Role of the Centrosome in Organizing the Interphase Microtubule Array: Properties of Cytoplasts Containing or Lacking Centrosomes

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ABSTRACT   To study the role of the centrosome in microtubule organization in interphase cells, we developed a method for obtaining cytoplasts (cells lacking a nucleus) that did or did not contain centrosomes. After drug-induced microtubule depolymerization, cytoplasts with centrosomes made from sparsely plated cells reconstituted a microtubule array typical of normal cells. Under these conditions cytoplasts without centrosomes formed only a few scattered microtubules. This difference in degree of polymerization suggests that centrosomes affect not only the distribution but the amount of microtubules in cells. To our surprise, the extent of microtubules assembled increased with the cell density of the original culture. At confluent density, cytoplasts without centrosomes had many microtubules, equivalent to cytoplasts with centrosomes. The additional microtubules were arranged peripherally and differed from the centrosomal microtubules in their sensitivity to nocodazole. These and other results suggest that the centrosome stabilizes microtubules in the cell, perhaps by capping one end. Microtubules with greater sensitivity to nocodazole arise by virtue of change in the growth state of the cell and may represent free or uncapped polymers. These experiments suggest that the spatial arrangement of microtubules may change by shifting the total tubulin concentration or the critical concentration for assembly.

Microtubules have many important functions in cells, including a role in cell motility, intracellular transport, determination of cell shape, and segregation of chromosomes in mitosis (10, 26). For all of these functions microtubules must have the proper location and orientation in the cell. The mechanisms that determine the location and orientation of microtubules are not well understood. In the last several years, however, a better picture of the overall arrangement of microtubules in cells has been developed by immunofluorescence microscopy using antibody to tubulin as well as by electron and light microscopy. These methods have demonstrated that microtubules originate near the nucleus and fan out toward the cell periphery in what has been termed the cytoplasmic microtubule complex (4, 11, 12, 28). Since the location of the microtubules in the cell is to some extent determined by their sites of origin, considerable attention has been directed toward study of these sites.

By electron microscopy the centrosome region of interphase animal cells seems to be the site where microtubule density is higher than in any other portion of the cell (25). However,

outside this region the overall arrangement of microtubules is not readily apparent from thin sections. The visualization of microtubules is better achieved by immunofluorescence of whole cells. These images suggest that many of the microtubules of the cell originate near the nucleus, perhaps in the vicinity of the centrosome. However, the exact origin of the microtubules cannot be distinguished on the light microscope level (40). Except in cells displaying very sparse microtubule arrays, such as neutrophils (30), it is difficult, even by electron microscopy of whole-mount preparations, to distinguish the points of origin of most microtubules. This is due to problems of resolution, superposition, and tracking. Therefore, although it is possible to say that some microtubules originate at the centrosome and that most microtubules originate near the nucleus, it is not possible to say that all or even most microtubules arise from the centrosome.

The strongest argument supporting the idea that microtubules are nucleated from sites associated with centrioles has come from regrowth experiments where the cytoplasmic microtubule complex is first depolymerized with drugs like

colchicine. When the drug is withdrawn the sites of reassembly can be visualized by immunofluorescence against a much reduced background of polymer (3, 5, 11, 22, 23, 36, 37, 39). In such studies most microtubules were seen to originate from distinct sites. In some cases the number was one to two per cell and corresponded to the number of centriole complexes. In some cases a larger number of nucleating sites were found (3, 32, 36, 37). In these experiments, however, the points of microtubule growth were only discernible in the initial stages of regrowth, until background from the polymerized microtubules obscured their origins. Thus, though it is fair to say that some microtubules originate from distinct sites, it is not possible to say that those sites are the origin of all the microtubules in the cell. There may be additional difficulties in comparing steady state with initial regrowth conditions.

In this paper we raise the general question of what role the centrosome plays in organizing the microtubules of a typical mammalian cultured fibroblast: the mouse L929 cell. The main experimental innovation has been to obtain enucleated cells, called cytoplasts, either containing or not containing centrosomes, and to study the organization of their microtubules. Using these cytoplast models, we have addressed the questions of whether all the microtubules of the interphase cell originate from the centrosome and whether centrosomal and free microtubules can co-exist in the same cytoplasm and have speculated about the mechanism of centrosome nucleation and stabilization of microtubules. Examination of the state of microtubule assembly in cells lacking a centrosome has allowed us to evaluate the role of the centrosome. We have obtained new information on how the centrosome functions in interphase cells and how it controls the polymer levels and spatial distribution of microtubules. We present the results and interpret them in terms of what is now known about microtubule polymerization.

MATERIALS AND METHODS

Cell Culture: The mouse fibroblast L929 cell line was grown in modified Eagle's medium with Earle's balanced salts, containing 5% calf serum, 100 μg/ml penicillin, and 100 μg/ml streptomycin. The cells were incubated in a humidified tissue culture incubator at 37°C with 5% CO_2. For studies on the effect of cell density on microtubule regrowth patterns, cells were seeded at different densities and allowed to grow for 48 h. All preparations were then examined at the same time.

Enucleation Procedure: Cytoplasts were prepared according to the procedure of Lucas et al. (21) with some major modifications. Cells were grown on glass coverslips (1.25 cm diam) that had been treated with concentrated sulfuric acid at ~60°C for 1 h, extensively washed with distilled water, rinsed with ethanol, and air dried. Then the coverslips were placed in Falcon 24-well tissue culture plates (Falcon Labware, Div. of Becton, Dickinson & Co., Oxnard, CA) and sterilized by UV irradiation. Cells were passaged onto the coverslips at the desired density and allowed to spread and grow for 2 d before enucleation.

Cytoplasts containing centrosomes were prepared in the following way. The coverslips were preincubated cell-side down for half an hour in sterile 15-ml Corex centrifuge tubes containing 2 ml of medium with 10 μg/ml cytochalasin B (Sigma Chemical Co., St. Louis, MO) at 37°C in a 5% CO_2 atmosphere. The enucleations were performed in a prewarmed JS13 rotor using a J221 Beckman centrifuge (Beckman Instruments, Inc., Palo Alto, CA) previously equilibrated at 37°C by spinning the empty rotor. The cells were centrifuged at 10,000 rpm for 1 h in the presence of cytochalasin B. The cytoplasts without centrosomes were prepared as above except that the medium contained 10 μg/ml nocodazole (Aldrich Co., Milwaukee, WI) as well as cytochalasin B throughout the procedure. After enucleation the coverslips were placed cell-side up in 24-well plates, rinsed five times with fresh media to remove the drugs, and returned to the incubator.

Immunofluorescence: The immunofluorescence procedure was optimized for double staining with the tubulin and centrosome antibodies. Coverslips were dipped once in prewarmed (37°C) PBS to rinse off the residual medium, dunked five times (total of 10 s) in prewarmed 80 mM PIPES, 5 mM EGTA, 1 mM $MgCl_2$, 0.5% Triton X-100, pH 6.8, and fixed in -20°C methanol for 5 min. They were then washed in PBS containing 1% BSA and 0.1% Tween 20 for several minutes at room temperature. The coverslips were subsequently covered with 20 μl of centrosome antibody (38) which was diluted 1/100 in the above buffer and incubated for 20 min. Without rinsing, the coverslips were further incubated for 20 min with 25 μl of previously mixed centrosome and tubulin antibodies, both diluted 1/100 in the buffer. After this incubation the cells were rinsed and covered for 30 min with 25 μl of fluorescein-labeled goat anti-mouse and rhodamine-labeled goat anti-human antibodies, both diluted 1/100. The same procedure was used for the actin-centrosome double staining. The coverslips were rinsed again, including one rinse with 10 μg/ml Hoechst, bisbenzimide, and mounted on slides in freshly prepared 90% glycerol, 0.1 M $NaHCO_3$, 2% propylgallate (13). In some experiments cells were directly fixed by plunging the coverslips into methanol at -22°C for 5 min after a brief rinse in PBS. The cells were photographed using a Zeiss photomicroscope III and Kodak Tri-X films developed in Diafine.

Antibodies: The monoclonal antitubulin, DM1 anti-α, was a generous gift from S. Blose at Cold Spring Harbor. The anticentrosome serum was a human serum from a patient with linear scleroderma (38). The rabbit antiactin antibody was prepared against frog skeletal muscle actin (15). Fluorescein-labeled goat anti-mouse and rhodamine-labeled goat anti-rabbit and human IgG were obtained from Cappel Laboratories (Cochranville, PA).

