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Nuclear Behavior and Differentiation in *Paramecium caudatum*, Analyzed by Immunofluorescence with Anti-tubulin Antibody

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ABSTRACT—To study the dynamics of the microtubules throughout the sexual cycle of *Paramecium caudatum*, we employed a monoclonal antibody (mAb) N356, that specifically recognized the 50-57 kDa axonemal proteins, equivalent to the tubulins of *P. caudatum*. The mAb decoration was observed on the micronuclei, as well as structures that consisted of microtubules not only in *P. caudatum* but also in other species *P. bursaria*, *P. tetraurelia* and *P. trichium.* While the micronucleus was consistently decorated with mAb, the macronucleus was decorated only during morphological changes, that is, at binary fission of the vegetative phase and at skein-formation during conjugation. During conjugation, the oral apparatus, with its associated microtubular structures, disappeared at meiosis I then reappeared at the end of the third division of the synkaryon. The current study led to several major findings on the behavior of the germinal micronucleus during conjugation: 1) the microtubules seemed to connect some of the haploid nuclei to the paroral region at the end of meiosis II; 2) mAb decoration disappeared from degenerating haploid nuclei, while it remained in the surviving nucleus; 3) mAb decoration also disappeared from macronuclear anlagen after the initiation of nuclear differentiation, while it remained in the other four post-zygotic micronuclei. The mAb decoration pattern could be used as an indicator of the fate of a micronucleus and suggests that both the intra-micronuclear and the cytoplasmic microtubules might have an important role in determining of the fate of micronuclei by translocating them to specific areas of the cytoplasm.

INTRODUCTION

Paramecium caudatum has two types of nuclei, a larger somatic macronucleus and a small germinal micronucleus. The germ line of this ciliate is governed by the micronucleus, while the macronucleus serves as the somatic nucleus, governing the vegetative and conjugational functions (Mikami, 1988). When conjugation is induced between two complementary mating types of *P. caudatum*, the micronucleus of each individual pair undergoes meiosis, and forms the fertilization nucleus (synkaryon). The synkaryon further divides and its products of division develop into both the macronuclear anlagen and a new micronucleus. These micronuclear events are accompanied by changes in morphology and/or the intracellular localization of the nuclei (Hiwatashi and Mikami, 1989). Especially at two stages of this process, the fate of nucleus strongly influenced by its position in the cytoplasm. One is the stage of degeneration of the meiotic products, one haploid nucleus positioned next to the paroral region survives while the other three meiotic products degenerate (Skoblo and Ossipov, 1968; Sonneborn, 1954; Wichterman, 1946). Yanagi and Hiwatashi (1985) proposed the possibility that the microtubules play a role in this process. Their proposal was based on the effects of the microtubule disrupting drug Vinblastine on *P. caudatum*, however, the morphological data to support this idea was not provided. The other stage occurs at the differentiation of both the macro- and micronucleus, where the position of the microtubular spindles of the postzygotic third division is important to determining the micro- and macronuclear differentiation (Grandchamp and Beisson, 1981; Mikami, 1980). These events raise the interesting question of what controls nuclear behavior and differentiation.

In order to better understand the control of nuclear behavior and differentiation in ciliates, it seems important to ascertain microtubule dynamics throughout the life cycle. Over the years role for microtubules in the mechanisms of nuclear behavior, such as division, localization, or migration of the nucleus, had been suggested (Cohen and Beisson, 1988; Eichenlaub-Ritter and Tucker, 1984; Fleury *et al.*, 1995; Iftode *et al.*, 1989; Jurand, 1976; Jurand and Selman, 1970; Kaneda and Hanson, 1974; Mikami, 1980; Stevenson, 1972; Stevenson and Lloyd, 1971; Tucker *et al.*, 1980, 1985; Yanagi and Hiwatashi, 1985). Moreover, recent findings of microtubule associated proteins in the ciliate nucleus and their behavior during the period of nuclear differentiation (Curtenaz *et al.*, 1997; Keryer *et al.*, 1989) suggest that microtubules

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might have important roles in the behavior and differentiation of the nucleus. In the present study, we describe the behavior of the microtubules as revealed by indirect immunofluorescence with mAb N356 during the sexual cycle of *P. caudatum* and discuss the role of the microtubules in relation to the mechanisms that control nuclear behavior and differentiation during and following meiosis.

MATERIALS AND METHODS

Materials

Stocks used in the current study were 27aG3sII (mating type V) and C103sII (mating type VI) that belong to syngen 3 of *Paramecium caudatum*, stock Sj2 belonging to syngen 1 of *P. bursaria*, an axenic culture of syngen 2 of *P. multimicronucleatum*, stock 51(mating type O and E) belonging to syngen 4 of *P. tetraurelia*, and stock OM4 of *P. trichium. Chlorella*-free *P. bursaria*, derived from the green cells of natural stock by rapid growth in the dark (stock Sj2, syngen 1) was kindly supplied by Dr. I. Miwa (Ibaraki University, Japan; Miwa *et al.*, 1996). The axenic culture of *P. multimicronucleatum* was kindly supplied by Dr. A. K. Fok and Dr. R. D. Allen (University of Hawaii, USA; Fok and Allen, 1979). The stock of *P. trichium* was kindly supplied by Dr. T. Watanabe (Tohoku University, Japan). Cells were cultured in diluted fresh lettuce juice medium diluted with Dryl's salt solution (Dryl, 1959) modified by the substitution of KH_2PO_4 for NaH_2PO_4 (pH 7.0) and inoculated with a nonpathogenic strain of *Klebsiella pneumoniae* as a food organism the day before use. Cultivation and experiments were carried out at room temperature (24±1°C).

Antibodies

MAb N356, raised against chick-brain microtubules and found to be specific for α-tubulin subunit of fibroblasts and chick brain, was purchased from Amersham (Little Chalfont, UK). The antibody was used at 1:25, and 1:50 dilutions for immunofluorescence labeling, and used at a 1:1,500 dilution for the immunoblotting. Fluorescein isothiocyanate (FITC)-conjugated affinity purified goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA), and was used at 1:200 dilution as a secondary antibody for immunofluorescence labeling. Alkaline phosphatase-conjugated affinity purified goat anti-mouse IgG was purchased from Cappel (Durham, NC) and was used at a 1: 5,000 dilution as a secondary antibody for immunoblots. All the antibodies were diluted with phosphate buffered saline (PBS, pH 7.2) containing 1% bovine serum albumin (BSA) and 0.02% sodium azide. All other chemicals were obtained from Sigma (St. Louis, MO).

Electrophoresis and Immunoblot Analysis

Cilia were isolated from 2-liter batches of cell suspensions. In order to demembranate the cells, cells were extracted in a solution containing, 10 mM EDTA, 20 mM KCl, 10 mM MOPS (pH 7.5), and 0.007% Triton X-100 for 25 min at room temperature. Detachment of the axoneme from demembranated cells was induced by a solution containing 20 mM $CaCl_2$ and 10 mM Tris-HCl with slight stirring for 10 min at 4°C, then washing twice with cold Tris-HCl buffer. The resulting 15.72 μg of pelleted axonemes was subjected to one dimensional Tris (hydroxymethyl) aminomethane dodecyl sulfate polyacrylamide gradient (5–20%) slab gel electrophoresis (Tris-DS-PAGE). Tris-DS-PAGE was performed according to Laemmli (1970), using phosphorylase b (94 kDa), bovine serum albumin (67 kDa), egg albumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), and α-lactalbumin (14 kDa) as molecular mass standards. After electrophoresis, axonemal proteins were transferred onto polyvinylidene difluoride (PVDF) membranes according to Towbin, *et al.* (1979), and which were soaked in 1% BSA, 150 mM NaCl, 20 mM Tris-HCl (pH 7.5), and 0.05% Tween 20 for 1 hr at room temperature for blocking. The PVDF membranes were incubated sequentially with a mAb N356 and a goat anti-mouse serum conjugated to alkaline phosphatase at room temperature for 60 min and 30 min, respectively, and developed by addition of nitroblue tetrazolium (50 mg/ml in 70% dimethyl-formamide) and 5-bromo-4-chloro-3-indolyl phosphate (50 mg/ml in 100% dimethyl-formamide) in alkaline phosphatase buffer containing 100 mM-Tris-HCl (pH 9.5), 100 mM NaCl, and 5 mM $MgCl_2$.

