Journal of Cell Science 110, 721-730 (1997)
Printed in Great Britain © The Company of Biologists Limited 1997
JCS3490

Role of microtubules and microtubule organizing centers on meiotic chromosome elimination in *Sciara ocellaris*

M. R. Esteban[1,*], M. C. C. Campos[2,*], A. L. P. Perondini[2] and C. Goday[1,†]

[1]Centro de Investigaciones Biológicas, Consejo Superior de Investigaciones Científicas, Velázquez 144, 28006 Madrid, Spain
[2]Departamento de Biologia, Universidade de S. Paulo, São Paulo, Brasil

*These two authors have contributed equally to this work
†Author for correspondence

SUMMARY

Spindle formation and chromosome elimination during male meiosis in *Sciara ocellaris* (Diptera, Sciaridae) has been studied by immunofluorescence techniques. During meiosis I a monopolar spindle is formed from a single polar complex (centrosome-like structure). This single centrosomal structure persists during meiosis II and is responsible for the non-disjunction of the maternal X chromatids. During meiosis I and II non-spindle microtubules are assembled in the cytoplasmic bud regions of the spermatocytes. The chromosomes undergoing elimination during both meiotic divisions are segregated to the bud region where they associate with bundles of microtubules. The presence and distribution of centrosomal antigens in *S. ocellaris* meiotic spindles and bud regions has been investigated using different antibodies. γ-Tubulin and centrin are present in the bud as well as in the single polar complex of first meiotic spindle. The results suggest that spermatocyte bud regions contain microtubule-organizing centres (MTOCs) that nucleate cytoplasmic microtubules that are involved in capturing chromosomes in the bud regions. The distribution of actin and myosin in the spermatocytes during meiosis is also reported.

Key words: Diptera, Sciaridae, Meiosis, Monopolar spindle, MTOC, Chromosome elimination

INTRODUCTION

The selective elimination of paternal chromosomes in sciarid flies during early development and meiosis constitutes a classical example of genomic imprinting (Rieffel and Crouse, 1966). In sciarids, during the early development of the embryo (3X;2A), one or both paternally inherited X chromosomes are eliminated in the somatic cells of the embryos destined to be females (2X;2A) or males (X;2A), respectively. An even more drastic case of elimination takes place during male meiosis, where the whole paternally derived chromosome complement is discarded. The peculiar chromosome behavior that leads to non-random segregation and chromosome elimination during male meiosis was first described by Metz (1925, 1926, 1927, 1933, 1938; Metz et al., 1926). Although, the majority of the information on this phenomenon derives from the species *Sciara coprophila* (Fig. 1), the main known features concerning chromosome movements during elimination in male meiosis are also true for other sciarids like *Trichosia pubescens* and *S. ocellaris*. During the first male meiotic division, the formation of a monopolar spindle has been described (Fig. 1A,B) (Metz, 1925, 1926; Metz et al., 1926; Basile, 1970; Amabis et al., 1979; Fuge, 1994). During this process, the chromosomes, which are asynaptic, remain scattered and never become aligned in a metaphase-like array; that is, primary spermatocytes proceed directly from prometaphase to an anaphase-like stage (Metz, 1925; Metz et al., 1926). During anaphase I, maternal chromosomes and L chromosomes (germ line limited chromosomes that are eliminated from the soma during embryogenesis) move polewards, while paternal chromosomes move away from the pole into a cytoplasmic bud to be eliminated (Metz et al., 1926; Smith-Stocking, 1936). At meiosis I, a single polar complex containing 'giant centrioles' encircled by dense material from which microtubules radiate has been described in *S. coprophila* and *T. pubescens* by electron microscopy (Phillips, 1967; Kubai, 1982; Fuge, 1994). In addition to the polar complex, irregular centriolar components have been identified in *T. pubescens* at the cytoplasmic region opposite the single pole (Fuge, 1994).

The mechanisms underlying the differential segregation of maternal and paternal chromosomes and how paternal chromosomes are captured into the bud, to be excised from the primary spermatocyte, are still poorly understood (reviewed by Gerbi, 1986). Electron microscopy data in *S. coprophila* indicate that maternal homologs have kinetochore structures but apparently do not bind kinetochore microtubules (Kubai, 1982). On the other hand, in *S. coprophila* it has been suggested that all paternal chromosomes display a reverse orientation during anaphase I; that is, they migrate to the cytoplasmic bud area apparently with their centromeres lagging rather than leading the direction of the movement (Abbott et al., 1981). The kinetochores of paternal chromosomes were found by electron microscopy to interact with microtubules emerging from the single pole, although normal forces on the centromeres by chromosomal fibers may not be exerted (Kubai, 1982).

During the second meiotic division in sciarid spermatocytes (Fig. 1C,D), the spindle follows the same axis as the first

meiotic spindle (Metz et al., 1926) and it has been considered to be bipolar and monocentric (Dubois, 1933; Abbott and Gerbi, 1981). By metaphase II, maternal autosomes and L chromosomes are arranged on the metaphase plate, while the X chromosome is seen 'precociously' at one pole of the spindle (Metz, 1925; Metz, 1934; Crouse, 1943). At anaphase II, autosomal chromatids separate and move conventionally to opposite poles. The chromatid group lacking the sex chromosome is eliminated in a cytoplasmic bud, while the daughter nucleus containing both chromatids of the X is retained as the future spermatid nucleus (Abbott and Gerbi, 1981). Recent data in *Trichosia* indicate that a unique polar structure (centrosome) is visible in meiosis II (Fuge, 1994). Therefore, as proposed by Gerbi (1986), in sciarids vestiges of a monopolar spindle are still present in the bipolar spindle of meiosis II.

A locus controlling the precocious sex chromosome behavior has been identified in *S. coprophila*; it is located in the proximal heterochromatin of the X chromosome, but not at the centromere (Crouse, 1960, 1977). How this locus governs the precocious chromosome behavior is still unknown. Based on classical cytological observations it had been suggested that this locus could control the earlier movement of the X chromosome with respect to the rest of chromosomes although more recent data (Abbott and Gerbi, 1981; Gerbi, 1986) suggest that in early meiosis II the X chromosome is already near the pole because its centromere is inhibited from normal function. The nature of the anchoring of the X chromosome to the pole has not been determined.

In the present report, *Sciara ocellaris* has been studied by immunofluorescence techniques, using anti-tubulin antibodies and Hoechst chromatin staining, to achieve a fine cytological characterization of spindle formation and the unusual chromosome behavior during male meiosis in sciarids. Our data show that contemporaneously with the formation of the monopolar first spindle, non-spindle microtubules are assembled in the cytoplasmic regions involved in bud formation. The chromosomes undergoing elimination during both meiotic divisions become associated with bundles of microtubules in the bud region. Moreover, the cytology shows that non-disjunction of the maternal X chromatids is achieved because the X chromosome remains associated with the polar complex from the first monopolar spindle through meiosis II. In view of these results, we have investigated the conservation and distribution of centrosomal proteins in the first and second meiotic spindles as well as in the bud region. To this end, a battery of antibodies (anti-γ-tubulin, Rb188, anti-centrin, 224 and MPM-2) that recognize centrosomal antigens in other systems have been used. The results show that centrosomal proteins are present both in the polar complex structure of the first spindle and in the cytoplasmic bud regions during meiosis. Centrosomal antigens are absent in the second meiotic spindle that is devoid of asters. The data suggest the existence of cytoplasmic microtubule-organizing centres (MTOCs) that nucleate bud microtubules during meiosis. Therefore, bud regions in *S. ocellaris* seem to be actively involved in the capture of the chromosomes that undergo elimination. We also report the distribution of actin and myosin in the spermatocyte during meiosis.

MATERIALS AND METHODS

Indirect immunofluorescence

Fixation of male meiotic divisions

Prepupae of *Sciara ocellaris* were dissected in KNT buffer (183 mM KCl, 47 mM NaCl, 10 mM Tris-HCl, pH 6.8) to remove the testes. The testes were squashed in a drop of the same buffer, frozen in liquid N_2 to remove the coverslips and immediately fixed in 100% methanol for 10 minutes at –20°C. After fixation, the slides were treated with acetone at –20°C for 1 minute, washed three times in PBS for 10 minutes at room temperature and permeabilized in 1% Triton X-100 in PBS for 10 minutes. They were subsequently washed in PBS three times for 5 minutes and incubated for at least 30 minutes at room temperature with 3% nonfat dried milk in PBS.

