

Tubulin Assembly Sites and the Organization of Cytoplasmic Microtubules in Cultured Mammalian Cells

B. R. BRINKLEY, S. M. COX, D. A. PEPPER, L. WIBLE, S. L. BRENNER, and R. L. PARDUE
Department of Cell Biology, Baylor College of Medicine, Houston, Texas 77030

ABSTRACT The number, distribution, and nucleating capacity of microtubule-organizing centers (MTOCs) has been investigated in a variety of cultured mammalian cells. Most interphase cells contain a single MTOC that is localized at the centrosome region and corresponds to the centriole and pericentriolar material. MTOCs, like centrioles, become duplicated during the S phase of the cell cycle and are equationally distributed to daughter cells in mitosis. Multiple MTOCs were rarely observed in cultured cells except in one cell line (neuroblastoma), which also displayed an equally large number of centrioles in the cytoplasm. The kinetics of microtubule assembly and the tubulin nucleating capacity of MTOCs was assayed by incubating tubulin-depleted, permeabilized 3T3 and simian virus 40-transformed 3T3 cells with phosphocellulose-purified 6S brain tubulin and microtubule assembly buffer. Initiation and assembly of 6S tubulin occurred in association with the cells' endogenous MTOCs, and the length, number, and distribution of microtubules generated about the organizing centers were regulated and cell specific. Our results are consistent with the notion that the specification of microtubule length, number, and spacial arrangement resides largely in the MTOCs and surrounding cytoplasm and not in the tubulin subunits.

When cultured cells are examined by indirect immunofluorescence using antibodies to purified tubulin, a delicate fluorescent network, the cytoplasmic microtubule complex (CMTC), can be observed at interphase (4, 5, 10, 20). When cells enter mitosis the CMTC is dismantled and replaced by another assemblage, the mitotic apparatus, which is composed of a completely different arrangement of microtubules. The CMTC and mitotic apparatus represent only two examples of microtubule arrays that are spatially and temporally organized in eukaryotic cells. Other more ordered assemblies include the axoneme of cilia and flagella, the manchette of spermatids, and the cytopharyngeal basket in some ciliates. From electron microscope studies it is apparent that microtubules are usually organized into patterns around discrete foci such as centrioles, basal bodies, and kinetochores of metaphase chromosomes. Such foci have been termed microtubule-organizing centers (MTOCs) (23) and, as the term implies, are thought to be both

tubulin initiation sites and organizing centers that serve as templates for the arrangements of specific microtubule arrays in cells. This seminal concept has been based largely upon morphological observation and has not received rigid experimental verification.

It has been proposed that microtubules, in concert with other cytoskeletal components, regulate cell form and the outgrowth of cell processes (24). Because cell form and patterns of process extension are inherited traits that are transmitted from parent to daughter cell (24, 28), MTOCs may qualify as endogenous cytoplasmic determinants of cell form. Accordingly, they would not only serve as foci for the initiation and anchorage of microtubules, but as templates for determining length, number, and spatial distribution of microtubules that grow from them. To qualify as cytoplasmic determinants, MTOCs must be duplicated and segregated during cell division and temporally "programmed" to generate specific microtubule arrays at appropriate times in the cell cycle or at specific stages of development.

At this juncture, relatively little is known about the molecular composition, number, location, and mode of replication of

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MTOCs in cells, and even less is known about their ability to organize the microtubule arrays that grow from them.

In the present report, we have used immunofluorescence procedures to localize MTOCs in the cytoplasm and to follow their replication and distribution in large populations of cultured mammalian cells. In addition, we utilized a permeabilized cell model with pure exogenous tubulin to assay MTOCs in two cell lines (the methods for these procedures have been published elsewhere; see references 3, 4, 6, 7, 17, and 22). We conclude that the centrosome (centrioles and pericentriolar material) constitutes the primary MTOC of interphase cells. These organelles serve as templates for the initiation and assembly of specific microtubule arrays in cells and retain their activity even in tubulin-depleted, lysed cells when exposed to exogenous brain tubulin. Thus, specificity of microtubule length, number, and spatial arrangement appears not to reside in the protein subunits of microtubules but in the organizing centers and their surrounding environment.

Visualization of MTOCs by Immunofluorescence

When interphase cells were examined by indirect immunofluorescence using tubulin antibody, an elaborate array of cytoplasmic microtubules, the CMTC, could be seen in most cells (Fig. 1). In well-flattened cells, one or two brightly fluorescent spots could be seen where microtubules appeared to converge. These spots were usually near the nucleus and were presumed to be the cell center or centrosome. In other cells, such regions were not always apparent. When the cell culture

was exposed to Colcemid for 2 h, the CMTC disappeared, leaving only a few short residual microtubules in the cytoplasm and an occasional bright fluorescent spot. When the exposed cells were washed and placed into Colcemid-free medium for 15–30 min, MTOCs could be detected as distinct sites in the cytoplasm from which short lengths of microtubules regrew in a radial pattern (Fig. 2). This procedure, described earlier by several investigators (5, 20, 29, 30), permits one to identify tubulin initiation sites or MTOCs and to determine their location and distribution in cells. By counting the initiation sites in a large number of cells, it is possible to establish a frequency distribution profile. Fig. 3 shows distribution profiles of MTOCs in Swiss mouse 3T3 and simian virus 40-transformed 3T3 (SV3T3) cell lines analyzed by this procedure. In every cell line examined, most cells in the population displayed one or two MTOCs. Some cells displayed multiple tubulin initiation sites but these cells were always in the minority. One exception was the neuroblastoma cell line N-115, which displayed a higher frequency of multiple assembly sites as previously reported by Spiegelman et al. (29).

To evaluate the effects of Colcemid treatment or fixation on the display of MTOCs, we varied the Colcemid concentration and used both methanol and formaldehyde. These treatments had no significant effect on the frequency of MTOCs as detected by immunofluorescence (3).