Fluorescence-microscopy

For indirect immunofluorescence experiments, *Paramecium* cells were fixed for 45 min at room temperature in buffered 3% formaldehyde solution containing 50 mM Hepes (pH 7.1), 10 mM EGTA, and 2 mM $MgCl_2$ in order to stabilize the microtubules during fixation. The fixed cells were then permeabilized with cold acetone at –20°C for 20 min, washed 3 times with PBS. Prior to the mAb labeling, the cells were incubated with 1% BSA in PBS for 30 min at room temperature for blocking. Then the cells were incubated sequentially with a primary and a secondary antibody (FITC-conjugated goat anti-mouse IgG) at room temperature for 60 min and 30 min, respectively. Unbound antibodies were washed away with excess PBS. To determine the exact localization of the nuclei in the cytosol, cells were stained with 1 μg/ml 4'6-diamidino-2-phenylindole dihydrochloride (DAPI) for 15 min at room temperature, then washed three times with PBS for 20 min each. Cells were examined either in a Carl Zeiss Axiovert 135 microscope or an Olympus IX70, equipped with epifluorescence illumination.

Induction of mating reaction

Conjugation was induced by mixing reactive two complementary mating types of *P. caudatum*, syngen 3, stocks 27aG3sll and C103sll. Two to three hours later mating pairs were isolated in modified Dryl's solution as described above. Cells were fixed at given times and processed first for immunofluorescence labeling and then for DAPI staining as described above.

RESULTS

The specificity of mAb N356

To examine the specificity of mAb N356 in *Paramecium*, the isolated axonemal proteins of *P. caudatum* were subjected to Tris-DS-PAGE and then electrophoretically transferred onto membrane for immunoblotting. After subjection to Tris-DS-PAGE, two major bands were detected: one was found near 200 kDa (estimated as 217–239 kDa); and the other was over the 50–57 kDa on the gel (Fig. 1, lane 1). The bands are broad, and seem to contain several proteins. As known from other *Paramecium* species, dyneins and tubulins are the major proteins in the axoneme: the typical dyneins have high molecular weights of about 200 kDa (Travis and Nelson, 1988) while α- and β-tubulin subunits comigrate at around 50–57 kDa on the gel (Cohen *et al.*, 1982). Thus, the major bands observed at the higher molecular weight area probably correspond to the axonemal dyneins. The mAb N356 reacted strongly and exclusively with the broad band in the 50-57 kDa area (Fig. 1, lane 2). Although the separation between α- and β-tubulins was obscure in this gel system, the band at the 50–57 kDa area does contains α-tubulin (Fig. 1, lane 1). Neither the major bands nor the minor bands (Fig. 1, lane 2) labeled with mAb N356, showing that this mAb specifically reacted with the presumptive tubulin containing area.

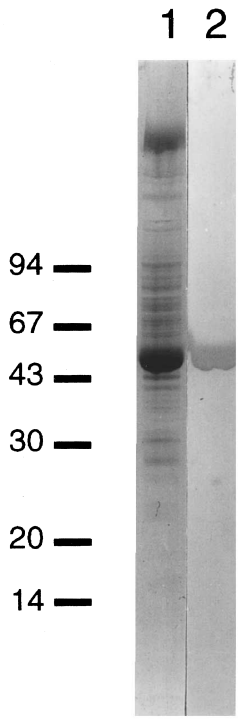

Fig. 1. Isolated axonemal proteins of *Paramecium caudatum* were stained with coomassie and α-tubulin specific monoclonal antibody N356. The isolated axonemal proteins were separated by Tris-DS-PAGE. Lane 1, Coomassie staining of isolated axonemal proteins. The amount of protein applied to lane 1 was 15.72 μg. Lane 2, Immunoblot of isolated axonemal proteins stained with the antibody N356. Molecular weight markers (kDa) are shown on the left.

Vegetative phase cells

A typical pattern of mAb N356 immunofluorescence labeling at interphase of *P. caudatum* is shown in Fig. 2A, in which all of the axonemal microtubules, the microtubules existing at the oral apparatus, cytopharyngeal microtubular ribbons, postoral fiber, the microtubules of the radiating arms of the contractile vacuole complexes, the cytoproct, and also the periphery of the micronucleus are strongly labeled with this mAb (Fig. 2B). To determine the exact location of the macro- and micronucleus, DAPI was employed (Fig. 2C, E). As shown in Fig. 2B, the interphase micronucleus was decorated with mAb N356. The mAb decoration was detected on the periphery and sometimes on the micronuclear matrix. However, the macronucleus in the interphase cell was not labeled with mAb (Fig. 2A, B). The mAb decoration on micronuclei persisted during interphase in *P. caudatum*, suggesting that the tubulin molecule was present throughout interphase, as has also been observed in *P. tetraurelia* (Jurand and Selman, 1970; Keryer *et al*. 1989).

Figure 2D shows the nuclei during vegetative division in *P. caudatum*. Both the macro- and the micronucleus are labeled with mAb N356 and both contain well developed spindles, suggesting that microtubules polymerization in both nuclei during vegetative division. As in *P. tetraurelia*, the intra-macronuclear microtubules in *P. caudatum* appeared very early during nuclear division (data not shown). At first microtubular bundles were assembled and then the spindles formed. Decoration of the macronucleus was consistently observed during cell division, but this decoration disappeared soon after the fission of the macronucleus. For the micronucleus mAb decoration persisted even after cell division.

This difference in the immunoreactivity between macro- and micronuclei was also true for other *Paramecium* species (Table 1). We examined four species in the current study, *P. bursaria*, *P. multimicronucleatum*, *P. tetraurelia*, and *P. trichium*. Although the mAb N356 decoration on the macronucleus was not detected during interphase, the decoration was always observed when the macronuclear spindle developed within the nucleus at karyokinesis. The micronuclei of all species except *P. multimicronucleatum*, consistently had mAb N356 decoration on their periphery during interphase and their division spindles were all strongly labeled with this mAb during cell division. In the case of *P. multimicronucleatum*, the mAb decoration of the micronucleus was observed in some preparations, but not in other preparations. Our inability to consistently decorate the micronucleus of *P. multimicronucleatum* could reflect insufficient permeabilization or other technical difficulties.

Early stages of conjugation

At the premeiotic S phase, the micronuclei of individual pairs round up (Fig. 3A, B). DAPI staining demonstrated that the micronuclear chromatin had an even distribution at this stage (Fig. 3B). The beginning of meiotic prophase, the micronucleus eleongated and became rod-shaped, which was accompanied by the development of the microtubules (Fig. 3C). The distribution of the chromatin became uneven at this stage (Fig. 3D). At late meiotic prophase, the micronucleus become elongated, curved and formed a crescent-shape. Immunofluorescence labeling with mAb N356 demonstrated that many microtubules were assembled at the periphery and also between the chromatin in the crescent-shaped micronuclei (Fig. 3E). The chromatin of the micronucleus seemed to condense in one specific part of the crescent (Fig. 3F). At prometaphase, a fine spindle appeared within the micronucleus (Fig. 3H), and the chromatin seemed to form a filamentous structure, the leptonema (Fig. 3I). The micronucleus elongated along with the extension of the spindle (Fig. 3K), and their chromosomes were separated to both ends of the dividing micronuclei by anaphase of meiosis I (Fig. 3L). At the telophase of meiosis II, longer separation spindles were observed (Fig. 3N) and the chromosomes became hard to detect (Fig. 3O).