Antibody incubation

Primary and secondary antibodies were diluted in 1% BSA in PBS and used as follows: (a) anti-β-tubulin (1:200) (Amersham) followed by FITC-conjugated anti-mouse IgG (1:50) (Southern Biotechnology Associates, Inc.) or Rhd-conjugated anti-mouse Ig (1:40) Dakoppats). (b) Rb188 antiserum (Whitfield et al., 1988) (1:10) followed by FITC-conjugated anti-rabbit (1:50) (Boehringer). (c) Anti-centrin antiserum 26/14-1 (Salisbury et al., 1984) (1:10) followed by FITC-conjugated anti-rabbit (1:50) (Boehringer). (d) MPM-2 (1:50) (Upstate Biotechnology Inc.) followed by FITC-conjugated anti-mouse IgG (1:50) (Southern Biotechnology Associates, Inc.). (e) Anti-γ-tubulin antiserum Rb 143 against *Drosophila* γ-tubulin (C. Gonzalez, unpublished) (1:100) followed by FITC-conjugated anti-rabbit (1:50) (Boehringer). (f) Anti-myosin (1:10) (Sigma) followed by FITC-conjugated anti-mouse IgM (1:50) (Southern Biotechnology Associates, Inc.). (g) Rhodamine-phalloidin (1:80) (Molecular Probes Inc.).

Slides of male meiotic divisions were incubated with primary antibody for 2 hours at room temperature or overnight at 4°C, washed three times in PBS for 10 minutes each, and incubated with secondary antibody for 45 minutes at room temperature. They were subsequently washed three times in PBS for 10 minutes. To perform double staining with a second primary antibody, the process was repeated before chromatin staining. A 0.5 μg/ml Hoechst 33258 solution in HB buffer (150 mM NaCl, 30 mM KCl, 10 mM Na_2HPO_4, pH 7) was used for simultaneous chromatin staining for 10 minutes at room temperature. Slides were washed briefly in HB buffer and mounted with antifading solution.

Microscopy and photography

Observations were made using a computer-controlled Zeiss Axioscope epifluorescence microscope equipped with a cooled charge coupled device (CCD) camera (Photometrics). FITC, Rhd-phalloidin and Hoechst fluorescence, detected by specific filter combinations, were recorded separately as gray scale images and when needed, digitally pseudocolored and merged.

RESULTS

Tubulin distribution during meiosis

The testes in sciarids are composed of many cysts and the cells within one cyst are localized at the periphery around an internal area (Metz et al., 1926; Phillips, 1970). The cells within one cyst are synchronous (Phillips, 1970). In primary spermatocytes of *Sciara ocellaris*, a species lacking L chromosomes, six autosomes and two X chromosomes are present ($2n$=8) (Dubois, 1933; Metz, 1938; Mori and Perondini, 1980).

The spherical premeiotic primary spermatocytes become pear-shaped during early prometaphase of meiosis I due to the formation of a cytoplasmic bud towards the central area of the cyst (Fig. 2A), as described previously for other species of sciarids (Metz et al., 1926; Kubai, 1982). Immunofluorescence analysis with anti-β-tubulin antibody reveals a relatively disperse cytoplasmic network of microtubules at this stage (Fig. 2A). As prometaphase proceeds

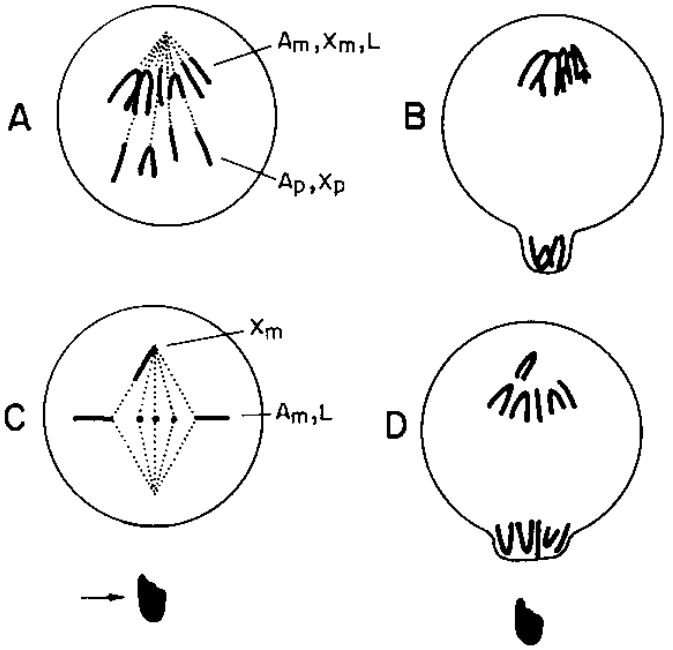

Fig. 1. Schematic diagram of the first (A,B) and second (C,D) spermatocyte division in *Sciara coprophila*. (A) Monopolar spindle at anaphase I-like stage. Maternal chromosomes (A_m, X_m) and L chromosomes (L) move polewards while paternal chromosomes (A_p, X_p) move away in the opposite direction. (B) Telophase I, showing paternal chromosomes segregated in a cytoplasmic bud. (C) Metaphase II, showing the precocious X-dyad (X_m) at one of the spindle poles while the rest of the chromosomes (A_m, L) are aligned at the equatorial plate. The arrow points to paternal chromosomes eliminated during telophase I. (D) Telophase II, showing the nullo-X-chromosome complement segregated into a cytoplasmic bud. (Modified from Dubois, 1933.)

(Fig. 2B), primary spermatocytes show an accumulation of tubulin staining in the cellular region located peripherally in the cyst where a polar complex is formed; microtubules emanate from it and extend to the cytoplasm to organize the monopolar spindle. In addition, an increase of tubulin staining is detected in the forming bud; this tubulin appears more dispersed than that in the polar complex and is not organized in a similar way. As expected, during the formation of the monopolar spindle the chromosomes are scattered reflecting the absence of synapsis and lack of a metaphase stage. These peculiarities have been described for other sciarids (Metz, 1925; Metz et al., 1926).

In anaphase I (Fig. 2C,D), microtubules emanating from the single polar complex are numerous and are often seen to end near the bud region. At this stage the tubulin in the bud region is organized in short microtubule bundles. Maternal chromosomes are located closer to the pole and still well-separated from each other. In contrast, paternal chromosomes segregate closely grouped towards the bud region, where microtubule-chromatin interactions become evident.

In late telophase I (Fig. 2E) the cytoplasmic bud region containing paternal chromosomes and microtubule bundles separates from the main body of the cell while the condensed and individualized maternal chromosomes maintain their position near the polar complex aster.

Our cytological results concerning the second meiotic division (Fig. 3) indicate that the polar complex observed during meiosis I persists through meiosis II and constitutes the only polar structure of the spindle in secondary spermatocytes (Fig. 3A-D), as has been suggested for *T. pubescens* (Fuge, 1994). The axis of the second meiotic spindle is also oriented towards the cytoplasmic bud region; the spindle is slightly asymmetrical, the hemispindle close to the polar complex being longer (Fig. 3A,B). The hemispindle near the bud region is not associated with a visible polar structure and no astral microtubules are observed (Fig. 3A-E). At metaphase II (Fig. 3A,B), the undivided X chromosome remains attached to the polar complex while the three autosomes congregate into a metaphase plate. In some cells, as in Fig. 3A, it is possible to distinguish three sets of microtubule bundles, presumably corresponding to the three autosomes.

During anaphase II the autosomal chromatids move in opposite directions (Fig. 3C). One set of autosomal chromatids reaches the 'former' pole containing the undivided X chromosome while the other set reaches the cytoplasmic bud region of the spermatocyte. During chromatid segregation the spindle morphology appears unaltered (Fig. 3C,D). Following segregation, and before the second meiotic spindle disorganizes, the set of autosomal chromatids reaching the bud area becomes associated with non-spindle bud microtubules (Fig. 3C,D). All the chromatin eliminated during meiosis I and II in the excised buds exhibits a persistent association with microtubule bundles even during spermatid differentiation (Fig. 3E,F). When spermatid differentiation starts, cells become elongated and a dense cytoplasmic microtubule cytoskeleton is observed (Fig. 3E). The organization of microtubules at this stage resembles the one seen in *Drosophila* (Cenci et al., 1994).

Centrosomal proteins distribution

Anti-γ-tubulin immunofluorescence

The polyclonal antibody that recognizes γ-tubulin in *Drosophila* stains the polar complex regions and bud regions during meiosis I in *S. ocellaris* spermatocytes (Fig. 4A). A unique γ-tubulin signal corresponding to the persistent polar complex of the first spindle is observed during meiosis II (Fig. 4B). Simultaneous staining with anti-β and anti-γ antibodies demonstrates the absence of γ-tubulin sites at the asterless tips of the metaphase spindles. Disperse γ-tubulin staining is detected in the bud regions containing paternal chromosomes associated with microtubule bundles from meiosis I (Fig. 4B,I). γ-Tubulin is detected in the bud cytoplasm throughout meiosis and it persists in the excised buds together with microtubules and eliminated chromatin. During spermiogenesis anti-γ-tubulin antibody stains the basal body site at the base of the sperm nucleus (Fig. 4C-D). Taken together, these observations strongly suggest a correlation between the presence of γ-tubulin antibody and the existence of organized microtubules.

