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Cytoplasmic microtubules in tissue culture cells appear to grow from an organizing structure towards the plasma membrane

(tubulin antibody/immunofluorescence microscopy/mitotic drugs/centrosphere/cilium)

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ABSTRACT A structure which appears to organize cytoplasmic microtubules in interphase mouse 3T3 cells can be visualized by immunofluorescence microscopy. Purified monospecific antibody against homogeneous tubulin from brain visualizes, in addition to cytoplasmic microtubules, a cytoplasmic polar structure as the focal point from which the microtubules seem to radiate. The structure is preserved after treatments that depolymerize cytoplasmic microtubules, i.e., exposure of cells to mitotic drugs or to low temperature. When cells recover from these treatments one end of each microtubule organizing structure acts as a nucleating center from which cytoplasmic microtubules grow toward the plasma membrane. Thus cytoplasmic microtubules assemble *in vivo* in an ordered unidirectional manner, and therefore the cell must be able to avoid the assembly of unwanted, unoriented, and disconnected microtubules. These results suggest that the assembly of tubulin into microtubules is regulated *in vivo.*

Maintenance of cell shape, cell motility, chromosome movement, and the intracellular movement of pigments and organelles are functions generally thought to involve microtubules (1, 2). In view of this complex set of functions the question of how cytoplasmic microtubules are organized and assembled is very important. The idea that centrioles or the centrospheric region could be connected with the regulation of the assembly of microtubules as "microtubular organizing centers" has often been discussed (2–6). In most cases the emphasis was put on spindle microtubules and flagella microtubules, where centrioles are obviously involved in the microtubular display itself. The problem of an "organizing center" for cytoplasmic microtubules, however, is a more difficult one. Centrioles have not been reported in all cells, nor has there been agreement as to whether they are present at all stages of the life cycle (4, 6). In addition, although some electron microscopic studies of tissue culture cells have shown that microtubules can radiate from the centrospheric region (7, 8), the comparatively few instances in which this has been documented have restricted the general acceptance of centrioles as microtubular organizing structures in mammalian cells during interphase.

Here we report studies on the display of cytoplasmic microtubules in tissue culture cells during interphase as visualized in indirect immunofluorescence microscopy. The use of a monospecific tubulin antibody has allowed us to recognize in interphase cells not only the complex array of fragile cytoplasmic microtubules described previously (9, 10), but also a tubulin-containing structure from which the cytoplasmic microtubules seem to radiate. This microtubular organizing structure is resistant to treatments which depolymerize cytoplasmic microtubules, i.e., mitotic drugs and low temperature. When cells recover from these treatments cytoplasmic microtubules polymerize from one end of this organizing structure towards the plasma membrane in an ordered unidirectional manner. These results indicate that the assembly of tubulin into microtubules is regulated *in vivo*.

RESULTS AND DISCUSSION

Identification of a microtubular organizing structure in interphase cells

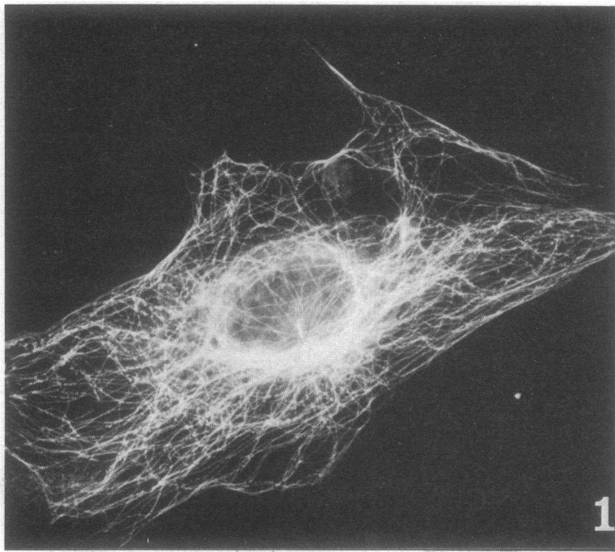
The anti-tubulin antibody was obtained against homogeneous tubulin from pig brain. The γ-globulin fraction of the rabbit serum was fractionated on tubulin coupled to Sepharose 4B (10–12). The purified monospecific antibody reacted specifically with homogeneous tubulin in immunodiffusion and immunoelectrophoresis (11, 12). Details of the immunological procedures and of the immunofluorescence microscopy have been published (9, 10, 12, 13).