RESULTS

Preparation of Cytoplasts with and without Centrosomes

Several laboratories (14, 24, 31, 33, 41, 42) have developed a technique for the efficient enucleation of mammalian cells by centrifugation in the presence of cytochalasin B. Zorn et al. (42) and others have reported that centrosomes are not removed with the nucleus but remain in the cytoplasts; the cytoplast is defined as the cell remnant containing all the normal cytoplasmic constituents except the nucleus (33, 41). Fig. 1A shows a phase-contrast image of an L929 cytoplast prepared in such a manner. Except for the absence of a nucleus, this cytoplast looks similar in size and morphology to normal cells. Fig. 1B shows an immunofluorescent micrograph of the same cytoplast stained with a human autoantibody specific for pericentriolar material (6). Almost all of these cytoplasts contain a fluorescent spot near the center of the cell, which often appears double (see also reference 19). Electron microscopy demonstrates that the antigen is localized on material surrounding the centrioles and is associated with centriolar satellites (6). L929 cells that have not been enucleated each contain a perinuclear spot stained with the anticentrosome antiserum (19).

We speculated that centrosomes might be held in the cytoplasts by the interphase microtubule network and that it should be possible to produce cytoplasts without centrosomes by first depolymerizing the microtubule network with antimicrotubule drugs. Consequently L929 cells were enucleated in the presence of both cytochalasin B and nocodazole, a drug which is similar to colchicine in its action on microtubules but which can be removed more rapidly from the cells (8, 18). After varying the time of drug treatment and the time of centrifugation, we found conditions that resulted in centrosome removal in ~30% of the cytoplasts. Such a preparation of cytoplasts is shown by phase-contrast microscopy in Fig. 1C and by immunofluorescence after staining with the anticentrosome antiserum in Fig. 1D. Cytoplasts with or without centrosomes can be unambiguously distinguished. Careful examination shows that there is no obvious difference in size between those cytoplasts containing a centrosome and those that do not. In addition, another cytoplasmic marker, the amount and distribution of actin as determined by immuno-

fluorescence, was also rather uniform irrespective of whether the cytoplasts did or did not have centrosomes (Fig. 1, E and F). The actin staining is diffuse in these cells, which have few stress fibers. Actin also is found in short spikes associated with membrane processes. Whole L929 cells display a similar actin pattern (data not shown).
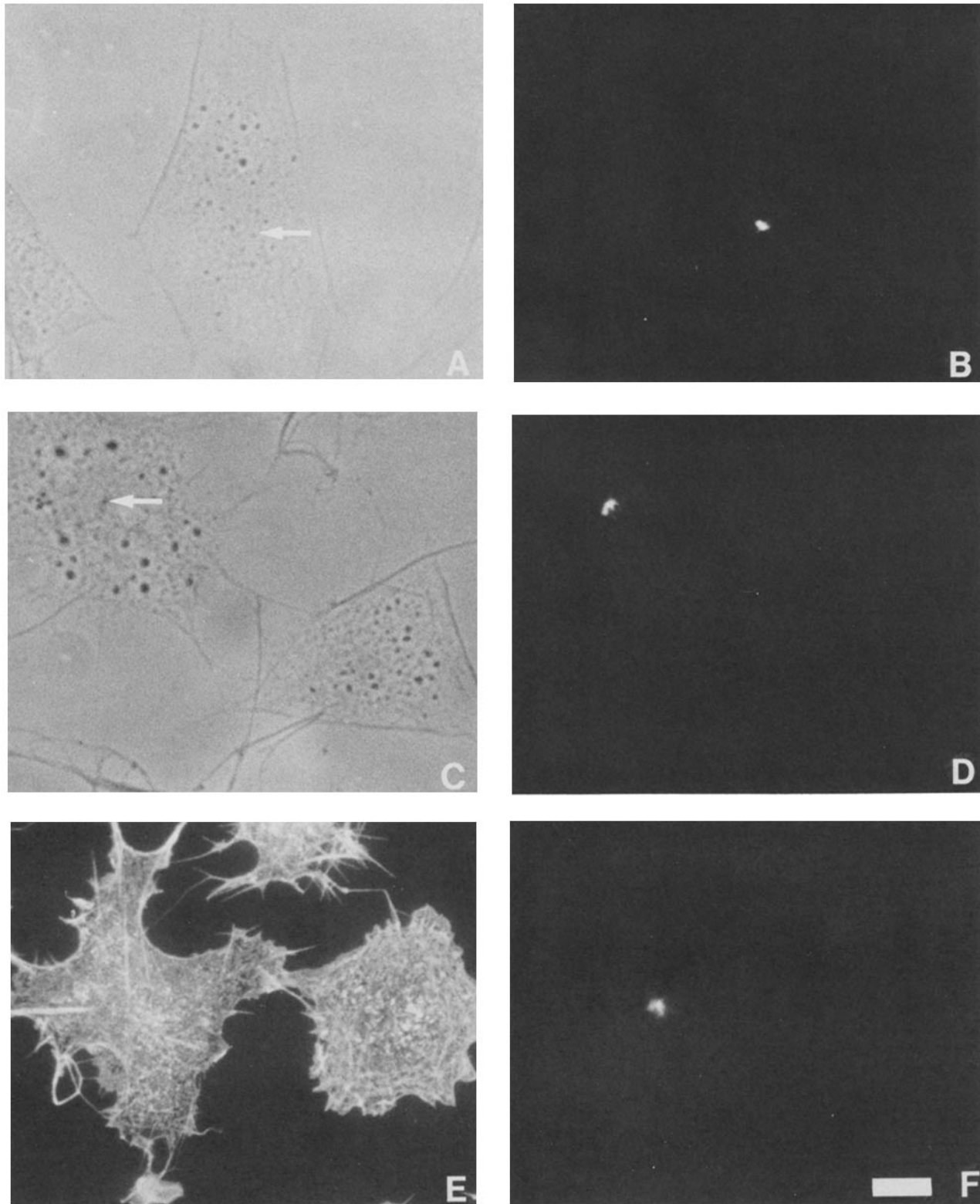

FIGURE 1   Cytoplasts with and without centrosomes. (A and B) Cytoplasts prepared by enucleation in the absence of nocodazole and fixed 18 h later: (A) Phase contrast; (B) immunofluorescence of the same sample as A with anticentrosome antibody. (C–F) Cytoplast prepared by enucleation in the presence of cytochalasin B and nocodazole and fixed 18 h later: (C) Phase contrast; (D) immunofluorescence of the same sample as C with anticentrosome antibody; (E) immunofluorescence with antiactin antibody (fluorescein channel); (F) same sample as E stained with anticentrosome antibody (rhodamine channel). Bar, 10 μm. × 1,000.

Microtubule Distribution in Cytoplasts with and without Centrosomes

CYTOPLASTS PREPARED FROM NONCONFLUENT CELLS: We will first examine the organization of microtubules in cytoplasts prepared from nonconfluent cells that have been allowed to recover for 18 h in drug free medium after enucleation. The microtubule network in cytoplasts with cen-

trosomes (Fig. 2, *A* and *B*) is very similar to that seen in whole cells. When cytoplasts are prepared with nocodazole, as shown above, a mixture of cytoplasts with and without centrosomes results. Before removal of nocodazole no microtubules are detected. However, when microtubules are allowed to regrow in the absence of nocodazole, two patterns of microtubule regrowth are observed, which generally correspond to the presence and absence of centrosomes. The extent of micro-
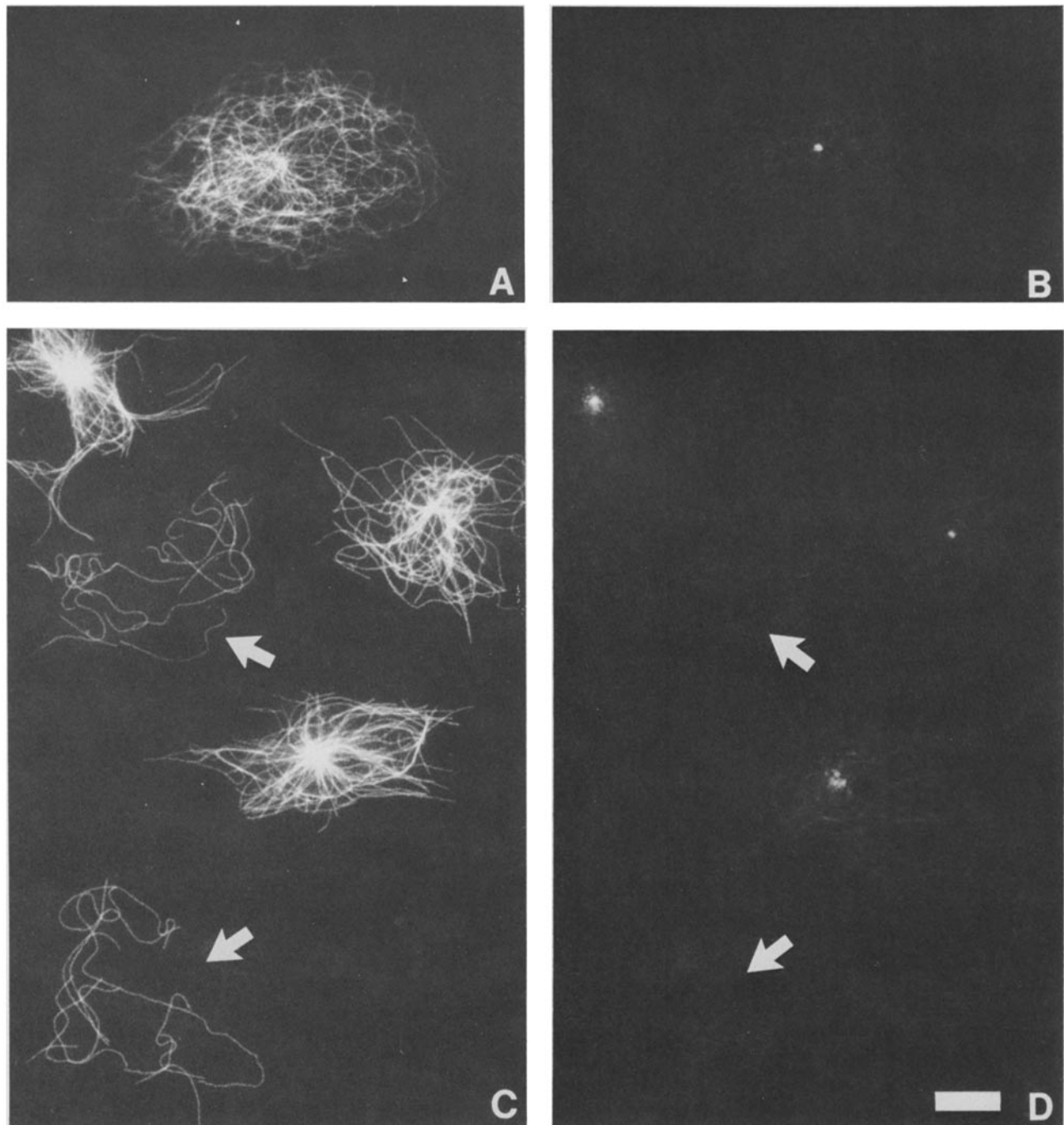