Rb188 immunofluorescence

We have tested in *S. ocellaris* the polyclonal antibody Rb188 that specifically recognizes a centrosome-associated antigen (CP190) in *Drosophila melanogaster* (Whitfield et al., 1988; Kellogg et al., 1989). In primary spermatocytes of *S. ocellaris*, Rb188 associates with the nucleus until early prometaphase I (Fig. 5A). As meiosis proceeds, Rb188 signal is progressively

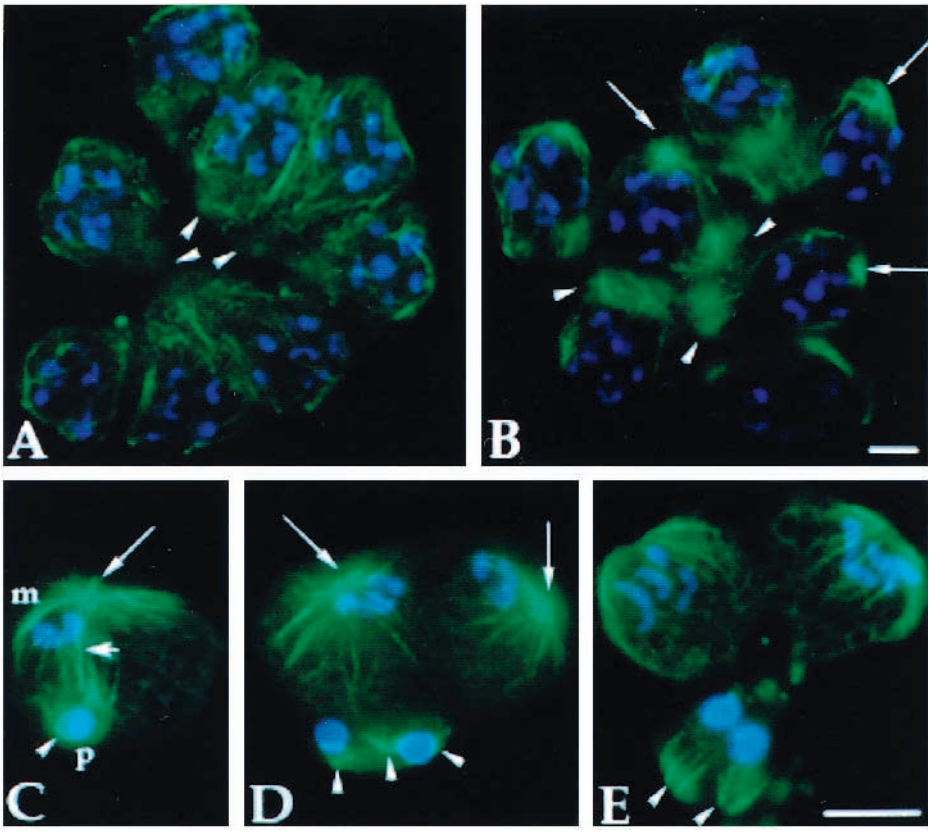

Fig. 2. Indirect immunofluorescence tubulin staining (green) and simultaneous Hoechst 33258 chromosome staining (blue) during first male meiotic division in *S. ocellaris*. (A) Cyst in early prometaphase. Cells are pear-shaped due to the formation of the cytoplasmic bud towards the central part of the cyst (arrowheads). Tubulin signal surrounds the nucleus and it is disperse in the cytoplasm. (B) Cyst in late prometaphase. The developing polar complex, located at the periphery of the cells, is brightly stained with the anti-tubulin antibody (arrows). An increased accumulation of tubulin is also visible at the bud regions (arrowheads). Chromosomes are condensed and well-separated. (C-D) Anaphase I. Numerous microtubules irradiate from the single polar complex (long arrows) and some of them end near the bud region (short arrow in C). Maternal chromosomes (m) are located near the polar complex; paternal chromosomes (p) migrate closely grouped towards the bud and interact there with short bundles of microtubules (arrowheads). (E) Late telophase I. The cytoplasmic bud containing paternal chromosomes and microtubule bundles (arrowheads) separates from the main body of the cell. Microtubules from the polar complex and maternal chromosomes are visible. Bars, 10 μm.

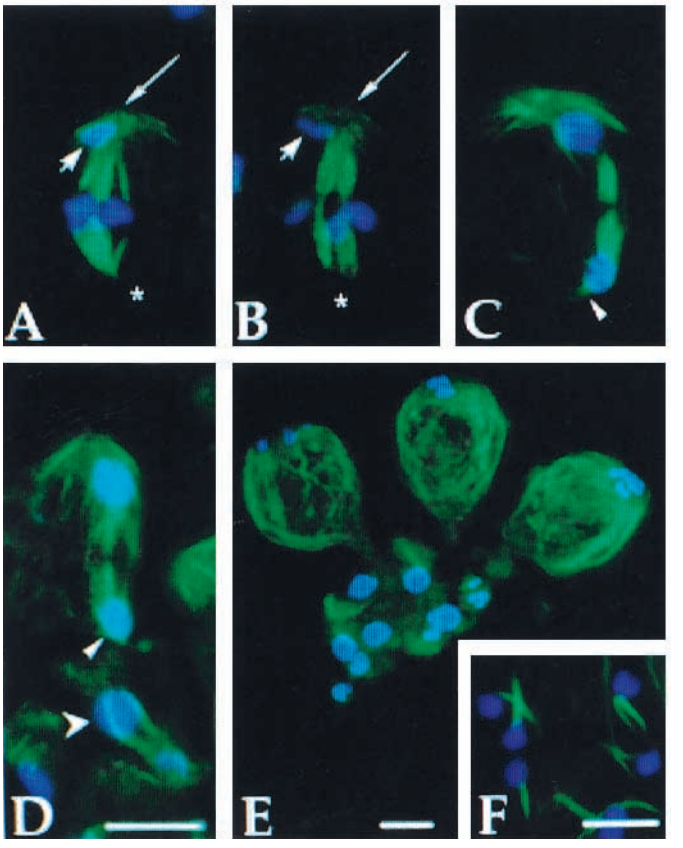

Fig. 3. Tubulin indirect immunofluorescence (green) and simultaneous Hoechst 33258 chromatin staining (blue) during the second male meiotic division of *S. ocellaris*. (A-B) Metaphase II. The single polar complex formed during first meiotic division persists through meiosis II (long arrows) and the undivided X chromosome (short arrows) are observed in close association with microtubules irradiated from this polar structure. The second meiotic spindle is bipolar and devoid of asters; this is clearly visible in the lower half spindle (*); the upper half spindle tip is located near the polar complex. The three autosomes (clearly visible in B) align at the equatorial plate and they are associated with three bundles of spindle microtubules (clearly visible in A). (C) Anaphase II. The autosomal chromatids migrate towards opposite directions. Following segregation, the upper chromatid group joins the undivided X chromosome and is larger than the chromatid group positioning towards the bud region. This latter chromatid set interact with bud-associated microtubules from this stage on (arrowhead). (D) Late anaphase II. The spindle becomes thinner prior to disorganization. The interactions between bud-associated microtubules and chromatin undergoing elimination persist (small arrowhead). The paternal chromosomes eliminated during first meiotic division are still associated with microtubule bundles (large arrowhead). (E-F) Early spermatid differentiation. A dense cytoplasmic microtubule network is visible (E). The eliminated chromatin during male meiosis is still interacting with short microtubule bundles (E,F). Bars, 10 μm.