Ultrastructural Correlates to MTOCs

To examine the ultrastructure of MTOCs, cells treated as described above were fixed and processed for transmission

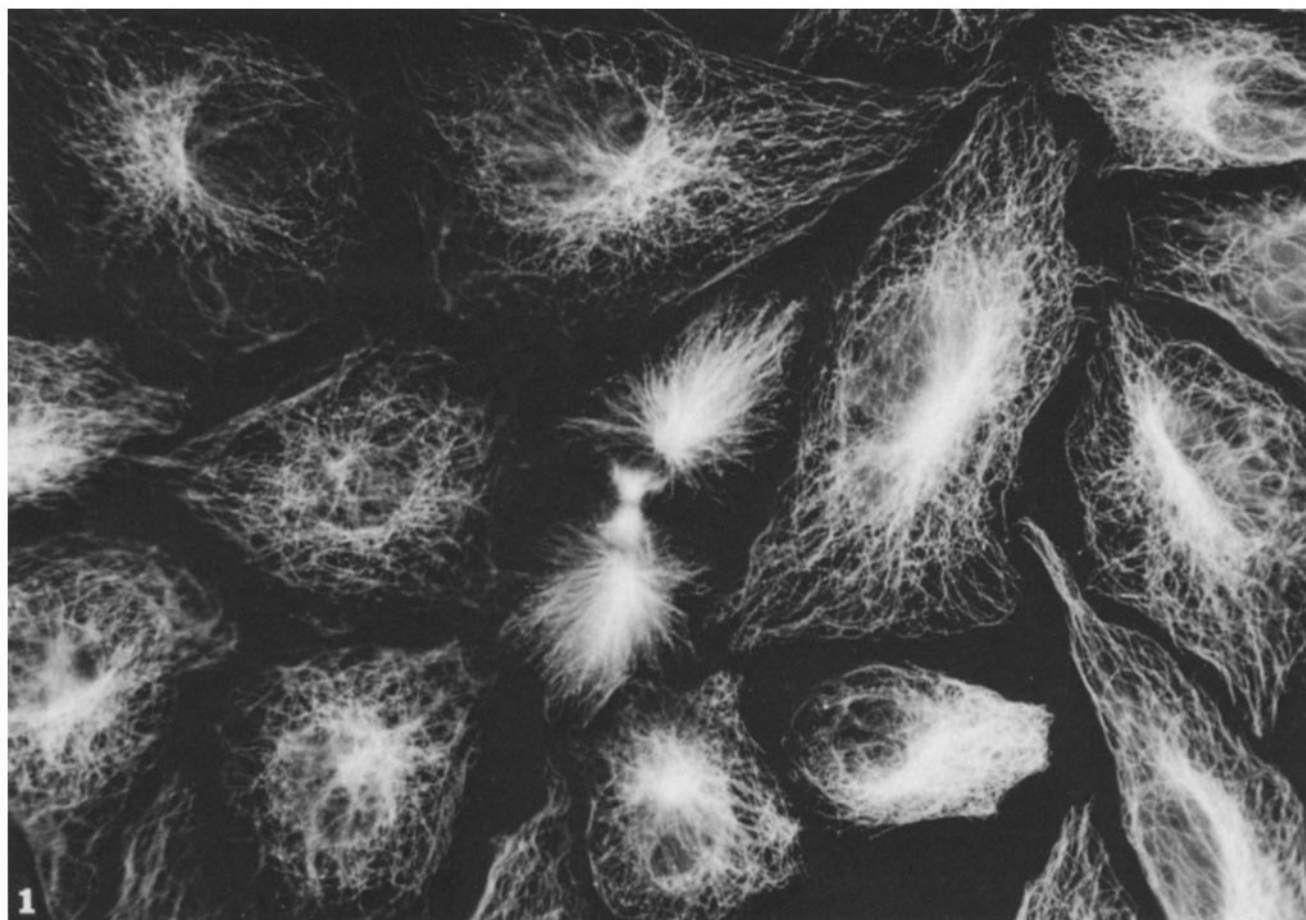


FIGURE 1 3T3 cells showing the cytoplasmic microtubule complex (CMTC). The bright cell in the middle is in telophase. $\times 1500$

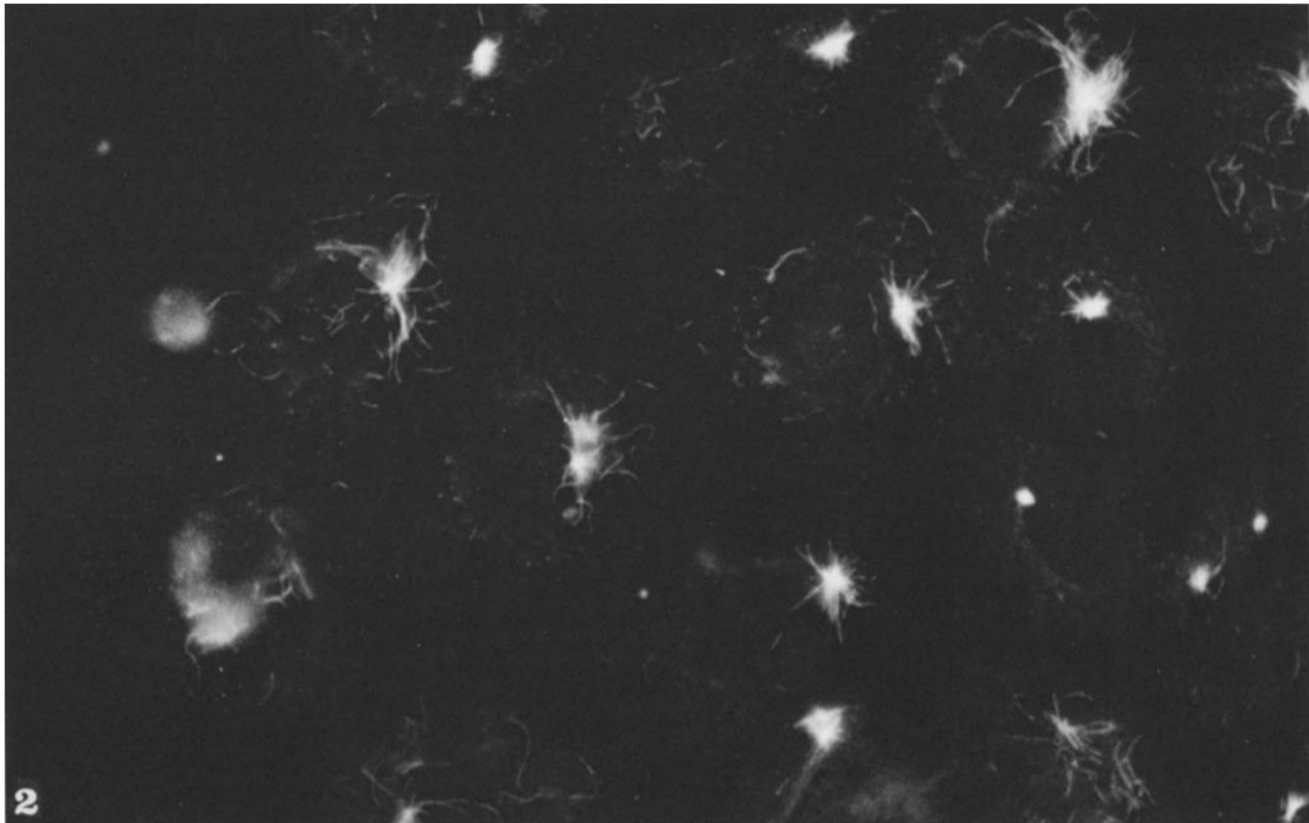
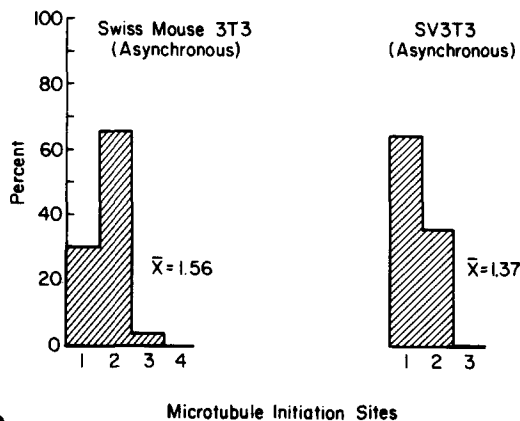


