POLARITY OF MICROTUBULES NUCLEATED BY CENTROSOMES AND CHROMOSOMES OF CHINESE HAMSTER OVARY CELLS IN VITRO

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ABSTRACT

The structural and growth polarities of centrosomal and chromosomal microtubules were studied by analyzing the kinetics of growth of these microtubules and those initiated by flagellar seeds. By comparing rates of elongation of centrosomal and flagellar-seeded microtubules, we determined whether the centrosomal microtubules were free to grow at their plus ends only, minus ends only, or at both ends. Our results show that centrosomal microtubules elongate at a rate corresponding to the addition of subunits at the plus end only. The depolymerization rate was also equivalent to that for the plus end only. Chromosomal microtubule elongation was similar to the centrosome-initiated growth. Since the data do not support the hypothesis that both ends of these spindle microtubules are able to interact with monomer in solution, then growth must occur only distal or only proximal to the organizing centers, implying that the opposite end is unavailable for exchange of subunits. Experiments with flagellar-seeded microtubules serving as internal controls indicated that the inactivity of the minus end could not be accounted for by a diffusible inhibitor, suggesting a structural explanation. Since there is no apparent way in which the distal ends may be capped, whereas the proximal ends are embedded in the pericentriolar cloud, we conclude that centrosomal microtubules are oriented with their plus ends distal to the site of nucleation. A similar analysis for chromosomal microtubules suggests that they too must be oriented with their plus ends distal to the site of initiation.

KEY WORDS microtubules · polymerization · polarity · spindle · mitosis

An unresolved problem in the cell division field is the polarity of spindle microtubules. This problem has two aspects. One is the intrinsic structural polarity and the other is the polarity of growth. As pointed out previously (5, 18), these properties are essentially independent of each other.

The unsettled status of this problem is amply attested to by the literature. Two models of mitosis (21, 27), as well as variations on them (19, 22, 24), stipulate an antiparallel structural polarity of polar and kinetochore microtubules in the half-spindle, whereas the recent model of Margolis et al. (16) stipulates a parallel arrangement, and the model of Bajer (2) considers polarity of microtubules unnecessary for spindle function. The site-specific formation of microtubules onto centrosomes and chromosomes in vitro (9–11, 17, 26, 28, 29) suggests that these organelles nucleate tubule assembly and that growth occurs by addition of subunits

at the free ends. However, proof of the site of addition is lacking and observations of spindle formation in vivo (14, 15, 23, 25) have led some workers to conclude that kinetochores may not nucleate but rather attach to microtubules formed elsewhere, presumably at the poles. Also, on the basis of UV microbeam experiments (7, 12) and observations of spindle formation (6), it has been suggested that the addition of subunits to chromosome fibers occurs at the kinetochore and not at the distal ends of the fibers. Loss of subunits from kinetochore tubules is generally thought to occur at their distal ends (13), but on the basis of interpretation of birefringence changes in anaphase, Forer (8) has suggested that subunits are lost from the kinetochore end.

Formally, the polarity problem is a combinatorial one with $2^4 = 16$ possibilities. These arise in the following way: We have two microtubule-organizing centers, namely, the centrosome and the kinetochore. For each organizing center, a microtubule may be attached with one of two structural polarities. Using a convention adopted previously (5), we assign plus and minus ends to a tubule and define the absolute intrinsic polarity by stipulating that the plus end is structurally equivalent to the end of the flagellar A tubule distal to the basal body. For each class of tubule, addition of subunits may be favored (i.e., have the greater rate constant) at either the plus or minus end. Similarly, loss of subunits may be favored at either plus or minus end and not necessarily at the same end as for addition of subunits. Thus, the four variables, organizing center, intrinsic tubule polarity, site of addition and site of loss of subunits, combine to generate 16 possible structural and growth polarity relations for the half-spindle. In a previous paper (5), we suggested on the basis of estimates of the rate of growth of microtubules onto organizing centers in vitro that centrosomal and chromosomal microtubules were antiparallel to each other and that their plus ends were pointed away from their site of origin. However, it was also noted that since the observations could not dissociate structural from growth polarity, we could not rigorously exclude other polarity relations.

Recently, we have refined our kinetic analysis in a study of the head-to-tail polymerization of microtubules and have identified the direction of head-to-tail assembly relative to the absolute structural orientation of a microtubule; that is, the "head" of a microtubule is its plus end (3). We have also established that the plus end is the end having the greater rate constant for dissociation.