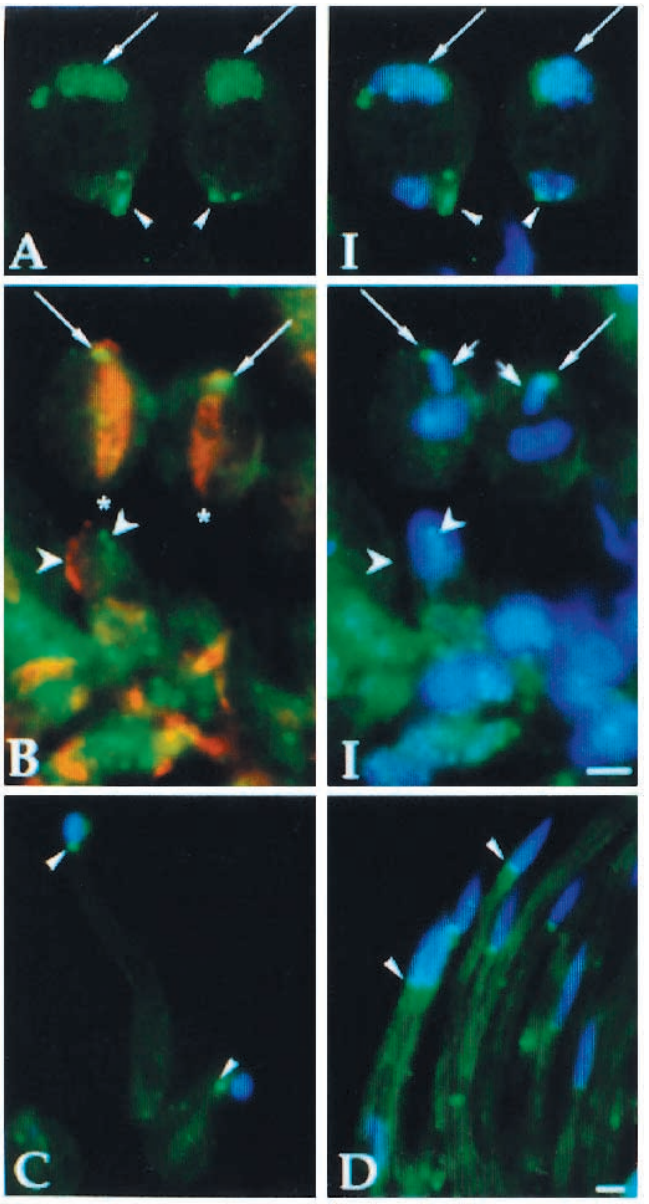

Fig. 4. (A) Indirect immunofluorescence staining with anti-γ-tubulin antibody (green) and simultaneous Hoechst 33258 chromatin staining (blue) during anaphase I in *S. ocellaris* spermatocytes. A broad fluorescent staining localizes at the polar complex region (long arrows); fluorescent signals are also clearly visible at the bud regions (arrowheads), where paternal chromosomes are already located (I). (B) Double-immunofluorescence labeling with anti-γ-tubulin antibody (green), anti-tubulin (red) and simultaneous Hoechst 33258 chromatin staining (blue) in metaphase II. Anti-γ-tubulin clearly stains the polar complex (long arrows) while no fluorescent signal is detected at the tip of the lower half spindle (*). In I, short arrows point to the maternal X chromosomes in close interaction with the polar complexes stained by the anti-γ-tubulin antibody. In the lower part of the image, a partial view of the central part of the cyst, it is possible to see the persistent association of short bundles of microtubules to paternal chromosomes as well as γ-tubulin signals (arrowheads) in the buds corresponding to meiosis I. (C-D) γ-Tubulin indirect immunofluorescence (green) and Hoechst 33258 chromatin staining (blue) during spermiogenesis in *S. ocellaris*. γ-Tubulin is present at the centriolar sperm basal body (arrowheads) both in early spermiogenesis (C) and in mature spermatozoa (D). Bars, 10 μm.

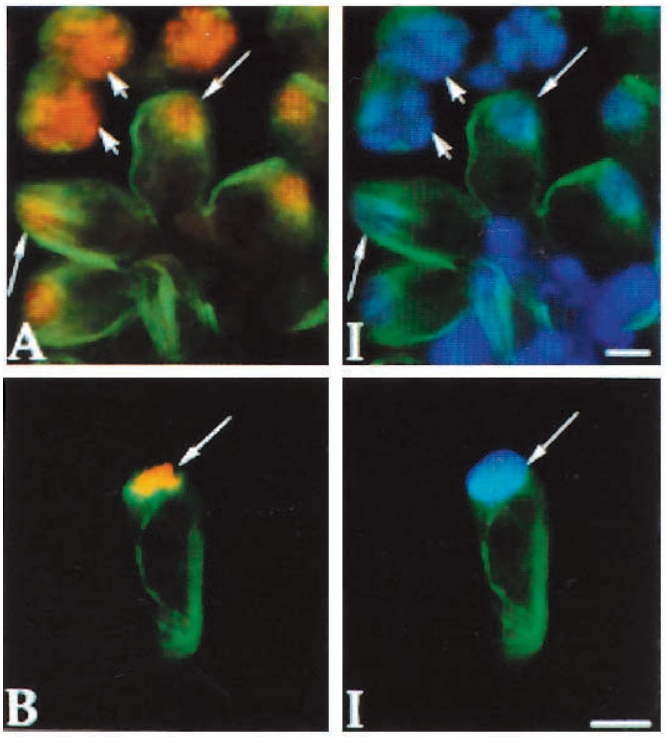

Fig. 5. Double-immunofluorescence labeling with Rb188 antibody (anti-CP190) (red), anti-tubulin (green) and Hoechst 33258 chromatin staining (blue) of male germ cells in meiosis I (A) and spermiogenesis (B) in *S. ocellaris*. (A) Rb188 signal associates with the nucleus during early prometaphase (short arrows). In anaphase I, Rb188 decorates the polar complex region (long arrows). (B) Rb188 staining is observed in close association with the future sperm nucleus (long arrows). Bars, 10 μm.

concentrated near the polar complex. In anaphase I, Rb188 decorates the large and unique centrosomal structure (Fig. 5A). The same pattern is observed during anaphase II (not shown). During spermiogenesis, the Rb188 staining was visualized in close association with the future sperm nucleus (Fig. 5B). These results show the conservation of the antigen recognized by Rb188 antibody in *Sciara ocellaris* and indicate that it is a component of the polar complex during male meiosis.

Anti-centrin immunofluorescence

Centrin is a highly conserved component of centrosomes and mitotic spindle poles of diverse organisms (Errabolu et al., 1994). In *S. ocellaris*, anti-centrin antiserum 26/14-1 staining accumulates at the bud region throughout male meiosis (Fig. 6). A remarkable accumulation of centrin is already observed during prometaphase I at the central region of the cysts, where buds are being formed (Fig. 6A). In addition, a discrete fluorescent signal identifies the position of the polar complex structure located at the opposite region of the cells. In anaphase I, the bud region is strongly decorated by the anti-centrin antibody and, in some cells, the polar complex region shows a disperse and faint anti-centrin staining (Fig. 6B). In metaphase II the pattern persists (Fig. 6C), as well as in anaphase II (not shown). During spermiogenesis, some discrete fluorescent spots are seen to be associated with the future sperm nucleus (not shown) while in mature spermatozoa a bright signal is

detected at the base of the spermatozoon nucleus corresponding to the basal body site (Fig. 6D).

224 immunofluorescence

224 monoclonal antibody was recently obtained in our laboratory and it specifically stains centrosomes during mitosis in organisms as distant as nematodes and mammals (Y. Panzera et al., unpublished results). The cytological pattern of 224 antibody during *S. ocellaris* male meiosis (Fig. 7A) indicates the presence of 224 centrosomal antigen both in the polar complex and in the bud region of the spermatocytes during chromosome elimination.

MPM-2 immunofluorescence

MPM-2 monoclonal antibody recognizes centrosome-associated phosphoproteins (Davis et al., 1983; Vandre et al., 1984). In *S. ocellaris* male meiosis MPM-2 antibody strongly decorates the polar complex while slightly weaker fluorescent signals are also observed in the bud region during chromosome elimination (Fig. 7B). These results indicate the presence of centrosomal phosphorylated epitopes in the polar complex and in bud regions throughout meiosis.

