.

A model organism particularly suitable for the elucidation of complex developmental processes is Drosophila melanogaster. Mutational dissection of Drosophila embryogenesis, followed by the molecular characterization of the relevant genes, has led to an increasing understanding of the fundamental mechanisms underlying embryonic development. Genetic analysis has also been applied to Drosophila spermatogenesis and many mutants have been isolated that disrupt specific aspects of this process (see for example Lifschytz and Hareven, 1977; Lifschytz and Meyer, 1987; Hackstein, 1991). Although these studies have clearly shown that the genetic dissection of spermatogenesis is feasible, very few genes involved in sperm development have been characterized at the molecular level, and our current conception of the mechanisms that control the formation of male gametes is still very poor. Recently, however, many new genes involved in Drosophila spermatogenesis have been identified by single P element mutagenesis (Castrillon et al., 1993; Fuller, 1993) and postmeiotic (for review see Lindsley and Tokuyasu, 1980) stages are currently available. Light microscope analyses, though more widely accessible, are unfortunately not as detailed and complete. The classic cytological procedure for light microscope analysis of Drosophila spermatogenesis is the observation of living preparations in phase contrast (see for example Lifschytz and Hareven, 1977; Castrillon et al., 1993; Fuller, 1993). The morphological differentiation of living cells is remarkably good and most cell types and stages, including the meiotic divisions, can be readily recognized. However, the fact that the structures observed in living cells cannot be discriminated by immuno- and histochemical staining techniques,
limits the utility of this material. On the other hand, classical fixation techniques, such as those described by Cooper (1965), allow a clear visualization of chromosomal material but not of other cellular structures, such as the cytoskeleton and the spindle.

An ideal fixation technique would be one that preserves the cellular morphology observed in vivo yet simultaneously allowing staining with chemical and immunological reagents. We have developed such a technique during the course of studies on the Y chromosome loops (Pisano et al., 1993). In this report we have exploited this technique to describe the arrangement and the organization of microtubules, chromatin and mitochondria in the various cell types arising during D. melanogaster spermatogenesis.

MATERIALS AND METHODS

Stocks
All the observations were made on larval or pupal testes of an Oregon-R stock that has been maintained in our laboratory for about 30 years. The flies were reared on standard Drosophila medium at 25±1°C; dissections and cytological procedures were performed at room temperature.

Fixation and staining procedures
Pupal and larval testes were dissected and fixed according to Pisano et al. (1993). Fixed preparations were washed three times (5 minutes each) in PBS, before incubation with the primary antibody for indirect immunofluorescence or Giemsa staining. Three different anti-tubulin antibodies were used for immunostaining: (1) an α-tubulin monoclonal antibody (Ab) raised against chick brain microtubules (Amersham; Blose et al., 1982) designated as A-Am; the ascites fluid containing this antibody was diluted 1:50 in PBS. (2) An anti-β-tubulin monoclonal Ab also raised against chick brain microtubules (Amersham; Blose et al., 1982) designated as B-Am; the ascites fluid with this antibody was diluted 1:30 in PBS. (3) The anti-α tubulin monoclonal Ab 3A5 raised against Drosophila embryonic tubulin (Piperno and Fuller, 1985), which was diluted 1:2 in PBS. In every case the slides were incubated for 45 minutes with 30 µl of diluted primary antibody in a humid chamber at room temperature. They were then washed three times in PBS (5 minutes each) and incubated for 45 minutes with the secondary antibody (sheep anti-mouse IgG, F(ab)2 fragment, conjugated with 5(6)-carboxy-fluorescein-N-hydroxysuccinimide ester (FLUOS) from Boehringer, Mannheim, cat. no. 1214616) diluted 1:15 in PBS.

After three washes in PBS (5 minutes each) the immunostained slides were stained with Hoechst 33258 according to Bonaccorsi et al. (1988). Giemsa staining was performed as previously described with 4% Giemsa (Bonaccorsi et al., 1988).

The slides were analyzed with a Zeiss III photomicroscope equipped with a mercury light source for incident illumination. FLUOS and Hoechst 33258 fluorescence were detected using the 0.9 (BP 450-490, FT 510, LP420) and the 0.1 (BP 365/11, FT 395, LP 397) Zeiss filter combinations, respectively. Microphotographs were taken with Kodak T-Max 100 film.

Bromo-deoxyuridine (BrdU) labeling
Dissected testes were incubated in 10 µg/ml BrdU (Sigma) dissolved in PBS. They were then washed for at least 3 minutes in testis buffer (Kremers et al., 1986), squashed and fixed as described above. Testis preparations were incubated for 1 hour with the ready-to-use Amersham solution (code RPN 202) containing an anti-BrdU monoclonal antibody, nuclease and 1% BSA. After washing in PBS for 5 minutes the slides were incubated with the secondary antibody (sheep anti-mouse IgG, F(ab)2 fragment, conjugated with FLUOS from Boehringer, Mannheim) diluted 1:15 in PBS, washed and mounted in PBS for observation.