FIGURE 2   Effect of centrosome removal on the microtubule patterns in cytoplasts prepared from cells plated at low density. Indirect double immunofluorescence using a monoclonal antitubulin antibody and a human anticentrosome auto-antibody. The cytoplasts are fixed 18 h after enucleation. (A) Typical microtubule pattern in a cytoplast prepared by enucleation in the presence of cytochalasin B alone. (B) Centrosome visualization in the same cytoplast as in A. (C) Microtubule pattern in cytoplasts prepared by enucleation in the presence of cytochalasin B and nocodazole. Arrows show cytoplasts lacking centrosomes (deduced from Fig. 2D). (D) Centrosome visualization in the same cytoplasts as shown in C. Arrows show the cytoplasts lacking centrosomes. Bar, 10 μm. × 1,000.

tubule regrowth in cytoplasts with centrosomes (Fig. 2, C and D) is very similar to that in the original whole cells. Most microtubules seem to arise from the centrosome and this can be visualized even more clearly in the flat cytoplasts. However, in cytoplasts without centrosomes a dramatic difference is observed in the microtubule arrays (also shown in Fig. 2, C and D). In most of these cytoplasts very few microtubules are present (Table I). Typically, some (approximately 10) long microtubules regrow randomly in these cytoplasts without an apparent organizing site. In some cytoplasts without centrosomes, however, a dense network of peripheral microtubules is also present. The significance of this is described in the following section.

The observed microtubule distribution seems to be a stable rather than a kinetically achieved pattern since similar results are observed in cytoplasts fixed at various time points between 30 min and 18 h after enucleation. Also, we have verified that the drug treatment alone does not induce any permanent modification of the microtubule pattern. Whole L929 cells treated with cytochalasin B and nocodazole but without centrifugation, which are allowed to recover for 30 min or longer, display a general pattern of microtubules similar to what is found in untreated cells (Fig. 3).

It seemed possible, though unlikely, that the cytoplasts without centrosomes had somehow lost much of their tubulin during the enucleation procedure, leading to the diminished regrowth. To address this possibility we incubated cytoplasts for 18 h in 5 μM taxol, a drug which induces spontaneous microtubule assembly by decreasing the critical concentration for polymerization (28). After treatment with taxol all cytoplasts fill up with an extensive mass of microtubules, often in the form of bundles (Fig. 4A). This is the same as the response

of whole cells (29). Cytoplasts containing a centrosome can again be identified with the anticentrosome antibody (Fig. 4B). In comparing the extent of microtubule assembly induced by taxol in cytoplasts with and without centrosomes, we found no obvious difference. Thus one cannot explain the reduced microtubule polymerization in cytoplasts without centrosomes by a reduced content of tubulin.

In all of these experiments we have extracted the cells with Triton X-100 before fixation with methanol. This eliminates the background produced by the presence of unpolymerized tubulin. Although this procedure produces especially clean microtubule patterns, there is a danger that some microtubules could be depolymerized during the extraction process.

TABLE I

Microtubule Distribution in Cytoplasts with and without Centrosomes

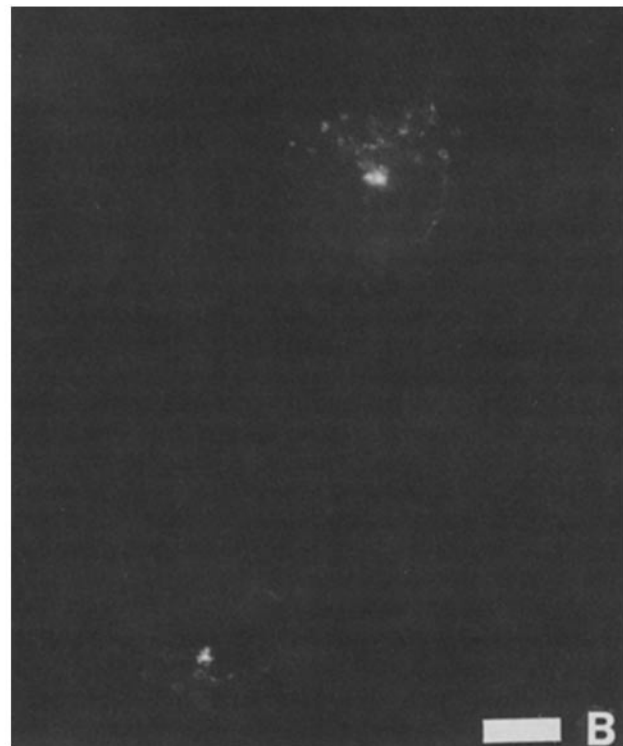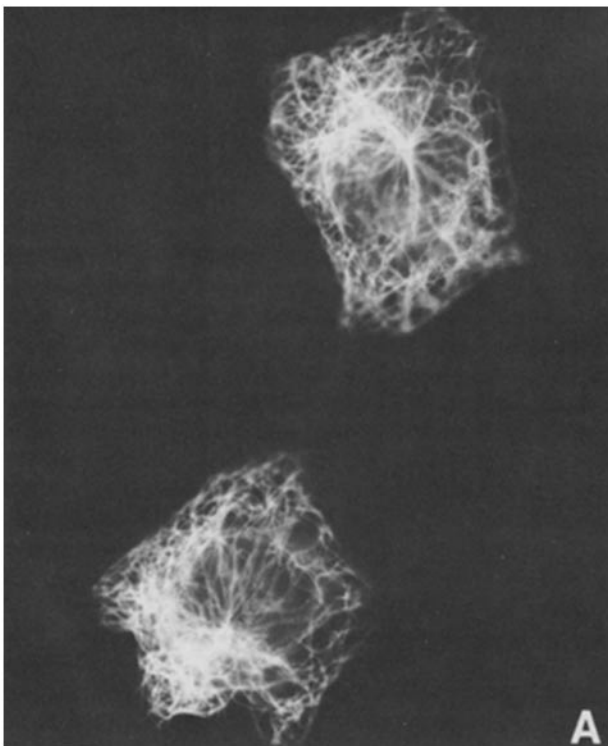| Culture conditions	Time of microtubule regrowth	Cytoplasts without centrosomes	Cytoplasts with less than 20 microtubules	
			Without centrosomes	With centrosomes
	h	%	%	%
Sparse	0.5	34	84	0
	18	ND	83	0
Confluent	18	39	0	0
Confluent (+ 0.1 μg/ml nocodazole)	18	28	100	0