Actin and myosin distribution

To analyze the distribution of microfilamentous proteins in spermatocytes during bud formation and chromosome elimination, rhodamine-phalloidin staining and anti-myosin II antibodies have been used. The immunofluorescence analysis with the F-actin probe phalloidin shows that a high concentration of actin is present at the bud regions during both meiosis I and II (Fig. 8A,B). During meiosis II the actin-labeling persists in the buds containing the paternal chromosomes eliminated during meiosis I (Fig. 8B). A different pattern is seen using anti-myosin II antibodies. During prometaphase I the antibody signal is preferentially associated with the nucleus, although brighter fluorescence is seen at the peripheral region of the cells, where the

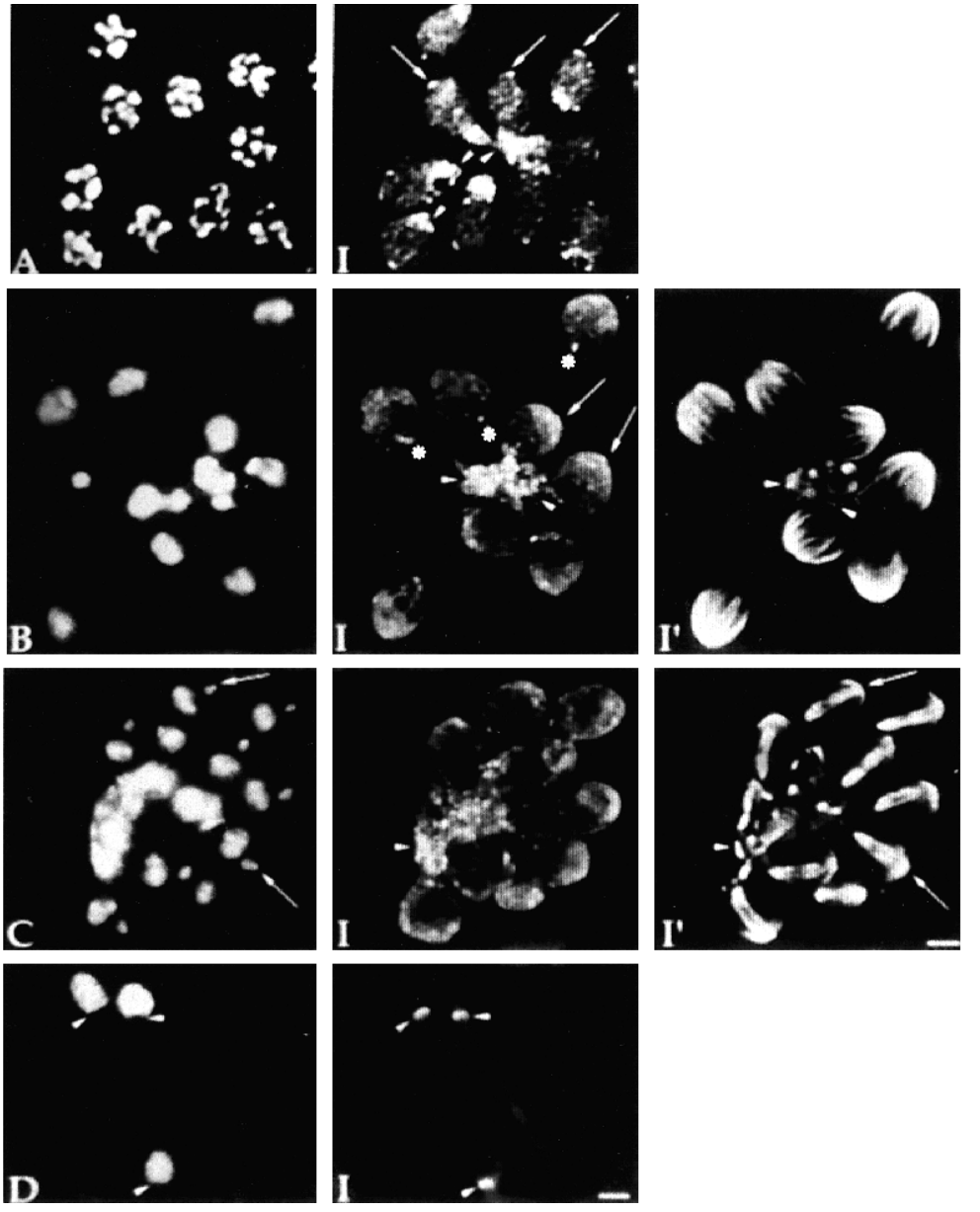

Fig. 6. Hoechst 33258 chromatin staining (A,B,C,D) and double-immunofluorescence labeling with anti-centrin (I) and anti-tubulin (I′) antibodies during male meiosis of *S. ocellaris*. (A) Cyst in prometaphase I. A high concentration of centrin is observed in the bud forming area towards the central part of the cyst (arrowheads in I). A discrete anti-centrin signal is also visible at the opposite region of the cell (long arrows), where the polar complex develops. (B) Part of a cyst in anaphase I. The anti-centrin antibody preferentially decorates bud regions (arrowheads in I); note in (I′) the corresponding tubulin staining pattern in the buds (arrowheads). The anti-centrin spot-like labeling seen in some cells (*) is due to their detaching during preparation. In some cells, the polar complex region shows a disperse and faint anti-centrin staining (arrows in I). (C) Part of a cyst in metaphase II. The anti-centrin labeling is strongly concentrated in bud regions, both formed during meiosis I and meiosis II (arrowhead in I); in I′ it is possible to see the tubulin staining pattern in the bud regions (arrowhead). In C, arrows indicate the undivided X chromosomes that remain attached to the polar complexes (arrows in I′). (D) Mature spermatozoa. Centrin is clearly present at the base of the axoneme, where the basal body is located (arrowheads). Bars, 10 μm.

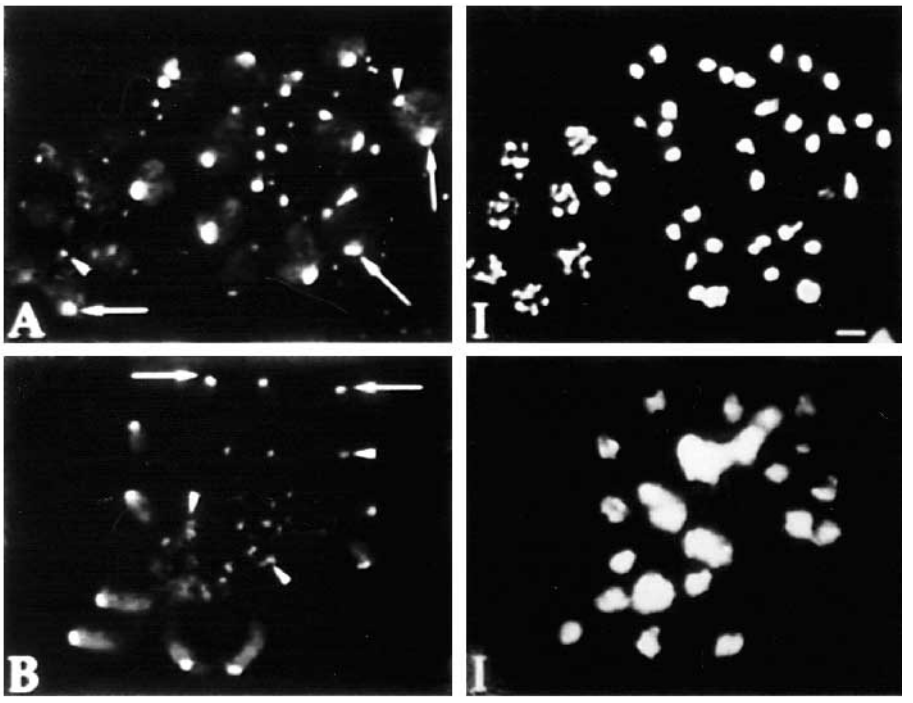

Fig. 7. (A) Indirect immunofluorescence labeling with 224 antibody (A) and simultaneous Hoechst 33258 chromatin staining (I) in *S. ocellaris* spermatocytes during prometaphase and anaphase I. The antibody signal is visible in both the polar complex (arrows) and cytoplasmic bud region (arrowheads). (B) MPM-2 indirect immunofluorescence staining (B) and simultaneous Hoechst 33258 chromatin staining (I) in *S. ocellaris* spermatocytes during anaphase II. This antibody clearly associates with the polar complex (arrows) and slightly weaker fluorescent signals are also visible in the central bud regions (arrowheads). Bar, 10 μm.

polar complex develops (Fig. 9A). During anaphase I the polar complex is brightly stained as well as the bud regions that contain paternal chromosomes (Fig. 9B). At anaphase II the staining of the polar complex persists together with the labeling at the bud regions (not shown). These results indicate that both actin and myosin are present at the cytoplasmic bud regions throughout meiosis but only myosin is also associated with the polar complex structure of the first meiotic spindle.

DISCUSSION

In sciarid males, a monopolar spindle structure has been classically described to direct the differential segregation of maternal and paternal chromosomes that leads to the elimination of paternal chromosomes in a cytoplasmic bud during meiosis I (reviewed by Gerbi, 1986). Our cytological analysis of male meiosis in *S. ocellaris* (summarized in Fig. 10) has revealed that, contemporaneous to the formation of the monopolar spindle, bundles of microtubules become organized in the cytoplasmic region involved in bud formation. We have also found that paternal chromosomes in the bud area become associated with these non-spindle microtubules. In this respect, in *Trichosia pubescens*, some bud-oriented microtubules were reported, in a single observation, to be associated with paternal chromatin at the electron microscopy level; however, light microscopy failed to show evidence of tubulin at the bud region in anaphase I (Fuge, 1994). In spite of this, it was proposed that the final transport of paternal chromosomes into the bud could be a microtubule-mediated process. The present results demonstrate the existence of bud-microtubules in *S. ocellaris* and lead us to suggest that the ultimate sequestering of paternal chromosomes into the bud is mediated by microtubules formed in the bud regions.