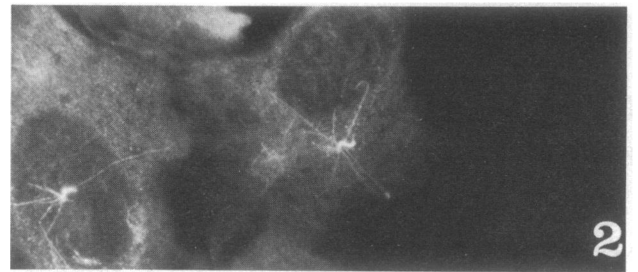
Fig. 1 shows the complex pattern of cytoplasmic microtubules visualized in 3T3 cells using the monospecific tubulin antibody in indirect immunofluorescence microscopy. The elaborate array of fragile tubules is typical for 3T3 cells. The majority of the tubules seem to extend radially from the perinuclear region toward the plasma membrane. Some tubules terminate close to the plasma membrane while others are curved and conform to the contour of the cells. This display of cytoplasmic microtubules has been described by us (9, 10) and confirmed independently by Brinkley *et al.* (14). The use of monospecific antibody rather than of total γ-globulins has improved the quality of the structural details. The nuclear fluorescence seen in our earlier studies in mouse 3T3 cells, but not in other cell lines (9), is absent when the monospecific antibody is used (10, 12). Thus it becomes possible to detect in many interphase 3T3 cells a tubulin-containing cylindrical structure in the perinuclear space which seems to organize the cytoplasmic microtubules (Fig. 1). This structure has the following properties.

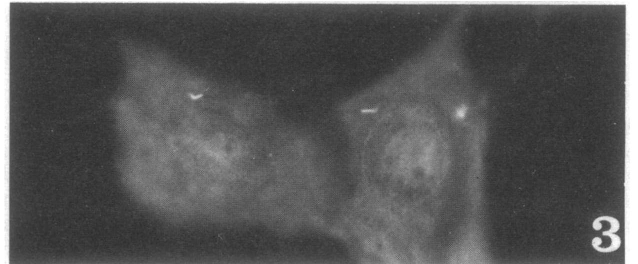
(*a*) It is located above or at the edge of the nucleus, and there are usually one or two such structures per cell. These organizing structures are also present in many enucleated 3T3 cells.

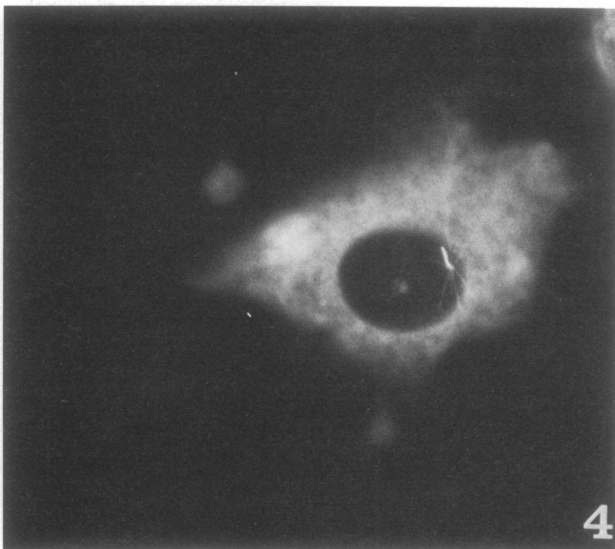
(*b*) It is a polar structure. The top of the structure is usually out of focus with and above the majority of the cytoplasmic microtubules, while the base of the structure appears as a focal point from which the majority of the cortical microtubules diverge.