FIGURE 3   Microtubule network of cells that have been incubated in cytochalasin B and nocodazole for 1.5 h and further incubated for 18 h in normal medium. Fluorescence microscopy. (A) Microtubule staining; (B) centrosome staining of the same cells as shown in A. Bar, 10 μm. $\times$ 1,000.
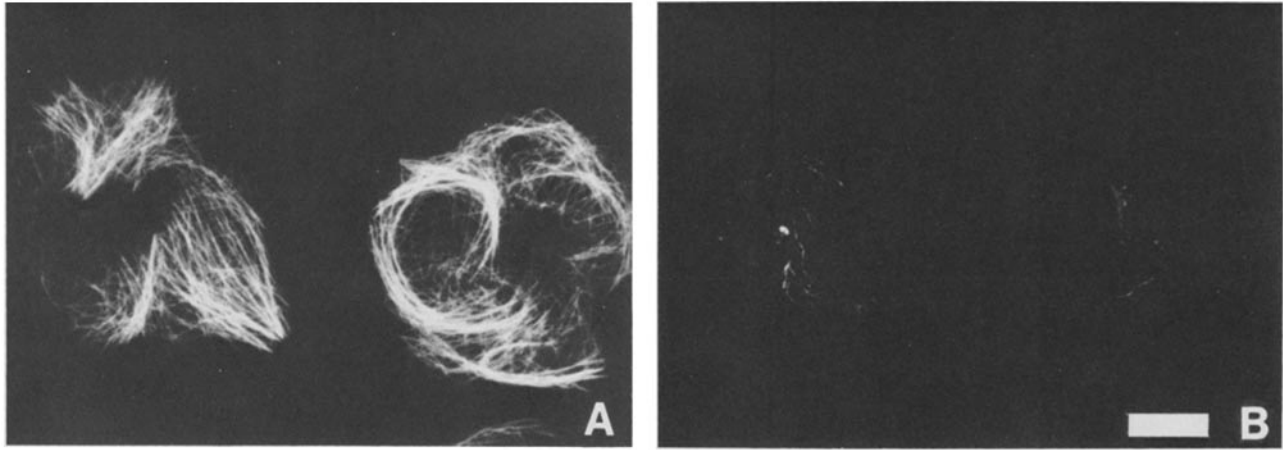
FIGURE 4 Microtubule pattern of nonconfluent cytoplasts prepared using cytochalasin B and nocodazole and further incubated in 5 µM taxol for 18 h after enucleation. Fluorescence microscopy. (A) Microtubule staining; (B) centrosome staining of the same cytoplasts as in A. The cytoplast on the right has no centrosome. Bar, 10 µm. × 1,000.

To address this possibility, we have examined the microtubule pattern in cytoplasts directly fixed with methanol for 5 min at −20°C. As expected, a higher fluorescent background is observed. Nevertheless, as in previous experiments, cytoplasts devoid of centrosomes generally had few unorganized microtubules, whereas cytoplasts with centrosomes had a centrally organized and much more extensive microtubule array.

CYTOPLASTS PREPARED FROM CONFLUENT CELLS: The striking difference in the extent of microtubule regrowth between cytoplasts containing centrosomes and those that do not is only evident from cytoplasts made from cells grown at nonconfluent density. Under confluent conditions a different pattern is observed and the distinction between cytoplasts with and without centrosomes is much less pronounced. As shown in Fig. 5, C and D, at confluent cell density, cytoplasts lacking centrosomes have extensive microtubule arrays, hardly distinguishable from cytoplasts in the same preparation having centrosomes, or cytoplasts prepared with centrosomes (Fig. 5, A and B). The number of cytoplasts without centrosomes having only a few random microtubules drops from 80% under sparse conditions to 0% under confluent conditions, whereas the overall yield of cytoplasts without centrosomes is not affected by cell density (Table I).

There is a small but real difference in the spatial distribution of microtubules in cytoplasts with and without centrosomes grown at high density, even though the mass of polymer is similar. In cytoplasts without centrosomes, the microtubules appear to be mostly peripheral. In cytoplasts with centrosomes, in addition to the peripheral microtubule, there are some that arise from a focus. Surprisingly most microtubules in cytoplasts with centrosomes do not seem to arise from the centrosome under these conditions.

Does this nonfocused distribution of the majority of microtubules in confluent cytoplasts reflect the situation in untreated cells or is it due to some aberrant pathway of assembly in cytoplasts? The microtubules in whole cells cannot be seen as clearly as they can in the very flat cytoplasts so it is impossible to answer the question unequivocally. However, we have found that cytoplasts whose microtubule arrays have not been depolymerized possess the same nonfocused microtubule distribution. In Fig. 5A confluent cells have been enucleated with cytochalasin B but not exposed to nocodazole. All cytoplasts have centrosomes but again they all possess

extensive nonfocused arrays. We have also examined the microtubule network during the enucleation procedure as the cells are being spun. Cells at intermediate stages of enucleation are partly distorted but the microtubule network is not strongly affected and the centrosome usually occupies its central position in the cell (data not shown). Therefore, we believe it is likely that the nonfocused microtubule array in cytoplasts reflects the situation in intact confluent cells.

Effect of Low Nocodazole Concentrations on the Regrowth and Stability of Centrosomal and Noncentrosomal Microtubules

The effect of nocodazole on microtubule regrowth and stability at steady state was studied in cytoplasts prepared from confluent and nonconfluent cells. We will only describe the results obtained in confluent cells, since the results obtained for nonconfluent cells are basically similar. We incubated cytoplasts in various concentrations of nocodazole during recovery from enucleation and compared the pattern of microtubules in cytoplasts containing a centrosome with those that did not. In the presence of 0.1–0.2 µg/ml nocodazole most of the noncentrosomal microtubules fail to regrow in cytoplasts with centrosomes, while the centrosome-bound microtubules reappear (Fig. 6, A and B). In cytoplasts without centrosomes a few microtubules reappear (Fig. 6C). In addition, whole cells treated with 0.1 µg/ml nocodazole also contain a simplified pattern of microtubules, originating mostly from the centrosome (Fig. 6, D and E), confirming observations previously reported (8).

When the nocodazole concentration is raised to 0.5 µg/ml, centrosomal microtubules shorten and become less numerous. In cytoplasts devoid of centrosomes, only two to five very short microtubules could be found on the average. At 1 µg/ml only a few (sometimes only one), usually very short microtubules, regrow from the centrosome. No free microtubules were found.

In another series of experiments the response of a complete microtubule array to various concentrations of nocodazole was studied. Cytoplasts obtained by enucleation in the presence of cytochalasin B and nocodazole (10 µg/ml) were allowed to recover overnight in the absence of drugs. This reconstituted the whole microtubule network. Nocodazole
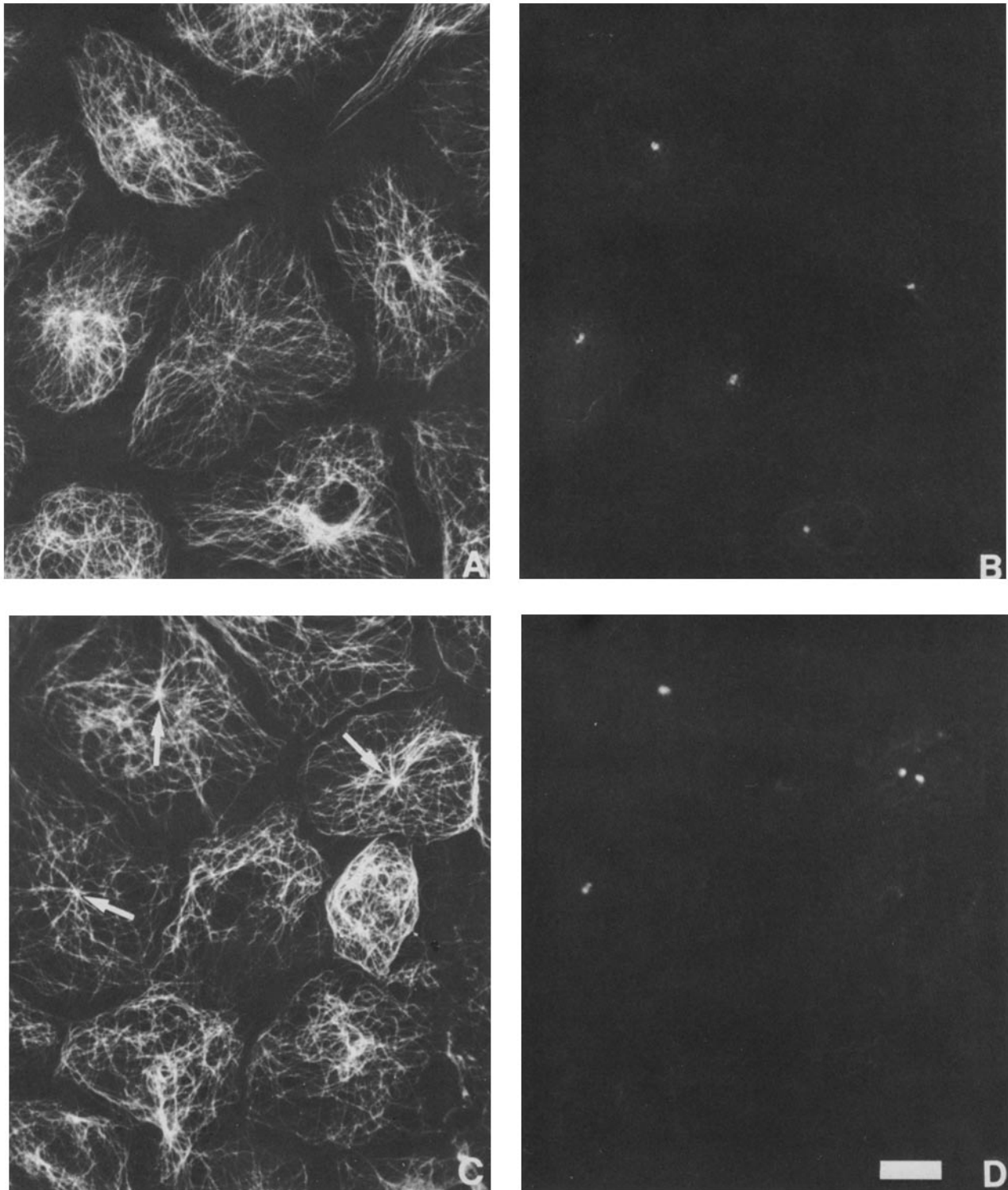
FIGURE 5 Effect of centrosome removal on the microtubule pattern of cytoplasts prepared from confluent cells. Cytoplasts were fixed 18 h after enucleation. (A) Microtubule staining of cytoplasts prepared by enucleation in the presence of cytochalasin B alone and cultured for 18 h in drug-free medium. (B) Centrosome staining of the cytoplasts shown in A. (C) Microtubule staining of cytoplasts prepared in the presence of cytochalasin B and nocodazole and cultured for 18 h in drug free medium. Arrows show cytoplasts with centrosomes. (D) Centrosome staining of the cytoplasts shown in C. Note that the cell on the right has a split centriole, with only one nucleating many microtubules (see C). Bar, 10 μm. X 1,000.

