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# The Microtubular System and Posttranslationally Modified Tubulin During Spermatogenesis in a Parasitic Nematode With Amoeboid and Aflagellate Spermatozoa

**AÏCHA MANSIR AND JEAN-LOU JUSTINE***

*Laboratoire de Biologie parasitaire, Protistologie, Helminthologie, Muséum national d'histoire naturelle, Paris, France*

**ABSTRACT** Using transmission electron microscopy and immunologic approaches with various antibodies against general tubulin and posttranslationally modified tubulin, we investigated microtubule organization during spermatogenesis in *Heligmosomoides polygyrus*, a species in which a conspicuous but transient microtubular system exists in several forms: a cytoplasmic network in the spermatocyte, the meiotic spindle, a perinuclear network and a longitudinal bundle of microtubules in the spermatid. This pattern differs from most nematodes including *Caenorhabditis elegans*, in which spermatids have not microtubules. In the spermatozoon of *H. polygyrus*, immunocytochemistry does not detect tubulin, but electron microscopy reveals two centrioles with a unique structure of 10 singlets. In male germ cells, microtubules are probably involved in cell shaping and positioning of organelles but not in cell motility. In all transient tubulin structures described in spermatocytes and spermatids of *H. polygyrus*, detyrosination, tyrosination, and polyglutamylation were detected, but acetylation and polyglycylation were not. The presence/absence of these posttranslational modifications is apparently not stage dependent. This is the first study of posttranslationally modified tubulin in nematode spermatogenesis. *Mol. Reprod. Dev. 49:150–167, 1998.* © 1998 Wiley-Liss, Inc.

**Key Words:** spermiogenesis; cytoskeleton; tubulin; posttranslationally modified tubulin; detyrosination; tyrosination; polyglutamylation; *Heligmosomoides polygyrus*

## INTRODUCTION

Microtubules are ubiquitous components of the cytoskeleton of eucaryotic cells that are associated with many cellular functions, including mitosis, cytokinesis, cytoplasmic organization, and cell motility. They display a wide biochemical diversity, which is generated by the differential expression of several alpha- and beta-tubulin isogenes and is increased by posttranslational modifications. Different posttranslational modifications have been described for alpha-tubulin: acetylation of Lys40 (L'Hernault and Rosenbaum, 1985), detyrosination of the C-terminal Tyr451 (Gundersen et al., 1984), and tyrosination at the same position (Kreis, 1987); for beta-tubulin: phosphorylation of Ser444 (Alexander et al., 1991); and for both subunits: polyglutamylation (Alexander et al., 1991; Eddé et al., 1990; Wolff et al., 1992) and polyglycylation (Redeker et al., 1994). Apart from acetylation, all these modifications concern the 10 carboxyl-terminal residues of alpha- and beta-tubulin.

Acetylation is a posttranslational modification by which an acetyl group is reversibly attached to lysine 40 of ϵ-amino groups of alpha-tubulin (L'Hernault and Rosenbaum, 1985). The addition and removal of the acetyl group are successively carried out by alpha-tubulin acetylase (Greer et al., 1985), which favours assembled tubulin as substrate, and by tubulin deacetylase (Maruta et al., 1986). Acetylated alpha-tubulin originally was found in cilia and flagella (Piperno and Fuller, 1985) and later was recorded from a variety of cells and organisms (Delgado-Viscogliosi et al., 1996; Iomini and Justine, 1997; Wolf, 1994). In many cells, acetylation concerns only certain subsets of microtubules, such as in mammal spermatids (manchette microtubules not acetylated versus axonemes acetylated; Fouquet et al., 1994). Acetylation is often but not always a component of stable microtubules (Schulze et al., 1987).

During posttranslational detyrosination/tyrosination, a tyrosine residue is added to or removed from the C terminus of the alpha-tubulin peptide chain (Thompson, 1982) by, respectively, tubulin carboxy peptidase and tubulin tyrosine ligase (Kreis, 1987). The former is more active on polymeric tubulin, in contrast to the latter, which is ATP dependent and more active on protomeric tubulin (Arce et al., 1978; Kreis, 1987). Tyrosination and detyrosination are both known in several organisms (Delgado-Viscogliosi et al., 1996; Warn et al., 1990). There is variation within a single organism (Jackson et al., 1995; Lessman et al., 1993).

*Correspondence to: Prof. Jean-Lou Justine, Laboratoire de Biologie parasitaire, Protistologie, Helminthologie, Muséum national d'histoire naturelle, 61 rue Buffon, F-75231 Paris cedex 05, France.
E-mail: justine@mnhn.fr

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Tyrosination is generally more abundant in unstable microtubules (Gundersen and Bulinski, 1986), whereas detyrosination is found mainly in stable microtubules (Schulze et al., 1987).

Polyglutamylation (Eddé et al., 1990), the major posttranslational modification of brain tubulin, is a reversible modification that affects both polymeric alpha- and beta-tubulin. This modification consists of the successive addition of one to at least six glutamyl units onto a glutamate residue (445 in alpha-tubulin and 435 in beta-tubulin). Polyglutamylation of tubulin was identified in mammal neurones (Eddé et al., 1990) and found in various nonneuronal cells or tissues (Wolff et al., 1992). In mammals, it is present in Sertoli cells and in axonemes and centrioles in spermatids and spermatozoa but not in the manchette, a transient structure of the spermatid composed of parallel singlet microtubules (Fouquet et al., 1994; Kann et al., 1995).

Polyglycylation consists of the addition of up to 34 glycyl units to the gamma carboxyl groups of Glu445 and Glu437 on the alpha and beta subunits of tubulin, respectively (Redeker et al., 1994). However, polyglycylation affects only beta-tubulin in bull sperm (Rüdiger et al., 1995). This modification is widely distributed in axonemes of phylogenetically diverging organisms (Bré et al., 1996; Bressac et al., 1995; Callen et al., 1994; Levilliers et al., 1995; Redeker et al., 1994) but has not been detected, however, in the protozoa *Euglena* and *Trypanosoma* (Bré et al., 1996).

Spermatogenesis represents a good model for the study of microtubule diversity (Bré et al., 1996; Fouquet et al., 1994; Fouquet and Kann, 1994; Hermo et al., 1991; Kann et al., 1995; Lessman et al., 1993; Piperno and Fuller, 1985; Rüdiger et al., 1995; Wolf, 1996; Wolf and Hellwage, 1995) because most spermatozoa have prominent motile microtubular organelles such as axonemes. In contrast, nematode spermatozoa have a unique motile system based on major sperm protein (MSP), a specific protein acting for pseudopod amoeboid motility (Scott, 1996; Theriot, 1996), and are always devoid of a flagellum. However, in the nematode *Heligmosomoides polygyrus*, which has the MSP system (Mansir and Justine, 1996), a microtubular system is present in spermatocytes and spermatids but disappears in the spermatozoon. Spermatogenesis in this species therefore represents an interesting model for studying tubulin in cells in which microtubules are not associated with motility.

By means of antitubulin antibodies and immunocytochemical techniques, we followed the development of the microtubular system and investigated posttranslational modifications of tubulin during nematode spermiogenesis. Tubulin was detected in a cytoplasmic network in spermatocytes, in the meiotic spindle, and in a prominent but transient microtubular system in spermatids. In contrast, the microtubular system in spermatozoa is reduced to the two centrioles only. Posttranslational modifications of tubulin such as tyrosination, detyrosination, and polyglutamylation were present in all stages, but acetylation and polyglycylation were not detected.

## MATERIALS AND METHODS

### Material

Samples of *H. polygyrus* (Dujardin, 1845) were obtained from laboratory white mice. Adults were collected 2 to 4 weeks after oral infection of mice with 300 larvae. Male germ cells were collected from males in all experiments.

Germ cells were obtained by dissecting each adult male in a drop of phosphate-buffered saline (PBS; Sigma Chemical Company, St. Louis, MO) on a pit slide previously washed with alcohol and acetone. The anterior end was cut off, and the genital system, which consists of a single elongated testis and a seminal vesicle, was isolated. The seminal vesicle was then incised to release mature spermatozoa. Immature germ cells were obtained by gently pressing the testis with thin needles. Cells adhered to the slide without coating.

### Antibodies Used for Immunoblots and Immunocytochemistry

Primary antibodies used for immunocytochemical experiments, including antigens, concentration, and references are listed in Table 1.

### Immunocytochemistry

**Fixation and permeabilization.** Slides with pits containing cells in a drop of PBS were kept in a humid chamber for 1 hr to allow cells to sink and adhere to the slide. The PBS was then removed and replaced by a drop of fixative.

Various fixation and permeabilization methods were tried:

1. 3.7% formaldehyde in PBS, 15 min, room temperature. The pit was rinsed with PBS (3 × 5 min). The cells were then permeabilized with (a) acetone, 5–10 min, room temperature, or (b) acetone, 5 min, −20°C, or (c) Triton X-100 in PBS, 0.01%, 0.1%, 0.2%, or 0.5%, 5–10 min, room temperature, or (d) SDS in PBS, 0.5%, 1%, or 3%, 5 min, room temperature, or (e) Triton X-100 0.5% plus SDS 0.5%, 1%, or 3% in PBS, 5–10 min, room temperature.
2. 0.7% glutaraldehyde in PBS, 15 min, room temperature. The pit was rinsed with PBS (3 × 5 min). The cells were then permeabilized with (a) 0.5% SDS in PBS, 15 min, room temperature, or (b) 0.5% SDS plus 0.5% Triton X-100 in PBS, 5–10 min, room temperature.
3. 100% ethanol, 4 min, room temperature, followed by acetone, 4 min, at −20°C. The pit was rinsed with PBS (3 × 5 min). The cells were then permeabilized with 0.5% Triton X-100 in PBS, 5–10 min, room temperature.
4. A mixture of 80% acetone plus 20% methanol, 6 min, at −20°C, followed by rinsing with PBS (3 × 5 min).