We have applied this refined kinetic approach to a study of microtubules nucleated by the centrosomes and chromosomes of Chinese hamster ovary (CHO) cells in vitro. The rates of elongation and depolymerization were compared to those of internal markers (flagellar axonemes) for subunit exchange at the plus and minus ends separately. A key finding is the inactivity of the minus end for both subunit addition and loss. Arguments are presented which indicate that the results are sufficient to dissect structural and growth polarities and permit a choice of polarity relation for the half-spindle.

MATERIALS AND METHODS

Cell culture

CHO cells were grown as monolayers in Ham's F-10 medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% fetal bovine serum, antibiotics, and 15 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES), pH 7.2, and were maintained at 37°C in a humid atmosphere with 5% CO_2.

Preparation of Centrosomes and Chromosomes from Mitotic Cells

Mitotic cells were obtained by treatment with 2 mM thymidine. After 12–15 h, they were resuspended in fresh medium for 3–5 h and then exposed to 0.1 μg/ml colcemid for an additional 3–5 h. Cells blocked in mitosis were collected by centrifugation in a tabletop centrifuge and lysed as described by Gould and Borisy (9) with some modification. Cells were resuspended in 10 vol of distilled water and then added to an equal volume of two-times concentrated extraction medium (10 mM piperazine-*N*-*N'*-bis[2-ethane sulfonic acid] [PIPES], 1 mM EGTA, 0.1 mM $MgCl_2$, 0.25% Triton X-100, pH 6.7). Lysis of cells and release of centrosomes and chromosomes were checked by phase-contrast microscopy. Mitotic cell lysates were used for microtubule assembly experiments. For experiments on assembly onto chromosomes, extraction medium containing 0.5 mM $MgCl_2$ was used and microtubule subunits were added as soon as possible to the cell lysate.

Microtubule Protein and Subunit Preparation

Microtubule protein was prepared from porcine brain tissue by two cycles of polymerization and depolymerization with differential centrifugation according to the procedure of Borisy et al. (4). The buffer used throughout the procedure was PEMG (100 mM PIPES, 1 mM EGTA, 0.1 mM $MgCl_2$, 1 mM GTP, pH 6.94). After freezing in liquid nitrogen, polymer was stored at −80°C. After the subsequent thaw, an additional cycle of purification was performed to remove protein inactivated during storage. Subunits were prepared from this material by high-speed centrifugation (1, 3). This material was generally frozen and stored for short periods (a week or less) without any detectable loss of activity (3).

Flagellar Seeds

Flagellar seeds were prepared by the procedure of Allen and Borisy (1), modified as described previously (3). The seeds were routinely frozen with liquid nitrogen and stored at −80°C for up to 1 mo.

Assembly Conditions

Solutions of microtubule subunits were thawed immediately before an assembly experiment and ranged from 0.7 to 2.0 mg/ml in protein concentration. Preparations of flagellar seeds were also thawed just before an experiment and diluted with PEMG to an appropriate concentration as determined by electron microscope examination. The proportions of subunits, flagellar seeds, and mitotic lysate were varied during the course of the work, but typically 500 μl of subunits and 20 μl of flagellar seeds were added to 200 μl of mitotic lysate in a 1.5-ml polypropylene tube and incubated at 30°C for various periods of time. Depolymerization experiments were done by first preparing mixtures and polymerizing for 5.5 min. Then an aliquot of 100 μl was removed, diluted with 1 ml of PEMG prewarmed to 30°C, and aliquots were removed at intervals thereafter. Microtubule polymerization and depolymerization reactions were quenched by addition of one-half volume 3% glutaraldehyde in PEMG.

Electron Microscope and Computer Analyses

Samples were centrifuged onto carbon-coated Formvar grids as described previously (3), rinsed in distilled water, stained with 1% phosphotungstic acid, and air dried. The grids were analyzed in a Philips 300 electron microscope. Microtubules were photographed on 70-mm film (Kodak), and lengths were determined from these negatives with the aid of a Numonics digitizer (Numonics Corp., Lansdale, Pa.) interfaced with a Hewlett-Packard 9825A computer (3) (Hewlett-Packard Co., Palo Alto, Calif.).

RESULTS

In a previous study on head-to-tail polymerization (3), we showed that the kinetics of elongation and depolymerization for the two ends of a microtubule could be studied individually through the use of flagellar microtubule seeds, whose ends could be distinguished morphologically. The stable flagellar seed served to separate the two ends of the labile cytoplasmic microtubules and make them available for independent analysis.