FIGURE 2 3T3 cells that have been exposed to Colcemid and then allowed to recover for 15 min. The bright spots are tubulin assembly sites (MTOCs). $\times 1500$



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FIGURE 3 Histogram showing the frequency distribution tubulin initiation sites in asynchronous 3T3 and SV3T3 cells (from reference 3).

electron microscopy. Examination of ultrathin serial sections showed that, in every case, sites of microtubule regrowth were characterized by the presence of centrioles and pericentriolar material (Fig. 4). In the case of neuroblastoma cells, which displayed a higher frequency of multiple tubulin initiation sites, multiple centrioles (up to 16 per cell) were found in the cytoplasm (Fig. 5). These were present in both serum-fed cultures and cultures induced to differentiate in serum-free medium. Although they were usually observed in a localized cluster, the centrioles were occasionally seen to be dispersed in the cytoplasm, possibly accounting for the multiple MTOCs observed by immunofluorescence. Further evidence that cen-

trioles represented sites of tubulin initiation was obtained when MTOCs were analyzed throughout the cell cycle.

Fate of MTOCs in the Cell Cycle

To investigate the disposition of MTOCs in the cell cycle, we examined patterns of microtubule regrowth in CHO cells in asynchronous growth, in confluent nondividing cultures (plateau), and at G_1 , S, and G_2 phases of the cell cycle. As shown in Fig. 6, asynchronous cultures displayed approximately equal numbers of cells with one or two MTOCs. Plateau-phase cultures contained predominantly single MTOCs. A similar distribution was found in G_1 and early-S-phase cells. As cells progressed further into S phase, most displayed two MTOCs, and by late G_2 (early prophase) $>90\%$ of the cells contained two MTOCs (data on G_2 not shown). These data suggest that MTOCs are duplicated during mid-S phase, a time when centrioles also become visibly duplicated (31).

Centrosomes vs. MTOCs

To further compare the distribution of MTOCs with centrosomes (centrioles plus pericentriolar material), we compared the distribution frequency of tubulin initiation sites with the staining pattern produced in interphase 3T3 cells by a human antoantiserum directed against the centrosome (2). For these comparisons, exponentially growing 3T3 cells were split into two equivalent populations and analyzed independently by the two probes. As shown in Fig. 7 the frequency distribution of centrosomes corresponds precisely with the frequency of MTOCs in 3T3 cells (compare with Fig. 3).

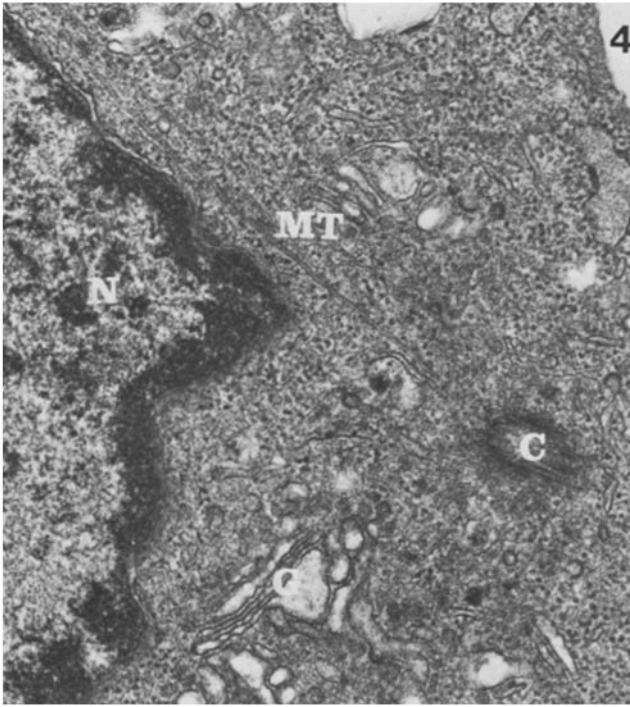


FIGURE 4 Electron micrograph of tubulin initiation sites in 3T3 cells. C, centriole; N, nucleus; MT, microtubule; G, Golgi. $\times 26,000$