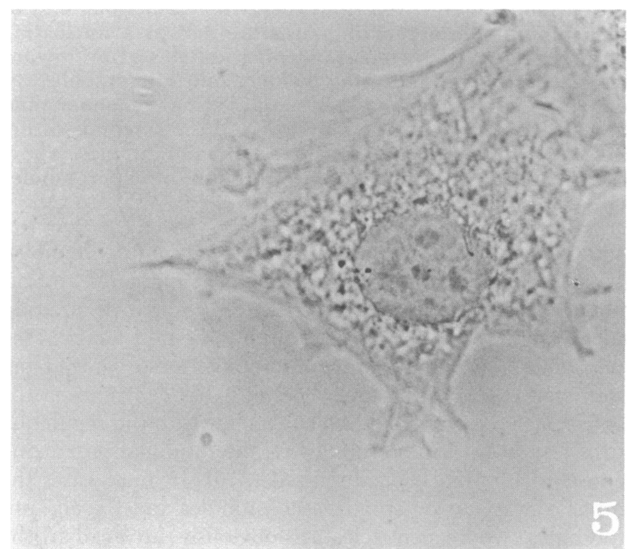
(*c*) It can be detected after cells have been exposed to mitotic drugs (Fig. 2) or to low temperature (Fig. 3). In both cases, as expected from electron microscopic studies (15, 16; for other references see refs. 1 and 2), the cytoplasmic array of microtubules is abolished. The organizing structure is still visualized in fluorescence microscopy, and often, as shown in Fig. 2 for colchicine-treated cells, a few residual fibers can be seen to diverge from its base, again emphasizing the

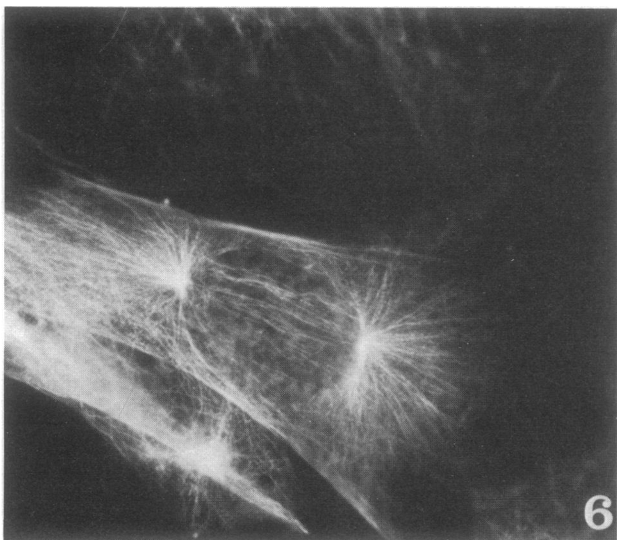


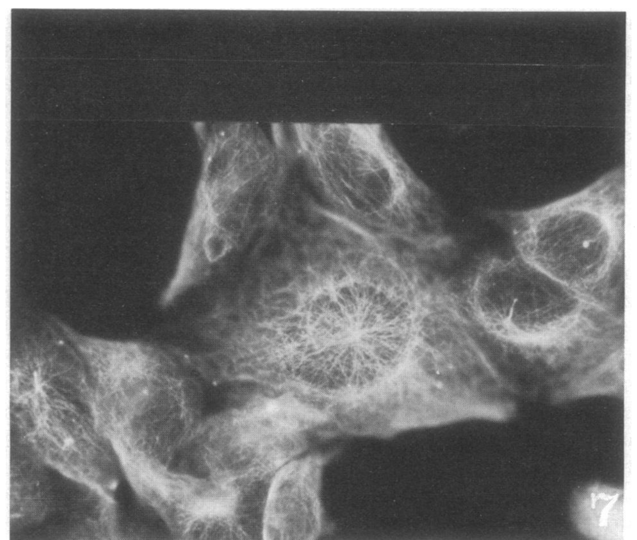












FIGS. 1–7. (*Legend appears at bottom of the following page.*)