In the present study, flagellar seeds were used as internal markers for the polarity of microtubules nucleated by the centrosomes and chromosomes of Chinese hamster ovary cells. The basic experimental design was to prepare cell extracts containing centrosomes and chromosomes and to add flagellar seeds and porcine brain microtubule subunits. Growth and depolymerization of microtubules nucleated by the mitotic organizing centers and the flagellar seeds were then compared by quantitative electron microscopy. Our objectives were to determine whether the microtubules nucleated by mitotic organizing centers grew and depolymerized at the plus end rate, at the minus end rate, or at a rate equal to the sum of the plus and minus end values. This information would define the relative growth polarities of centrosomal and chromosomal microtubules. It would also determine whether the site-initiated microtubules exchanged subunits at one end or two, and whether net loss and net gain of subunits occurred at the same or opposite ends.

In a previous study (9) where we demonstrated nucleation of microtubules by pericentriolar material, growth was analyzed at a single time point and no effort was made to determine the kinetics of elongation. For this objective to be feasible, a better documented preparation was required. To obtain reliable length data from which rates of elongation and depolymerization may be calculated, we found it clearly necessary to trace individual microtubules and to identify their ends.

A typical centrosome after incubation with subunits gave rise to a radial display of microtubules as shown in Fig. 1. After this brief time of polymerization, the microtubules were short enough so that most of their ends were within the frame of the micrograph. At higher beam illumination, a centriole was observed at the focus of the microtubules.

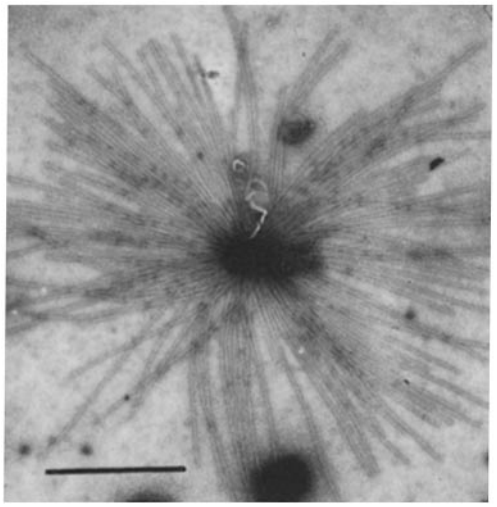

FIGURE 1 Centrosome-initiated microtubule assembly in vitro. A lysate of colcemid-blocked Chinese hamster ovary cells was incubated with 0.73 mg/ml porcine brain microtubule subunits at 30°C for 8 min. Microtubules are relatively uniform in length, suggesting that nucleation is rapid and that elongation occurs at a constant rate. Bar, 5 μm. × 3,700.

The centrosome in Fig. 1 nucleated 165 microtubules, and this number corresponds well to the value reported for polar microtubules in vivo (20). Nucleation was apparently rapid since the number of microtubules per centrosome was roughly constant for samples assayed at 5, 10, and 15 min after mixing the reactants together (data not shown). Additional experiments indicated that nucleation was completed by 2 min. The length distribution of centrosomal microtubles was approximately Gaussian (Fig. 2), centered about a mean which increased linearly with time (see Fig. 5).

Flagellar seeds added to the centrosome preparation also nucleated microtubules. The presence of residual cell extract did not appear to have detectable effects on the pattern of seeded assembly. As previously described (1, 3), the added tubules were longer on the plus (distal) end than on the minus (proximal) end, and at low concentrations of subunits (0.2–0.4 mg/ml), growth was observed only at the plus end. The explanation for this behavior is that the critical concentration for the plus end is lower than for the minus end (3). Hence, at a protein concentration above the critical concentration for the plus end but below the critical concentration for the minus end, growth will occur only on the plus end.

Fig. 3 shows an electron micrograph of microtubules nucleated at low protein concentration (0.35 mg/ml) by a centrosome and a flagellar seed in close proximity to each other. Under these conditions, growth occurred only at the plus end. The plus end of the flagellum was identified by its typical fraying into outer doublets, whereas the minus end characteristically remained intact. Direct inspection of the micrograph shows that the lengths of the centrosomal microtubules are comparable to those at the plus end of the flagellar seed. This result eliminates the possibility that centrosomal microtubules grow at the minus end only. However, since growth at the minus end of the flagellar seed was negligible, the experiment could not distinguish between the possibilities of growth at only the plus end or at both the plus and minus ends.