RESULTS AND DISCUSSION

Our methanol-acetone fixation technique (Pisano et al., 1993; see Materials and Methods) results in good preservation of the cell morphology observed in live material by phase-contrast optics. As shown in Fig. 1, most of the morphological details that can be seen in living cells are also visible in fixed preparations, suggesting that fixation artifacts are minimal. The only intracellular structures that are substantially affected by fixation are the Y chromosome loops. These filamentous structures are usually faint and labile in living preparations but become clearly apparent after fixation (Bonaccorsi et al., 1988; Pisano et al., 1993).

We have immunostained fixed preparations from both larval and pupal testes with three different anti-tubulin antibodies (A-Am, B-Am and 3A5; see Materials and Methods). It has been shown that A-Am recognizes most of the Drosophila α-tubulin isoforms (Matthews et al., 1989), while B-Am reacts with both the β- and the testis-specific β2-tubulin (Kimble et al., 1989); the monoclonal antibody 3A5 reacts with α-tubulin (Piperno and Fuller, 1985). These antibodies gave identical results with all the cell types of D. melanogaster spermatogenesis. Thus, we will not distinguish between them in describing the various immunostaining patterns.

Following tubulin immunostaining, testis preparations were treated with Hoechst 33258 and viewed under a fluorescence microscope with filter combinations that detect either fluorescein or Hoechst fluorescence. This enabled us to examine microtubule and chromatin organization in the same cell and to correlate the behavior of these cellular components with the other structures that can be seen under phase-contrast microscopy. Based on these observations we have proposed a stage subdivision of spermatocyte growth, meiotic divisions and early spermatid differentiation. Each stage is defined only by morphological criteria and corresponds to a facet of the process that can be unambiguously distinguished from both the preceding and the successive one.

Spermatogonia
Cytological analysis of the cells located at the apex of the testis is difficult because in squashed preparations these cells are usually very close to each other and very similar in morphology. Thus, we have been unable to discriminate between germ-line stem cells, primary spermatogonia and the progenitors of the cyst cells. However, cysts containing 2, 4, or 8 secondary spermatogonia can be easily recognized and analyzed. Secondary spermatogonia of these cysts appear identical when in the same phase of cell cycle, with nuclei almost entirely occupied by chromatin (Fig. 2). Hoechst staining permits an easy discrimination between G1 nuclei and those that have completed DNA synthesis, which have roughly doubled the chromatin content (Fig. 2). In most cases, the cyst cell nuclei can be readily identified because of their elongated appearance with the chromatin often divided into a main larger mass and a smaller, more or less separated element. In addition, the
chromatin content of these nuclei is comparable to that of post-
synthetic spermatogonia, indicating that cyst cells are in an
extended G2 phase of the cell cycle (Fig. 2).

Interphase spermatogonia exhibit a dense cytoplasmic
network of microtubules that undergoes a dramatic reorgan-
ization in preparation for mitosis, culminating in spindle
formation. Spermatogonial mitoses are relatively rare, sug-
gestting that mitotic division of these cells is very rapid. The
metaphase spindle consists of two relatively narrow and
elongated hemispindles that shorten progressively during
anaphase and telophase. At telophase the hemispindles become
broader as they flatten against the reforming nuclei; a midbody
connecting the two daughter nuclei is often visible at this time.
These structures progressively disappear as the next interphase
begins and a new cytoskeleton takes shape in the cytoplasm
surrounding each nucleus (Fig. 2).

Premeiotic DNA synthesis
The four gonial divisions generate 16 primary spermatocytes
that are morphologically indistinguishable from the parental
secondary spermatogonia in the G1 phase (we designate this
stage as S0; an early S0 stage is shown in Fig. 2). These
primary spermatocytes soon enter a program of growth accom-
panied by a series of characteristic changes in nuclear mor-
phology that result in a 25-fold increase in nuclear volume
(Tates, 1971; Lindsley and Tokuyasu, 1980; Fuller, 1993).

An important question about spermatocyte growth concerns
the timing of premeiotic DNA synthesis (cf. Olivieri and
Olivieri, 1965; Lindsley and Tokuyasu, 1980). Olivieri and
Olivieri (1965) injected one-day-old males with tritiated
thymidine and examined their testes by autoradiography for
thymidine incorporation at several post injection (p.i.) time
intervals. They detected DNA synthesis in spermatogonial
cysts as early as 30 minutes p.i. In contrast, thymidine incor-
poration into 16-cell primary spermatocyte cysts was first
observed at 8 hours p.i. The same results were obtained by
Sato (quoted by Lindsley and Tokuyasu, 1980), using a higher
specific activity tritiated thymidine. Because only the
youngest spermatocytes were labelled at 8 hours p.i., the
labelling of these cells could result from thymidine incorpo-
ration occurring during the S phase of the 8-cell-cyst sper-
matogonia. Thus, these observations indicate either that pre-
meiotic DNA synthesis occurs throughout the growth phase
of D. melanogaster spermatocytes, or that these cells do not
incorporate tritiated thymidine for some unknown reason.