The oral apparatus disappeared during meiosis I (Fig. 3G, J, M). Fig. 3G shows the conjugated area of the mating pair during the crescent stage of conjugation, in which mAb N356 decorated the rows of penniculi and the quadrulus of the oral apparatus. However, the associated microtubular structures such as the cytopharyngeal ribbons and the postoral fiber had already disappeared. By the promethaphase of meiosis I, the oral apparatus had completely disappeared (Fig.

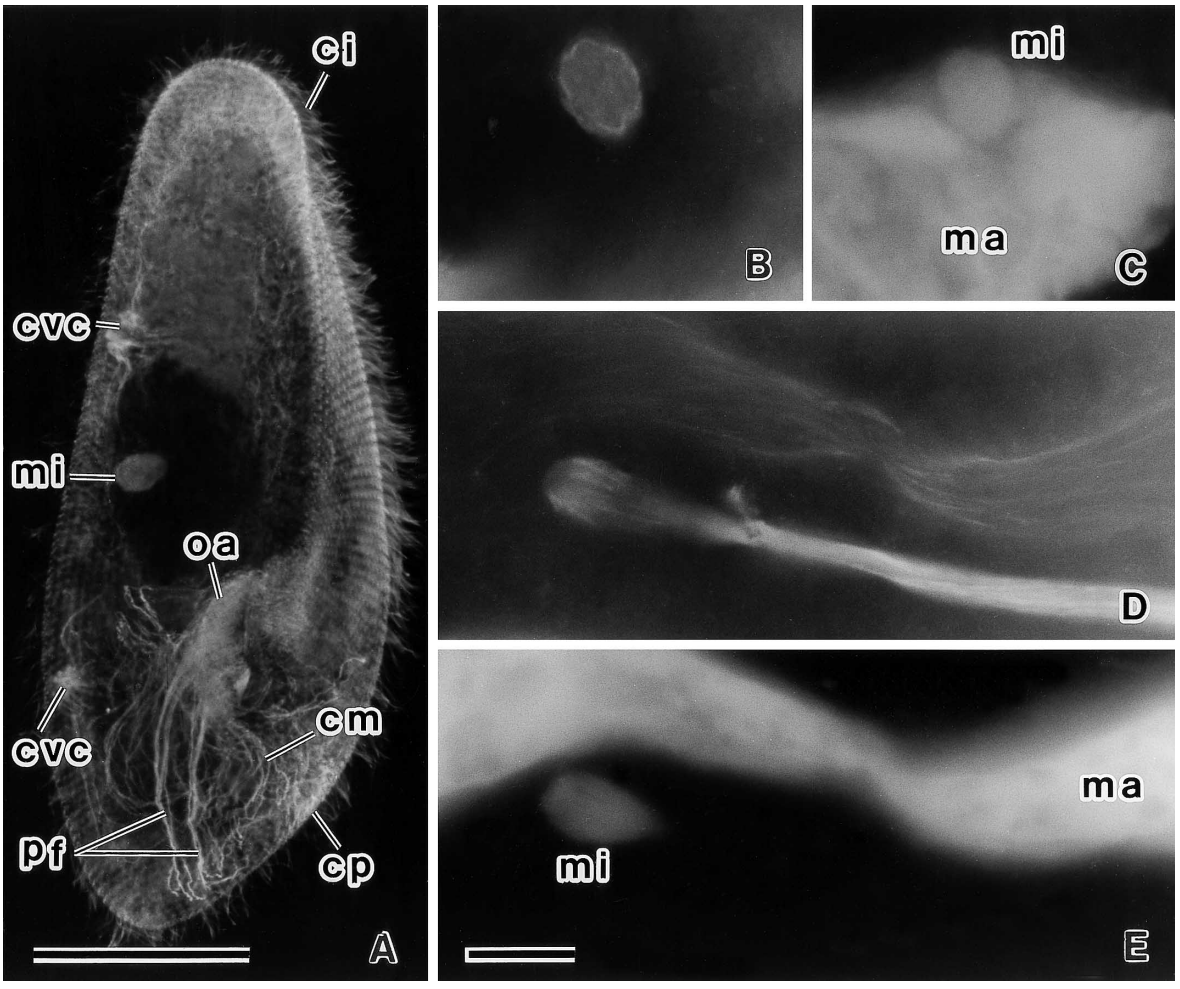

Fig. 2. Morphology of *Paramecium caudatum* revealed by indirect immunofluorescence labeling with anti α-tubulin antibody (mAb N356) and DAPI staining. A. A typical interphase labeling pattern with mAb N356. B and C. Enlarged image of a micronucleus of an interphase cell stained with mAb N356 (B) and with DAPI (C). D and E. Enlarged image of the equatorial region of a divider showing a typical labeling pattern with mAb N356 (D) and DAPI (E) on both a macro- and a micronucleus. The somatic cilia (ci), the oral apparatus (oa), the cytopharyngeal microtubules (cm), the postoral fiber (pf), the radial canal microtubules of the contractile vacuole complexes (cvc), the cytoproct (cp) and also the periphery of the micronucleus (mi) were labeled with mAb. Although the macronucleus (ma) was not labeled during interphase, it was labeled during vegetative division (D). Bars: in A, 50 μm; in E, for B–E, 10 μm.

Table 1. The mAb N356 decoration on the nuclei during the vegetative cell cycle in *Paramecium* species.

Species	Interphase		Cell division	
	Macro	Micro	Macro	Micro
P. caudatum	–	+	+	+
P. bursaria	–	+	+	+
P. multimicronucleatum	–	+/–	+	+/–
P. tetraurelia	–	+	+	+
P. trichium	–	+	+	+

Macro, macronucleus; Micro, micronucleus; +, detected; +/–, detected but not in all the preparations; –, undetected.

3J). A conical area (arrowhead) remained where the former oral apparatus had existed. This is the so-called paroral region. A microtubule thread (arrow) is always found at the posterior end of the paroral region (arrowhead) at late meiosis I (Fig. 3M). These microtubules are always observed at the end of the migration period of the haploid nuclei, but never after the separation of the conjugated cells.

The migration period of the haploid nuclei

During meiosis, four haploid micronuclei were formed within each cells of a pair, and were brightly labeled with immunofluorescence for mAb N356, suggesting the existence of relevant epitopes in all meiotic products (Fig. 4A). It is note worthy that fine microtubular filaments (arrows, Fig. 4A) seem to anchor haploid nuclei to the cytoplasmic surface of the paroral region. The DAPI staining of the same sample showed that none of the haploid nuclei had moved into the paroral region yet, suggesting that this mating pair was in the nuclear migration period. The two nuclei having a microtubular connection with the paroral region in Fig. 4A must be the translocating nuclei.