To decide between these possibilities, we carried out experiments at a higher protein concentration where significant growth would be expected at the minus end. Fig. 4 shows a frequency histogram of microtubule lengths for the plus and minus ends of internal flagellar seeds and for centrosomes. Within experimental error, the lengths of the centrosomal and plus end microtubules were equal to each other, and greater than the minus end tubules by a factor of three. If both ends of a centrosomal microtubule were free to grow, then the mean tubule length would be 12 μm instead of the 9 μm observed. Therefore, the results suggest that only one end of a centrosomal microtubule is able to add subunits, and the identity of that end is plus.

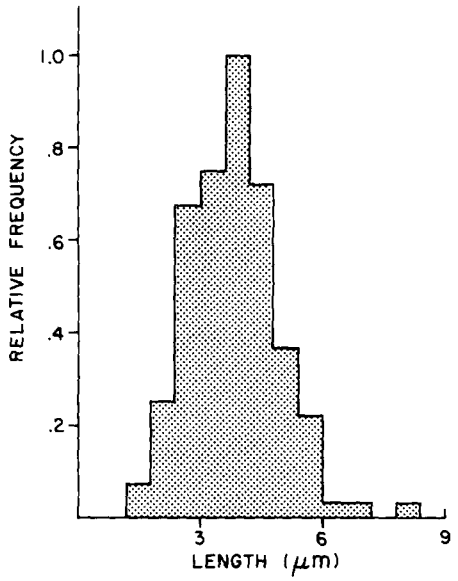

FIGURE 2 Length distribution of centrosomal microtubules. Centrosomes were incubated with microtubule subunits (0.85 mg/ml) at 30°C for 5 min. Mean length = 3.8 ± 1.1 μm (n = 116).

The previous result was obtained at a single time and thus was dependent on the kinetics of both nucleation and elongation. To establish definitively that centrosomal microtubules elongate at the plus end rate, we required a time-course of polymerization. Fig. 5 shows the results from such an experiment. For each time point, the lengths of centrosomal and flagellar seeded microtubules were determined and frequency histograms were constructed, and the mean lengths plotted. As can be seen, the data are fitted well by straight lines that pass through the origin, indicating that nucleation occurred rapidly and that thereafter the tubules grew at a constant rate. Within experimental error, the rate of growth of centrosomal microtubules was equal to that for the plus end.

The time-course of depolymerization was also analyzed. Microtubules were first grown onto centrosomes and flagellar seeds as before. The prep-