To examine the occurrence of the premeiotic DNA
synthesis dissected larval testes were incubated at 25°C in
PBS containing bromo-deoxyuridine (BrdU). At different
times after the beginning of the incubation, testes were fixed
with our usual procedure, and treated with an anti-BrdU
antibody to detect BrdU incorporation. The first clearly
labelled cysts were observed after 3 hours incubation.
Labelled nuclei were observed in 2-, 4- and 8-cell spermatogonial cysts, as well as in 16-cell cysts containing very young spermatocytes morphologically indistinguishable from secondary spermatogonia in the G2 stage (Fig. 3). Cysts containing spermatocytes even slightly larger than gonial cells were invariably unlabelled.

The spermatocyte labeling in 16-cell cysts cannot be due to an immunostaining artifact because the cyst cell nuclei, which are in an extended G2 phase, do not react with the anti BrdU antibody (Fig. 3). Moreover, the fact that three hours are the minimum time required for labeling of both spermatogonia and spermatocytes strongly suggests that spermatocyte labeling is the direct consequence of BrdU incorporation into these cells, and not the result of BrdU uptake during the last spermatogonial S phase. Thus, although we cannot explain why the tritiated thymidine experiments failed to detect premeiotic S phase, we conclude that premeiotic DNA synthesis occurs in very young primary spermatocytes. The early occurrence of this synthesis may facilitate the elevated transcriptional activity of primary spermatocytes, which have embarked on the production of most, if not all, mRNA needed for spermiogenesis (for review see Fuller, 1993).

**Spermatocyte growth**

Primary spermatocytes that have just completed DNA synthesis (stage S1, Fig. 4a) are similar in size and morphology to the G2 secondary spermatogonia. As a primary spermatocyte begins its growth, the nucleus progressively assumes an eccentric position within the cytoplasm (stage S2, Fig. 4b,c). This so-called polar spermatocyte stage (Tates, 1971) is characterized by an asymmetrical distribution of the mitochondria within the cytoplasm, with these organelles clustered at the pole of the cell opposite to the nucleus (Cooper, 1965; Tates, 1971). In our cytological preparations polar spermatocytes exhibit a dense network of cytoskeletal microtubules and a cloud of Hoechst-fluorescent, dotted material clustered in a cytoplasmic region opposite to the nucleus (Fig. 4b,c). These fluorescent dots most likely correspond to the Hoechst-stained mitochondrial DNA, thus identifying the cytoplasmic location of these organelles.

During the polar spermatocyte stage there is a substantial increase in nuclear diameter accompanied by changes in chromatin organization within the nucleus. In young polar spermatocytes (stage S2a, Fig. 4b), the chromatin appears as a compact mass occupying the center of the nucleus. As the polar spermatocytes grow, chromatin subdivides into three masses...
or clumps that remain closely apposed to the inner nuclear envelope (stage S2b, Fig. 4c; see also Cooper, 1965). The space between these clumps increases with nuclear growth, so that in mature spermatocytes most of the karyoplasm is not occupied by the chromatin. Two of these chromatid clumps are larger and denser than the other and probably correspond to the somatically-paired large metacentric autosomes (Cooper, 1965). The third chromatid mass appears to be composed by 3-5 fluorescent elements that are likely to correspond to the X chromosome, the portions of the Y chromosome not involved in loop formation, and the tiny fourth chromosomes (Cooper, 1965).

As nuclear growth continues, the nucleus progressively resumes a central position within the cell and the mitochondria disperse uniformly into the cytoplasm (stage S3). This stage is also characterized by the first appearance within the karyoplasm of the Y chromosome loops. In D. melanogaster there are three lampbrush-like loops, formed by the kl-5, kl-3 and ks-1 fertility factors (Bonaccorsi et al., 1988). We have previously shown that these structures develop asynchronously during spermatocyte growth, with the kl-5 and ks-1 loops appearing earlier than the kl-3 loop (Bonaccorsi et al., 1988). The youngest apolar spermatocytes (stage S3, Fig. 4d) have a centrally located nucleus surrounded by a symmetric network of microtubules, and exhibit two phase-dark bodies that correspond to the primordia of the kl-5 and ks-1 loops (Bonaccorsi et al., 1988).