At a later stage of the nuclear-migration period, one nucleus was found within the paroral region of one cell of the pair, the other haploid micronuclei were gathered around their

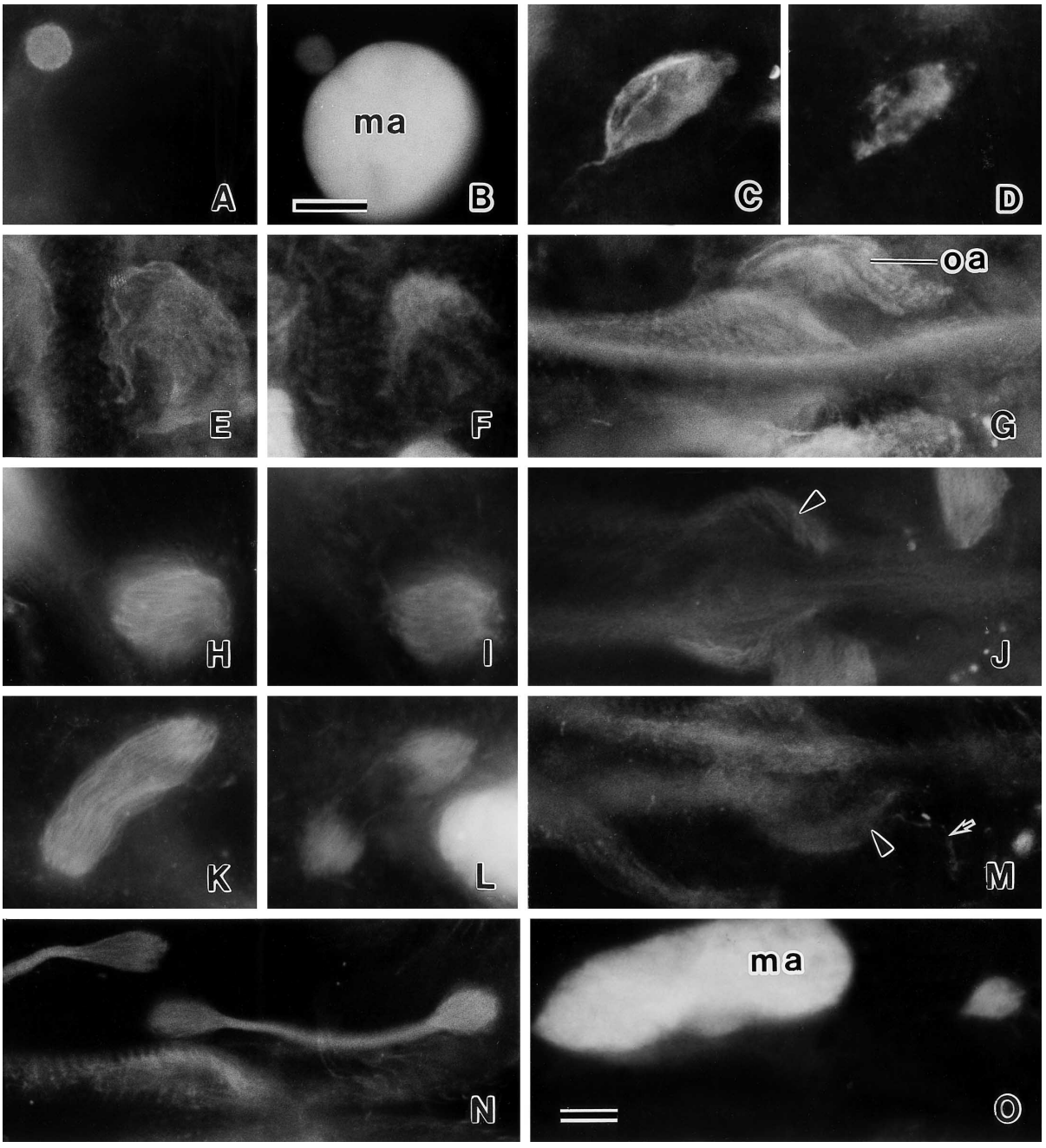

Fig. 3. Morphology of the micronucleus and the oral region during meiosis in *Paramecium caudatum* revealed by anti α-tubulin antibody (mAb N356) and DAPI staining. A and B. The same macro- and micronucleus in premeiotic S phase revealed by mAb N356 (A) and DAPI (B) staining. Double stained images of the micronucleus in early meiotic prophase (C and D) , late meiotic prophase (so-called crescent stage, E and F), promethaphase (H and I), anaphase of meiosis I (K and L), and telophase of meiosis II (N and O). FITC and DAPI images are shown in C, E, H, K, N and D, F, I, L, O, respectively. The images of the oral region revealed by FITC are in the crescent stage (G), promethaphase (J), and anaphase of meiosis I (M) stages. In G, J, M, N, O, the cell's anterior is to the left. ma, macronucleus; oa, oral apparatus. Bars: in B, for A–M, 10 μm; in O, for N and O, 10 μm.

paroral regions (Fig. 4C, D). The fine microtubular filament is barely visible at the posterior portion of the paroral region in the upper cell in this micrograph (small arrow, Fig. 4C). This microtubular filament appeared to be derived from the paroral region at a different focus level (data not shown) and extended close to a degenerating nucleus located posterior to the paroral cone (big arrow). Some of these haploid nuclei located next to the paroral region still contained immunofluorescence, however, two haploid nuclei in the lower cell, indicated by the arrowheads (Fig. 4C, D) had definitely lost their mAb decoration. In the lower cell of this mating pair, one haploid nucleus already moved into the paroral region, which implies that the two haploid nuclei indicated by arrowheads (Fig. 4C, D) must be destined to be degenerating nuclei. The mAb decoration,