was then added at the concentrations of 0.1 and 0.2 μg/ml and the cytoplasts were observed 2 h later. The results obtained (data not shown) were identical to those obtained by regrowth of microtubules in the presence of 0.1–0.2 μg/ml nocodazole (e.g., see Fig. 6, A–C). These experiments show that without the centrosome, very few microtubules can either regrow or are stable in the presence of 0.1–0.2 μg/ml nocodazole. However, under these conditions, many microtubules
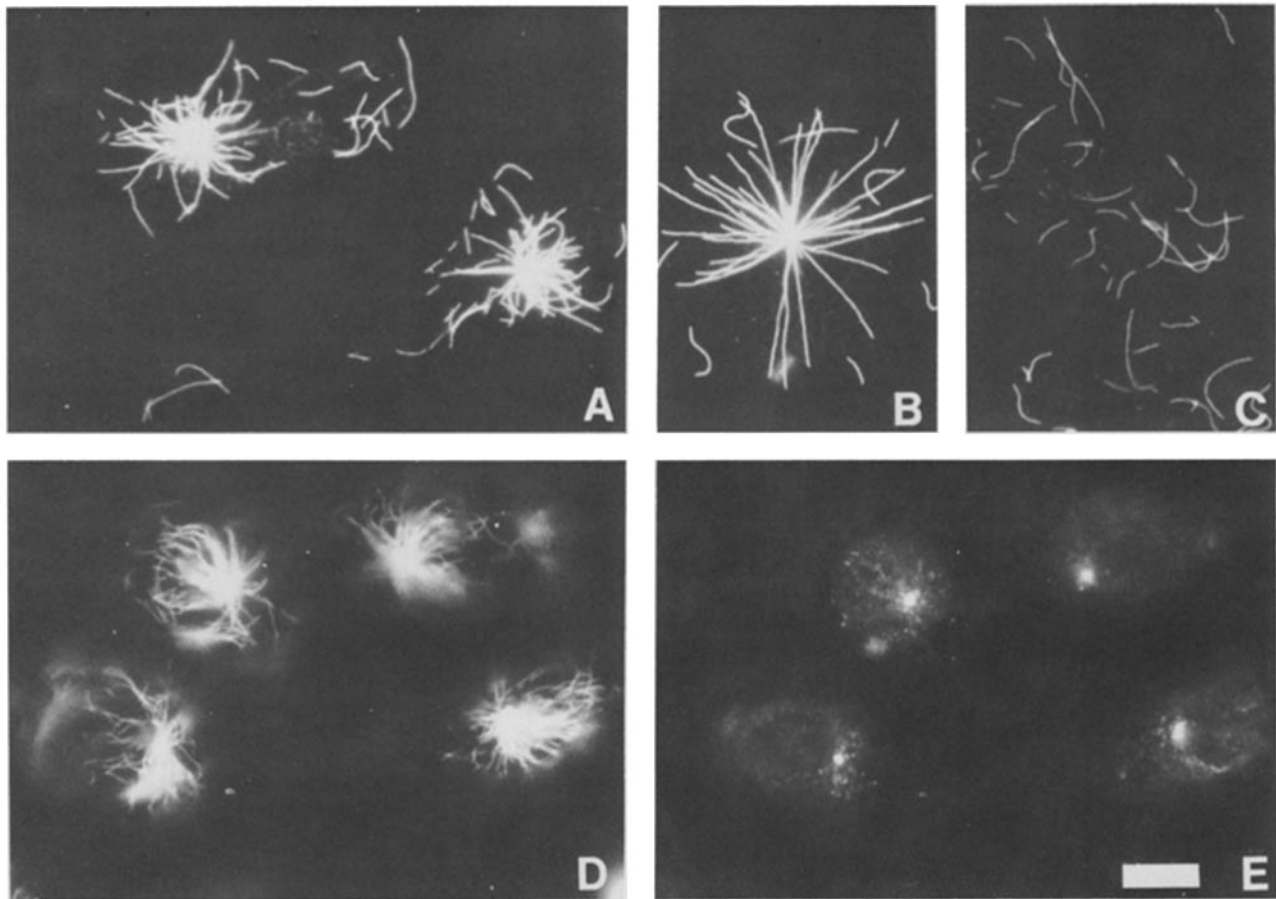
FIGURE 6 Regrowth of centrosomal and noncentrosomal microtubules in 0.1 µg/ml nocodazole. In A–C, cytoplasts were prepared from confluent cells in the presence of cytochalasin B and nocodazole (10 µg/ml). After a 2-h incubation in 0.1 µg/ml nocodazole they were stained with antibodies to tubulin and centrosomes. Only tubulin staining is shown in A–C. (A) Typical microtubule pattern. Many microtubules regrow from the centrosome. Their length is heterogeneous. Very few free microtubules are visible in the cells. (B) Occasional figure found in the cytoplast population. In this cytoplast, about 32 very long microtubules nucleated by the centrosome could be counted. (C) Two cytoplasts lacking a centrosome are shown. A few, randomly organized microtubules are formed. Their length is variable. (D) Cells preincubated in cytochalasin B and nocodazole (10 µg/ml) for 1.5 h, further incubated for 2 h in 0.1 µg/ml nocodazole, and fixed. Microtubule staining: most microtubules originate from the centrosome. Because of the thickness of the nucleus, the whole cell cannot be brought to focus at the same plane. (E) Centrosome staining of the cells shown in D. Bar, 8 µm. × 1,300.

arise from the centrosome. The average number and length of these centrosomal microtubules is stable.

Kinetics of Centrosomal and Noncentrosomal Microtubule Regrowth In Vivo

When one examines the kinetics of microtubule regrowth in cytoplasts, the distinction between centrosomal and noncentrosomal microtubules is clearly apparent. If cytoplasts are allowed to recover in drug-free media for 2 min, almost no regrowth is observed. At 3 min, many very short microtubules start to grow from the centrosomes. In some but not all cytoplasts, both with and without centrosomes, a few short peripheral microtubules are visible as well (Fig. 7A). At 5 min, centrosome microtubules are very extensive while the number and length of peripheral microtubule polymerization is variable from one cytoplast to another. A typical image is shown in Fig. 7B. By 7 min after drug removal, centrosomal microtubules have almost reached their final length and number (Fig. 7C). Still very few peripheral microtubules have formed. In centrosome-free cytoplasts the final pattern of peripheral growth is only reached at 30 min. Although the

kinetics of regrowth seem heterogeneous in this cytoplast population, the growth of microtubules attached to the centrosome is always favored over the peripheral microtubules, suggesting that these latter microtubules may not be nucleated by the same mechanism. In an additional interesting observation, most cytoplasts have split centrosomes when examined a short time after nocodazole removal, as has been previously reported for whole cells (34). One of the two centrioles always seems to nucleate more microtubules than the other (Fig. 7), as has been observed in polymorphonuclear leucocytes with split centrosomes (30).