Maturation of the apolar spermatocytes is characterized by a further increase in nuclear size and by the elaboration of the Y loops. The kl-5 and ks-1 loops continue to expand and soon lose their initial compactness, revealing their underlying filamentous structure. At this stage (stage S4, Fig. 4e), a third cluster of filamentous material, the kl-3 loop, also becomes apparent. The three loops enlarge steadily and reach their apparent maximum size in mature spermatocytes (stage S5, Fig. 4f). These cells are the largest cells produced during Drosophila spermatogenesis. Their nuclei exhibit three fully grown Y chromosome loops that differ both in the type of thread they contain and in their relative degree of compactness: the kl-5 and ks-1 loops are more compact and contain a coarse thick thread, while the kl-3 loop consists of a thinner, less twisted and folded filament (Bonaccorsi et al., 1988). The Y loops of mature spermatocytes occupy most of the nucleus and often overlap, so that in many cases they cannot be distinguished from each other. However, they can be easily identified by Giemsa staining at pH 10 or by immunostaining with various antibodies that specifically decorate each of these structures (Bonaccorsi et al., 1988; Pisano et al., 1993).

The disintegration of the Y loops marks the end of the growth phase of primary spermatocytes and the beginning of the first meiotic division. When the loops begin to fall apart into pieces (stage S6, Fig. 4g), the nuclei remain at more or less the same size as in the mature spermatocytes. However, in S6 nuclei the nucleolus has already disappeared; the chromatin has begun to condense in preparation for meiosis but is still close to the inner nuclear envelope. The microtubular system of these cells has also changed from that of mature spermatocytes in that the microtubules are now more concentrated around the nucleus than in the rest of the cytoplasm (Fig. 4g).

Traditionally, the spermatocyte growth phase is considered as the meiotic prophase. However, as already observed by Cooper (1965) in D. melanogaster and by Kremer and coworkers (1986) in D. hydei, spermatocyte growth is not accompanied by the typical progressive condensation and synopsis of meiotic chromosomes that can be seen in the meiotic prophase of most organisms. After premeiotic DNA synthesis the chromosomes are dispersed within the nucleus and become organized into three clumps as primary spermatocytes enlarge. Although there is no direct evidence that each clump contains a couple of paired homologs, this conclusion is generally accepted by students of Drosophila spermatogenesis (for reviews see Cooper, 1965; Fuller, 1993). In addition, it has been suggested that homologous chromosome association in the clumps may be the consequence of the persistence of 'somatic pairing' during gonial mitoses and throughout spermatocyte development (Metz, 1926; Cooper, 1965; reviewed by Fuller, 1993).

Although each clump is likely to consist of a bivalent, no leptotene- or zygotene-like chromosomes can be discerned within these structures by aceto-orcein or Hoescht staining (Cooper, 1965; present results). Thus, the clumps represent a diffuse state of meiotic chromosomes, analogous to the state of interphase chromatin. This decondensed state of spermatocyte chromosomes persists until the M1 stage (see below), when chromatin condenses very rapidly, just prior to the onset of the first meiotic metaphase. According to Cooper (1965), when the bivalents become visible by orcein staining, they have already attained a degree of condensation equivalent to that of diakinetin chromosomes.

The absence of the leptotene-pachytene stages in primary spermatocytes is probably related to the lack of synaptonephal complex (Meyer, 1964) and meiotic crossing over in D. melanogaster males. Interestingly, D. melanogaster females, that exhibit a normal synaptonephal complex and undergo meiotic recombination, also exhibit a canonical meiotic prophase that lasts about 80 hours and comprises chromosome condensation stages equivalent to leptotene/zygotene and pachytene (King, 1970; Carpenter, 1975; Davrin and Sunner, 1976, 1977). Thus, D. melanogaster possesses the genetic information necessary for progressive meiotic chromosome condensation, but this information is exploited only during female meiosis. Meiotic chromosome condensation in males is very rapid and is brought about in a fashion similar to that observed in mitotic cells. This observation suggests the intrigu-
Fig. 4. Primary spermatocyte growth. (Row a) A complete cyst of young primary spermatocytes in the S1 stage; the arrows point to the cyst cells. (Row b) Polar spermatocytes in the S2a stage; the arrows indicate the asymmetric cap of mitochondria. (Row c) Polar spermatocytes in the S2b stage; the arrows point to the clusters of mitochondria. (Row d) A young apolar spermatocyte in the S3 stage; note the primordia of the kl-5 (A) and ks-1 (C) Y chromosome loops, and the three chromatin clumps. (Row e) A mature primary spermatocyte in the S4 stage in which some filaments of the kl-3 loop (B) have become apparent. (Row f) A mature primary spermatocyte in the S5 stage showing fully developed Y chromosome loops; note that the nucleolus (arrow) is surrounded by the sex chromosome chromatin. (Row g) A primary spermatocyte in the S6 stage with disintegrating Y loops. Bar, 10 µm.
ing possibility that meiotic chromosome contraction in males exploits the same genetic program that governs chromosome condensation during mitosis.