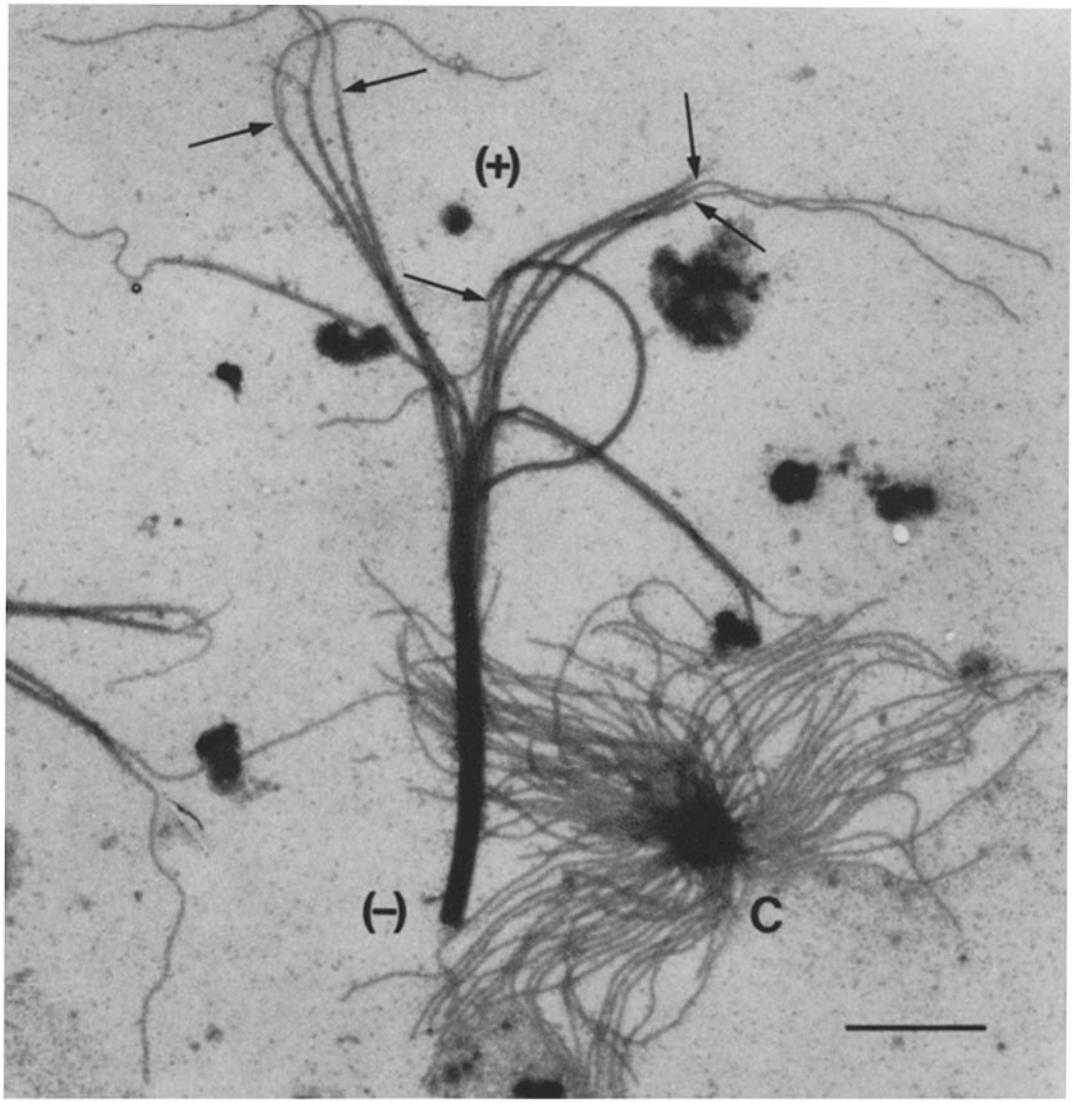

FIGURE 3 Comparison of centrosome and flagellar seed-initiated microtubules. Centrosomes and flagella were mixed together and incubated with 0.35 mg/ml microtubule subunits at 30°C for 8 min. Note that the added tubules at the plus end (+) of the flagellar seeds (arrows) are approximately of the same length as the centrosomal microtubules, and that under these conditions there was no minus end (−) growth. *C*, centrosome. Bar, 2 μm. × 9,600.

aration was then diluted at 30°C with a 10-fold excess of buffer to lower the subunit concentration below the critical concentration. As shown previously for flagellar-seeded tubules (3), depolymerization as studied by this procedure is linear with time over the period of interest. The results are presented in Fig. 6. At zero time of depolymerization, the centrosomal and flagellar-seeded tubules had the lengths attained by 5.5 min of growth at 30°C. After dilution in buffer, the tubules shortened, the rate of which was greater at the plus than at the minus end of the flagellar-seeded tubules as shown previously (3). The dotted line is drawn through the algebraic sums (▲) of plus and minus lengths and represents the predicted values for a microtubule that was able to associate and

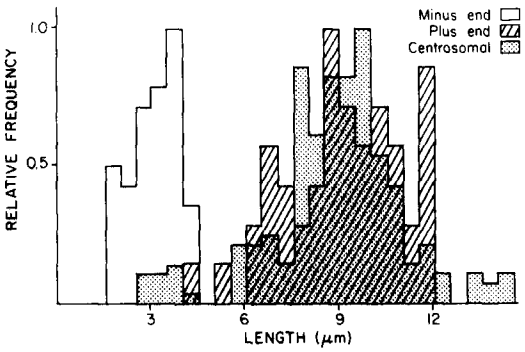

FIGURE 4 Comparative length distribution of centrosomal microtubules and plus and minus end tubules initiated by flagellar seeds. Centrosomes and flagellar seeds were incubated with 1 mg/ml microtubule subunits at 30°C for 5 min. The relative frequency of lengths is plotted for all three classes of microtubules. Note that the plus end (hashed) and centrosomal (stippled) microtubules were similar in length, whereas minus end tubules (open boxes) are threefold shorter.