**TABLE 1. Primary Antibodies Used for Immunocytochemical Experiments, Including Antigens, Concentration, and References**

| Antibody | Antigen used for antibody production | References | Concentration |
|---|---|---|---|
| Mab anti-alpha-tubulin | Chicken embryo brain microtubules | Clone DM1A (Blose et al., 1984), Sigma | 1:200 |
| Mab anti-beta-tubulin | Rat brain tubulin | Clone TUB2.1 (Gozes and Barnstable, 1982), Sigma | 1:100 |
| Mab anti-beta-tubulin | Chicken embryo brain microtubules | Clone DM1B (Blose et al., 1984) | 1:25–1:100 |
| Mab antiacetylated alpha-tubulin | Acetylated alpha-tubulin from the outer arm of sea urchin sperm axonemes | Clone 6-11B1 (Piperno and Fuller, 1985), Sigma | 1:25–1:500 |
| Mab antityrosinated tubulin | Carboxyl-terminal amino acids of synthetic peptide alpha-tubulin. | Clone TUB1A2 (Kreis, 1987), Sigma | 1:50 |
| Mab antipolyglutamylated alpha- and beta-tubulin | Synthetic peptide of the C-terminal sequence Glu441–Gly448 modified by addition of two glutamyl units at Glu 445 | Clone GT 335 (Wolff et al., 1992) | 1:100 |
| Mab antipolyglycylated alpha-tubulin | *Paramecium* axonemal tubulin | Clone TAP 952 (Bré et al., 1996; Callen et al., 1994) | 1:4[a] |
| Mab antipolyglycylated alpha- and beta-tubulin | *Paramecium* axonemes | Clone AXO 49 (Bré et al., 1996; Callen et al., 1994) | 1:100 |
| Mab antitubulin | *Trypanosoma brucei* tubulin | Clone 1B41 (Gallo and Précigout, 1988) | 1:1[a] |
| Mab antitubulin | Bovine brain tubulin | Clone 16D3 (Gallo and Anderton, 1983) | 1:1[a] |
| Mab antitubulin | Bovine brain tubulin | 3F3 (Gallo and Anderton, 1983) | 1:40–1:200 |
| Pab anti-beta-tubulin | Chicken embryo microtubules | T-3526, Sigma | 1:50–1:750 |
| Pab anti-beta-tubulin | Amino-terminal sequence of beta-tubulin | C 140 (Arevalo et al., 1990) | 1:25–1:50 |
| Pab antidetyrosinated tubulin | Synthetic peptide | GLU poly (Kreis, 1987) | 1:25 |

[a]1B41, 16D3, and TAP 952, cell culture supernatants; other monoclonal antibodies, ascite fluids.

**TABLE 2. Various Antitubulin Antibodies Tested on *H. polygyrus* Male Germ Cells**

| Antibody, antigen | Clone or reference | Spermatocytes | Round spermatids (stage 1) | Elongate spermatids (stages 2–4) | Spermatozoa |
|---|---|---|---|---|---|
| Mab anti-alpha-tubulin | DM1A | + | + | + | − |
| Mab anti-beta-tubulin | TUB2.1 | + | + | + | − |
| Mab anti-beta-tubulin | DM1B | + | + | + | − |
| Pab anti-beta-tubulin | C 140 | + | + | + | − |

*Note:* +, labeling; −, no labeling.

**Immunochemical labeling.** Nonspecific antigens were blocked with 20 mg/ml bovine serum albumin (BSA; Sigma) in PBS (BSA-PBS) for 45–90 min at room temperature. Different antitubulin antibodies (see Table 1), diluted with BSA-PBS, were applied for 90 min at room temperature. After washing in PBS (3 × 5 min), fluorescein isothiocyanate–labeled goat antimouse or goat antirabbit antibody (Nordic) according to the first antibody, 1:40 in PBS, were applied for 40 min at room temperature. The pits were rinsed (PBS, 3 × 5 min). To visualize the nucleus, the pits were incubated with propidium iodide in PBS (10 µg/ml) or Hoechst 33258 in PBS (1 µg/ml) for 10 min. After a final rinse (PBS, 3 × 5 min), mounting was done in Citifluor (Citifluor, Ltd., London, UK), and slides were sealed with nail enamel. Controls were done by omitting the first antibody or by using nonrelevant mouse antibody; they were negative and thus are not further illustrated nor mentioned.

For double labeling of tubulin and F-actin, the monoclonal anti-alpha-tubulin (DM1A; Sigma) was applied and followed by the fluorescein isothiocyanate–conjugated antimouse antibody and followed by tetramethyl rhodamin isothiocyanate (TRITC) phalloidin (5 mg/ml in PBS, 20 min; Sigma).

For double labeling of tropomyosin and tubulin, the monoclonal anti-alpha-tubulin (DM1A; Sigma) was applied and followed, in turn, by the fluorescein isothiocyanate–conjugated antimouse antibody, the antitropomyosin polyclonal antibody developed against tropomyosin from chicken gizzard (T-3651; Sigma) at 1:100 for 40 min, and the antirabbit TRITC-labeled goat antibody at 1:40 for 40 min.

**Epifluorescence microscopy.** Observations were made with a Nikon Optiphot epifluorescence microscope equipped with a mercury lamp and three single-band Nikon filters for FITC channel (B-2A), TRITC

**TABLE 3. Antibodies Against Posttranslational Modifications of Tubulin Tested on *H. polygyrus* Male Germ Cells**

| Antibody, antigen | Clone or reference | Spermato-cytes | Round spermatids | Elongate spermatids | Spermatozoa |
|---|---|---|---|---|---|
| Pab antidetyrosinated tubulin | GLU poly | + | + | + | − |
| Mab antityrosinated tubulin | TUB 1A2 | + | + | + | − |
| Mab antipolyglutam-ylated alpha-tubulin | GT 335 | + | + | + | − |
| Mab antiacetylated alpha-tubulin | 6-11B1 | − | − | − | − |
| Mab antipolyglycylated alpha-tubulin | TAP 952 | − | − | − | − |
| Mab antipolyglycylated alpha-tubulin | AXO 49 | − | − | − | − |

*Note:* +, labeling; −, no labeling.

channel (G-2A), and Hoechst channel (UV-1A) and with a double-band (FITC/TRITC) Omega filter (XF 52).

### Analysis of Proteins in Germ Cells

**Preparation of protein extracts.** Two protein extracts were prepared from spermatozoa and male germ cells. Protein extracts of spermatozoa were prepared from 640 adult males individually dissected. Each worm was placed in tiny drop of PBS, and the male genital system was obtained by cutting off the anterior end, the seminal vesicle was opened with fine needles, and spermatozoa were collected with a micropipette as they poured out in minimal volume. Protein extracts of male germ cells were prepared from testis and spermatozoa from 435 specimens. The cells or specimens were collected in PBS with 4 mM DTT and protease inhibitors (1 mM PMSF, 2 mM benzamidine, 20 µg/ml aprotinin, 20 µg/ml pepstatin, 100 µg/ml trypsin inhibitor). They were resuspended in hot Laemmli sample buffer (Laemmli, 1970) and boiled for 4 min.

**Electrophoresis and immunoblot.** Protein extract were electrophoresed in one dimension on 12% SDS polyacrylamide gels (Bio-Rad Mini-Protean II Dual slab) (Laemmli, 1970). After electrophoresis, gels were either stained with Coomassie brillant blue (R-250) or were blotted to nitrocellulose membrane (Hybond-C, Amersham) using a liquid transfer chamber (transfor E series, Hoefer) for 1 hr at 200 V. To ensure that efficient transfer of proteins had occurred, the blots were stained with 0.2% Ponceau S (Sigma) in 3% trichloroacetic acid. After washing, the membrane was saturated in an agitator with 5% skimmed milk in Tris-buffered saline (TBS) overnight at 4°C. After washing three times with TBS (10 min), the membrane was incubated with DM1A (1:5000), TUB2.1 (1:5000), 6-11B1 (1:2000 to 1:10,000), TUB1A2 (1:5000), GT 335 (1:1000 to 1:20,000), TAP 952 (1:100), AXO 49 (1:100), T-3526 (1:400), C 140 (1:1000 to 1:25,000), or GLUpoly (1:5000) diluted in PBS containing 0.2% BSA. A polyclonal anti-MSP antibody (Burke and Ward, 1983) was used as a positive control for spermatozoa protein extracts (1:1000 in BSA-PBS). After extensive washings with the same buffer, the secondary antibody (goat anti-mouse or goat antirabbit) conjugated with alkaline phosphatase (Nordic), 1:15,000, was applied for 2 hr at room temperature. The labeling was revealed in nitro-blue tetrazolium (50 mg/ml, 0.67%; Sigma) and 5-bromo-4-chloro-3-indoyl phosphate (50 mg/ml, 0.33%; Sigma) in revelation buffer (Tris 0.1 M, NaCl 0.1 M, $MgCl_2$ 5 mM), with exposure times of 1–5 min. The membrane was then rinsed in revelation buffer, dried, and photographed.

### Transmission Electron Microscopy

The genital tract was dissected and placed in cold (4°C) fixative. Specimens were fixed for 1 hr in 2% glutaraldehyde in a buffer solution of 0.1 M sodium cacodylate, 0.1 M sucrose, and 0.2 mM $CaCl_2$, pH 7.2, at a temperature of 4°C. After washing in the same buffer, the samples were postfixed for 1 hr in 1% osmium tetroxide in the same buffer, dehydrated in ethanol and propylene oxide, and embedded in epoxy resin (Spurr, 1969). Ultrathin sections were contrasted with a double-lead-stain technique (Daddow, 1986) and observed with a Hitachi H-600 microscope.

### Scanning Electron Microscopy

Male germ cells were obtained by dissecting adult male worms in a drop of PBS on a cover glass. Cells were allowed to sink and adhere to the glass for 2 hr and then were fixed in 2% glutaraldehyde in a 0.1 M sodium cacodylate buffer, pH 7.2, for 30 min. After rinsing, dehydration was performed in an ethanol series. Cells were coated with gold and observed with a JEOL 840 microscope.