Microtubule Patterns in Putative Daughter Cells

We have found it useful to compare centrosomal and noncentrosomal microtubule arrays in cytoplasts with similar morphology. Occasionally, cells can be seen that lie close to each other and have a rough mirror symmetry, in agreement with the observations of Albrecht-Buehler (1) and Solomon (35). We may surmise that these are sister cells, and some retain a cytoplasmic bridge, although proof would require some record of their previous history. The cytoplasts of such
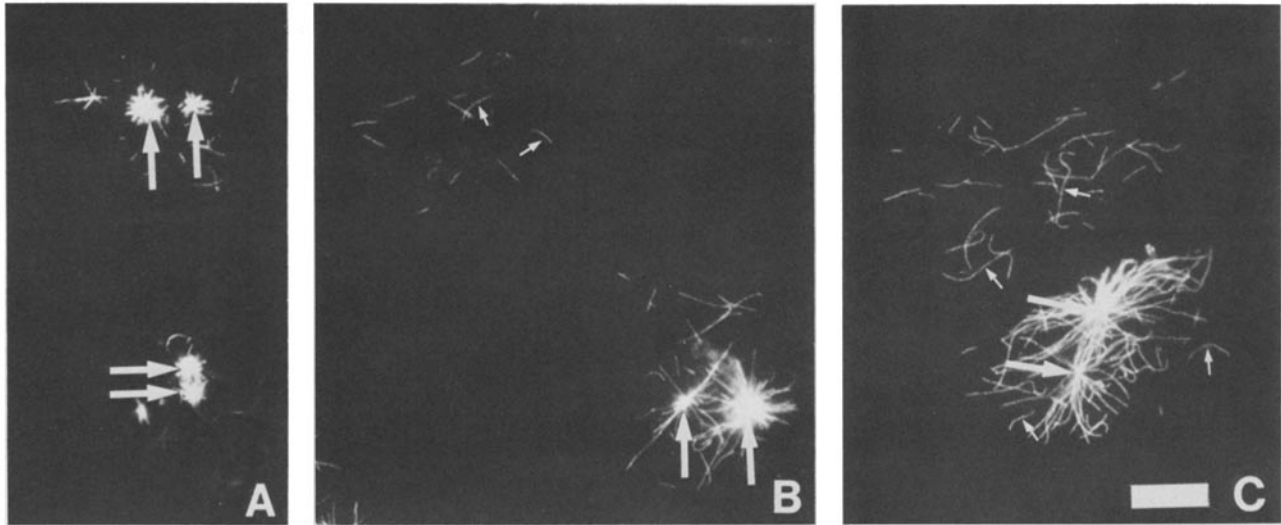
FIGURE 7 Kinetics of microtubule regrowth in cytoplasts with and without centrosomes. Cells were enucleated in the presence of cytochalasin B and nocodazole and allowed to recover from enucleation for 1 h in 10 µg/ml nocodazole without cytochalasin B. The cytoplasts were fixed and stained at the indicated time after transfer to regular medium. Microtubules are visualized; centrosome staining was done but not shown. (A) 3 min after nocodazole removal. Two cytoplasts are shown. The centrosome is split in both (arrows). Very short microtubules scattered in the cytoplast are also visible (mainly in the cell at the top of the figure). (B) 5 min after the nocodazole removal. Cell at the bottom of the figure has a split centrosome (large arrows). Cell at the top has no centrosome. Short free microtubules are visible (small arrows). (C) 7 min after nocodazole removal. Cell at the bottom has a split centrosome (large arrows). Cell at the top has no centrosome. Centrosome microtubules have almost reached their full length. Some free microtubules could be seen in the cytoplasts with and without centrosomes (small arrows). Bar, 10 µm. × 1,000.

"sister cells" retain their shape and relative orientation. This is not surprising since Solomon has shown that the daughter cell symmetry is preserved through microtubule depolymerization and regrowth (35).

On coverslips seeded at low density and cultured for 48 h before enucleation, it was easy to find two closely apposed cytoplasts with similar overall shape. Even if, in all cases, they are not sisters, a comparison of the microtubule distributions in these similar cytoplasts is instructive. This is illustrated in Fig. 8. The centrosome staining is not shown. In Fig. 8 A are two cells having no centrosomes. Both cells contain six to eight microtubules. Fig. 8 B shows another two cytoplasts lacking centrosomes but possessing an extensive network of peripheral microtubules. In Fig. 8 C a pair of cytoplasts, one possessing (above) and one lacking (below) a centrosome, is shown. The cytoplast lacking a centrosome has only peripheral microtubules; the cytoplast with a centrosome has superimposed on the peripheral array a centrally nucleated array. Fig. 8 D shows two cytoplasts each possessing a centrosome. Both cytoplasts have peripheral as well as centrally nucleated microtubules. These observations strongly suggest that centrosomal and noncentrosomal microtubules can occupy spatially distinct positions in the same cell.

Centrosomal and Noncentrosomal Microtubules in Whole Cells

We have documented that centrosomal and noncentrosomal microtubules can co-exist in cytoplasts. To determine if this were also the case in nucleated L929 cells, we have observed their microtubule patterns in relation to the localization of the centrosome.

In cells seeded at low density, the microtubule network of most cells is clearly focused on the centrosome (Fig. 9, A and B). In some cells, however, it is not (Fig. 9, C and D). At

confluency, all the microtubules do not seem focused on the centrosome in any cell (Fig. 9, E and F). This impression is supported by the observation of confluent cells treated by 0.1 µg/ml nocodazole (Fig. 6, D and E). In this case, microtubules originate only from the centrosome and the appearance of the network is different from what is observed in Fig. 9 E.

DISCUSSION

Microtubule organizing centers, such as centrosomes in interphase and mitotic cells, are believed to be important in inducing the polymerization of microtubules and in anchoring them in specific locations. In this paper we have observed what happens when this organizing center is removed from a mouse fibroblast cell. The nucleus as well as the centrosome can be removed from the cell by centrifugation in the presence of cytochalasin B and nocodazole. Although removal of the nucleus may have some unknown effects on the cytoskeleton, the resulting cytoplasts seem to have a normal microtubule distribution, while the flatness of the cytoplasts makes the microtubule arrays more easily visualized than in whole cells. However, this procedure is only partially effective in removing centrosomes. Even though the entire microtubule array is depolymerized in all cells by the nocodazole treatment, only 30% of the cells are actually freed of centrosomes. The cytoplasts containing centrosomes could be identified unambiguously with a human autoimmune serum that reacts specifically with centrosomes (38). The cytoplasts, though distinguishable by the presence or absence of the centrosome, were similar in their overall size and shape, actin content, or content of polymerizable tubulin.

We have used these cytoplast preparations to study the characteristics of tubulin polymerization in vivo when the centrosome is present and when the centrosome is absent. The results obtained help define the role of the centrosome
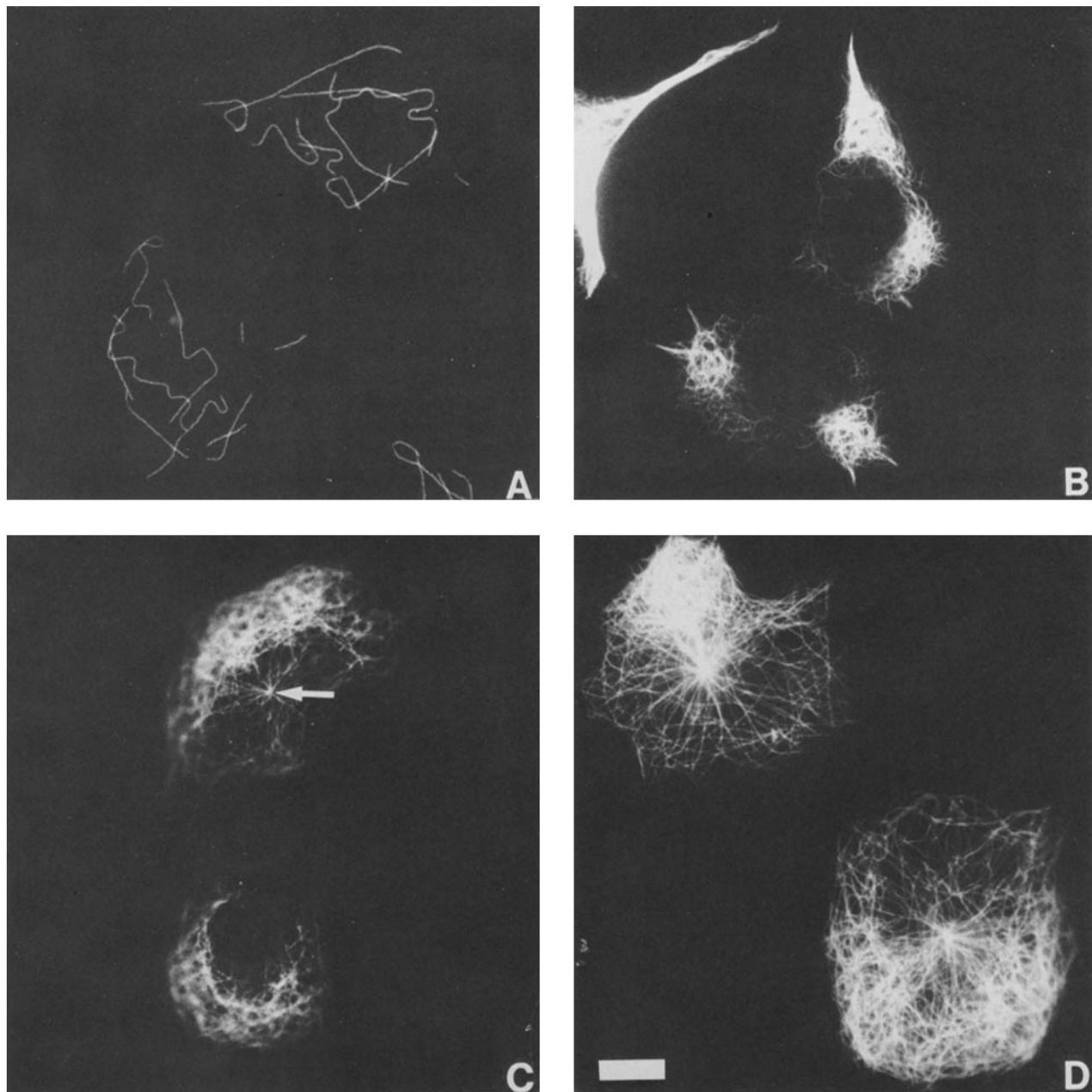
FIGURE 8 Microtubule patterns in cytoplasts made from putative daughter cells. Cells were seeded at a very low density and were cultured for 48 h before enucleation. Cytoplasts were prepared in the presence of cytochalasin B and nocodazole. Microtubule regrowth was carried out overnight in normal medium. Microtubule staining by immunofluorescence microscopy; centrosome staining was done but not shown. (A) Example of two cytoplasts without centrosome both containing very few microtubules. (B) Two cytoplasts lacking a centrosome containing a peripheral network of microtubules. (C) Two cytoplasts in which one lacks the centrosome (bottom) but still contains a peripheral network of microtubules and the other (top) contains the centrosome (arrow). (D) Two cytoplasts containing a centrosome with both peripheral and central microtubules. Bar, 10 μm. × 1,000.