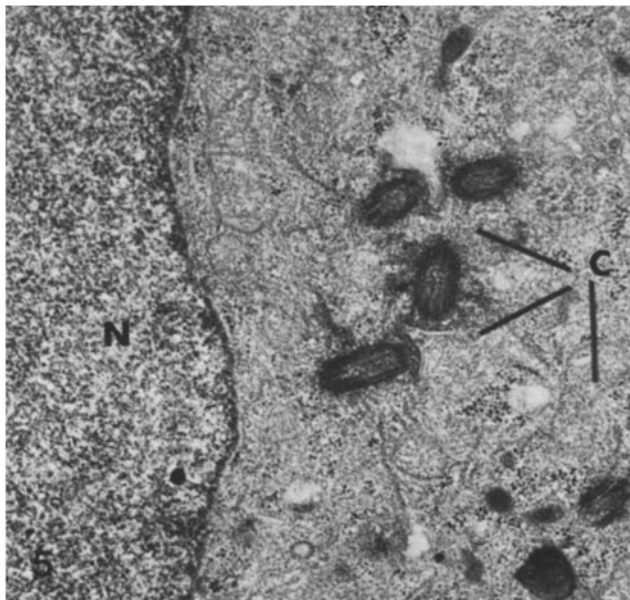


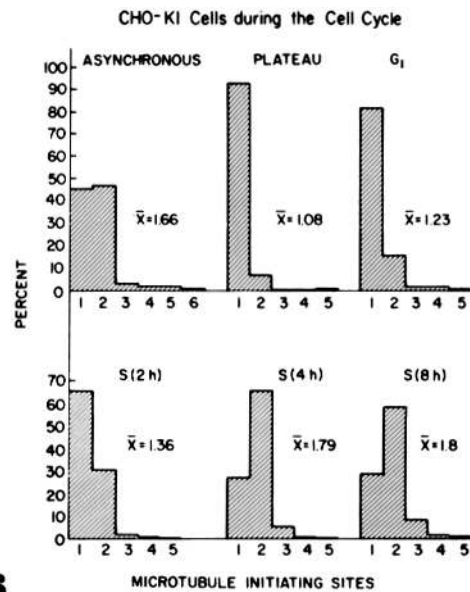
FIGURE 5 Electron micrograph of neuroblastoma cell showing profiles of four centrioles (C) in one section. As many as 16 centrioles could be detected in these cells. C, centriole; N, nucleus. $\times 26,000$

Analysis of MTOCs in Permeabilized Cells

These experiments were designed to assay the nucleating capacity of MTOCs and attempt to reconstitute the CMTC in lysed, detergent-extracted cells using purified bovine brain tubulin. 3T3 cells were treated with Colcemid to disrupt endogenous microtubules and then lysed with Triton X-100 in microtubule assembly buffer (details concerning methods are published elsewhere; see references 7 and 22). When these cells were stained with tubulin antibody, only a few short fluorescent

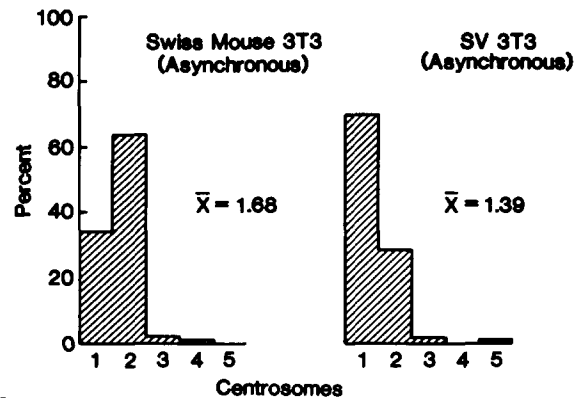
microtubules were present along with one or two bright fluorescent spots representing residual MTOCs in the cytoplasm (Fig. 8a). When the permeabilized cells were incubated in microtubule reassembly buffer for 15 min at 37°C containing a concentration of 1.0 mg/ml 6S bovine brain tubulin and then stained with tubulin antibody, numerous long straight or slightly curved microtubules were seen extending radially from discrete organizing centers (Figs. 8d and 9). In most cases it was possible to identify apparently individual microtubules with one end associated with a MTOC and the other ending near the cell surface. Some microtubules appeared to penetrate through the lysed cell surface and extend for a short distance outside the cell. Most of them, however, terminated inside the cell near the site of the plasma membrane. Only rarely did the reconstituted microtubules bend and extend along the cell cortex as seen in the intact CMTC.

The success of this procedure was dependent upon the use of phosphocellulose-purified tubulin in the reassembly mixture. If microtubule-associated proteins, (MAPs) were present, only short microtubules formed and these were randomly distributed inside and outside of the lysed cells.



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FIGURE 6 Frequency distribution of MTOCs in CHO-K1 cells during the cell cycle (from reference 3).



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FIGURE 7 Histogram showing the frequency distribution of centrosomes as determined by indirect immunofluorescence using human autoantiserum.