## RESULTS

### Efficiency of Methods and of Antibodies

**Specificity of the antibodies.** To assess the validity of the immunocytochemistry results and to test the specificity of the various antitubulin antibodies, protein extracts of germ cells or spermatozoa of *H. polygyrus* were analysed. On Western blots, all general monoclonal and polyclonal antibodies to alpha-tubulin (DM1A) and to beta-tubulin (TUB21, C 140) reacted in male

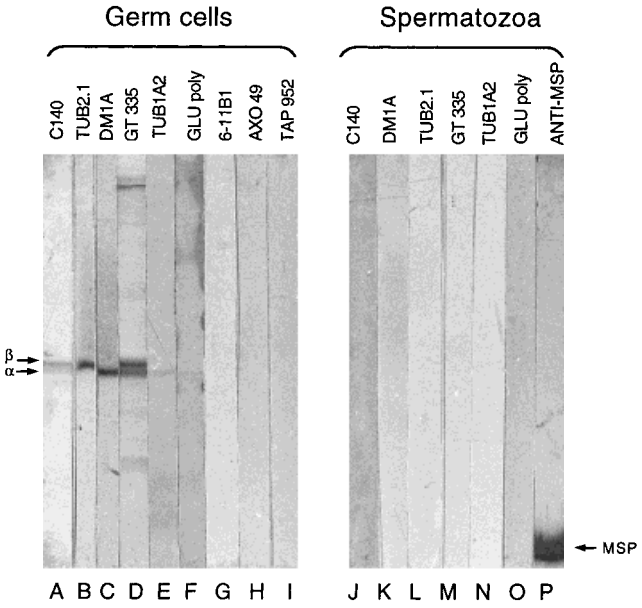


**Fig. 1.** Immunoblots of germ cell proteins of *H. polygyrus*. The antibodies used are indicated on the top. Germ cell proteins were extracted from all cells from the genital tract, including spermatogonia, spermatocytes, spermatids, and spermatozoa. Spermatozoa proteins were extracted from only the spermatozoa, collected in the male genital tract. **A–F:** Germ cells, labeling showing the presence of tubulin, and the presence of the posttranslational modifications, polyglutamylation (GT 335), tyrosination (TUB1A2), and detyrosination (GLU). Note that GT 335 labels two bands, indicating that polyglutamylation affects both alpha and beta subunits. **G–I:** Germ cells, absence of labeling with antibodies against acetylation (6-11B1) and polyglycylation (AXO 49 and TAP 952). **J–O:** Proteins from spermatozoa, absence of labeling with all antitubulin antibodies. **P:** Positive control, labeling of the MSP band (14 kDa) showing the presence of sperm proteins.

germ cell protein extracts with a polypeptide that by its molecular weight could be identified as tubulin (Fig. 1, lanes B, C, and A). In the same protein extracts, antibodies against polyglutamylation, detyrosination, and tyrosination labeled a band with the same molecular weight (see Fig. 1, lanes D, E, and F), but the antiacetylated and antipolyglycylated tubulin monoclonal antibodies did not react (see Fig. 1, lanes, G, H, and I).

The antibody GT 335 directed against glutamylated tubulin reacted also with high-molecular-weight polypeptides (see Fig. 1, lane D); this result has been noted already in other organisms (Bré et al., 1994; Delgado-Viscogliosi et al., 1996). The staining intensity with Glu-poly and TUB1A2 (see Fig. 1, lanes E and F) was not as great as that obtained with the general anti-alpha-tubulin antibody (see Fig. 1, lane C).

In protein extract of spermatozoa, no reactivity was seen with all antitubulin antibodies used (see Fig. 1, lanes J–O). The presence of sperm protein was assessed by a strong reactivity with the polyclonal anti-MSP (lane P), which reacts with the major sperm protein of this species (Mansir and Justine, 1996).

**A test for various antibodies against tubulin in nematode spermiogenesis.** Monoclonal antitubulin antibodies such as 16D3, 1B41, and 3F3 and the polyclonal antitubulin antibody T-3526 did not label any stage of *H. polygyrus* spermatogenesis, probably because of their specificity restricted to certain organisms. Because we expect to continue this study, here performed on a single model species, by comparative studies on other species, these negative results are useful for further studies.

**Efficiency of the various methods of fixation and permeabilization used.** The method of fixation-permeabilization by acetone-methanol (method 4) did not produce labeling. Preservation of microtubules in germ cells of *H. polygyrus* required the use of formaldehyde-containing fixatives followed by triton and SDS (method 1e), which gave good results in term of clarity of labeling and reproducibility. Most of the images presented in this paper were obtained by this method. The methods with glutaraldehyde (method 2) or with ethanol acetone produced a labeling of microtubular structures, but individual microtubules could not be resolved, the labelling was not sharp, and results were sometimes not reproducible.

## The Microtubular Structures of *H. Polygyrus* Male Germ Cells

Immunofluorescence labeling of *H. polygyrus* male germ cells was performed with the same panel of antibodies used to analyse tubulin on Western blots. Results obtained with DM1A exemplify the structure of the microtubular system during spermatogenesis. An expected result was that the antibodies against beta-tubulin (monoclonal or polyclonal) gave a labeling (see Figs. 8 and 10E–H) similar to the anti-alpha-tubulin.

Spermatocytes are round cells with a round nucleus centrally located. An extensive microtubule network in the cytoplasm is visible. The first division of meiosis shows heavily labeled centrosomes at the beginning and centrosomes and spindle labeled at the end (Fig. 2A–F).

**Fig. 2.** Spermatogenesis of *H. polygyrus*, immunolocalization of tubulin. All labeling is with the anti-alpha-tubulin antibody. **A,B:** Contrast in the labeling between spermatocytes with cytoplasmic labeling and spermatids with perinuclear labeling. **C–F:** Spermatocyte, first meiosis division. Labeling of centrosomes and spindle. **G,H:** Spermatid, stage 1, labeling around nucleus and in nuclear fossa. **I:** Spermatids, stage 1, various orientations showing labeling around nucleus and in nuclear fossa and a few microtubules in cytoplasm. **J:** Spermatid, stage 2, beginning of formation of microtubule bundle, originating from nuclear fossa. **K:** Spermatids, stage 2, beginning of nuclear elongation. **L,M:** Spermatid, stage 2, short microtubule bundle, originating from nuclear fossa. **N:** Spermatid, stage 3, microtubule bundle. **O,P:** Spermatid, stage 3, tropomyosin labeling at the stage of microtubule bundle. Tropomyosin is located in a cylinder around the bundle. **Q:** Spermatid, stage 3, with attached droplet. **R:** Spermatid, stage 4, after discarding of droplet. Long microtubule bundle extending from the nuclear fossa to the anterior extremity of the cell. **S,T:** Spermatid, stage 4, late elongate spermatid, long microtubule bundle. **U,V:** Spermatid, stage 4, late microtubule bundle, progressively disappearing. **W:** Spermatid, stage 4, microtubule bundle almost disappeared. **X,Y:** Mature spermatozoon, stage 5, absence of tubulin labeling. A, C, E, G, I, J, K, L, N, O, Q, R, S, U, W, X, tubulin labeling; P, tropomyosin labeling; B, D, F, H, M, T, V, Y, corresponding nuclear labeling. A–K, O, P, U–W, ×1450; L–N, Q–T, X, Y, ×2900.

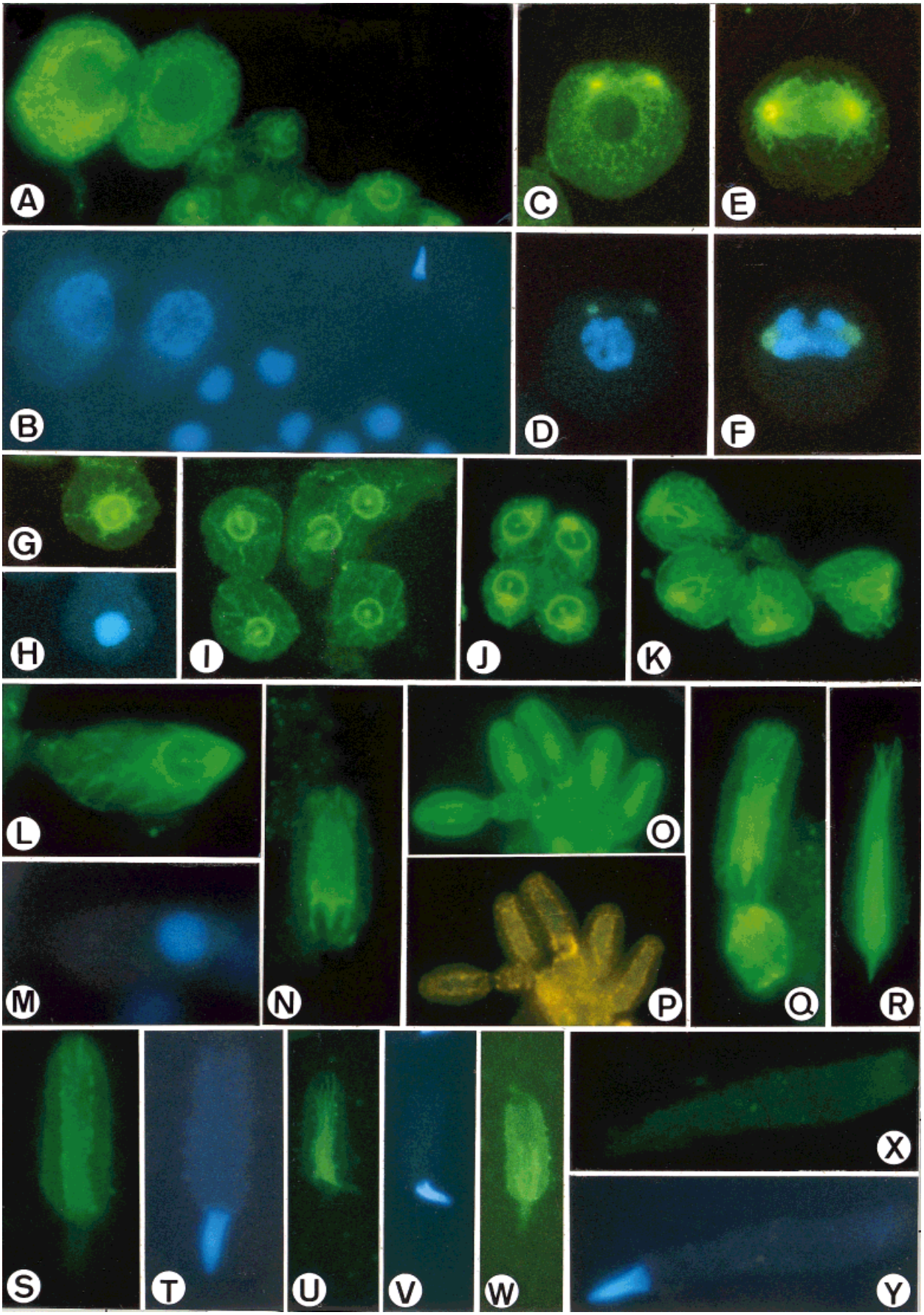


Fig. 2.