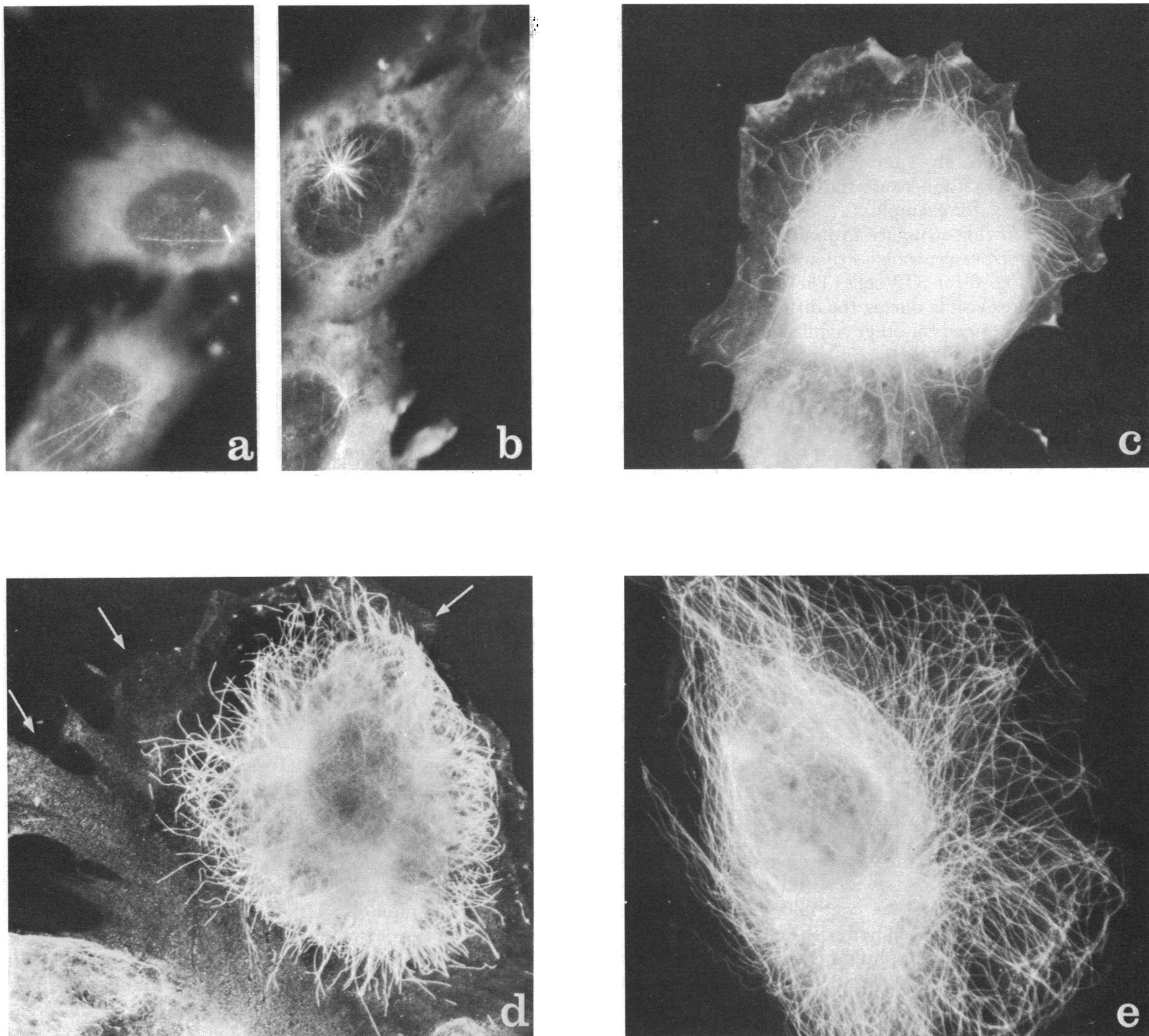


FIG. 8. Recovery of cytoplasmic microtubules in 3T3 cells after Colcemid. Visualization is in immunofluorescence microscopy using antibody against tubulin. Colcemid was present at 0.5 μg/ml. After 1 hr the cells were washed twice with growth medium and allowed to recover for the number of minutes indicated: (a) 0, (b) 30, (c) and (d) 50, (e) 75. Note the microtubular organizing structure in (a), the cytaster in (b), the "incomplete tubules" which extend only part of the way to the plasma membrane in (c) and (d) and the full recovery of the complete cytoplasmic microtubular system in (e). The background in (c) and (d) has been overexposed so that the cell boundary can be seen. The arrows in (d) indicate the boundary of the cell. The magnification in a, b, c, and e is ×900 and in d ×640.

polar nature of the structure. Structures similar to that shown in Fig. 2 are also found after treatment of 3T3 cells with three other mitotic drugs: Colcemid (see below and Fig. 8), griseofulvin (12) and drug R17934 from Janssen Co. (our unpublished results).