Meiotic divisions

As the Y loops disintegrate the nucleus shrinks and its diameter eventually becomes about 20% shorter than that of mature spermatocytes (stage M1, Fig. 5a,b). A distinguishing feature of the M1 stage is the presence within the nucleus of a few compact granules derived from Y-loop disintegration. The microtubular system of these M1 cells has already completed its reorganization: the network of microtubules around the nucleus has disappeared and two prominent asters have become apparent. During the M1a stage (Fig. 5a), the asters, initially located on the same side of the nucleus, migrate to opposite poles, while remaining closely apposed to the outer nuclear envelope. By the M1a stage, the chromatin has already attained a high degree of condensation and the three major bivalents are clearly visible. The fourth chromosome bivalent is not always clearly identifiable. However, we often observed a dotted element associated with the smallest bivalent, suggesting that the fourth chromosomes lie close to the sex chromosomes, retaining their apparent vicinity during interphase. In the M1a stage the bivalents tend to be peripherally located opposite poles, while remaining closely apposed to the outer nuclear envelope. By the M1a stage, the asters, initially located on the same side of the nucleus, migrate to opposite poles, while remaining closely apposed to the outer nuclear envelope. By the M1a stage, the asters, initially located on the same side of the nucleus, migrate to opposite poles, while remaining closely apposed to the outer nuclear envelope. By the M1a stage, the asters, initially located on the same side of the nucleus, migrate to opposite poles, while remaining closely apposed to the outer nuclear envelope. By the M1a stage, the asters, initially located on the same side of the nucleus, migrate to opposite poles, while remaining closely apposed to the outer nuclear envelope. By the M1a stage, the asters, initially located on the same side of the nucleus, migrate to opposite poles, while remaining closely apposed to the outer nuclear envelope.

The M1a nuclei are round or ovoid and the cytoplasm around them has a rather homogeneous appearance under phase-contrast microscopy. In the later M1b stage (Fig. 5b), the nuclei become irregularly shaped and are surrounded by phase-contrast dense, elongated elements. Most likely these structures correspond to the system of parafusorial and astral membranes that surround the spindle and the poles during male meiosis (Tates, 1971; Church and Lin, 1982). During the M1b stage the asters are more prominent than in the M1a stage, and a few spindle fibers radiating from the asters appear to reach the bivalents, which occupy variable positions within the nucleoplasm. The demarcation between the nucleus and the cytoplasm progressively disappears as meiosis proceeds, while additional spindle fibers become connected to the bivalents, which can be seen at different distances from the spindle poles (stage M2; Fig. 5c). The M1b and M2 stages almost certainly correspond to the prometaphase of the first meiotic division, a phase in which the bivalents, connected to the spindle poles by kinetochore microtubules, undergo complex motions necessary for proper metaphase orientation (Church and Lin, 1982, 1985).

Prometaphase of the first meiotic division is completed when the bivalents congress to a metaphase plate (stage M3; Fig. 5d). During metaphase, the bivalents cluster in a single Hoechst-bright structure equidistant from the poles and are connected to the spindle poles by two symmetrical sets of microtubules. In addition to these pole-to-chromosome microtubules the metaphase spindle exhibits bundles of microtubules that do not encounter the chromosomes. These microtubules appear to originate from the poles, run outside the congregated bivalents and end either in the opposite pole or distally to the metaphase plate (Fig. 5d).

Nearly all the cysts with cells in the M3 stage also contain early anaphase I cells (stage M4a; Fig. 5e). Interestingly, in most of these early anaphases the segregating bivalents have already migrated a considerable distance towards the poles, suggesting that initial anaphase chromosome movement is very rapid. The spindle organization of early anaphase I cells is very similar to that of metaphase I cells in the M3 stage, consisting of two closely apposed umbrella-shaped half spindles. However, as anaphase proceeds the spindle microtubules reorganize: the density of microtubules in the region between the segregating sets of chromosomes (central spindle) progressively increases, while the number of microtubules located in the proximity of the chromosomes decreases. By mid-anaphase (stage M4b, Fig. 5f) the microtubules emanating from each pole overlap in the central region of the spindle, so that the two opposed umbrella-shaped half spindles of early anaphase are now fused in a unique oval structure. By late anaphase/early telophase (stage M4c, Fig. 5g,h) there is a further reduction in the number of microtubules emanating from the centrosomes, accompanied by an apparent increase of the density of central spindle microtubules. Concomitant with this spindle reorganization, there is a further increase in the distance between the two daughter nuclei, suggesting that the M4a and M4b-c stages correspond to anaphase A and anaphase B, respectively.