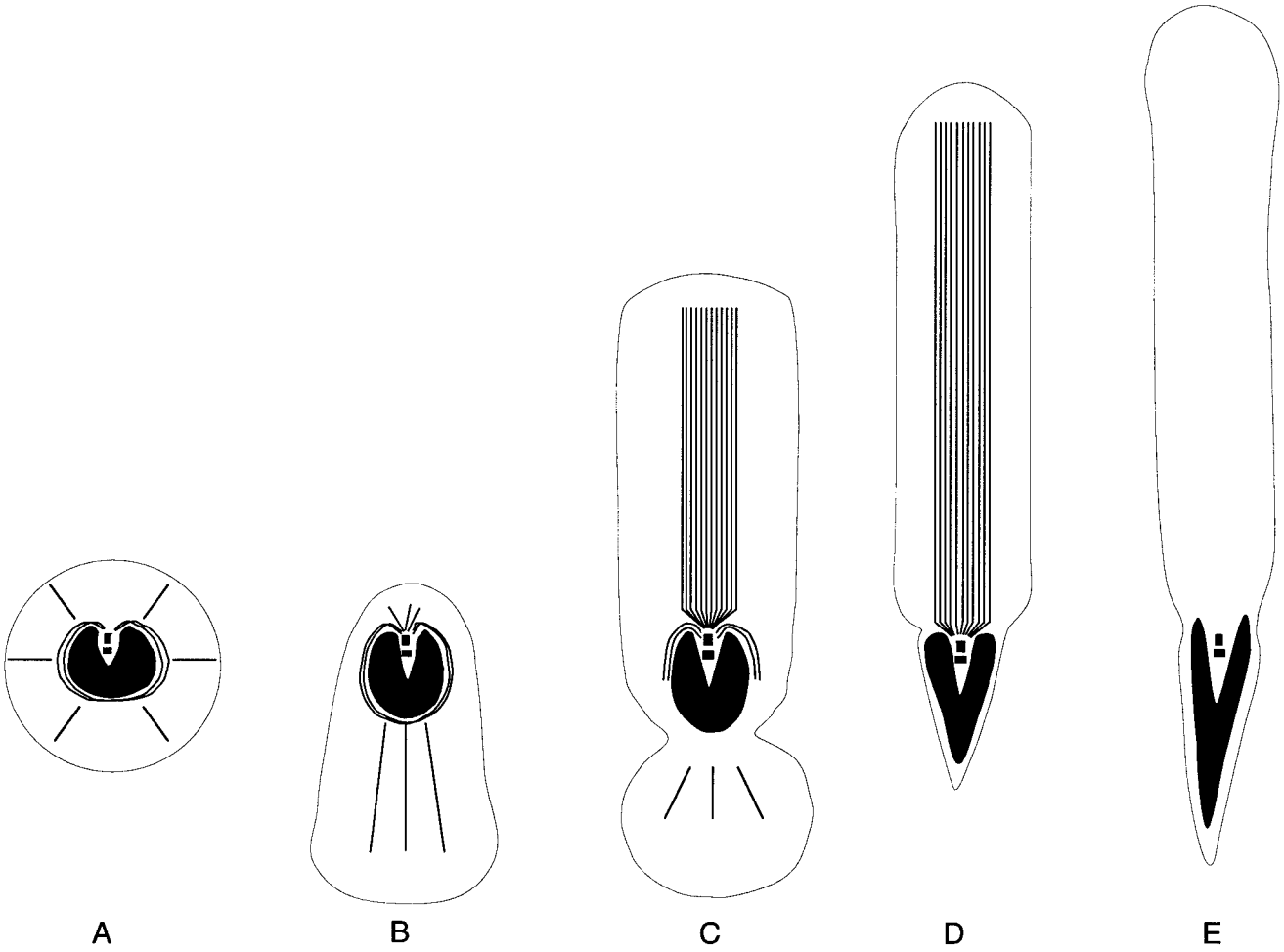


**Fig. 3.** Diagram of the microtubular system during spermiogenesis of *H. polygyrus*. **A:** Stage 1: round spermatid. **B:** Stage 2: spermatid with droplet. **C:** Stage 3: elongate spermatid with droplet. **D:** Stage 4: elongate spermatid without droplet. **E:** Stage 5: mature spermatozoon. In the mature spermatozoon, no tubulin can be detected by immunocytochemistry.

Spermatids show several distinct stages, which can be grouped in five main chronological steps, shown in Fig. 3.

**Stage 1: Round spermatid** (Fig. 3A). Round spermatids have a round nucleus with a nuclear fossa. Tubulin labeling is located as a perinuclear sheath, an intense labeling in the fossa, and a few microtubules radiating from the sheath in all directions (see Fig. 2G, I).

**Stage 2: Spermatid with droplet** (Fig. 3B). The nucleus is slightly more elongate. The nuclear fossa gives rise to a small bundle of parallel microtubules. The cytoplasm opposite the fossa, and linked to the rachis, elongates to form the cytoplasmic droplet. Microtubules originating from the sheath are visible in this droplet (see Fig. 2J–L).

**Stage 3: Elongate spermatid with droplet** (Fig. 3C). The bundle of microtubules originating from the fossa elongates dramatically. The microtubules around the nucleus disappear progressively. The cytoplasmic droplet, at the opposite end to the bundle, shows a heavy tubulin labeling. The nucleus is slightly more elongate (see Fig. 2N–Q). At this stage, tropomyosin is detected with a characteristic pattern (Mansir and Justine, 1996) as a cylinder around the microtubule bundle (see Fig. 2P).

**Stage 4: Elongate spermatid without droplet** (Fig. 3D). The bundle reaches its maximal length. The microtubules around the nucleus have disappeared. The nucleus is now piriform. The spermatids are not linked to the rachis and have lost the cytoplasmic droplet (see Fig. 2R). At the end of this stage, the microtubular bundles disappear gradually by the apical extremity (see Fig. 2S, U, W).

**Stage 5: Mature spermatozoon**(Fig. 3E). In the mature spermatozoon, no tubulin can be detected by immunocytochemistry and immunoblot (see Figs. 1 and 2X).

### Assessment of the Immunocytochemistry Results by Electron Microscopy

Scanning electron microscopy (Fig. 4) reveals that the late spermatid is an elongate cell, 12 µm in length,

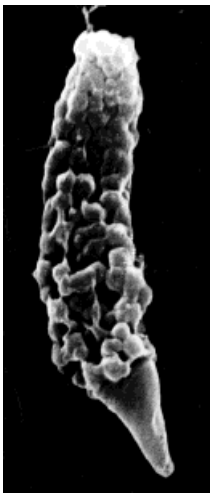

**Fig. 4.** Spermatid, scanning electron microscopy. The spermatid is an elongate cell with a piriform nucleus. Spermatozoa in the male tract have a similar morphology but are more elongate (×5000).

with the posterior extremity bearing a piriform nucleus. Spermatozoa have a similar morphology but are more elongate (18 µm).

Transmission electron microscopy (Figs. 5–8) was used to assess the presence of microtubules in various stages of spermatogenesis, detected by immunocytochemistry.

Spermatocytes, identified by the presence of synaptonemal complexes, are round cells with a high nucleocytoplasmic ratio (see Fig. 5A). The nucleus has a nuclear envelope. Numerous microtubules are present in the cytoplasm (see Fig. 5B). Spermatocytes are attached to a central rachis (see Fig. 5C).

Late spermatocytes (see Fig. 5D) show a well-developed cytoplasm containing numerous fibrous bodies spread throughout the cytoplasm, which represent a transitory storage organelle for MSP. Microtubules are present in the cytoplasm as a network lining the cell membrane (see Fig. 5E) and as more central cytoplasmic microtubules (see Fig. 5F).

Round spermatids, or stage 1 (see Fig. 6A), are still attached to a rachis. The round nucleus shows a triangular fossa in which two perpendicular centrioles lie in an electron-dense matrix (see Fig. 6B). The nuclear envelope is absent. Fibrous bodies and mitochondria are ordered at the periphery of the cell, but the zone facing the fossa is devoid of organelles.

Microtubules clearly originate from the centrioles in the nuclear fossa (see Fig. 6C).

Elongating spermatids, or stages 3–4 (see Fig. 6C–F), show a more electron-dense nucleus, still devoid of nuclear envelope. Fibrous bodies are arranged along the periphery and leave an empty space in the middle of the cell. Microtubules grow from the centrioles in the nuclear fossa (see Fig. 6F) and occupy the central cytoplasm as a bundle of parallel elements (see Fig. 6C–F), as observed in longitudinal (see Fig. 6C) and transverse sections (see Fig. 6D, E).

Spermatozoa, or stage 5 (see Fig. 7), are elongate cells (see Figs. 4 and 7A) with a piriform nucleus. The extremity opposite the nucleus shows the fibrous cytoplasm characteristic of the MSP cytoskeleton (see Fig. 7A). Various sections of spermatozoa in the seminal vesicle (see Fig. 7B) never show cytoplasmic microtubules. However, two perpendicular centrioles (see Fig. 7C–E) are present in the nuclear fossa, embedded in an electron-dense matrix. These two centrioles show an outstanding feature: They are each made up of 10 singlets (see Fig. 7E) ordered along an ellipse, 140 × 160 nm.

### The Transient Microtubular Structures Are Posttranslationally Modified During Spermatogenesis in *H. polygyrus*

In this system where microtubular structures are only transient during spermatogenesis, we investigated the existence of posttranslational modifications of tubulin. Six antibodies directed against modified forms of tubulin were used (see Table 1), and results were compared with those of a monoclonal antibody specific for the alpha subunit of tubulin (DM1A). Glutamylation (Fig. 9), detyrosination (Fig. 10), and tyrosination (Fig. 10) were detected, and the immunofluorescence pattern was the same as for DM1A. The monoclonal antibody GT 335 directed against polyglutamylated tubulin gave a good labeling, which was intense in the centrosomes in spermatocytes (see Fig. 9). The polyclonal antidetyrosinated tubulin also labeled heavily the centrosomes and perinuclear microtubules in spermatocytes (see Fig. 10A,B). Spermatozoa were not labeled by any of the antibodies used. The antibodies against acetylated tubulin and polyglutamylated tubulin (AXO 49 and TAP 952) also gave no labeling.