An interesting question is how to explain the different kinetic behavior of maternal and paternal chromosomes. According to

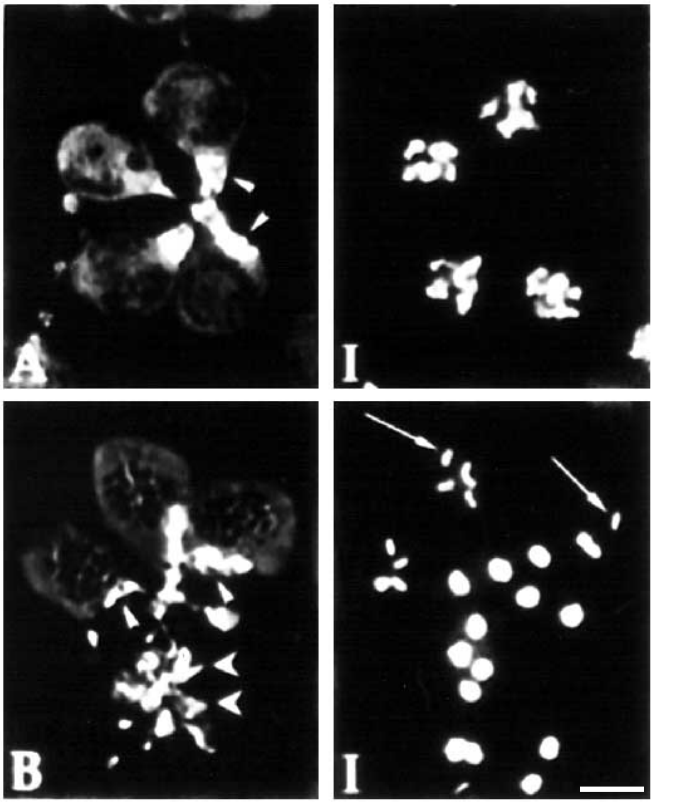

Fig. 8. Immunofluorescence staining with Rh-phalloidin (A,B) and Hoechst 33258 chromatin staining (I) of male spermatocytes of *S. ocellaris*. (A) Partial view of a cyst in prometaphase I. A large concentration of actin is visible at the cellular bud-forming regions located towards the central part of the cyst (arrowheads). (B) Partial view of a cyst in metaphase II. Actin labeling is observed in the developing buds regions (small arrowheads). Large arrowheads point to actin labeling of buds containing previously eliminated chromatin (I). In I note X chromosomes (arrows). Bar, 10 μm.

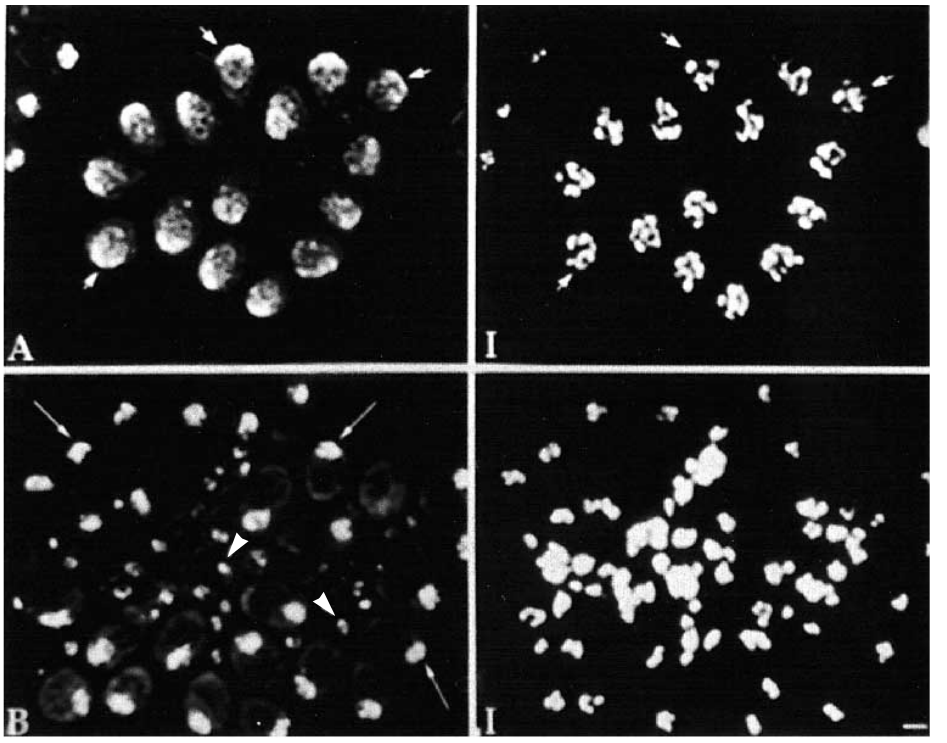

Fig. 9. Myosin indirect immunofluorescence labeling (A,B) and Hoechst 33258 chromatin staining (I) of male spermatocytes of *S. ocellaris*. (A) Cyst in prometaphase I. The whole nuclear area is diffusely stained, although bright fluorescence is seen at the peripherical region of the cell, where the polar complex is forming (arrows). (B) Cyst in anaphase I. The anti-myosin antibody strongly decorates the polar complex region (arrows) and weaker fluorescent signals are also visible in the bud region. Bar, 10 μm.

the classical view, all chromosomes in the primary spermatocyte attach to spindle fibers emanating from the single pole (Metz and Moses, 1926; Metz, 1926). However, more recent EM data in *S. coprophila* suggest that maternal chromosomes move to the pole unattached to spindle fibers while the kinetochore microtubules of paternal chromosomes may prevent them from moving towards it (Kubai, 1982). From our analysis it is not possible to exclude the possibility that both sets of chromosomes interact with microtubules radiating from the single pole. The possibility that paternal chromosomes could be already segregated by prophase I from the rest of the chromosomes, and that they do not really move at anaphase I, has also been raised (Kubai, 1982; reviewed by Gerbi, 1986). In view of our cytology, it is also possible that paternal and maternal chromosome sets in *S. ocellaris* may be already separated in the prometaphase nucleus. If so, paternal chromosomes may locate at the nuclear area opposite from the single polar complex and already be near the cytoplasmic region that will develop a bud. Assuming this hypothesis, it is also possible that microtubules emanating from the single pole and reaching the kinetochores of paternal chromosomes could be responsible for either preventing them from moving towards the pole or, alternatively, for pushing them further away. In this respect, we have observed that polar microtubules often end close to the bud region where paternal chromosomes migrate at the end of anaphase I. This suggests that the location of paternal chromosomes near the bud

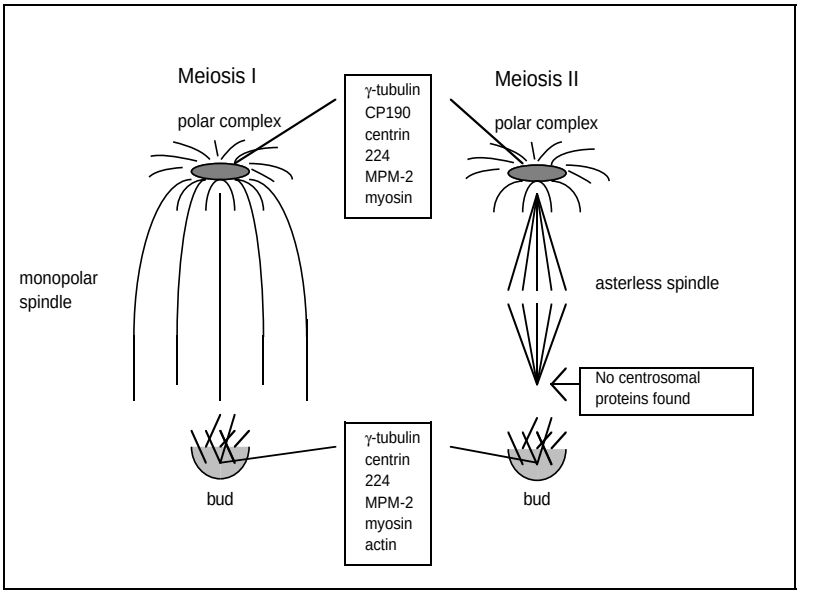

Fig. 10. Schematic diagram summarizing tubulin distribution and location of the antigens analyzed during meiosis I and II in *Sciara ocellaris*.

area is most probably mediated, at least initially, by microtubules nucleated from the single polar complex.