FIGURE 9 Microtubule patterns found in whole cells cultured at low and high cell density. Microtubule and centrosome staining. (A) Tubulin staining of a cell at low cell density where most microtubules seem to originate from the centrosome. (B) Centrosome staining of the same cell as in A. (C) Tubulin staining of a typical cell at low cell density where most microtubules do not seem to originate from the centrosome (arrow). (D) Centrosome staining of the cell shown in C, same focal plane. (E) Tubulin staining of confluent cells. The microtubule pattern is similar to C but uniform in all cells. Only a few microtubules seem to radiate from the centrosome (arrow). (F) Centrosome staining of the cells shown in E. In one cell, the centrosome is out of focus (arrow). Bar, 10 μm. × 1,000.
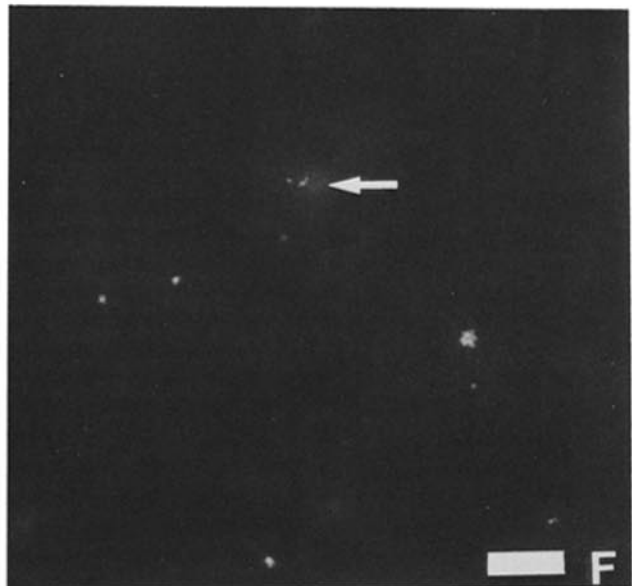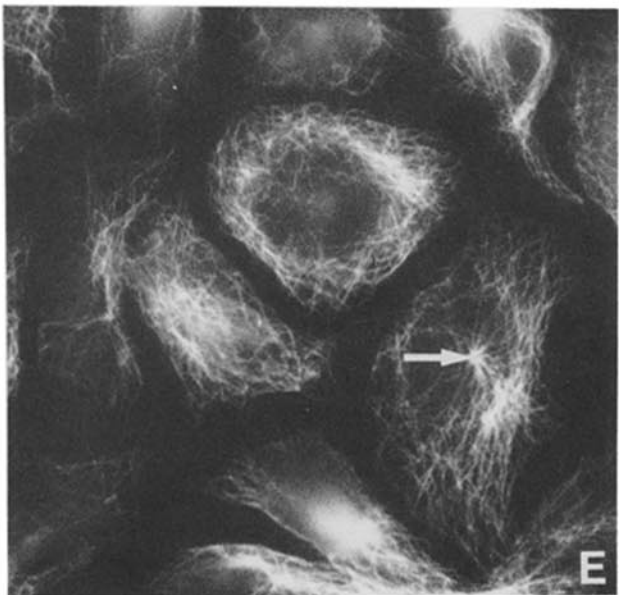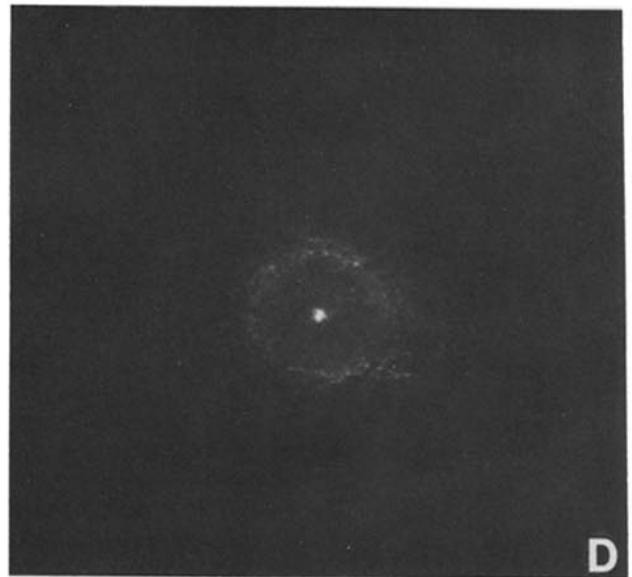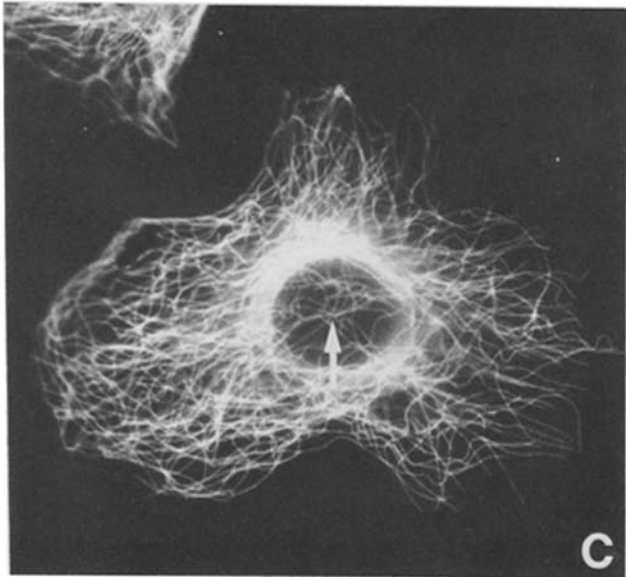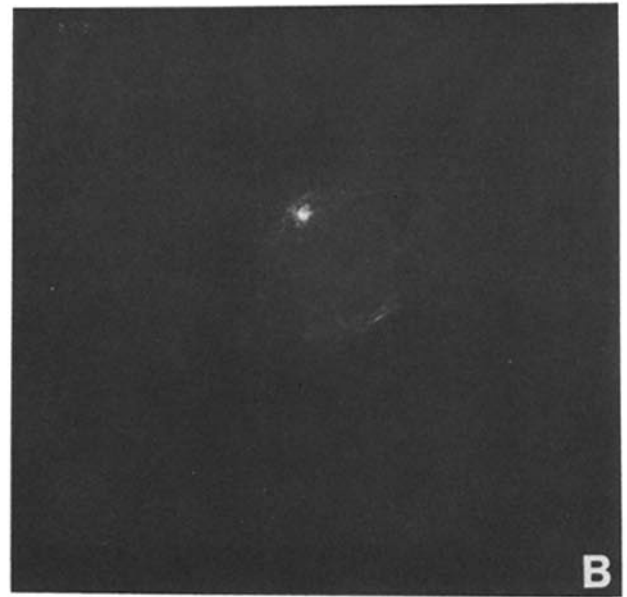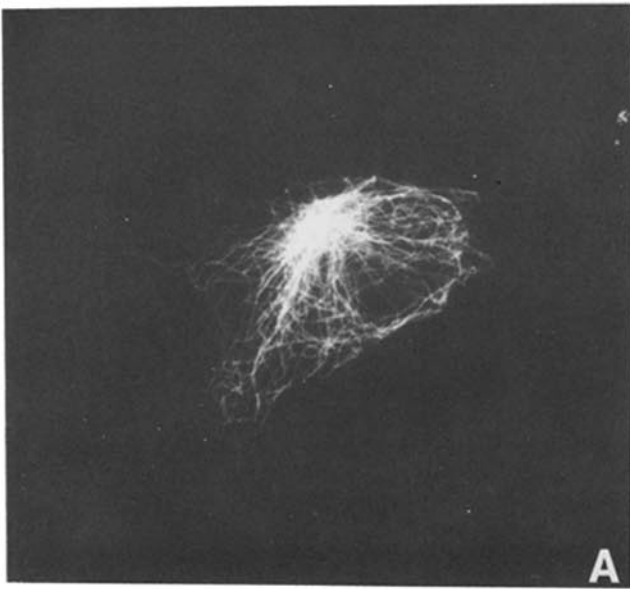
in nucleating and organizing the interphase microtubule array. Specifically, the following questions have been addressed: (a) Do microtubules regrow in cytoplasts devoid of centrosomes? (b) Do centrosomal microtubules co-exist with noncentrosomal microtubules? (c) How does the centrosome nucleate microtubules? .