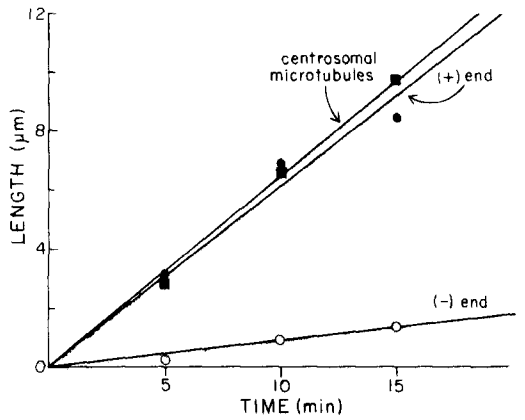

FIGURE 5 Time-course of elongation of centrosomal and flagellar seed-initiated microtubules. Centrosomes and flagellar seeds were incubated with 0.63 mg/ml microtubule subunits and samples were removed every 5 min. The rates of plus (●) and centrosomal (■) microtubule elongation are the same, whereas elongation at the minus (○) end is sevenfold slower.

dissociate subunits at both ends during the experiment. The centrosomal microtubules were initially the length of the plus end tubules and shortened at a rate equal to that of the plus end. This demonstrates that disassembly as well as assembly of centrosome-initiated tubules is restricted to one end and that the end is plus.

A similar analysis was attempted for microtubules nucleated by chromosomes. Chromosomes were prepared essentially according to a procedure described previously (10) with the modification that the magnesium concentration was raised to 0.5 mM and extracts were incubated with microtubule protein as soon after lysis as possible. Under these conditions, microtubules were initiated from the kinetochore region, but the chromosome morphology was variable.

The two chromosomes shown in Fig. 7 indicate the range of morphology found. The first (Fig. 7 *a*), isolated at a final concentration of 75 mM PIPES, shows good recognizable chromosome structure, but the exact position and structure of the kinetochore itself cannot be identified. The second chromosome (Fig. 7 *b*) was isolated at lower ionic strength (50 mM PIPES) and the chromosome structure is somewhat dispersed, but the

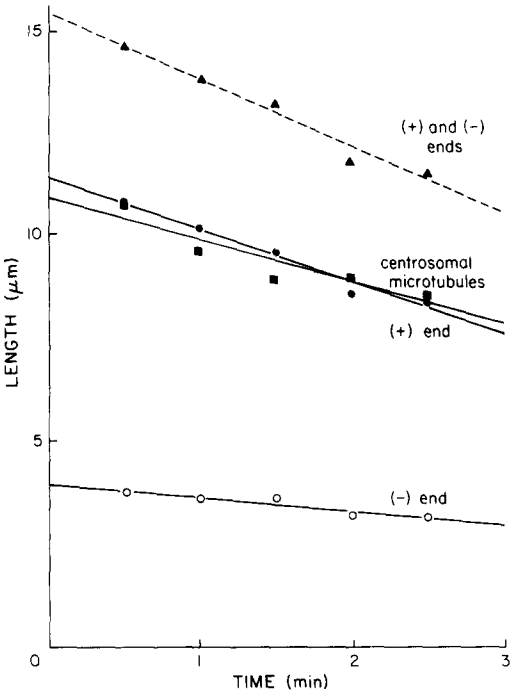

FIGURE 6 Depolymerization of centrosomal and flagellar seed-initiated microtubules. Centrosomes and flagellar seeds and microtubule subunits were mixed and incubated as described in Fig. 5. At 5.5 min, an aliquot of the reaction mixture was diluted 10-fold with buffer to induce depolymerization. Aliquots were removed every 30 s, and the lengths of all three classes of microtubules were determined. The data were fitted by a least-squares regression. Note that the plus end (●) and centrosomal (■) microtubules shorten at similar rates and faster than the minus (○) end. The triangles (▲) are the algebraic sums of plus and minus end lengths, and represent the length expected if both ends of a microtubule were free to exchange subunits.