Concerning non-disjunction of the maternal X chromatids at meiosis II, the emerging picture is that the X chromosome does not undergo a 'precocious' segregation with respect to the autosomes, as has been assumed in sciarids (Crouse, 1943; Metz, 1925, 1934). Instead, the present data demonstrate the possibility advanced by Abbott and Gerbi (1981) for *S. coprophila* that 'a precocious chromosome-pole interaction may be established in meiosis I and maintained throughout meiosis II'. As hypothesized by Gerbi (1986) for *S. coprophila*, it is also possible that in *S. ocellaris* the locus controlling the differential behavior of the X chromosome somehow modifies its centromeric function. We believe that the maintainance throughout meiosis of a single polar structure originated at the beginning of meiosis I constitutes a general mechanism in sciarids that assures the inclusion of the non-disjoined X chromatids in the sperm nucleus. The cytology of meiosis II has also suggested that at the end of anaphase II, microtubules present in the bud region are involved in capturing the chromatid set lacking the sex chromosome destined to be eliminated. Thus, even though the mechanisms of chromosome/chromatid segregation differ between the two meiotic divisions, the microtubule-mediated process ensuring chromatin elimination seems to be common to both.

To investigate the molecular nature of the polar complex structure as well as the cytoplasmic bud regions involved in the organization of non-spindle microtubules we have analyzed the distribution in *S. ocellaris* of different antigens that are known components of functional centrosomes in other organisms. As summarized in Fig. 10 the antigens recognized by anti-γ-tubulin, anti-centrin, anti-CP190, anti-MPM-2 and 224 antibodies are all constituents of the polar complex. Moreover, except for CP190 antigen, the same antigens are also present in the cytoplasmic bud regions throughout meiosis. Thus, the distribution of these centrosomal antigens is correlated with the presence of tubulin and assembled microtubules. γ-Tubulin, a highly conserved member of the tubulin superfamily, is considered to be essential for microtubule nucleation and assembly (Oakley and Oakley, 1989; reviewed by Oakley, 1995). This protein has been found to be located in ring structures, containing other proteins as well, within the pericentriolar material of the centrosomes and to be a minus-end nucleator of microtubule assembly (Moritz et al., 1995; Zheng et al., 1995). The presence of a high concentration of γ-tubulin antigen in the polar complex is most probably due to the considerable amount of pericentriolar material associated with the giant centrioles in sciarids. Consistently, γ-tubulin is also detected at the centriolar sperm basal body that, according to Phillips (1966), derives from the caudad migration at the end of meiosis II of the single giant centriole located at the polar complex. Furthermore, γ-tubulin is also present throughout meiosis in the bud regions where, as described for *Trichosia*, atypical centriolar components were seen, in some electron microscope observations, to associate with growing microtubules and with a mass of dense electron-scattering material. However, these organelles were considered incapable of acting as polar centres and consequently not to be functional (Fuge, 1994).

The present data on the distribution of γ-tubulin in *Sciara* male meiotic cells strongly supports the existence of MTOCs responsible for the assembly and polarity of microtubules in the cytoplasmic bud regions. We believe that these MTOCs foci are functional throughout meiosis and direct the assembly of microtubules that participate in the process of chromatin elimination. This is further reinforced by the fact that, as we have shown, in addition to γ-tubulin, other centrosomal antigens have been detected in both the polar complex and bud regions. Among them, the remarkable concentration of anti-centrin labeling found at the bud regions of the cysts in *Sciara* is, we think, especially relevant with respect to the existence of active MTOCs foci in the buds. In lower eukaryotes, the calcium-binding protein centrin plays an important role in basal body positioning and orientation, centrosome segregation and microtubule severing (Baum et al., 1986, 1988; McFadden et al., 1987; Salisbury et al., 1988; Sanders and Salisbury, 1989). Similar functions have been suggested for centrin in higher eukaryotes (Errabolu et al., 1994). The presence of centrin at the basal body of mature spermatozoa is also consistent with previous reports showing a high content of centrin at the base of the flagellar apparatus (Salisbury et al., 1986).

DMAP190 (CP190), together with DMAP60 (CP60) and γ-tubulin have been reported in *Drosophila* as components of a centrosomal complex involved in the interaction of the centrosome with microtubules (Raff et al., 1993). Recent studies of CP190 protein in *Drosophila* suggest its specific association with the centrioles (Whitfield et al., 1995). In *Sciara* CP190 is only present in the polar complex and apparently not in the bud regions, in contrast to the anti-γ-tubulin labeling of both cellular regions. This may indicate that CP190 antigen is a structural constituent of an 'orthodox' centrosome, but it is not present, or is somehow modified and not detected, in the irregular centriolar structures that are presumably present in the bud regions.

From our results, centrosomal antigens are present at the single polar complex structure throughout meiosis I and II in *S. ocellaris*. We have also demonstrated, by contrast, that the tips of the second meiotic spindle in *Sciara* are devoid of centrosomal antigens. This leads to the question of how this atypical spindle is originated. A number of examples of spindles generated in the absence of centrosomes has been reported for other meiotic systems (reviewed by Fuge, 1994; Dietz, 1966; Mckim and Hawley, 1995; Therkauf and Hawley, 1992). As suggested by Fuge (1994) for *Trichosia*, the autosomes may participate in the organization of the second meiotic spindle in sciarids.

Bud formation in sciarid spermatocytes is a cellular process highly correlated with the segregation and migration of chromosomes during both meiotic divisions. The final discarding of chromosomes is the result of an extremely asymmetrical division of the cell which is achieved by bud excision. In different eukaryotic organisms, from yeast to humans, there are several examples of asymmetric cell division as a mechanism to generate different cell types (reviewed by Way et al., 1994). In this respect, the meiotic process in sciarid flies can be seen as an extreme case of nuclear and cytoplasmic differential partitioning linked to cellular differentiation. In the budding yeast *Saccharomyces cerevisiae* it is known that actin has a decisive role in the emergence and organization of bud sites in the dividing cell (Adams and Pringle, 1984). The present cytological analysis suggests that actin microfilaments may be involved in the formation of the buds in *S. ocellaris* spermatocytes, and most probably in the ultimate excision process. In contrast to actin, myosin labeling indicates a lower concentration of this protein in the buds. In addition, myosin antigen also associates with the polar complex during both meiotic divisions. This is in accord with reported data indicating that

myosin is a component of isolated centrosomes of various origins (Komesli et al., 1989; Bailly et al., 1992) but its possible role in centrosome functions remains unknown.

We are grateful to Drs W. Whitfield, J. L. Salisbury and C. Gonzalez for kindly providing Rb188, anti-centrin 26/14-1 and anti-γ-tubulin antibodies, respectively. We thank Drs S. Pimpinelli, P. Ripoll and L. Sanchez for helpful suggestions and comments on the manuscript. This work has been supported by a UE project C11*-CT94-0071.

REFERENCES

Abbott, A. G. and Gerbi, S. A. (1981). Spermatogenesis in *Sciara coprophila*. II. Precocious chromosome orientation in meiosis II. *Chromosoma* **83**, 19-27.

Abbott, A. G., Hess, J. E. and Gerbi, S. A. (1981). Spermatogenesis in *Sciara coprophila*. I. Chromosome orientation on the monopolar spindle of meiosis I. *Chromosoma* **83**, 1-18.

Adams, A. E. M. and Pringle, J. R. (1984). Relationship of actin and tubulin distribution to bud growth in wild-type and morphogenetic-mutant *Saccharomyces cerevisiae*. *J. Cell Biol.* **98**, 934-945.

Amabis, J. M., Reinach, F. C. and Andrews, N. (1979). Spermatogenesis in *Trichosia pubescens* (Diptera: Sciaridae). *J. Cell Sci.* **63**, 199-213.

Bailly, E., Bordes, N., Bornens, M. and Klotz, C. (1992). A high molecular weight centrosomal protein of mammalian cells is antigenically related to myosin-II. *Cell Motil. Cytoskel.* **23**, 122-132.

Basile, R. (1970). Spermatogenesis in *Rhynchosciara angelae*. *Rev. Bras. Biol.* **30**, 29-38.

Baum, P., Furlong, C. and Byers, B. (1986). Yeast gene required for spindle pole duplication: Homology of its product with Ca^{2+}-binding proteins. *Proc. Nat. Acad. Sci. USA* **83**, 5512-5516.

Baum, P., Yip, C., Goetsch, L. and Byers, B. (1988). A yeast gene essential for regulation of spindle pole duplication. *Mol. Cell. Biol.* **8**, 5386-5397.