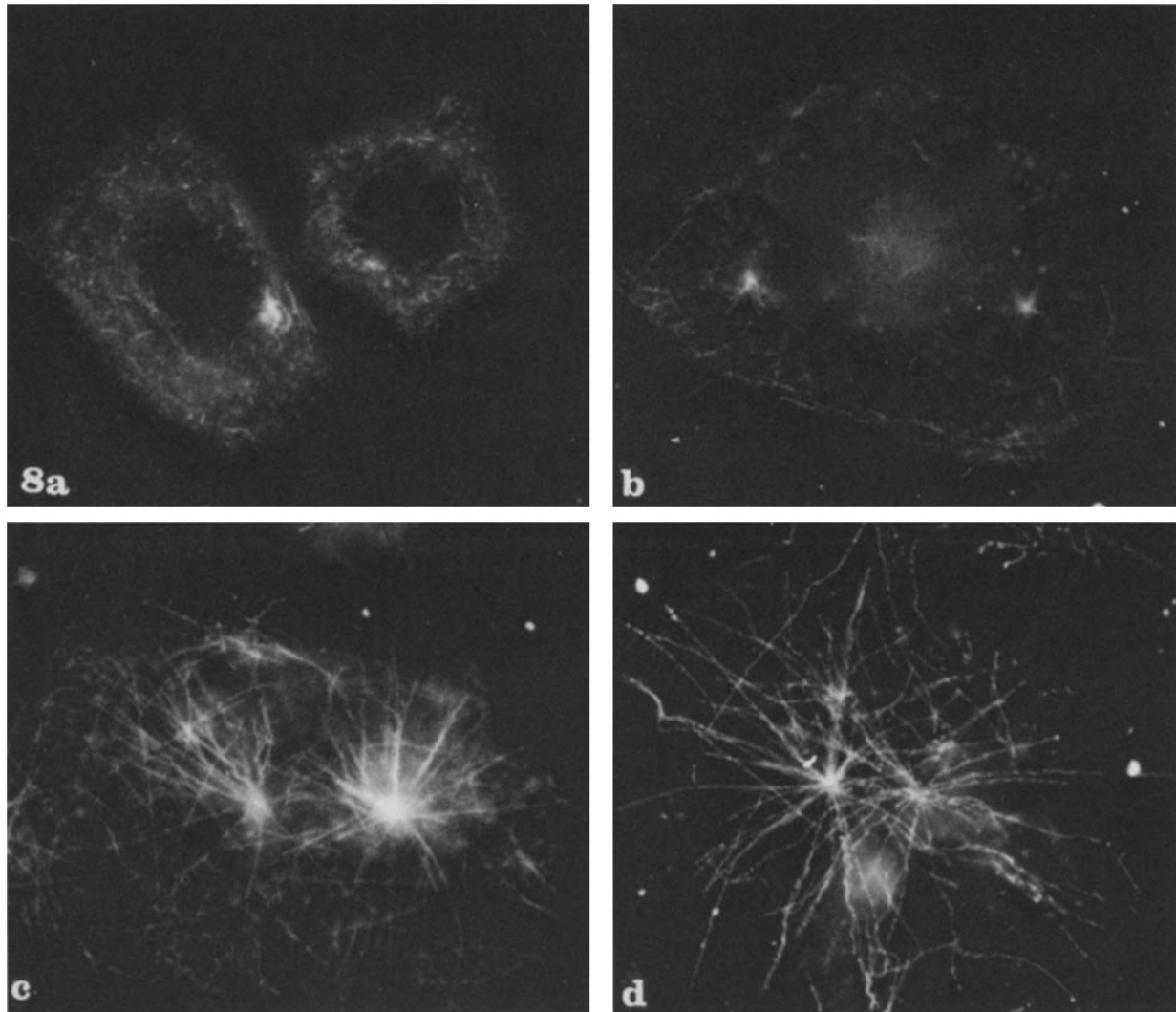


FIGURE 8 Initiation and assembly of bovine brain tubulin at MTOCs of lysed 3T3 cells. (a) Colcemid treated but not incubated with tubulin; (b) incubated with tubulin at a concentration of 0.25 mg/ml; (c) incubated with tubulin at a concentration of 0.45 mg/ml; (d) incubated with tubulin at 1.0 mg/ml. $\times 1800$

Because of the clarity of individual microtubules in these preparations it was possible to count and measure the length of tubules associated with MTOCs. Fig. 8a and b shows lysed cells that were incubated for 15 min with varying concentrations of tubulin. Essentially no growth of microtubules could be seen in cells incubated with protein concentrations <0.3 mg/ml. Above this concentration, microtubules could be detected and a measure of the elongation rate determined by comparing average microtubule length after 15 min of incubation at different protein concentrations. When microtubule growth was plotted as a function of protein concentration (Fig. 10), several interesting features were observed. Microtubule length increased during the incubation period with increasing concentrations of tubulin up to ~ 1.0 mg/ml and then ceased to increase further. From this data, the critical concentration (C_c) for initiation was estimated to be 0.3 mg/ml, a value comparable to that of the C_c in vitro (1). The number of microtubules per organizing center also reached a peak at ~ 1.0 mg/ml. At a higher tubulin concentration, the number of microtubules per organizing center actually decreased, for some unknown reason.

Similar kinetics were obtained by maintaining a fixed tubulin

concentration and varying the time of incubation (Fig. 11). After 15 min of incubation at 0.9 mg/ml tubulin, microtubule length stabilized and failed to increase with further incubation up until 32 min. A reduced rate of elongation was observed with 0.5 mg/ml tubulin.

Fidelity of MTOCs in the Lysed Cell Model

To compare the number of MTOCs in the lysed cell model with that in intact cells, MTOCs were counted in 300 randomly selected 3T3 cells that had been lysed and incubated with pure tubulin. As shown in Fig. 12, the number of MTOCs in the lysed cell model was equivalent to that in intact cells as determined by counts of sites where microtubules regrew after reversal from Colcemid. Thus, we conclude that all of the MTOCs are active in the lysed-cell model.

Comparison of MTOCs in 3T3 and SV3T3 Cells

In previous studies we have reported differences in the expression of cytoplasmic microtubules between normal and transformed cells (4, 11, 19). Therefore, we wished to use the lysed cell system to compare the microtubules assembled in

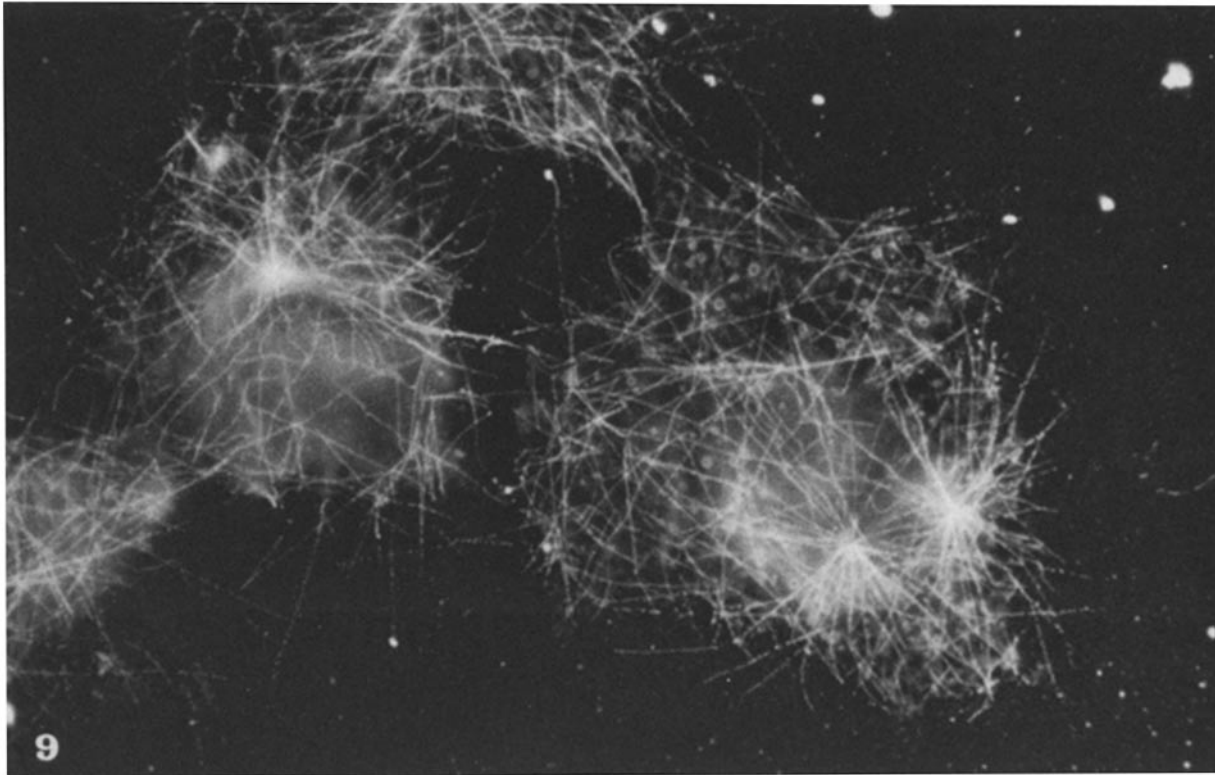


FIGURE 9 Microtubule pattern in 3T3 cells after incubation with pure tubulin. Note distinct initiation sites and radiating microtubules. $\times 1250$