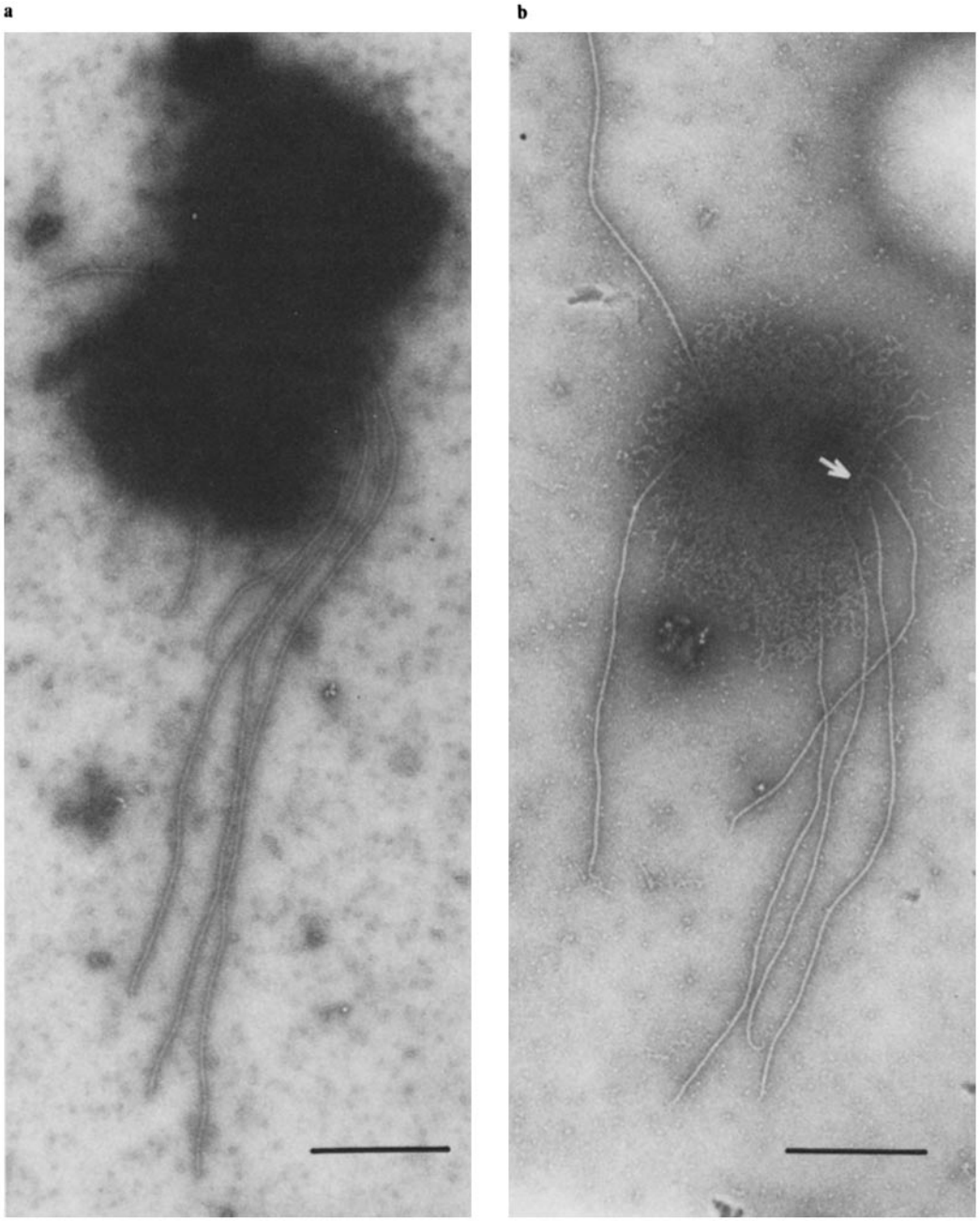

FIGURE 7 Chromosome-initiated microtubule assembly. Mitotic lysates were incubated with either (*a*) 50 mM PIPES (0.7 mg/ml microtubule subunits, 6 min, 30°C) or (*b*) 75 mM PIPES (1.2 mg/ml microtubule subunits, 4 min, 30°C). Note that although the kinetochore itself is more evident (arrows) in *a*, the chromosome seems more intact in *b*. Bar, 2 μm. × 10,000.

kinetochore is evident, appearing as a bar (arrow). The kinetochore microtubules can be seen to terminate at the bar and their exact length can be determined.

However, as reported previously (10), the mean number of microtubules per kinetochore was low and many chromosomes failed to initiate any microtubules. The initiation activity of the chromo-

somes appeared to be labile, declining rapidly after preparation of the mitotic lysates.

Due to these problems, we were unable to collect sufficient data from single experiments to generate statistically significant plots for the time-course of polymerization. Therefore, we pooled the data from different experiments by expressing the lengths as a ratio to the length of tubules grown onto the plus end of the flagellar seeds that served as internal controls. These values were plotted in a frequency histogram (Fig. 8) and the result is a Gaussian distribution centered about a value of $0.90 \pm 0.27 > (n = 300)$. This value is sufficiently close to 1 that we conclude that kinetochore-initiated microtubules also grow at one end only and that that end is plus.

DISCUSSION

The use of flagellar seeds as markers for the growth polarity of microtubules has enabled us to study the nucleation and elongation of centrosomal and chromosomal microtubules relative to a characterized internal standard (3). Assuming that the microtubules initiated by centrosomes and chromosomes are equivalent to those grown onto the flagellar seeds, then a comparative analysis of the rates of growth and depolymerization permits one to infer which end or ends of the spindle microtubules exchange subunits with monomer.