## DISCUSSION

In this report we studied the behaviour of microtubules and the distribution of different posttranslational modifications of tubulin during spermatogenesis in *H. polygyrus*. The microtubular structures observed are all transient, since they disappear at the inception of the spermatozoal stage. In mature sperm, the labeling was seen neither with anti-posttranslationally modified tubulin antibodies nor with the general antitubulin antibodies. This is certainly due to the disappearance of tubulin in either polymerised or nonpolymerised form, which is demonstrated by immunoblot. However, centrioles are present in mature spermatozoa, but this small quantity of tubulin is not detected by immunoblotting.

The microtubule network of spermatocytes in *H. polygyrus* is probably involved in shaping of the cell, and microtubules in the meiotic spindle are obviously involved in chromosome movement (Dustin, 1984). In spermatids, microtubules are of two kinds: Some microtubules are located around the nucleus, and others form a longitudinal bundle. The microtubules around the nucleus are analogous to the manchette known in various animals, especially annelids, insects, and mam-

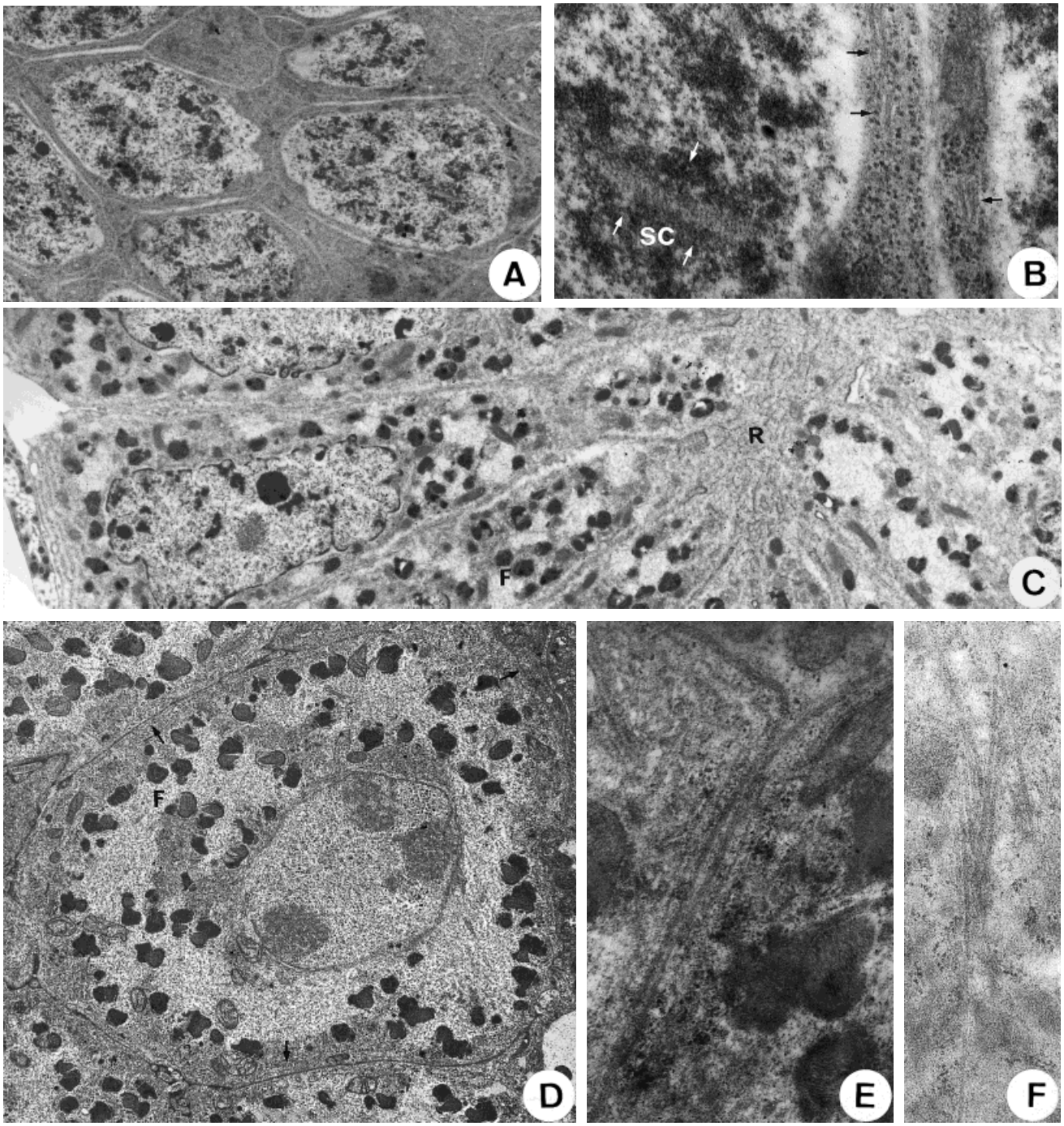


**Fig. 5.** Spermatocytes, transmission electron microscopy. **A:** General view of spermatocytes I. **B:** Spermatocytes I, nucleus with synaptonemal complex (SC) in the nucleus and microtubules (arrows) in the cytoplasm. **C:** Spermatocytes attached to the rachis (R). The cytoplasm contains fibrous bodies (F). **D:** Spermatocyte. Cytoplasm contains fibrous bodies (F) and peripheral microtubules (arrows). **E:** Peripheral microtubules of spermatocyte. **F:** Microtubules in the cytoplasm. A, ×6000; B, ×40,000; C, ×5000; D, ×6300; E, ×34,000; F, ×40,000.

mals. We have demonstrated in *H. polygyrus*, by ultrastructural and indirect immunostaining studies of spermatogenesis, a spatial and temporal association between microtubules and cellular and nuclear elongation. However, there is no direct proof that the manchette participates in the elaboration of the nucleus shape (Courtens and Loir, 1981; Fawcett et al., 1971; Kessel, 1966; Sherron et al., 1985). The relationships between the perinuclear microtubules and the nucleus in *H. polygyrus* are different from the other models because the nuclear envelope is absent (the absence of a nuclear envelope is a characteristic of spermatozoa in most

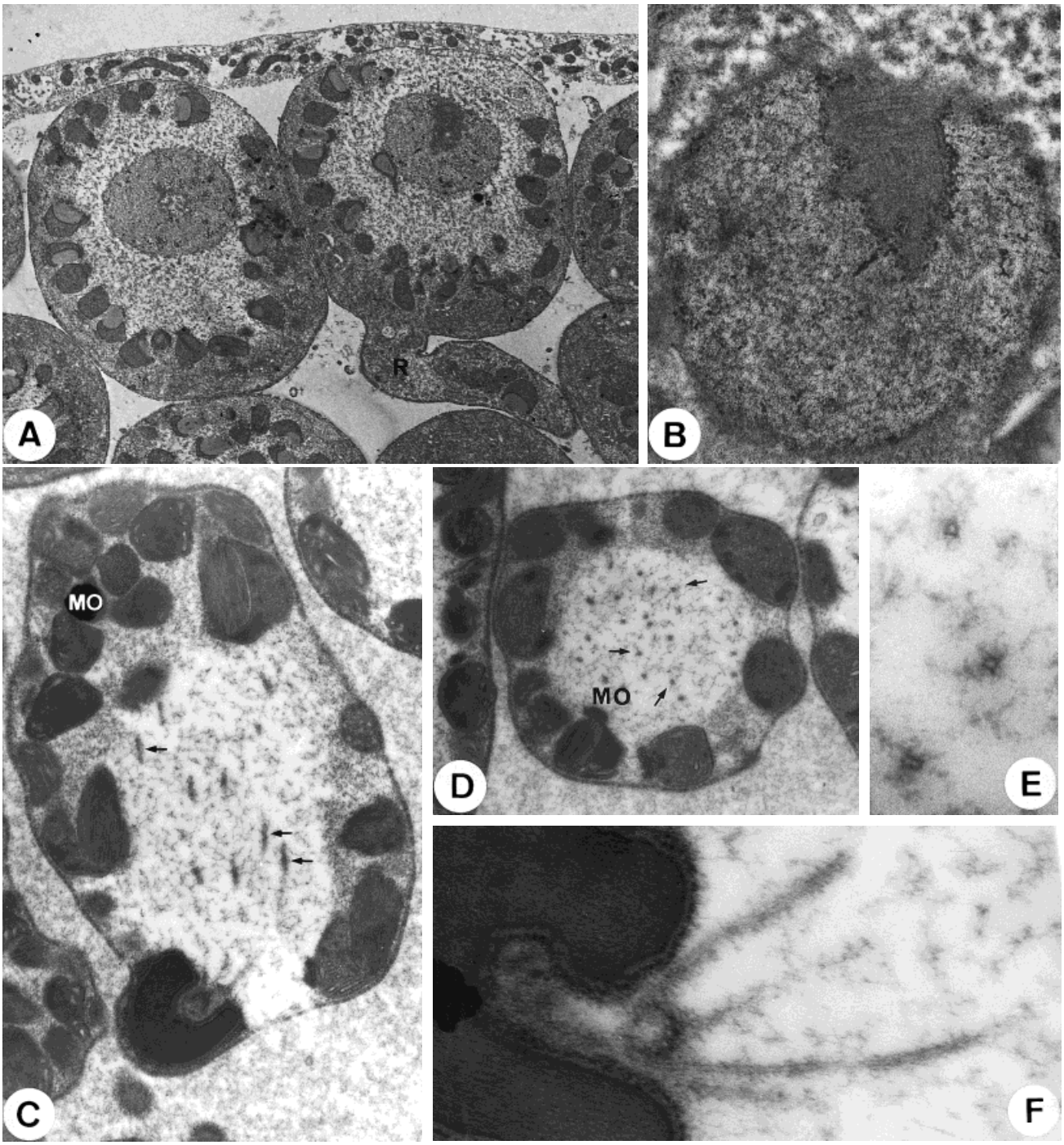


**Fig. 6.** Round and elongate spermatids, transmission electron microscopy. **A:** Round spermatids with rachis (R). **B:** Nucleus of round spermatid, with fossa containing a dense matrix and the two perpendicular centrioles. **C:** Early elongate spermatid, longitudinal section. Longitudinal microtubules (arrows) diverging from the centrioles in the nuclear fossa. MO, membranous organelles. **D:** Elongate spermatid, transverse section. Microtubules (arrows) occupy the center of the cell which is devoid of other organelles. Membranous organelles (MO) occupy the periphery of the cell. **E:** Microtubules, as in D; higher magnification. **F:** Microtubules diverging from centriole in nuclear fossa. N, nucleus. A, ×6000; B, ×35,000; C, ×17,500; D, ×14,300; E, ×70,000; F, ×50,000.

nematodes (references in Noury-Sraïri et al., 1993). Microtubules in the bundle of the central cytoplasm originate from the centrioles in the fossa. The polymerization of the bundle appears to be simultaneous with the lengthening of the spermatid, and the depolymerization with the end of this lengthening. These observations suggest a role of the microtubule bundle in elongation of the spermatid.