(*d*) The structure is visible in phase microscopy after cells are processed by our standard procedure for immunofluorescence microscopy, which includes fixation of cells with 3.7% formaldehyde. An example of the same cell photographed in fluorescence and in phase microscopy is shown in Figs. 4 and 5. A thickening at the base of the structure is often observed in phase microscopy (Fig. 5). The organizing structures are also visible in phase microscopy after cells are treated with Kane's fixative and digitonin, a procedure

FIGS. 1–7 (*on preceding page*). Microtubular organizing structure visualized in interphase cells with monospecific antibody against tubulin in immunofluorescence microscopy (Figs. 1–4, 7) and in phase microscopy (Fig. 5). The procedures for cell growth and for immunofluorescence microscopy were as in our previous studies (9, 10, 13).

Fig. 1. Interphase 3T3 cell. *Fig. 2.* 3T3 cell treated with colchicine at 1 μg/ml for 1 hr. *Fig. 3.* 3T3 cell held on ice for 15 hr. *Figs. 4 and 5.* 3T3 cell treated with colchicine as in Fig. 2, and then photographed in fluorescence microscopy (Fig. 4) and in phase microscopy (Fig. 5). Note the thickening at the base of the structure in Fig. 5. *Fig. 6.* 3T3 cell during mitosis, stained with antibody against tubulin. Note the centriolar structures. *Fig. 7.* 3T3 cell transformed by simian virus 40 (SV101). The magnification in Figs. 1–6 is ×900, in Fig. 7 ×600.

which renders centrioles and other tubulin-containing structures visible in phase microscopy (15). However, preservation of structural details of both the organizing structure and of cytoplasmic microtubules appears better if the formaldehyde treatment is used directly.

(*e*) The organizing structure in 3T3 cells has an apparent length of approximately 3 μm. Often it is difficult to measure the length exactly because the structure seems bent or curved (see Fig. 2, for example).

(*f*) The organizing structure in interphase cells is clearly distinguishable from centriolar structures in mitotic cells as illustrated in Fig. 6 for 3T3 cells. The various tubulin-containing structures visible during the different stages of mitosis have been described for other cell lines by means of tubulin-specific antibody, both by Fuller *et al.* (11) and by us (13).

(*g*) Finally, structures similar to that shown in Fig. 1 have been seen in interphase cells of a variety of cell lines in tissue culture. These include 3T3 cells transformed by simian virus 40 (Fig. 7), the Don line of Chinese hamster lung cells, C6 rat glial cells, and secondary embryonic cells from mouse and chicken. However, the length and exact shape of the structure as well as the percentage of cells showing such structures seems to be different in different cell lines.

The properties described for the polar structure shown in interphase cells in Figs. 1–5 and 7 suggests that it organizes microtubules and that it is associated with the centrospheric region. Thus, electron microscopic studies have documented that in some cases microtubules can radiate from the centrosphere (3, 7, 8), that centrioles can be observed in enucleated cells (17), and that the centriolar regions are much more resistant to the action of mitotic drugs than are cytoplasmic microtubules (15, 16).

In vitro studies have also suggested that centrioles may play a role in organizing microtubules (18). Thus, it seems reasonable to assume that the structure we see is in the centrospheric region, and, since we see no separate structure which can be identified as the centriole, that our structure probably is in intimate association with, and/or includes, the centriole. Thus, the structure might correspond either to the centrosphere, i.e., the centriole and associated structures, or to a cilium growing out from the centriole which would then be acting as a basal body (16).

It is possible to compare the results obtained with fluorescence microscopy with earlier electron microscopy studies. On the one hand, the length of the structure seen in immunofluorescence and in phase microscopy (3 μm) is much greater than the length of centrioles seen in electron microscopy [e.g., 0.5 μm in HeLa cells (19)] although the diameter would be approximately correct if the contribution of the antibody molecules is allowed for. It should be remembered, however, that structures described as centrioles appear much larger by light microscopy than by electron microscopy, a discrepancy which has been commented on, but not resolved (6). On the other hand, comparatively little is known about cilia in established tissue culture lines.