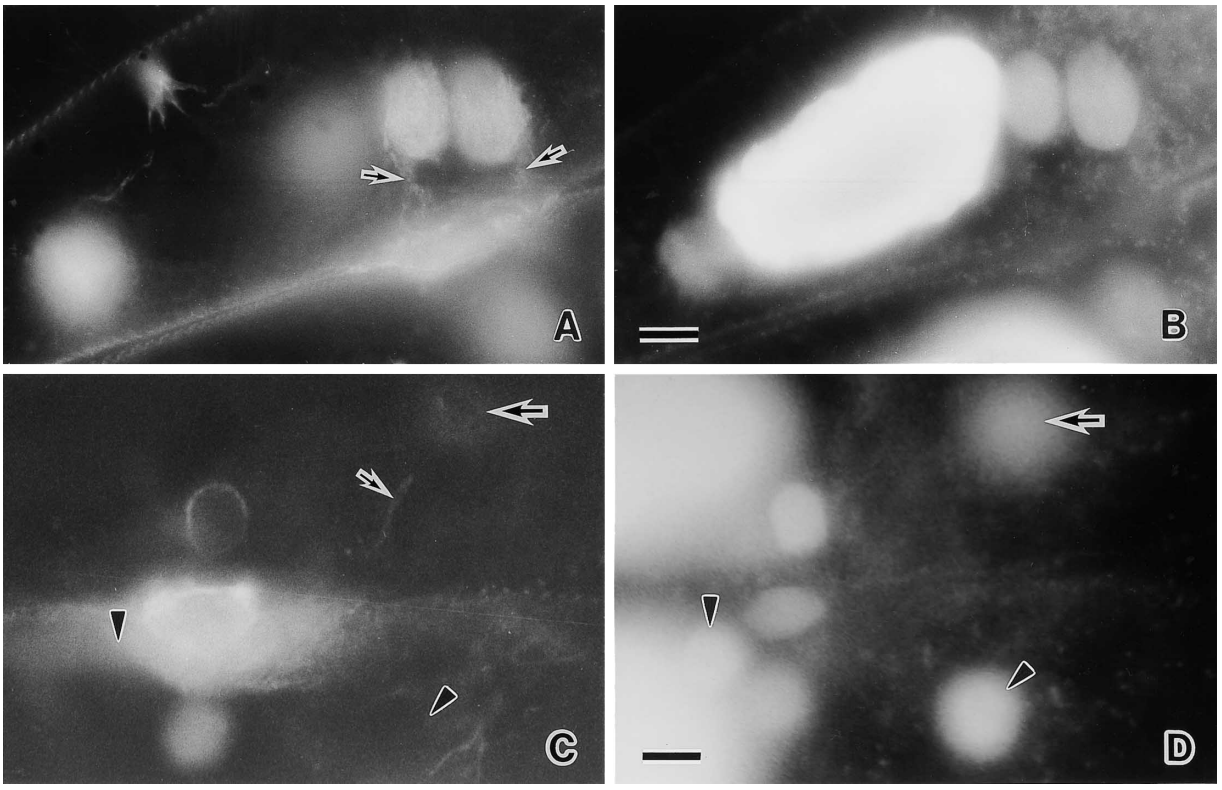

Fig. 4. Micronuclei at the paroral region after meiosis II in *Paramecium caudatum* as revealed by anti α-tubulin antibody (mAb N356) and DAPI staining. The cell anterior is directed to the left. A and B are the same cell double stained with mAb-FITC and DAPI, respectively. Microtubules connecting the haploid micronuclei to the paroral region are indicated by arrows in A. C and D, are the same cell double stained with mAb-FITC and DAPI, respectively. Larger arrows in C and D indicate a haploid nucleus which seems to be connected to the paroral region with microtubules (arrow in C). Two other haploid micronuclei, indicated by arrowheads in D, have lost mAb decoration (arrowheads in C). Bars: in B, for A and B, 10 μm; in D, for C and D, 10 μm.

therefore, seems to disappear from such degenerating nuclei before they undergo picnosis.

Mitosis of synkaryon

The surviving micronucleus mitotically divides in order to form two gamete pronuclei, the stationary and the migratory pronuclei. The migratory nucleus moves into the partner cell and fuses with the partner's stationary nucleus to form the zygotic-nucleus, the synkaryon. The synkaryon further divides three times. MAb N356 decoration was observed in dividing synkaryons as shown in Fig. 5A, D, G, I. Figure 5A shows indirect immunofluorescent images of the first division of the synkaryon. Enlarged images of the lower synkaryon, revealed by immunofluorescence and DAPI in Fig. 5B, C. The mitotic spindles, labeled with mAb, appeared first at the equatorial region of a synkaryon (Fig. 5B). The separation of the conjugated pairs then occurred after the first division of the synkaryon. Figures 5D, E, F show two dividing nuclei at the anaphase of the second mitotic division. The separation spindles (Fig. 5E) and the chromosomes (Fig. 5F) were observed in both dividing nuclei. Then, the third division occurred in the micronucleus. Their mitotic spindles was shown as in Fig. 5G. The macronucleus then began to undergo a morphological change (Fig. 5H). The most drastic morphological change of the macronucleus occurred later during the third division of the synkaryon (Fig. 5J). The macronucleus change from an ovoid shape into a loosely wound skein (the so-called "skein formation" stage) and these skeins have mAb N356 decoration (Fig. 5I). The mAb decoration was mainly on the periphery of the skein (Fig. 5K).

Four dividing post-zygotic nuclei and their separation-spindles could be detected by their bright immunofluorescence (Fig. 5I). They elongated along the longitudinal axis of the cell and extended through much of cell's length. As a result the daughter micronuclei were positioned near the two poles of the cell (Fig. 5L). All of the nuclear products resulting from the final division of the micronuclei still retain their mAb decoration but the long threads of the separation spindles have disappeared (Fig. 5K).

The oral apparatus reappeared during anaphase of the third division of the synkaryon (data not shown) and became intensely fluorescent at the same time that the separation spindles disappeared at the end of the third division (Fig. 5K).

Nuclear differentiation period

The difference between the first and the second divisions when compared to the third division is produced by the elongation of the nuclear spindles which are extended along with the longitudinal axis of the cell. The third division is considered to be the critical stage of nuclear differentiation and

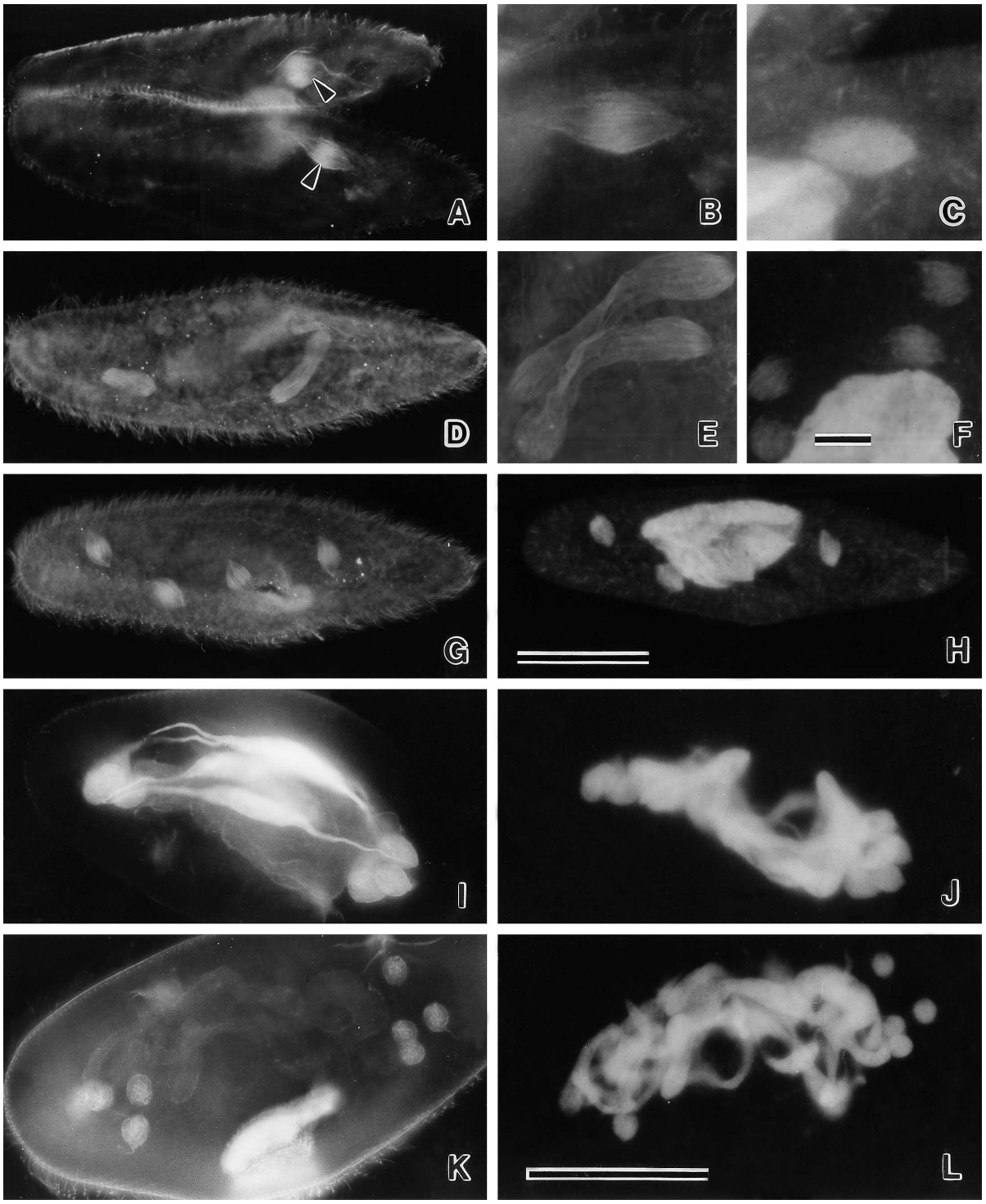

Fig. 5. Nuclear behavior during post-zygotic divisions in *Paramecium caudatum* revealed by anti α-tubulin antibody (mAb N356) and DAPI staining. FITC and DAPI images are shown in A, B, D, E, G, I, K, and C, F, H, J, L, respectively. In all micrographs the cell anterior is directed to the left. A. The first mitotic division of the synkaryon in a conjugating cell. Arrowheads indicate the dividing synkaryon. B and C. Enlarged image of the lower synkaryon. D. The second mitotic division of a synkaryon occurs after the separation of the conjugated pairs. E and F. Enlarged image of the second division of a synkaryon. G and H. Third division of the synkaryon. I and J. A later stage of the third division, in which well developed separation spindles are evident, and the macronucleus has changed its ovoid shape into a loosely wound skein. K and L. Cell in the critical stage of nuclear differentiation. Bars: in F, for B, C, E, F, 10 μm; in H, for A, D, G, H, 50 μm; in L, for I, J, K, L, 50 μm.

resulting in four macronuclear anlagen and four presumptive micronuclei in each exconjugant cell of *P. caudatum* (Grandchamp and Beisson, 1981; Hiwatashi and Mikami, 1989; Maupas, 1889; Mikami, 1980).