The dense bundle of microtubules in the central spindle formed during stage M4b-c is progressively squeezed and eventually attains an hourglass shape (telophase I, stage M5, Fig. 5i). In many cases, and more frequently in broken cysts, these hourglass-shaped spindles are bent at the mid-body. During telophase I (stage M5, Fig. 5i), the two daughter nuclei become clearly demarcated from the cytoplasm and often exhibit a dark dot, which stands out from the phase-contrast clear nucleoplasm. In favourable preparations these ‘round bodies’ (Grond, 1984) can also be seen in spermatid nuclei, but they disappear soon after the initiation of spermatid elongation (Lindsley and Tokuyasu, 1980). The nature and the biological role of these intranuclear structures, also called the protein bodies (Kremer et al., 1986) or the nucleolus-like bodies (Lindsley and Tokuyasu, 1980), are still largely unknown (Tates, 1971; Lindsley and Tokuyasu, 1980; Grond, 1984; Kremer et al., 1986).

As meiosis proceeds, telophase I nuclei enlarge and the second division asters become apparent (stages M6a and M6b; Fig. 6). Actually centrosome separation occurs earlier and sometimes two distinct centrosomes are already visible in late anaphase I (stage M4c; see Fig. 5i). These centrosomes, which contain a single centriole (Tates, 1971), progressively nucleate the aster microtubules and migrate to the cell poles. In telophases with hourglass-shaped spindles, two close foci of microtubule nucleation can often be seen. As the central spindle progressively disappears, these initial asters become more prominent and move to opposite poles, while remaining closely apposed to the outer nuclear envelope (stage M6a, not shown). When the asters have completed their migration to the poles, secondary spermatocyte nuclei have considerably enlarged and their chromatin appears to be relatively decondensed, occupying the center of the nucleus (stage M6b, Fig. 6a). The M6b cells are relatively rare, indicating that this stage, which probably corresponds to interphase II, has a short duration.

As the chromosomes condense, in preparation for meiosis II, three Hoechst-bright elements become apparent (stage M7, Fig. 6b). These elements (the two major autosomes and the X or the Y chromosome) in many cases lie very close to each
other due to the small size of secondary spermatocyte nuclei, so that only one or two Hoechst-bright spots can be observed. Soon after the chromosomes have condensed, the cytoplasmic nuclear demarcation disappears and additional spindle fibers penetrate the nucleus, while the chromosomes are found at variable distances from the spindle poles (stage M8; Fig. 6b).
These features suggest that during the M7 and M8 stages chromosomes undergo the prometaphase motions necessary for proper orientation and congression.

The subsequent stages of the second meiotic division are analogous to those of the first meiotic division. Thus, chromosomes eventually congregate into a metaphase plate (stage M9, Fig. 6c), while the density of both kinetochore and polar microtubules increases. During early anaphase II (stage M10a, Fig. 6d), the chromosomes move rapidly towards the poles and the spindle retains a metaphase-like configuration. Later in
**Fig. 6.** The second meiotic division in *D. melanogaster* males.

(Row a) A secondary spermatocyte nucleus in the M6b stage. (Row b) Two prometaphase secondary spermatocyte nuclei in the M7 stage (top) plus a prometaphase nucleus in the M8 stage (bottom). (Row c) A metaphase II in the M9 stage. (Row d) An early anaphase in the M10a stage. (Row e) A mid-anaphase in the M10b stage. (Row f) A late anaphase in the M10c stage. (Row g) Two telophases in the M11 stage. (Row h) A telophase in the M11 stage with a bent spindle. Note that in e-h Hoechst staining reveals the arrangement of mitochondria. Bar, 10 µm.

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anaphase, the daughter nuclei continue to move apart, while the density of fibers in the central spindle progressively increases (stages M10b, Fig. 6e; M10c, Fig. 6f). As for meiosis I, we propose that stages M10a and M10b-c correspond to anaphase A and B, respectively.

During telophase II (stage M11, Fig. 6g,h), the central spindle is progressively squeezed, attaining a hourglass shape, while the two daughter nuclei become demarcated from the cytoplasm. Many telophase II spindles are bent at an angle just as telophase I spindles. However, while most anaphase spindles of broken cysts are bent, spindle bending does not occur, or is much less pronounced, in complete cysts.

Our observations have shown that meiotic nuclei undergo cyclic changes in the nuclear-cytoplasmic demarcation. Late prophase/early prometaphase spermatocytes in the M1 stage exhibit a clear demarcation between the nucleus and the cytoplasm. This demarcation disappears as the cells enter the M2 stage; the absence of a nuclear-cytoplasmic boundary persists through metaphase and anaphase I (stages M3+M4) until telophase I (stage M5), when a sharply outlined nucleus becomes visible again. Nuclear demarcation is maintained during interphase, prophase and early prometaphase II (stages M6 and M7), and disappears again when secondary spermatocytes enter late prophase II (stage M8), to reappear at telophase II (stage M11). These cyclic changes in nuclear appearance cannot be due to the breakdown of the nuclear membrane, which remains largely intact during male meiosis (Tates, 1971). Instead, they most likely reflect transformations of other components of the nuclear envelope such as the nuclear lamina. This suggestion is supported by recent data of White-Cooper and coworkers (1993), who analyzed lamin immunostaining during Drosophila spermatogenesis, and showed that premeiotic primary spermatocytes have a prominent nuclear lamina that breaks down in cells undergoing meiosis.