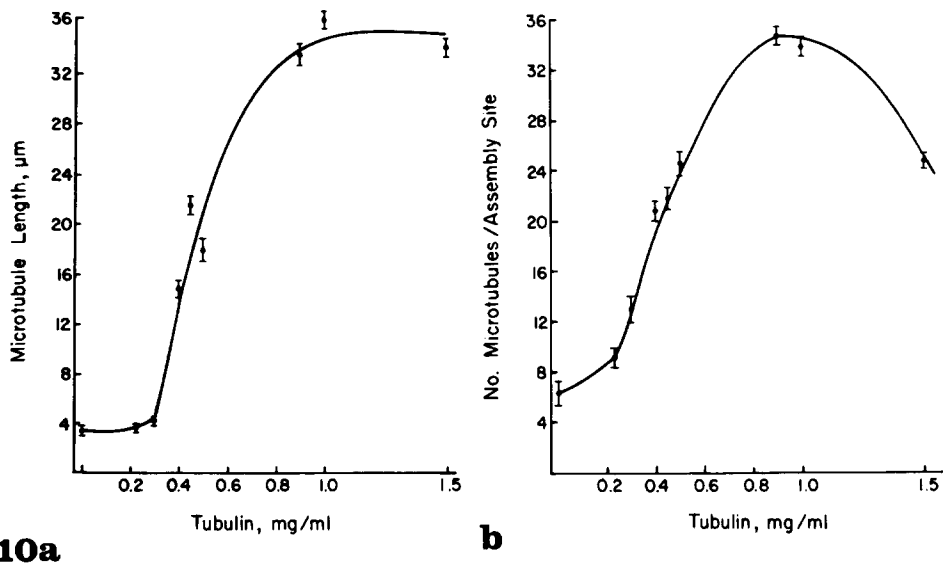
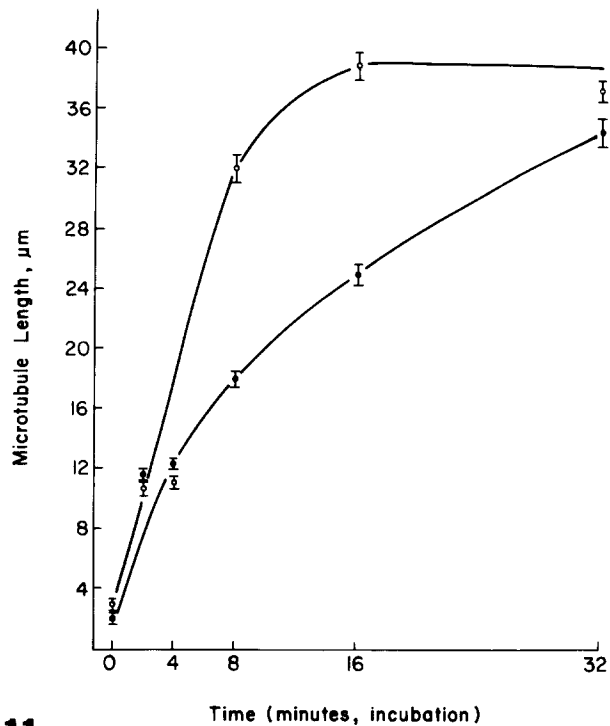


FIGURE 10 (a) Microtubule elongation vs. tubulin concentration. (b) Number of microtubules/initiation site reaches a peak at tubulin concentration of 1.0 mg/ml.

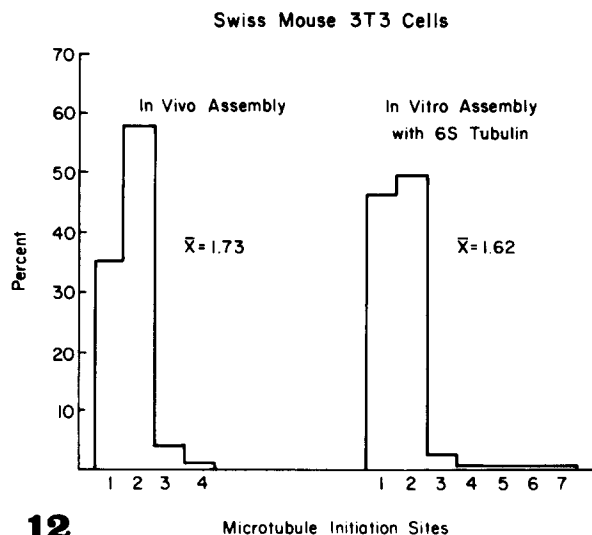
3T3 and SV3T3. When SV3T3 cells were lysed and exposed to pure tubulin under identical conditions used for 3T3 cells, microtubule assembly occurred in association with organizing centers. When SV3T3s were examined by indirect immunofluorescence, their microtubules appeared to be considerably shorter than those reconstituted in 3T3 cells (Fig. 13 *a* and *b*). When the initiation and elongation of microtubules were analyzed in SV3T3, the kinetics of assembly were similar to those of 3T3 cells and the number of initiation sites per cell was identical. However, the average length of microtubules assem-

bled in the transformed cells was two to three times less than in 3T3 cells (Fig. 14 *a*). Also, the number of microtubules per initiation site(s) in SV3T3 cells was 30–40% smaller (Fig. 14 *b*). A striking difference is also illustrated in the length distribution of microtubules grown about organizing centers as shown in Fig. 15. It is interesting and perhaps significant that in both 3T3 and SV3T3 cells microtubules appeared to terminate near the cell boundary. The shorter microtubules in SV3T3 cells therefore correlated with these cells being considerably smaller in diameter than 3T3 cells.