Our current immunofluorescence study demonstrates that mAb N356 decoration disappeared from the macronuclear

anlagen during this nuclear differentiation period, while the presumptive micronuclei retained their immunoreactivity for the mAb. Therefore the epitopes must be lost or drastically decreased in amount in the macronuclear anlagen during this period. Since only the presumptive micronuclei seemed to retain their immunoreactivity for the mAb, the impression that the microtubules might somehow be involved in nuclear differentiation we strengthened. To determine whether or not the microtubules are directly related to nuclear differentiation, we followed the time course of the disappearance of immunoreactivity for the mAb N356 in the post-zygotic micronuclei when compared to their DAPI staining.

During the first 30 min following the critical stage (the third division of the synkaryon), the mAb N356 decoration was observed in all eight post-zygotic nuclei (Fig. 6A). There was no distinct difference in their diameters (Fig. 6A, B). At 1 hr following the critical stage, some of the post-zygotic nuclei started swelling (two arrowheads in Fig. 6C). The swollen nuclei had immunofluorescence like the other nuclei. The diameter of the nuclei grew to more than 1.5 times that of other nuclei (Fig. 6C, D). At 4 hr, only 4 nuclei were recognized to contain immunofluorescence (Fig. 6E). Some anlagen (arrows) were morphologically distinguishable by their sizes when stained with DAPI (Fig. 6F). The number of nuclei labeling with mAb did not change even after 10 hr, but the macronuclear anlagen (arrows) became more and more evident by their increased size (Fig. 6G, H).

These results indicate that the morphological change of the macronuclear anlagen started before the disappearance of mAb decoration. To quantify the disappearance of mAb decoration from anlagen, the number of cells containing mAb decorated anlagen was determined compared to DAPI stained anlagen in all exconjugant cells (Fig. 7). As shown in Fig. 7, a decrease in the number of cells containing the mAb deco-

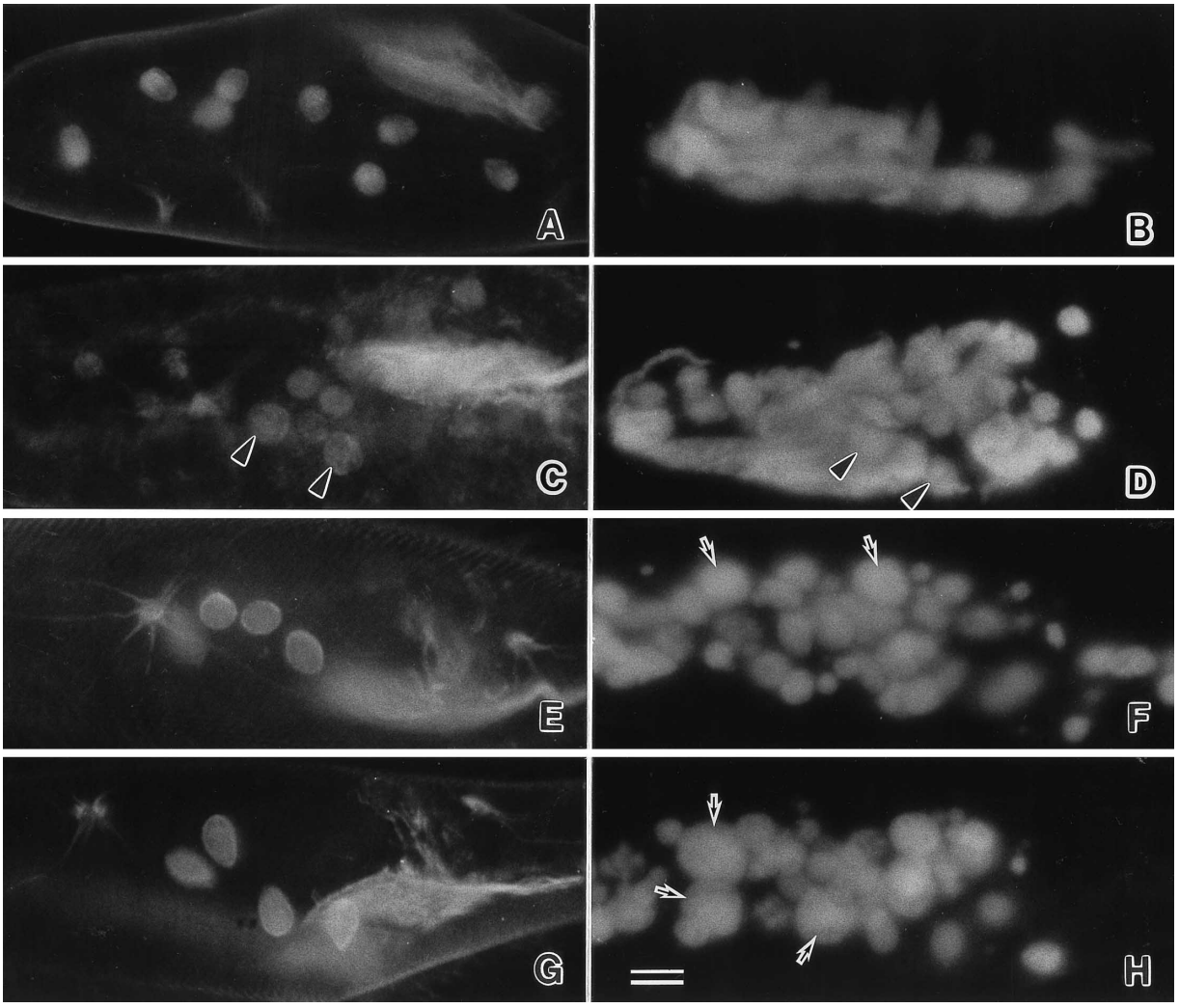

Fig. 6. Macronuclear differentiation in *Paramecium caudatum* revealed by anti α-tubulin antibody (mAb N356) and DAPI staining. In all micrographs the cell anterior is directed to the left. FITC and DAPI images were shown in A, C, E, G and B, D, F, H, respectively. A and B. A cell fixed at 30 min after the critical stage of nuclear differentiation. C and D. A cell fixed 1 hr after this critical stage. E and F. A cell fixed 4 hr after this critical stage. G and H. A cell fixed 10 hr after this critical stage. Arrowheads in C and D indicate two mAb-decorated early macronuclear anlagen that are relatively larger than the other micronuclei. Arrows in F and H indicate macronuclear anlagen. Bar, 10 μm.