Figs 5 and 6 clearly show that tubulin immunostaining detects a prominent fibrous structure that connects the two daughter nuclei during both the first and the second meiotic ana-telophase. This structure is recognized by three different anti-tubulin antibodies, which also react with the metaphase spindle and the cytoskeleton. Thus, this bundle of fibrous material is undoubtedly a bundle of spindle microtubules. However, in a recent cytological analysis of the first meiotic division of Drosophila males, Casal and coworkers (1990) failed to detect a central spindle by tubulin immunostaining. In these studies dissected testes were treated with taxol and fixed with formaldehyde, after an ethanol pre-fixation; the preparations were then stained by indirect immunofluorescence with either an anti-α tubulin antibody (Sera Lab, Accurate Chemicals, Westbury/NY, YL1/2; Kilmartin et al., 1982) or the same anti-β tubulin antibody employed here (Amersham; Blose et al., 1982; see Materials and Methods). Using this method they were able to detect metaphase and early anaphase spindles but not ana-telophase central spindles. The reason for these results is currently unclear. Their fixation procedure might have selectively disrupted the central spindle of anaphelaphase. Alternatively, and more likely, the taxol treatment might have interfered with spindle reorganization during anaphase, preventing the formation of the central spindle.

The central spindles of the first and the second meiotic divisions exhibit a remarkably similar behavior during anaphase. At mid-anaphase I (stage M4b) or II (stage M10b) the central spindle shows an extended zone of microtubule overlap at its equator. However, while the anaphase spindle elongates, this apparent overlap zone does not shorten as seen in organisms such as diatoms, where spindle elongation occurs by microtubule sliding in the overlap zone (for review see Candé, 1989). This observation suggests that anaphase B spindle elongation in Drosophila male meiosis is brought about by mechanisms that do not involve microtubule sliding in the central spindle. Alternatively, if such a sliding does occur, it must be accompanied by microtubule growth, so that the extension of the overlap zone would not decrease as the spindle poles move apart.

An interesting feature of male meiosis is the precise partition of mitochondria between the daughter cells at each meiotic division. Electron microscopy studies have shown that at prometaphase mitochondria aggregate around the nucleus, lining up parallel to the spindle axis. This arrangement persists through telophase until the occurrence of cytokinesis, thus mediating precise partition of these organelles (Tates, 1971; reviewed by Fuller, 1993). Due to their DNA content, mitochondria can be also identified by light microscopy in favourable Hoechst-stained preparations (Figs 5 and 6). In late meiotic prophase and early prometaphase I (stage M1) mitochondria appear to be uniformly distributed within the cytoplasm. However, as cells enter late prometaphase I (stage M2) mitochondria aggregate around the equator of the nucleus. This arrangement persists through metaphase, early and mid anaphase up until the formation of the central spindle. Then mitochondria line up along this structure and remain associated with it until the occurrence of cytokinesis, which divides them into two equal groups (Fig. 5). The same mitochondrial behavior is observed during meiosis II (Fig. 6), so that the four meiotic products receive the same amount of mitochondria.

The timing of meiotic division

Previous cinematographic studies on live cells have shown that about 72 minutes elapse from the time that primary spermatocytes enter the M1 stage to the time when anaphase begins (Church and Lin, 1985). M1 cells, selected on the basis of their characteristic morphology, were filmed for 1.5 hours, allowing precise determination of the timing of the initial meiotic events (Church and Lin, 1985). Within 30 minutes from their identification M1 cells undergo several morphological changes that culminate in the appearance of chromosomes; this indicates that a maximum of 30 minutes elapses between the formation of M1 cells and the appearance of the bivalents. From the time that chromosomes become visible to the beginning of anaphase I there are about 42 minutes. During the first 20 minutes of this interval the bivalents undergo a series of complex saltatory prometaphase motions; in the subsequent 22 minutes they exhibit a persistent bipolar orientation that is maintained until the onset of anaphase (Church and Lin, 1985).