The percentage of centrioles that bear cilia appears to be different for different cell lines (20). Thus, in Chinese hamster lung cells 4–12% of the centrioles show evidence of ciliogenesis (16, 20). In 3T6 cells very careful electron microscopic studies by Wheatley (20, 21) suggest that between 50% and 75% of centrioles may be acting as basal bodies, and that in these cells cilia can be at least 2 μm long. These findings, as well as the fact that we have seen cells in which the structure clearly protrudes out of the cell surface, would be consistent with the idea that we are visualizing cilia in 3T3 cells. This interpretation together with the fact that some cell lines do not have cilia (20) makes it likely that the centrospheres rather than the cilia act as the organizing center for cortical microtubules.

Apparent direction of growth of microtubules *in vivo*

Do cytoplasmic microtubules have a preferred direction of growth? To try to answer this question we have exposed 3T3 cells to mitotic drugs in order to depolymerize the cytoplasmic microtubules. The cells were then allowed to recover and the regrowth of cytoplasmic microtubules was monitored by immunofluorescence microscopy. Fig. 8 shows the results obtained by treating 3T3 cells with Colcemid for 1 hr at 0.5 μg/ml and then removing the drug. Immediately after the Colcemid treatment only the organizing structures are seen (Fig. 8a). Thirty minutes after removal of the Colcemid, microtubules are seen polymerizing from one pole of the organizing structures, giving the impression of a cytaster (Fig. 8b). At 50 min after removal of the drug numerous microtubules can be seen in most cells "stretching" into the previously microtubule-free cytoplasm (Fig. 8c and d). They extend only part way across the cytoplasm, stopping at a point intermediate between the nucleus and the plasma membrane. These "incomplete tubules" become longer with increasing time after removal of the drug. Sixty to 90 min after removal of Colecemid the microtubules have reached the plasma membrane and the cells have an appearance similar to that shown in Fig. 8e.

Essentially, the same direction of assembly from the microtubular organizing center towards the plasma membrane is seen during recovery of 3T3 cells from the influence of colchicine (1 μg/ml for 1 hr). However, the time necessary for recovery is longer. Thus, "incomplete tubules" corresponding to those shown in Fig. 8c and d are seen only after 18 hr and full recovery is recognized after approximately 24 hr. Recovery of cytoplasmic microtubules from cold treatment is a very rapid process and is nearly complete after 15 min at 37°. Thus, it is more difficult to document intermediate stages after cold treatment than after treatment with drugs. Nevertheless, the direction of microtubular assembly is the same in all cases, i.e., tubules grow from the organizing structure toward the plasma membrane.

We have also examined 3T3 cells which have been trypsinized and replated. Also in this case microtubules seem to grow toward the cell membrane, stretching the cytoplasm in the process of the transformation of a rounded cell to a fibroblastic morphology. These results strongly support the idea that microtubules are involved in the determination of the cell shape (1, 2).

The direction of growth observed for cortical microtubules in interphase cells argues that microtubule assembly *in vivo* occurs in an ordered unidirectional manner. Pictures such as Fig. 8d show that almost every incomplete microtubule originates in the perinuclear region. In general, microtubules do not appear to polymerize either from the plasma membrane or freely in the cytoplasm away from the perinuclear area housing the organizing structure. Thus, the cell must have two crucial mechanisms controlling microtubule assembly. One is a "positive control" regulating assembly in a directed way with the organizing structure as "origin." The other must forbid assembly outside this pathway and can be thought of as a "negative control," preventing unwanted, unoriented microtubule assembly. This latter inhibitory mechanism could be a specific or an unspecific one,

but it is required if the pool of free tubulin is not compartmentalized within the cell. Finally, any polymerization process poses the problem of the polarity of growth. For microtubule assembly *in vivo* the question of one or two growing points remains to be answered. Thus, it is not known if tubulin is added to growing microtubules at one or both ends or, if only at one end, at which one.

Note Added in Proof. Brinkley *et al.* (22), using immunofluorescence microscopy, have also suggested that microtubules appear to focus at the centrosphere.

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