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FIGURE 11 Microtubule elongation in 3T3 cells vs. time of incubation at two concentrations of 6S tubulin (○, 0.9 mg/ml; ●, 0.5 mg/ml) (from reference 7).



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FIGURE 12 Comparison of frequency of MTOCs in 3T3 cells in vivo and in the lysed cell system.

DISCUSSION

The organization of microtubules in eukaryotic cells ranges from highly ordered, as seen in axonemes of cilia and flagella and axopodia of some protozoans, to the relatively dispersed cytoplasmic networks seen in cell monolayers. In every case, however, microtubules are not assembled and arranged as free entities but as interacting arrays associated with a distinct MTOC. The MTOCs are structurally defined components that are duplicated in the cell cycle and distributed equally to daughter cells during division. It is reasonable to view MTOCs as heritable cytoplasmic determinants that are programmed to specify patterns of microtubules that grow from them. The

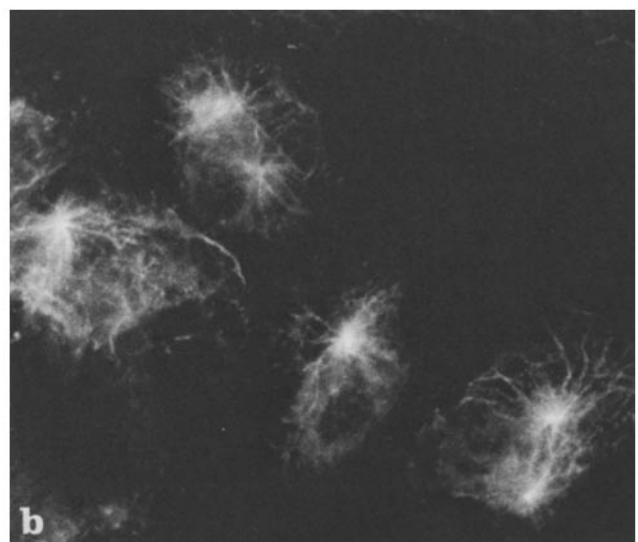
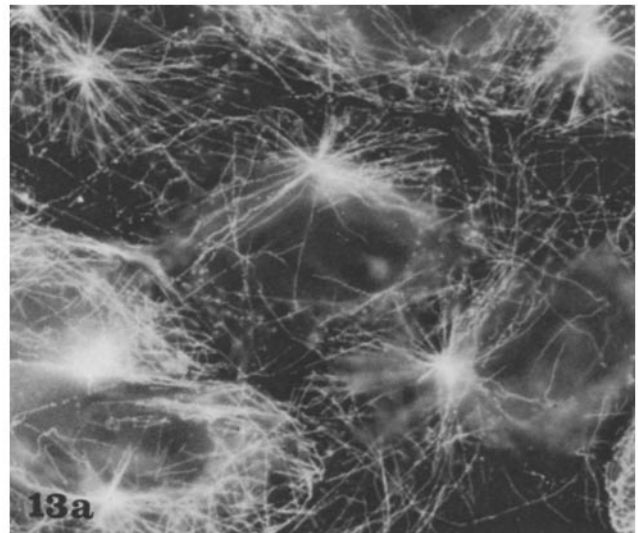


FIGURE 13 Analysis of tubulin initiation and elongation in lysed 3T3 and SV3T3 cells exposed to bovine brain tubulin under identical conditions. (a) Immunofluorescence of 3T3; (b) immunofluorescence of SV3T3. $\times 1000$

organization and distribution of microtubules may in turn influence the organization of other components of the cytoplasm and the overall shape of cells. Utilizing various immunofluorescence techniques, it is now possible to observe MTOCs in populations of cultured cells and to evaluate their capacity to initiate and specify microtubules under defined experimental conditions.

Although it has been proposed that cultured cells contain numerous MTOCs (29, 30), our study suggests that most cells display a single MTOC near the cell nucleus that corresponds morphologically to the centrosome and contains centrioles and pericentriolar material. During the G_1 phase of the cell cycle, each cell contains a single MTOC. Two functional MTOCs appear in the mid-S phase and remain functional throughout G_2 . In our study, multiple MTOCs were rarely seen in cultured cells. Our findings in this regard are in agreement with reports by Sharp et al. (26) and Watt and Harris (34) and are at variance with a report by Spiegelman et al. (29) who found an average of eight tubulin initiation sites in human fibroblasts, using very similar immunofluorescence procedures. These investigators also reported multiple MTOCs in neuroblastoma

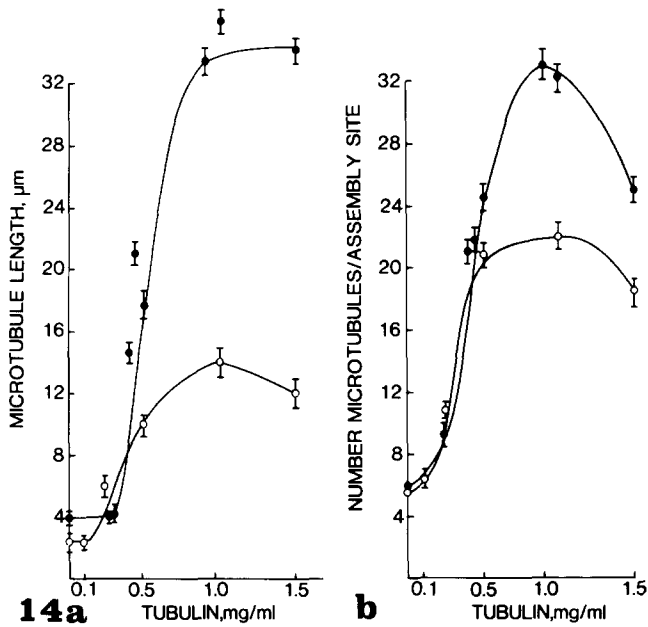


FIGURE 14 (a) Comparison of length vs. tubulin concentration in 3T3 (●) and SV3T3 (○); (b) comparison of the number of microtubules/assembly site as a function of tubulin concentration (from reference 7).