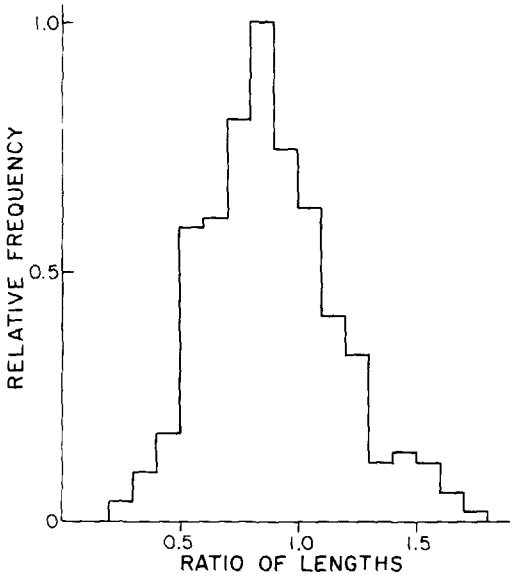

FIGURE 8 Distribution of chromosomal/plus end length ratios. The lengths of chromosomal microtubules were divided by the mean length of plus end microtubules initiated by internal flagellar seeds. The ratios were then plotted in a frequency distribution. Mean = 0.90 ± 0.27 ($n = 300$).

The results indicate that mitotic organizing centers (centrosomes and kinetochores) initiate microtubules rapidly (within 2 min) in vitro and that subsequently (up to 15 min) the microtubules elongate at a constant rate as do the microtubules seeded by flagella. Comparison of the rates of elongation shows that organizing center microtubules grow at the plus end rate.

The data are of sufficient precision to exclude not only growth at the minus end rate, but also growth at a rate corresponding to the sum of plus and minus end rates. This is an important result because it signifies that microtubules initiated by centrosomes and chromosomes are only able to add subunits at one end, and that the other end is unavailable for subunit incorporation.

The finding that only one end is available to add subunits permits us to dissociate growth from structural polarity. Subunits are added only at the plus end, which we know from previous work (3) to be equivalent to the end of flagellar A tubules distal to the basal body. The question, then, is whether the plus end is distal or proximal to the organizing center. To deduce the orientation of the plus end, we should consider the inactivity of the minus end.

Suppose the minus end were distal to the organizing center. Why would the end be inactive? Inactivation of the minus end might be accomplished by a specific diffusible inhibitor emanating from the organizing center or present in the cell extract. However, these possibilities are excluded by the observation that growth occurs at the minus end of the internal flagellar seeds. The exclusion of a diffusible inhibitor suggests a structural explanation. Since the distal ends of centrosomal or chromosomal microtubules are indistinguishable from those seeded by flagella, there appears to be no indication that they are modified or capped in some way. We turn, then, to the proximal end. Suppose the minus end were proximal to the organizing center. Its inactivity might be easily accounted for in structural terms by being embedded in the organizing material and being rendered unavailable for incorporation of subunits. Hence, the plus end would be distal and growth would occur by addition of subunits at the distal end.

Similar reasoning for the data on depolymerization of centrosomal microtubules indicates that loss of subunits occurs only at one end, and that

the site for loss is the same as the site for addition, namely the plus end. Similarly, the inactivity of the minus end towards depolymerization cannot be explained on the basis of a diffusible inhibitor since the minus end tubules of internal flagellar seeds depolymerized normally. We conclude that the minus end too is rendered unavailable for loss of subunits by its proximal relation to the organizing center. Hence, by this line of reasoning as well, the plus end is distal and depolymerization would occur by loss of subunits at the distal end.

It should also be noted that irrespective of the assignment of orientation relative to the organizing center, the results indicate that net addition of subunits under polymerizing conditions and net loss of subunits under depolymerizing conditions occur at the same end. Thus, we conclude that the polarity relation for the half-spindle is that centrosomal and chromosomal microtubules are antiparallel as previously suggested (5) and that subunit exchange is restricted to their distal ends.

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Note Added in Proof: The rate of growth of individual microtubules onto metaphase chromosomes, in vitro, has been studied by dark-field light microscopy (Summers, K., and M. Kirschner, 1979, *J. Cell Biol.* **83**:205–217). From these data they also have concluded that chromosomal microtubules grow at the plus end rate.

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