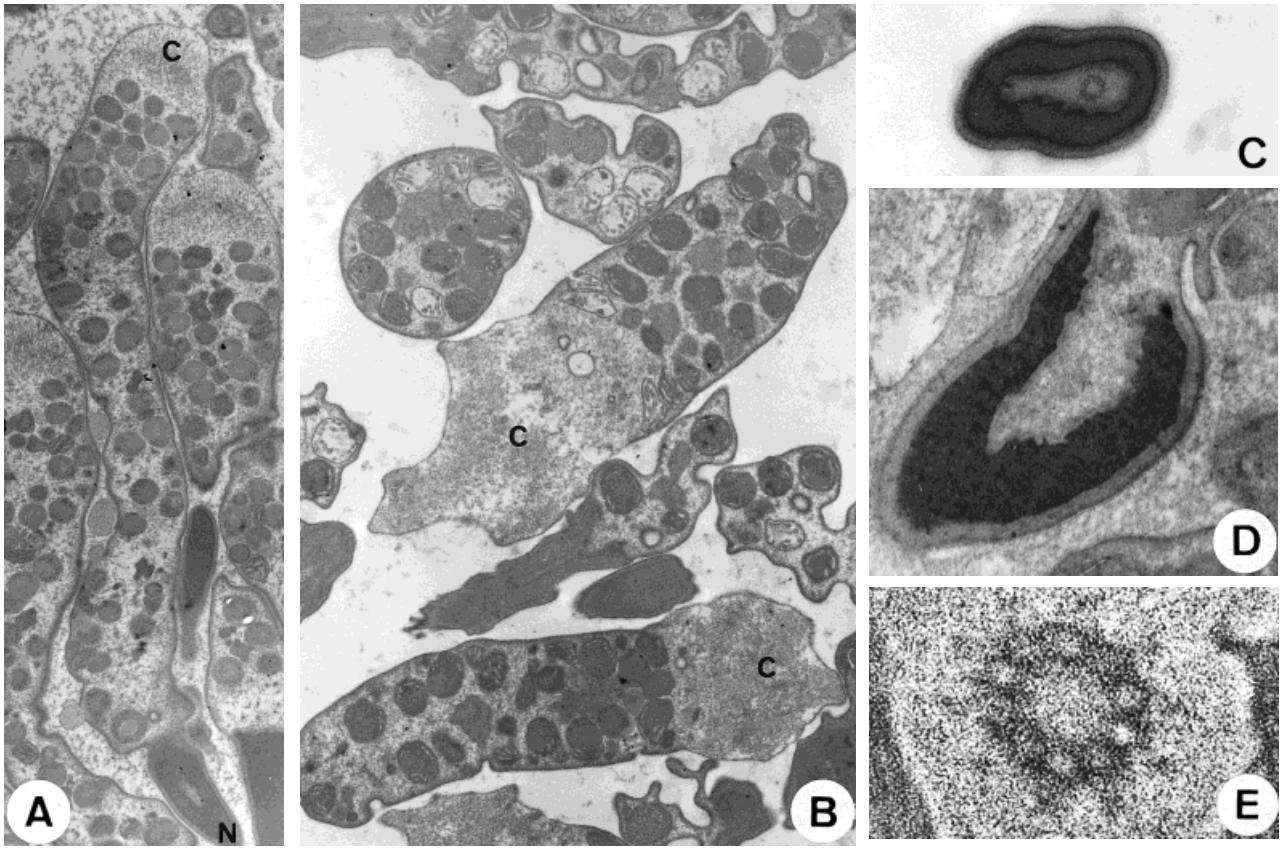


**Fig. 7.** Spermatozoa in testis, transmission electron microscopy. **A:** Spermatozoon, with anterior cap (C) and posterior nucleus (N). **B:** Sections of spermatozoa. C, anterior cap. No microtubules are visible. **C,D:** Centrioles in fossa of nucleus; C, transverse section of nucleus; D, longitudinal section of nucleus. **E:** Centriole showing 10 singlets. A, ×6500; B, ×7700; C, ×26,000; D, ×30,000; E, ×160,000.

In nematode spermatozoa, microtubules are usually absent (Noury-Sraïri et al., 1993) and particularly in *Ascaris* (Favard, 1961) and *Caenorhabditis* (Wolf et al., 1978), the two nematodes in which the spermatozoon and its MSP-based cytoskeleton have been intensively studied (Scott, 1996; Theriot, 1996). In *C. elegans,* the mitotic spindle is discarded with the residual body (Ward, 1986). However, there are a few nematode species that have microtubules in mature sperm. *Gastromermis* (Poinar and Hess-Poinar, 1993) has longitudinal microtubules and microtubules in pseudopod spikes. *Aspiculuris* (Lee and Anya, 1967) has a bundle of longitudinal microtubules associated with the nucleus. *Ekphymatodera* (Cares and Baldwin, 1994) and some other heterodorids have cortical microtubules in mature spermatozoa. In the strongylid group, to which *H. polygyrus* belongs, bundles of microtubules have been described in spermatids of *Nippostrongylus brasiliensis* (Jamuar, 1966; Wright and Sommerville, 1985) and *Nematodirus battus* (Martin and Lee, 1980), and in *Ancylostoma caninum,* microtubules are abundant during nuclear elongation but disappear later (Ugwunna and Foor, 1982). Although it is not clear in the literature based on electron microscopy whether the microtubules disappear in mature spermatozoa, it is likely that our observations on *H. polygyrus,* which are based on both electron microscopy and immunocytochemistry studies, may be generalized for other species of strongylids in which microtubular bundles have been described by electron microscopy. Clearly, microtubules are abundant in spermatids but completely absent in spermatozoa, with the exception of the centrioles.

Centrioles of nematode spermatozoa never conform to the usual nine triplet pattern but are generally made up of nine singlets (see references in Mansir and Justine, 1995) or nine doublets (*Gastromermis*) (Poinar and Hess-Poinar, 1993). The centrioles of *H. polygyrus* are thus exceptional not by the fact that they are made up of singlets but because they have a 10-fold radial symmetry. Although 9-fold symmetry is predominant in eucaryotic cells, centriolar or axonemal systems are known with 3-fold (Prensier et al., 1980), 6-fold (Schrével and Besse, 1975), 8-fold (Reger and Florendo, 1970), 12-fold (Baccetti et al., 1973; Van Deurs, 1973), 13-fold (Dallai et al., 1994; Yin et al., 1985), 14-fold (Baccetti et al., 1973; Dallai et al., 1992), and 16-fold (Dallai et al., 1992) symmetry, but the 10-fold symmetry has been found only in the axoneme of an insect (Dallai et al., 1996) and in the sperm centrioles of *H. polygyrus.* Despite their unique structures, the centrioles of *H. polygyrus* spermatozoa seem to normally act as MTOC in the spermatid.