Cenci, G., Bonaccorsi, S., Pisano, C., Verni, F. and Gatti, M. (1994). Chromatin and microtubule organization during premeiotic, meiotic and early postmeiotic stages of *Drosophila melanogaster* spermatogenesis. *J. Cell Sci.* **107**, 3521-3534.

Crouse, H. V. (1943). Translocations in *Sciara*; their bearing on chromosome behavior and sex determination. *Univ. Mo. Res. Bull.* **379**, 1-75.

Crouse, H. V. (1960). The controlling element in sex chromosome behavior in *Sciara*. *Genetics* **45**, 1429-1443.

Crouse, H. V. (1977). X heterochromatin subdivision and cytogenetic analysis in *Sciara coprophila* (Diptera, Sciaridae) I. Centromere localization. *Chromosoma* **63**, 39-55.

Davis, F. M., Tsao, T. Y., Fowler, S. K. and Rao, P. N. (1983). Monoclonal antibodies to mitotic cells. *Proc. Nat. Acad. Sci. USA* **80**, 2926-2930.

Dietz, R. (1966). The dispensability of the centrioles in the spermatocyte divisions of *Pales ferrugines* (Nematocera). *Heredity* **19** (suppl.), 161-166.

Dubois, A. M. (1933). Chromosome behavior during cleavage in the eggs of *Sciara coprophila* (Diptera) in the relation to the problem of sex determination. *Z. Wiss. Biol. Abt. B-Z Zellforsch. Mikrosk. Anat.* **19**, 595-614.

Errabolu, R., Sanders, M. A. and Salisbury, J. L. (1994). Cloning of a cDNA encoding human centrin, an EF-Hand protein of centrosomes and mitotic spindle poles. *J. Cell Sci.* **107**, 9-16.

Fuge, H. (1994). Unorthodox male meiosis in *Trichosia pubescens* (Sciaridae) – chromosome elimination involves polar organelle degeneration and monocentric spindles in first and second division. *J. Cell Sci.* **107**, 299-312.

Gerbi, S. A. (1986). Unusual chromosome movements in sciarid flies. In *Germ Line-Soma differentiation* (ed. W. Hennig), pp. 71-104. Berlin: Spinger Verlag.

Kellogg, D. R., Field, C. M. and Alberts, B. M. (1989). Identification of microtubule-associated proteins in the centrosome, spindle, and kinetochore of the early *Drosophila* embryo. *J. Cell Biol.* **109**, 2977-2991.

Komesli, S., Tournier, F., Paintrand, M., Margolis, R., Job, D. and Bornens, M. (1989). Mass isolation of calf thymus centrosomes: The identification of a specific configuration. *J. Cell Biol.* **110**, 405-415.

Kubai, D. F. (1982). Meiosis in *Sciara coprophila*: structure of the spindle and chromosome behavior during the first meiotic division. *J. Cell Biol.* **93**, 655-669.

McFadden, G., Schulze, D., Surek, B., Salisbury, J. and Melkonian, M. (1987). Basal body reorientation mediated by a Ca^{2+}-modulated contractile protein. *J. Cell Biol.* **105**, 903-912.

McKim, K. S. and Hawley, R. S. (1995). Chromosomal control of meiotic cell division. *Science* **270**, 1595-1601.

Metz, C. W. (1925). Chromosome behavior in *Sciara* (Diptera). *Anat. Rec.* **31**, 346-347.

Metz, C. W. (1926). An apparent case of monocentric mitosis in *Sciara* (Diptera). *Science* **63**, 190-191.

Metz, C. W. and Moses, M. S. (1926). Sex determination in Sciara (Diptera). *Anat. Rec.* **34**, 170.

Metz, C. W., Moses, M. S. and Hoppe, E. N. (1926). Chromosome behavior and genetic behavior in *Sciara* (Diptera). I. Chromosome behavior in the spermatocyte divisions. *Z. indukt. Abstamm.-u. Vererbungsl.* **42**, 237-270.

Metz, C. W. (1927). Chromosome behavior in *Sciara* (Diptera). II. Genetic evidence of selective segregation in *S. coprophila*. III. Absence of parthenogenesis in *S. coprophila*. *Z. indukt. Abstamm.-u. Vererbungsl.* **45**, 184-201.

Metz, C. W. (1933). Monocentric mitosis with segregation of chromosomes in Sciara and its bearing on the mechanisms of mitosis. *Biol. Bull. Mar. Biol. Lab. Woods Hole* **64**, 334-347.

Metz, C. W. (1934). Evidence that in *Sciara* the sperm regularly transmits two sister sex chromosomes. *Proc. Nat. Acad. Sci. USA* **20**, 31-36.

Metz, C. W. (1938). Chromosome behavior, inheritance and sex determination in *Sciara*. *Am. Nat.* **72**, 485-520.

Mori, L. and Perondini, A. L. P. (1980). Errors in the elimination of X-chromosome in *Sciara ocellaris*. *Genetics* **94**, 663-673.

Moritz, M., Braunfeld, M. B., Sedat, J. W., Alberts, B. and Agard, D. A. (1995). Microtubule nucleation by γ-tubulin-containing rings in the centrosome. *Nature* **378**, 638-640.

Oakley, B. R. (1995). A nice ring to the centrosome. *Nature* **378**, 555-556.

Oakley, C. E. and Oakley, B. R. (1989). Identification of γ-tubulin, a new member of the tubulin superfamily encoded by *mipA* gene of *Aspergillus nidulans*. *Nature* **338**, 662-664.

Phillips, D. M. (1966). Observations on spermiogenesis in the fungus gnat *Sciara coprophila*. *J. Cell Biol.* **30**, 477-497.

Phillips, D. M. (1967). Giant centriole formation in *Sciara*. *J. Cell Biol.* **33**, 73-92.

Phillips, D. M. (1970). Insect sperm: their structure and morphogenesis. *J. Cell Biol.* **44**, 243-277.

Raff, J. W., Kellog, D. R. and Alberts, B. M. (1993). *Drosophila* γ-tubulin is part of a complex containing two previously identified centrosomal MAPs. *J. Cell Biol.* **121**, 823-835.

Rieffel, S. M. and Crouse, H. V. (1966). The elimination and differentiation of chromosomes in the germ line of *Sciara*. *Chromosoma* **19**, 231-276.

Salisbury, J., Baron, A., Surek, B. and Melkonian, M. (1984). Striated flagellar roots: Isolation and partial characterization of a calcium-modulated contractile protein. *J. Cell Biol.* **99**, 962-970.

Salisbury, J., Baron, A., Coling, D., Martindale, V. and Sanders, M. (1986). Calcium-modulated contractile proteins associated with the eucaryotic centrosome. *Cell Motil. Cytoskel.* **6**, 193-197.

Salisbury, J., Baron, A. and Sanders, M. (1988). The centrin-based cytoskeleton of *Chlamydomonas reinhardtii*: distribution in interphase and mitotic cells. *J. Cell Biol.* **107**, 635-641.

Sanders, M. and Salisbury, J. (1989). Centrin-mediated microtubule severing during flagellar excision in *Chlamydomonas reinhardtii*. *J. Cell Biol.* **108**, 1751-1760.

Smith-Stocking, H. (1936). Genetic studies on selective segregation of chromosomes in *Sciara coprophila* Lintner. *Genetics* **21**, 421-443.

Therkauf, W. E. and Hawley, R. S. (1992). Meiotic spindle assembly in *Drosophila* females: behavior of nonexchange chromosomes and the effects of mutations in the nod kinesin-like protein. *J. Cell Biol.* **116**, 1167-1180.

Vandre, D. D., Davis, F. M., Rao, P. N. and Borisy, G. G. (1984). Phosphoproteins are components of mitotic microtubule organizing centers. *Proc. Nat. Acad. Sci. USA* **81**, 4439-4443.

Way, J. C., Wang, L, Run, J.-Q. and Hung, M. (1994). Cell polarity and the mechanism of asymmetric cell division. *BioEssays* **16**, 925-931.

Whitfield, W., Millar, S. E., Saumwebber, H., Frash, M. and Glover, D. M. (1988). Cloning of a gene encoding an antigen associated with the centrosome in *Drosophila*. *J. Cell Sci.* **89**, 467-480.

Whitfield, W. G. F., Chaplin, M. A., Oegema, K., Parry, H. and Glover, D. M. (1995). The 190 kDa centrosome-associated protein of *Drosophila melanogaster* contains four zinc finger motifs and binds to specific sites on polytene chromosomes. *J. Cell Sci.* **108**, 3377-3387.

Zheng, Y., Wong, M. L., Alberts, B. and Mitchison, T. (1995). Nucleation of microtubule assembly by a γ-tubulin-containing ring complex. *Nature* **378**, 578-583.

(Received 22 October 1996 – Accepted 13 January 1997)