We have determined the frequencies of the various meiotic stages in fixed testis preparations. The frequency of each stage should be proportional to its duration in live material. Thus, if one ascertains the frequency and the duration in vivo of a given meiotic stage, the duration of all the other stages can be easily calculated from their relative frequencies. Using these criteria, we have estimated the lengths of the meiotic phases (Table 1); calculations were made by grouping together stages that are easily distinguishable from both the preceding and the
following ones, and by assuming that stages M1-M3 last approximately 72 minutes (Church and Lin, 1985). For example, we have grouped stages M1a and M1b, which include late prophase and early prometaphase I, and are characterized by the presence of a clear nuclear-cytoplasmic demarcation; this demarcation is absent in the next group of stages (M2 and M3), that correspond to late prometaphase and metaphase I. Similarly, stages M6-M7, that comprise interphase II and early prometaphase II, exhibit a clear nuclear-cytoplasmic demarca-

<table>
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<tr>
<th>Number of cells</th>
<th>202</th>
<th>128</th>
<th>280</th>
<th>214</th>
<th>117</th>
<th>274</th>
<th>1,215</th>
</tr>
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<tbody>
<tr>
<td>Relative frequencys</td>
<td>16.6</td>
<td>10.5</td>
<td>23.1</td>
<td>17.6</td>
<td>9.6</td>
<td>22.6</td>
<td>100</td>
</tr>
<tr>
<td>Estimated duration (minutes)</td>
<td>44</td>
<td>28</td>
<td>61</td>
<td>47</td>
<td>26</td>
<td>60</td>
<td>266</td>
</tr>
</tbody>
</table>

The relative frequencies of the various stages were obtained by scoring 40 late larval/early pupal testes. E, early; L, late; prometa, prometaphase.

**Fig. 7.** Spermatid differentiation in *D. melanogaster*. (Row a) Partial cyst with spermatids at various differentiation stages. 1, stage T1; 2, stage T2; 3, stage T3 with nuclei smaller than nebenkerns. (Row b) Onion stage spermatids (stage T4); note the characteristically dense cytoskeleton. Bar, 10 µm.
diffuse chromatin. (c) Spermatids with elongated mitochondrial derivatives showing a dense chromatin mass flattened against the side of the nucleus underlying the growing axoneme. Bar, 10 μm.

The timing of D. melanogaster male meiosis has been also estimated by time-lapse cinematography of living pupal testes (Cross and Shellenbarger, 1979). This analysis showed that between the stage of ‘elongated nuclei’ during the first meiotic division and the stage defined as ‘toward the end of the second division’ there is a time interval of about 130 minutes. The ‘elongated nuclei’ probably correspond to cells in mid-anaphase I (stage M4b-c), while the ‘end of the second division’ is likely to correspond to telophase II (stage M11). We have calculated that the time elapsing between the beginning of anaphase I (stage M4a) and the end of telophase II (stage M11) is 194 minutes. Since the interval considered by Cross and Shellenbarger is obviously more restricted than ours, the two estimations appear to be comparable.

Spermatid differentiation

In telophase II cells, the mitochondria associated with the central spindle move towards the nuclei, forming an irregular mass on one side of the nucleus (stage T1; Fig. 7a). This mass quickly assumes a crescent-like shape (stage T2; Fig. 7a), which then results in a spherical shape (stage T3; Fig. 7a). Spermatids in the T3 stage consist of a spherical mitochondrial aggregate associated with a smaller, spherical nucleus. As spermatid differentiation proceeds, the nucleus enlarges and mitochondria progressively fuse to form a complex organelle, called the nebenkern, containing multiple layers of interlaced mitochondrial membranes (Tates, 1971; Tokuyasu, 1975). Because cross sections of this mitochondrial derivative resemble an onion, this stage of spermatid development has been named the onion stage (Bowen, 1922; Tates, 1971; Tokuyasu, 1975). Light microscopy and Hoechst staining do not allow discrimination between T3 mitochondrial aggregates and onion-stage nebenkerns. They both appear as phase-dense, round structures that exhibit a dotted Hoechst fluorescence, due to the presence of mitochondrial DNA. However, onion stage spermatids (stage T4; Fig. 7b) can be easily distinguished from those in the T3 stage because they have a nucleus of the same size or slightly larger than the nebenkern, and a dense cytosomekeleton with characteristic bundles of microtubules.

The onion-stage spermatid nuclei contain a very compact chromatin mass that can be easily visualized after both Hoechst and Giemsa staining (Figs 7b and 8a). When the elongation process begins, the nuclei remains spherical, while the nebenkern assumes an oval shape (stage T5, Fig. 8b). At stage T5 the nuclear chromatin loses its previous compactness and becomes more diffuse within the nucleoplasm. However, as elongation proceeds, chromatin condenses again, forming a dense mass flattened against the fenestrated side of the nucleus underlying the elongating axoneme (Fig. 8c). This extra cycle of chromatin decondensation and recondensation has been already described by light microscopy in D. hydei (Kremer et al., 1986). Although the precise biological meaning of this phenomenon is currently unclear, it has been correlated with the molecular reorganization of the chromatin that occurs in spermatid nuclei (Das et al., 1964; Hausteck-Jungen and Harl, 1982).

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REFERENCES


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