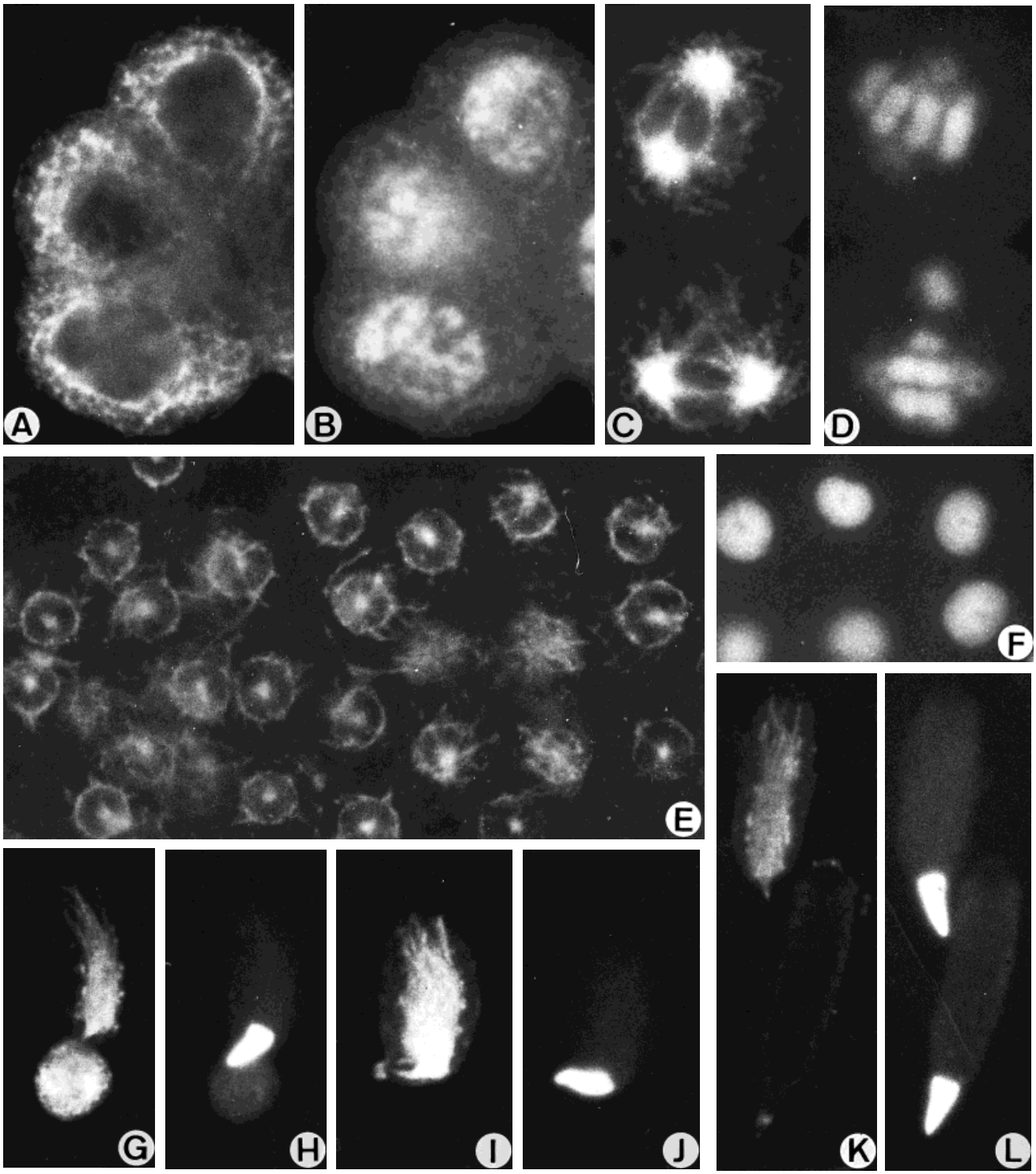


**Fig. 8.** Spermatogenesis, immunocytochemical localization of beta-tubulin. **A,B:** Spermatocytes. Tubulin located in all cytoplasm. **C,D:** Meiosis. Tubulin located in aster and spindle. **E,F:** Spermatids, stage 1. Tubulin located around the round nucleus and in the nuclear fossa. The nuclei labeled by nuclear dye (F) show a dark center corresponding to the fossa. **G,H:** Spermatid, stage 3, bundle of longitudinal microtubules originating from nuclear fossa. Note droplet containing tubulin. **I,J:** Spermatid, stage 4, after release of droplet. **K,L:** Spermatid, stage 4, and spermatozoon stage 5 together. Note complete absence of tubulin labeling in spermatozoon. A, C, E, G, I, K, tubulin labeling; B, D, F, H, J, L, corresponding nuclear labeling. A–L, ×2500.

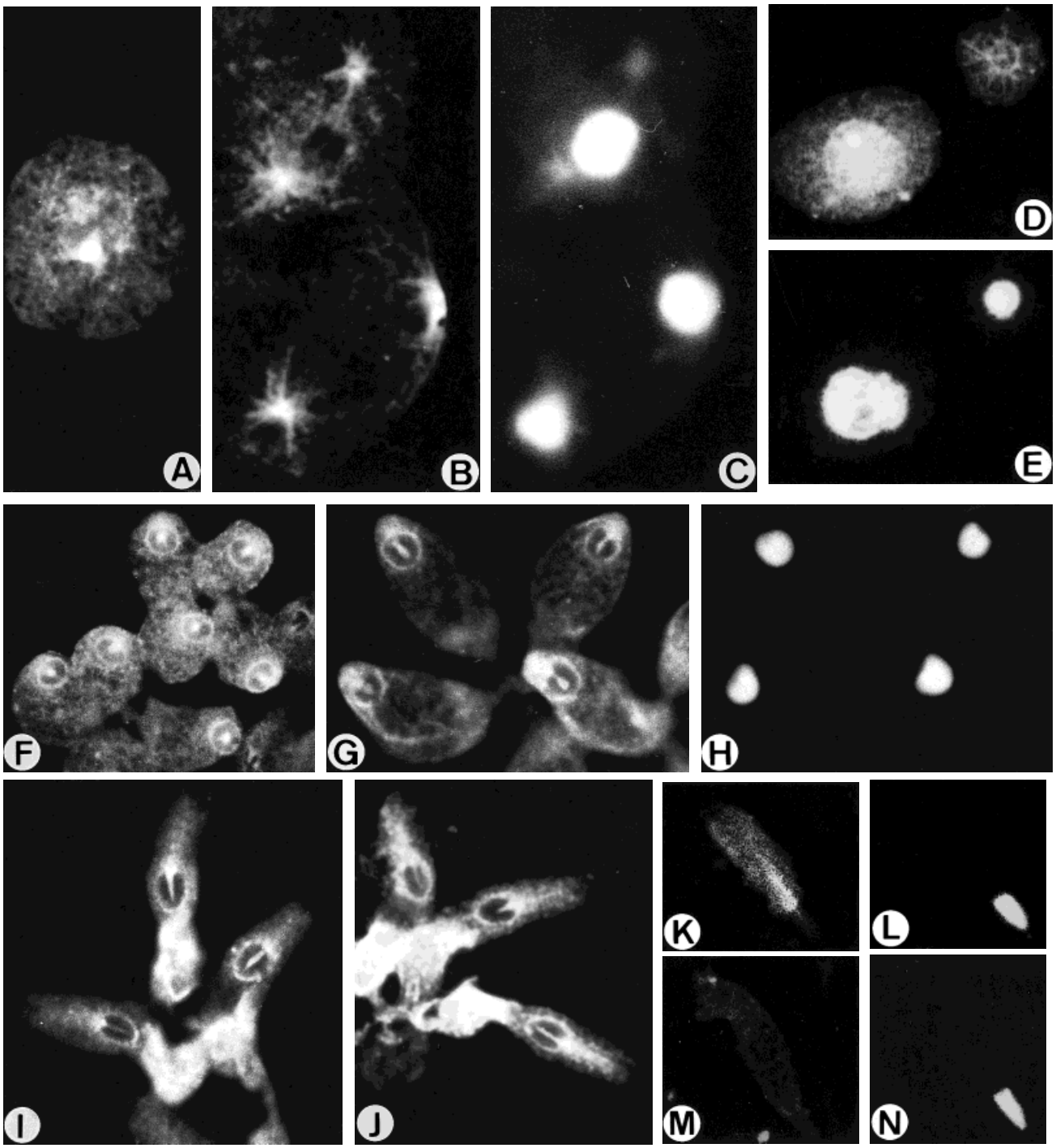


**Fig. 9.** Spermatogenesis, immunocytochemical localization of polyglutamylated tubulin. **A:** Mitosis of spermatocyte. Polyglutamylated tubulin is present at the level of the centrioles. **B,C:** Mitosis. Polyglutamylated tubulin is located in aster and spindle. **D,E:** Spermatocyte and spermatid. Note difference of size and contrasting tubulin pattern: Polyglutamylated tubulin is located in all cytoplasm in spermatocyte and mostly around nucleus in spermatid. **F:** Spermatids, stage 1, heavy tubulin labeling. **G,H:** Spermatids, stage 2, droplet at the opposite of nuclear fossa. **I:** Spermatids, stage 3, attached to rachis. Bundle of microtubules originating from nuclear fossa, and droplet with heavy tubulin labelling. **J:** Spermatids, stage 3, attached to rachis, slightly more advanced stage. **K,L:** Spermatid, stage 4, separate from rachis, bundle of microtubules disappearing. **M,N:** Spermatozoon, stage 5. Note absence of tubulin labelling. A, B, D, F, G, I, J, K, M, tubulin labeling; C, E, H, L, N, corresponding nuclear labeling. A–C, ×1600; D, E, K–N, ×1400; F, ×1300; G–J, ×1800.

Microtubules in transient microtubular structures in male germ cells of *H. polygyrus* are posttranslationally modified. A characteristic of these posttranslational modifications is that they are not dependent on the developmental stages of spermatogenesis: Polyglutamylation, tyrosination, and detyrosination are present