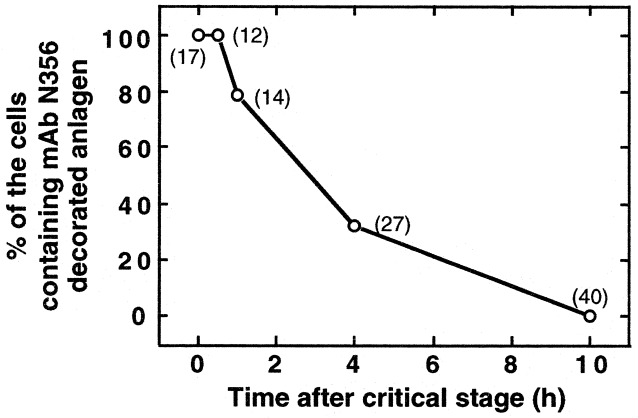

Fig. 7. The reactivity of the postzygotic micronuclei against the anti-α-tubulin antibody (mAb N356) was followed after the critical stage of nuclear differentiation by comparing DAPI staining and immunofluorescence images. The number of cells containing mAb-labeled macronuclear anlagen was counted at each time point and the percentage was plotted against the time elapsed since the critical stage. Values in parenthesis refer to the number of cells counted in each observation.

rated macronuclear anlagen started at 1 hr after the critical stage. By 4 hr after the critical stage, the number had decreased to approximately 30% of the total, then had completely disappeared by 10 hr. Thus, the disappearance of the mAb decoration from anlagen occurred mainly 1–4 hr after the critical stage. On the other hand, the morphological change was observed mostly within 1 hr after the critical stage (Fig. 6C). This suggests that the signal inducing differentiation has been received in the nuclei by 1 hr and the disappearance of mAb decoration from the anlagen will ensure.

DISCUSSION

To assess the possible role of the microtubular cytoskeleton either in its control of nuclear position or in nuclear differentiation during the development of *Paramecium*, we employed a mAb N356 directed against the α-tubulin subunit of chick brain, and immunocytochemical techniques applied to whole cells. This mAb specifically reacted with a major 50–57 kDa band on western blots of *P. caudatum* (Fig. 1, lane 2). The known molecular weights of both α- and β-tubulin of *Paramecium* are in this range and tubulins are the major proteins in the *Paramecium* axoneme (Cohen *et al.*, 1982). As in other ciliates, α-tubulin of *Paramecium* has a slightly higher electrophoretic mobility than that of β-tubulin (Cohen *et al.*, 1982; Grain, 1986; Suprenant *et al.*, 1985). The α-tubulin band was, therefore, expected to be found slightly below β-tubulin on the gel, however, separation of the α- and β-tubulin bands in the current gel system was not possible (Fig. 1, lane 1). Nevertheless, since the mAb strongly and exclusively labeled the broad 50–57 kDa band on the gel, we conclude that mAb N356 crossreacted with tubulins in *P. caudatum*.

MAb N356 decoration of the micronucleus

The existence of the microtubules in the interphase micronuclei has been documented by electron microscopy in *P. aurelia* species complex (Jurand, 1976; Jurand and Selman, 1970; Stevenson and Lloyd, 1971). In the interphase micronuclei of the *P. aurelia* species complex, the microtubules are always present in somewhat irregular sheets beneath the nuclear envelope (Jurand, 1976; Jurand and Selman, 1970). It is has been speculated that these sheet act as a storage of partly polymerized tubulin (Jurand, 1976). This idea was immunocytochemically strengthened by identifying a spindle-associated protein around such microtubule germs in *P. tetraurelia* (Keryer *et al.*, 1989). Recently, the presence of such a microtubule-organizing center (MTOC)-associated protein was also shown in a hypotrich ciliate, *Euplotes octocarinatus* (Curtenaz *et al.*, 1997). A polyclonal antibody raised against distinct sequences of γ-tubulin of *Aspergillis*, also reacted with the interphase micronucleus of this cell and was localized as numerous foci scattered through the nucleoplasm during interphase. This γ-tubulin polyclonal decoration on the micronucleus was continuously observed during the entire cell cycle. In the ciliates, such diffuse MTOCs in the micronuclear nucleoplasm seem to persist during interphase and so they may have an important role in the nucleation of the spindle during both vegetative and sexual divisions (Curtenaz *et al.*, 1997; Keryer *et al.*, 1989).

The current immunofluorescence study has demonstrated that mAb N356 decorated the periphery of the interphase micronucleus of *P. caudatum* as it does other *Paramecium* species as well (Fig. 2, Table 1). In the case of *P. caudatum*, the mAb decoration on the micronucleus was continuously observed throughout cell division and the conjugation period. The spindles were found to have a bright immunofluorescence as was reported in *P. tetraurelia* (Keryer *et al.*, 1989). The tubulin molecules, therefore, seemed to persist throughout the cell's life cycle, at least they do in two species, *P. tetraurelia* and *P. caudatum.*

MAb decoration on the oral apparatus

During conjugation of *Paramecium*, the breakdown of the old oral apparatus and the reconstruction of the new one takes place (reviewed by Ng, 1986). Although the time-course for the breakdown and the reconstruction of the oral apparatus have not been clarified, the times for the cessation and resumption of feeding activity were recently reported by Mikami (1992). By using a homopolar doublet cell, Mikami (1992) showed that a conjugating cell loses its ability to form digestive vacuoles at the crescent stage of the micronucleus, from 5–6 hr after the initiation of conjugation, and regains this ability at the end of the third division of the synkaryon.

In the current study, the oral apparatus, traced by the mAb N356 decoration of the ciliary rows of penniculi and the quadrulus, is shown to disappear during meiosis I (Fig. 3G, J, M). Recently, Nishihara *et al.* (1999) also reported that these oral membranelle and their related infraciliature seemed to be labeled with the mAb N356 in *P. bursaria*. Prior to the dis-

appearance of the oral apparatus, the oral apparatus associated microtubular structures, such as cytopharyngeal ribbons and the postoral fiber disappeared during the crescent stage of the micronucleus (Fig. 3G). The oral apparatus had completely disappeared by prometaphase of meiosis I (Fig. 3J), but then, reappeared at the end of the third division of the synkaryon. Reappearance of the oral apparatus was accompanied by intense label with mAb (Fig. 5K), suggesting that reorganization of the oral apparatus and probably also of both cytopharyngeal ribbons and the postoral fiber, occurred at this time period. The cytopharyngeal ribbons and the postoral fiber are known to be involved in food vacuole formation: the cytopharyngeal ribbons transport the donor membrane to the cytopharynx and the postoral fibers transport the newly separated food vacuole from the cytopharynx to the cytosol (Allen, 1974; Fok and Allen, 1988). Disorganization and reorganization of the oral apparatus and its associated microtubular structures during conjugation are critical to the cell's feeding activity. Our current immunocytochemical observations on the oral apparatus are consists with time-course studies of phagosome development and function, and thereby confirm Mikami's observation.

Microtubules anchor the haploid nuclei to the paroral region

In *Paramecium* it is thought that the survival or degeneration of nuclei after meiosis II depends on their position in the cytoplasm. This is based on the fact that only one nucleus is located in the paroral region, and that only this nucleus survives and undergoes mitosis (Skoblo and Ossipov, 1968; Sonneborn, 1954; Wichterman, 1946). Yanagi and Hiwatashi (1985) reported that sometimes one of the haploid nuclei located farther from the paroral region can move into the paroral region where it survives, degeneration which is the fate of the other haploid nuclei. Thus they speculated that an active transport-system might be involved in this nuclear migration. This idea was supported by the inhibitory effect that Vinblastine had on nuclear migration during anaphase of meiosis II. The action of Vinblastine, being a microtubule reactive drug implies that microtubules are involved in moving the nucleus into the paroral region of *Paramecium* (Yanagi and Hiwatashi, 1985). However, no morphological evidence had been published supporting microtubules connecting the micronuclei to the paroral region.