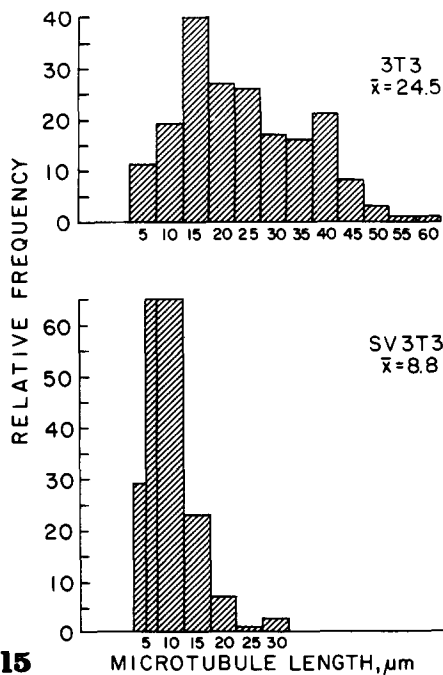


FIGURE 15 Length distribution of microtubules extending from MTOCs in 3T3 and SV3T3 cells selected at random.

(strain N-115) cells (30). Although we were not able to confirm their observation in fibroblasts, we did observe an unusually high frequency of MTOCs in N-115 cells. When these cells were examined by electron microscopy, an unexpected result was obtained. Instead of the usual centriole pair, these cells displayed as many as 16 centrioles per cell. Multiple centrioles have been independently described in mouse neuroblastoma cells by Sharp et al. (26). Although the centrioles usually existed in one or two clusters that functioned as localized organizing centers, some cells displayed dispersed centriole pairs that served as individual nucleating sites. Thus, the

multiple MTOCs seen in N-115 cells appear to correlate with the presence of an equally large number of centrioles. In our studies, multiple centrioles were not normally seen in fibroblast and epithelial cells, and we have no explanation for the results of Spiegelman et al. (29). A similar conclusion has been reached independently by Sharp et al. (26) and Watt and Harris (34).

Several lines of evidence suggest that MTOCs function to regulate the specificity of microtubules organized around them. The number, length, and distribution of microtubules generated by MTOCs of cultured cells may be cell cycle-dependent (4, 33). MTOCs of interphase cells generate microtubule arrays that differ from those generated in the mitotic cells. Telzer and Rosenbaum (33) reported that MTOCs of HeLa cell extracts were competent to initiate microtubule assembly only when the MTOCs were from mitotic cells. Under the conditions of our experiments the centrosome remains competent to initiate microtubules at all stages of the cell cycle. The differences between our findings and those of Telzer and Rosenbaum may be attributable to differences in the lysis and extraction procedure used. We also suggested that the differences may have occurred because MTOCs of HeLa cells, like those of SV3T3 and perhaps many other transformed cells, are attenuated in their capacity to initiate cytoplasmic microtubule assembly during interphase.

Our results with the lysed-cell system suggest that microtubules that initiate at MTOCs elongate to defined lengths and cease to elongate further even in the presence of higher protein concentrations or increased time of incubation. Not all microtubules grown from a selected MTOC achieved the same length during the incubation period, and a broad distribution of lengths was observed about each organizing center. In general, most microtubules terminated near the cell margins. The direction of elongation appeared to be from the organizing center outward, and polymerization always occurred within the plane of the cytoplasm. Although we have no direct information on microtubule polarity in our system, Kirschner (15) has proposed a model in which the minus ends of stable microtubules are embedded in the MTOC while the positive, fast-growing ends are distal to the organizing centers. Recent observations by McIntosh et al. (18) confirmed this arrangement in mitotic asters and other MTOCs where the polarity of microtubules was defined by a morphological marker. Thus, the polarity of microtubules appears to be defined by the MTOC, but at this juncture we know very little about how the lengths of microtubules are regulated.

Several models for regulating polymer length have been proposed (13, 14, 16, 21). Lengths achieved at steady-state may result from the availability of tubulin subunits. Obviously, this explanation does not apply in our system because the availability of soluble 6S tubulin was unlimited. Microtubule length may be controlled by a vernier mechanism involving another polymer such as microfilaments or intermediate filaments. Although filaments were abundant in our system, we have no information on their distribution with respect to microtubules. Another possible mechanism for limiting growth and controlling length would be to cap the growing end with another molecule. Because microtubules generally terminate near the cell margin, a capping mechanism may exist near the cell surface to regulate length.

The argument for length regulation in our system is reinforced by the finding that microtubules assembled in 3T3 cells were two to three times longer than those generated in SV3T3 cells under identical conditions of incubation. Although the

shorter length of microtubules in SV3T3 cells corresponds to a smaller cytoplasmic area in these cells, microtubules can be induced to elongate to approximately the same length as in 3T3 cells when incubated with cAMP (32). These experiments suggest that elongation may be regulated by the phosphorylation of a substrate, possibly tubulin or an MAP protein, by a cAMP-dependent protein kinase. Interestingly, microtubule elongation in 3T3 cells was not inducible by cAMP under the same conditions (32). These findings are consistent with the proposal that cytoplasmic microtubule organization is altered in transformed cells (4, 11, 19) and that cAMP can produce reverse transformation in a microtubule-dependent manner (4, 12). Whether or not the attenuation of microtubules in transformed cells is related to the action of a transforming factor such as pp60^{src} (9) or a similar transformation-associated kinase activity from sarcoma virus-transformed cells (25) remains to be determined. Other factors such as increased calmodulin-tubulin ratios in transformed cells may also limit microtubule polymerization (8).

In summary, our investigation supports the following general conclusions: (a) Most cultured mammalian cells contain a single MTOC associated with the centrosome region. (b) After cell division, G₁-phase cells generally contain a single MTOC that corresponds to a centriole pair and surrounding pericentriolar material. (c) The number of MTOCs, like centrioles, appears to double during the S phase. (d) The final length and number of microtubules assembled about MTOCs appear to be cell specific and independent of tubulin concentration. It is highly unlikely, however, that all of the specificity for microtubule arrays is programmed into the MTOC. The characteristics of microtubules emanating from MTOCs may also be determined in part by the surrounding cytoplasmic environment.

These experimental results are consistent with the classic notion that the centrosome defines the cell center and imparts order and arrangement to the surrounding cytoplasm, organelles, and inclusions. The temporal and spatial assembly and display of cytoplasmic microtubules provide a structural basis for regulating cell form and process formation. In turn, the sequential replication of centrioles and associated structures and their equal distribution to daughter cells provides a lineage of programmed organizing centers, thereby assuring a continuity of endogenous morphological determinants to cell shape and process formation, as described in the preceding article by Solomon (see also reference 27 and 28).

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