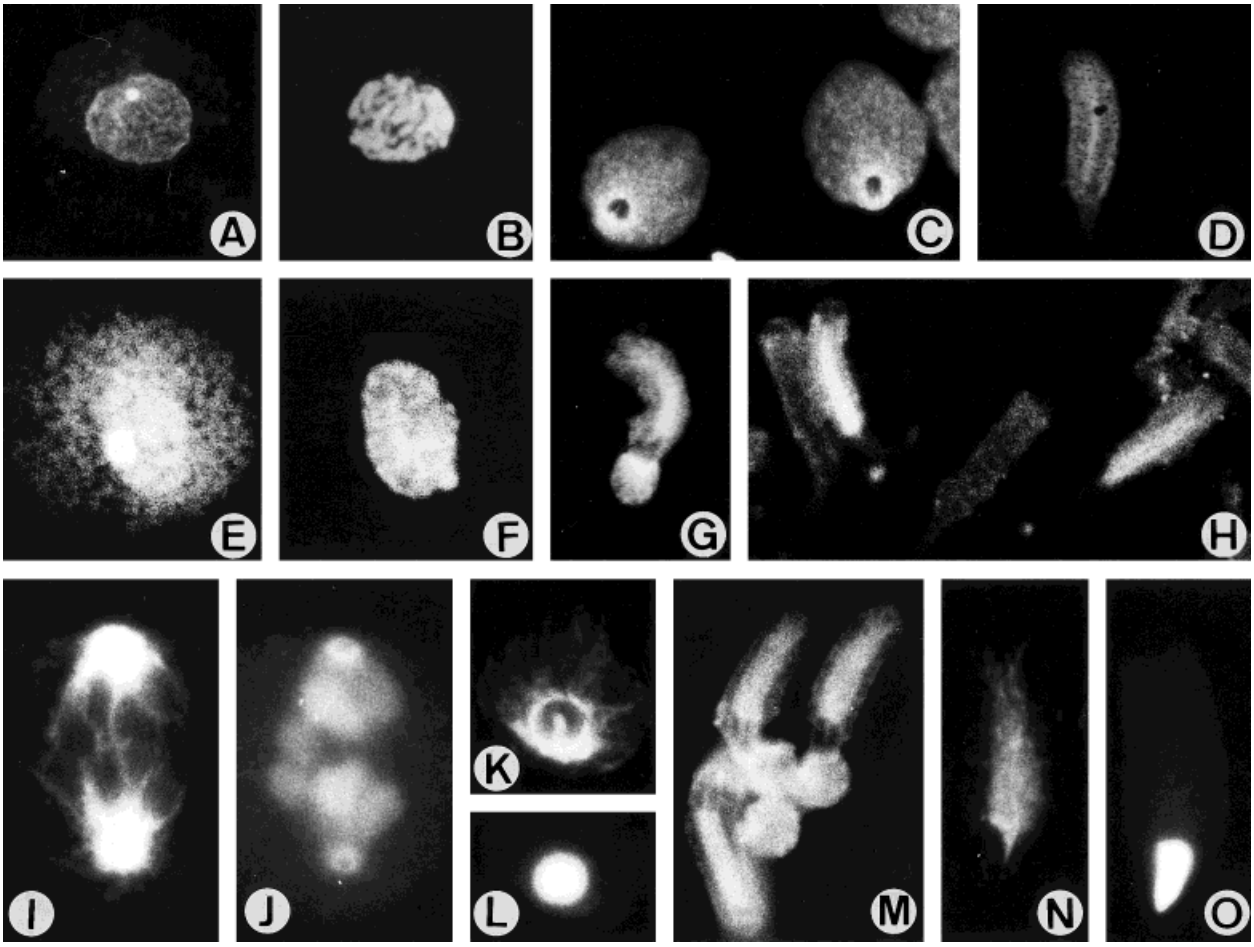


**Fig. 10.** Spermatogenesis, immunocytochemical localization of tubulin with various antibodies. **A–D:** GLU antibody (detyrosinated tubulin). **A,B:** Spermatocyte, labeling concentrated on centrosome region. **C:** Spermatids, stage 2, labeling around nucleus. **D:** Spermatid, stage 3, labeling of microtubule bundle in central zone of cytoplasm. A–D, ×1250. **E–H:** C 140 polyclonal antibody (beta-tubulin). **E,F:** Spermatocyte, perinuclear labeling of tubulin. **G:** Spermatid, stage 3, labeling of microtubule bundle in central zone of cytoplasm and droplet. **H:** Spermatids, stage 4, and mature spermatozoa, stage 5. Contrast between spermatids with heavy labeling and spermatozoa without labeling. E, F, ×1400; G, H, ×1800. **I–O:** TUB 1A2 (tyrosinated tubulin). **I,J**: Mitosis of spermatocyte, labeling of centrosomes and spindle. **K,L:** Spermatid, stage 2, perinuclear labeling. **M:** Spermatids, stage 3, labeling of longitudinal microtubule bundle and droplet. **N,O:** Spermatid, stage 4, labeling of microtubule bundle. A, C, D, E, G, H, I, K, M, N, tubulin labeling; B, F, J, L, O, corresponding nuclear labeling. I, J, ×2250; K, L, ×1400; M–O, ×1800.

from spermatocytes to spermatids in various microtubular structures such as cytoplasmic microtubules, meiotic spindles, perinuclear microtubules, and microtubule bundles of spermatids. Posttranslational modifications that are absent (acetylation, polyglycylation) were not found in any of these structures and stages. Acetylation, detyrosination, polyglutamylation, and polyglycylation modifications of tubulin are generally restricted to subpopulations of microtubules that often belong to the most stable microtubular systems (Bulinski and Gundersen, 1991; Delgado-Viscogliosi et al., 1996; Gurland and Gundersen, 1995; MacRae, 1992; Wolff et al., 1994). However, in our model, apart from the centrioles, none of the microtubular structures observed in male germ cells of *H. polygyrus* can be qualified as stable, and axonemes, for example, are completely lacking. The microtubular systems observed are transient, but no information is available about the duration of the spermatogenic stages, the turnover of microtubules, and the lifetime of each microtubular structure.

Acetylation and polyglycylation of tubulin are absent in male germ cells of *H. polygyrus*. Antibodies specific to acetylated and polyglycylated tubulin gave negative results in immunocytochemistry and immunoblot experiments, showing that these isoforms are absent in the different transient microtubular systems encountered during spermatogenesis. A single published record of acetylated tubulin in the nematode is available and concerns mechanosensory neurones of *Caenorhabditis elegans* (Siddiqui et al., 1989). Acetylation has been found in numerous organisms, including protozoa (Delgado-Viscogliosi et al., 1996), platyhelminth sperm (Iomini and Justine, 1997; Raikova et al., 1997), insect sperm (Warn et al., 1990; Wolf, 1994), sea urchin sperm (Levilliers et al., 1995; Stephens, 1992), and mammal sperm (Piperno and Fuller, 1985). There are cell models in which acetylated tubulin is absent, such as cultured

cells PtK2 (Piperno et al., 1987), rat spermatids (Hermo et al., 1991), and a diatom (Machell et al., 1995). The manchette microtubules in mammal spermatids that appear analogous to the microtubules around the nucleus of *H. polygyrus* are not acetylated (Fouquet et al., 1994), and this is comparable with our results. Acetylation of microtubules is apparently not indispensable in eukaryotic cells, since directed mutagenesis experiments producing nonacetylable tubulin in *Chlamydomonas* (Kozminski et al., 1993) and *Tetrahymena* (Gaertig et al., 1995) resulted in cells without acetylated tubulin but apparently showing no phenotypic difference from normal cells. However, a low degree of acetylation has been correlated in one case of sperm abnormalities and retinal degeneration in humans (Gentleman et al., 1996). Acetylation is often but not always a component of stable microtubules (Schulze et al., 1987).

During polyglycylation, the number of glycyl residues in glycyl chains added to alpha- and beta-tubulin subunits ranges from 3 to 40 among species (Mary et al., 1996; Weber et al., 1996) and is related to microtubule stability (Bré et al., 1996). An involvement of polyglycylated tubulin in axoneme motility has been suggested (Bré et al., 1996), since monoclonal antibodies that recognize polyglycylated tubulin specifically inhibit the reactivated motility of sea urchin spermatozoa. Polyglycylation is more abundant in A tubules than in B tubules of the peripheral doublets of the axoneme (Multigner et al., 1996). The glycyl chain is generally detected on microtubules located close to membranes (Fleury et al., 1995) and is interpreted as anchors between axonemal microtubules and membranes (Redeker et al., 1994); the absence of polyglycylation in *H. polygyrus* spermatids is consistent with this interpretation, since axonemes are absent and microtubules are widely separated from the cell membrane.

Polyglutamylation, detyrosination, and tyrosination of tubulin are present in male germ cells of *H. polygyrus*. Polyglutamylation apparently has never been mentioned in a nematode before the present study. In our experiment we have used the monoclonal antibody GT 335, which recognizes a basic motif of tubulin polyglutamylation independently of the number of glutamyl units present in the lateral chain (Wolff et al., 1992). Polyglutamylation was shown to involve only alpha-tubulin in nonnervous tissues such as sea urchin sperm (Mary et al., 1996) and only beta-tubulin in axonemes of the protozoa *Paramecium* (Fleury et al., 1995; Levilliers et al., 1995), *Paraustyla, Tetrahymena,* and *Euplotes* (Bré et al., 1994) and in mouse spleen, lung, or testis (Wolff et al., 1992). Polyglutamylation concerns both alpha- and beta-tubulin in mouse nervous tissues (Wolff et al., 1992) and in mammalian sperm (Fouquet et al., 1994). Our immunoblotting experiments show that polyglutamylation involves alpha- and beta-tubulin subunits in germ, nonnervous cells of *Heligmosomoides,* suggesting that two distinct polyglutamylation enzymes could exist in the testis. The labeling obtained with antipolyglutamylated tubulin is heavier around the centrioles in spermatocytes; this was not unexpected because polyglutamylation is generally associated with most stable microtubule structures like centrioles and axonemes of mammal spermatids (Fouquet et al., 1994; Kann et al., 1995). In contrast, the manchette of mammal spermatids, a transient unstable microtubular structure that is apparently analogous with the microtubules around the nucleus of the spermatid of *H. polygyrus,* is not polyglutamylated (Fouquet et al., 1994; Kann et al., 1995). Antibodies against polyglutamylated tubulin inhibit the movement of axonemes, and thus polyglutamylation is thought to play a role in dynein-based motility (Gagnon et al., 1996). Polyglutamylation regulates the binding of kinesin to microtubules (Larcher et al., 1996), thus suggesting a role of polyglutamylated microtubules in intracellular transport. The presence of polyglutamylated tubulin in the transient microtubules of the spermatids of *H. polygyrus* is consistent with a role of these microtubules in movement of organelles, particularly the movement of the MSP-containing "fibrous bodies" during spermiogenesis (Mansir and Justine, 1996). Tyrosination has been detected in ferm sperm cells (Hoffman and Vaughn, 1995) and germ cells of insects (Wilson et al., 1994; Wolf, 1994). Detyrosination and tyrosination were both present in male germ cells of *H. polygyrus,* as is the case in various other species (Delgado-Viscogliosi et al., 1996; Gurland and Gundersen, 1995; Warn et al., 1990). In 3T3 polarized cells, vimentin intermediate filaments coalign with detyrosinated microtubules, while the bulk of tubulin is tyrosinated, and detyrosination of tubulin acts as a signal for recruitment of vimentin IFs to MTs (Gurland and Gundersen, 1995). Detyrosination is found mainly in stable microtubules (Schulze et al., 1987), including microtubules associated with intracellular transport and cell polarity (Bulinski and Gundersen, 1991), but toad erythrocytes possess stable microtubules showing tyrosination but not detyrosination (Gundersen and Bulinski, 1986) and thus are an exception to this generalization. From these somewhat contradictory examples, it appears that neither tyrosination nor detyrosination may be used as an unequivocal marker of microtubule stability or nonstability. The presence of both tyrosinated and detyrosinated tubulin in the transient microtubules of the spermatids of *H. polygyrus* represents a new example of the simultaneous presence of these two modifications in a microtubular system.

This is the first study of posttranslational modifications of tubulin in nematode spermatogenesis. The observations show that polyglutamylation, tyrosination, and detyrosination are present, whereas acetylation and polyglycylation are absent. The posttranslational modifications present are apparently not stage dependent, being present in all microtubular structures from spermatocyte to spermatid.

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