In the current study, microtubules were observed linking the paroral region to some of the haploid nuclei. Microtubules were observed first around the posterior end of paroral region during meiosis I (Fig. 3M). This was followed by an increase in microtubules between the haploid nuclei and cytoplasmic surface of the paroral region during the migration period (Fig. 4A). As microtubular connections were detected between several haploid nuclei and the paroral region (Fig. 4A, C), it appears that all haploid nucleus but also the other three haploid nuclei are capable of having microtubular connections with the paroral region. These findings are consistent with the observation of Yanagi (1987) that removal of the nucleus from the paroral region results in another nucleus moving into paroral region where it will survive and divide. This means that any one of the four haploid nuclei could attach to the "migration machinery". The current findings, therefore, support the idea proposed by Yanagi and Hiwatashi (1985) that microtubules are involved as the machinery in the migration of the micronucleus to the paroral region in *Paramecium*.

The exact origin of these microtubules is unknown, however, the following three possibilities exist: 1) they are remnants of the micronuclear separation-spindles of second meiosis; 2) they are perinuclear microtubules (microtubules are known to polymerize on the cytoplasmic surface of haploid nuclear membranes in *Tetrahymena* (Gaertig and Fleury, 1992)) or 3) they are newly polymerized microtubules on the paroral region. If the divided haploid nuclei still have a remnant of the separation spindle and this remnant is located next to the paroral region, it might give the appearance of a nuclear connection with microtubules. The separation-spindle is, however, usually a thicker bundle than that of the connecting microtubules we observing (compare Fig. 3N and 4A), and connecting microtubules are also more consistently observed than would be expected if due only to chance. The depolymerizing end of the microtubules should be free of attachments and thereby unable to promote migration of the micronucleus to the paroral region. For *Tetrahymena*, Gaertig and Fleury (1992) reported that the perinuclear microtubules anchor some of the haploid nuclei during the migration period. At first the microtubules appeared on the cytoplasmic surface of the nuclei where they anchor the nucleus to the fusion zone, a region functionally analogous to the paroral region in *Paramecium*. In *Paramecium*, however, no morphological observations on perinuclear microtubules have been published, and we also failed to find such perinuclear microtubules. On the other hand, a thread of microtubules was always found at the posterior portion of the paroral region following the disappearance of the oral apparatus (Fig. 3M). This led to the impression that new microtubules may first develop at the paroral region and then finally make contact with the micronuclei. To determine both the origin of these microtubules and how these microtubules connect the nuclei to the paroral region will require further electron microscopic studies. These are now in process.

For nuclear migration in *Tetrahymena*, another cytoskeletal system has been considered as a possible candidate. This consists of the 14-nm intermediate filaments. The intermediate filament protein, now known to be the multifunctional protein, citrate synthase, appears as a filamentous meshwork at the junction between the two conjugating cells after second meiosis (Numata, 1996; Numata *et al.*, 1985). This meshwork surrounds the functional nucleus during the conjugation period (Numata *et al.*, 1985). The 14-nm intermediate filament was, therefore, considered to be involved in micronuclear behavior during conjugation in *Tetrahymena.* The same kind of protein could also be involved in nuclear migration in *Paramecium*, if these cells are found to have such intermediate filaments.

Disappearance of the mAb N356 labeling from degenerating haploid nuclei

The mAb N356 decoration disappeared in the degenerating haploid nuclei following meiosis at a time when these haploid nuclei undergo picnosis. During picnosis volume of the nucleus decreases (data not shown) along with a loss of intranuclear labeling with mAb (Fig. 4C). Finally, the nuclei themselves disappear. The disappearance of the mAb decoration was also observed in the macronuclear anlagen, however, the volume of the anlagen increased (Fig. 6) as a new macronucleus was generated. It is, therefore, obvious that the disappearance of the mAb decoration from the anlagen precedes totally different subsequent events in these two nuclei.

Disappearance of the mAb N356 labeling from macronuclear anlagen

In *P. tetraurelia*, Keryer *et al.* (1989) immunocytochemically identified a spindle associated protein in the micronuclei that becomes decorated with mAb CTR532 (mAb CTR532 recognizes centrosomal and spindle associated components in mammalian cells as well as 170 kDa micronuclear protein in *Paramecium*). This decoration was consistently observed during both the vegetative phase and the conjugation period. The decoration with mAb CTR532 completely disappeared in macronuclear anlagen shortly after the macronuclear anlagen's brief positioning at the posterior pole of the cell (Keryer *et al.*, 1989). Our current immunofluorescent study with mAb N356 has demonstrated the disappearance of the microtubules from the macronuclear anlagen after the critical stage. This is consistent with the observation of the disappearance of mAb CTR532 decoration in *P. tetraurelia* (Keryer *et al.*, 1989), and leads to the impression that a spindle associated protein lie that in *P. tetraurelia* might also exist in *P. caudatum*. We therefore carried out a time course study to determine when mAb decoration disappeared from the post-zygotic nuclei during the differentiation period (Fig. 7). Based on our results, the disappearance of the mAb decoration from post-zygotic nuclei occurred after the onset of the differentiation signal (Figs. 6, 7). This suggested that the changes occurring in the microtubules of the post-zygotic nuclei during the macronuclear differentiation period were not directly related to the initiation of macronuclear differentiation.

A similar but distinct immunocytochemical observation was obtained in a hypotrich ciliate *Euplotes* by using anti-γ-tubulin antibody (Curtenaz *et al.*, 1997). This antigen disappeared from young macronuclear anlagen, however, the immunofluorescent labeling on the macronucleus reappeared in the anlagen at a later stage, after chromosome polytenization had been completed in the anlagen. The mAb labeling on the macronucleus subsequently disappeared again when anlagen had elongated into the peculiar C-shaped mature macronuclei (Curtenaz *et al.*, 1997). The behavior of the microtubules and their associated proteins in this hypotrich ciliate, therefore, seems to differ from that observed in *Paramecium* species.

Elimination of supernumerary presumptive micronuclei

Micronuclear number must be reduced to arrive at the uni-micronucleate condition that persists after the stage of macro- and micronuclear differentiation. The elimination of supernumerary micronuclei had been suggested by morphological observations (Klitzke, 1914; Maupas, 1889) but was confirmed by nuclear transplantation studies (Mikami, 1982). However, there seems to be no significant difference among the four anterior presumptive micronuclei at the stage of macro- and micronuclear differentiation. In fact, when any three of the micronuclei were microsurgically removed, the remaining nucleus was able to divide at each post-conjugational fission and persisted as a micronucleus in the vegetative phase (Mikami, 1980). The evidence indicates that the determination of which micronucleus will degenerate has not occurred at this critical stage. In the current study, the DAPI staining and immunofluorescent labeling demonstrated that all four presumptive micronuclei persisted for at least 10 hr after this critical stage (Fig. 6G), thus the presumptive micronuclei were not eliminated for at least 10 hr after the critical stage. How and when a presumptive micronucleus is chosen to survive have get to be determined.

In summary, microtubules play a critical role in micronuclear division during both the vegetative and the sexual phase of the cell cycle. The disappearance of the microtubules in both degenerating haploid micronuclei and in the anlagen of the macronucleus, therefore, gives a good indication of the fate of these nuclei following meiosis. Microtubules themselves may not be directly involved in determining of micro- and macronuclei, however, their growth into fairly long spindles at the critical stage insures that the nuclei are located in specific areas of the cytoplasm. The current findings of microtubular anchors between the nucleus and the paroral region also suggests that microtubules apparently help to determine the fate of nuclei by positioning them in appropriate place.

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