Although previous reports have suggested that microtubules regrow from the centrosomes after depolymerization by cold or drugs (3, 5, 11, 23), Albrecht-Buehler (2) has shown that cell fragments without centrosomes do contain microtubules. Moreover, DeBrabander et al. (9) have shown that free microtubules regrow in addition to centrosomal microtubules in early stages after nocodazole reversal in nucleated cells. The results reported here clearly show that microtubules can regrow in cytoplasts devoid of centrosomes.

The generally accepted picture that all of the microtubules in a cell originate from the centrosome is seriously challenged by the cytoplast experiments. Most cytoplasts containing centrosomes do display centrally nucleated microtubule arrays consistent with the accepted picture. When the centrosome is absent in these cytoplasts, the extent of microtubule assembly is greatly diminished. However, in some cytoplasts containing centrosomes from nonconfluent cells and in virtually all of the cytoplasts containing centrosomes from confluent cells, the small central aster of microtubules is superimposed on a larger mass of peripheral microtubules. These are likely to be noncentrosomal, since when the centrosome is missing from confluent cytoplasts the peripheral mass of microtubules is still found. This is especially clear in cytoplasts from putative daughter cells which retain a high degree of similarity in shape. Here, if one cytoplast contains a centrosome, it has both a small aster and a peripheral mass of microtubules, while its companion, having no centriole, retains the peripheral mass (Fig. 8). Basically the same observations can be made for whole cells, where it is often clear that many microtubules are not attached to the centrosome (Fig. 9).

These observations suggest that cells can have both centrosomal and noncentrosomal microtubules. This raises three questions: How does the centrosome affect microtubule assembly? What is the origin of the noncentrosomal microtubules? How can centrosomal and noncentrosomal microtubules co-exist in the same cytoplasm?

In answer to the first question, we have shown that in a given population of sparse cytoplasts, the extent of microtubule assembly depends strongly on the presence of the centrosome. This seems to be the result at steady state, since the fraction of the cytoplasts that display very few microtubules in the absence of the centrosome does not increase between 30 min and 18 h. The diminished number of microtubules in the cytoplasts without centrosomes cannot be due to a lowered concentration of polymerizable tubulin, since taxol can induce a similar extent of polymerization in all cytoplasts.

We can most easily explain this centrosome dependence of polymer formation in sparse cytoplasts in terms of a mechanism of microtubule stabilization involving the capping by the centrosome of the end of the microtubule with the higher critical concentration for assembly. As discussed previously (16, 20), since microtubules appear to have different critical concentrations for polymerization at their two ends, blocking the end with the higher critical concentration with a structure such as the centrosome means that the monomer-polymer equilibrium will be determined by the end with the lower critical concentration. This would lead to an amount of

polymer in the cell greater than can be achieved for microtubules having two free ends. In the absence of the centrosome, the monomer-polymer equilibrium will be determined by a kinetically weighted average of the critical concentration of the two ends. This would lead to decreased polymer formation. If the total tubulin concentration in the cell is just above this average value of the critical concentration for free microtubules, only a small amount of polymer will form. This model predicts what is in fact found in sparse cytoplasts, that in the absence of the centrosome, a stable endpoint is reached where there is much less polymer in the cell. If the total tubulin concentration were below that needed for spontaneous polymerization, cytoplasts without centrosomes would be unable to polymerize any microtubules.

In answer to the second question, as to the origin of the noncentrosomal microtubules, we have two properties to consider. The extent of noncentrosomal microtubules depends on growth conditions (sparse versus confluent) and is differentially affected by nocodazole. It therefore may be that the concentration of tubulin is higher in cells such as confluent cells with extensive noncentrosomal polymerization. (In this context tubulin concentration refers to the overall tendency of tubulin to polymerize, which includes contributions from the actual tubulin concentration, concentration of associated factors, posttranslational modifications of tubulin and factors, and physiological conditions such as the concentration of Ca^{++}, nucleotides, pH, etc.) Since the noncentrosomal microtubules appear under conditions of higher tubulin concentrations, one explanation is that they may arise by spontaneous polymerization. The relative amount of spontaneous polymerization in a cell could be affected by changes in the monomer pool or by any of the several possible changes in microtubule physiology that would result in a change in the equilibrium constant for polymerization. Such changes seem to occur when growth conditions are modified as suggested by our results. This is also suggested by a recent observation made on 3T3 cells (27). In these cells, colcemid eliminates only a fraction of the microtubules and the amount of residual microtubules (mainly centrosomal) seems higher in quiescent than in growing cells.

Are the noncentrosomal polymers truly uncapped? Though their increased sensitivity to nocodazole suggests that they may be, it is also possible that the noncentrosomal microtubules interact with a weak or leaky capping structure that would still allow for some subunit exchange (for a fuller explanation, see references 16 and 17). Such a mechanism would serve to give the noncentrosomal microtubules a spatial organization but still make them especially sensitive to nocodazole. The fact that in many cytoplasts these polymers seem to be preferentially localized at the periphery of the cell may suggest that their location is determined by some specific nucleation mechanism.

As discussed earlier, a closed system at steady state would tend toward the assembly of the most stable polymers and the loss of the less stable ones (16, 20). In particular, where there is a difference in critical concentration for the two ends of a microtubule, an initial mixture of capped and uncapped microtubules should tend to a state where only capped ones remain. Such a succession has been observed in vivo (9). However, the complete replacement of uncapped with capped polymers assumes that the capped microtubules can grow indefinitely long or that there is no control over the number of microtubules that are capped by the centrosome. There are

several nonspecific constraints on polymer growth such as inhibition of assembly by bending and compression (16) as well as by specific interactions. If, as seems reasonable, some limit exists on the extent of centrosomal microtubule growth, free microtubules could co-exist with centrosomal microtubules. In nonconfluent cells, the total tubulin concentration may be low enough or the monomer-polymer equilibrium unfavorable enough that after the capped polymers reach their limit of growth, uncapped microtubules would not be stable.

An alternative explanation for the centrosome-dependent growth of microtubules has been recently proposed by De-Brabander (7). In his view, induction of assembly and anchoring of microtubules can be separable properties of the microtubule organizing centers. Induction of assembly is due to the centrosome being surrounded by a region in which the critical tubulin concentration is lower than elsewhere in the cytoplasm. Once formed, microtubules may become either truly capped for some of them, or partially stabilized for others, at the minus end in any case. Although basically an extension of the simple capping model (20), this model adds a requirement for lowering the critical concentration locally and a notion of heterogeneity for the centrosomal microtubules. These two parameters help to explain the co-existence of centrosomal and noncentrosomal microtubules in certain cells if one assumes that the latter form spontaneously because of a low critical concentration. If the critical concentration is low everywhere in the cytoplasm, the advantage for the centrosome is indeed lost. This would also explain why the number of microtubules emanating from the centrosome seems low in the cytoplasts or cells containing free microtubules (see Figs. 5 and 9), a result similar to what has been observed with taxol (9).

The foregoing discussion makes great use of the concept of critical concentration in explaining the microtubule distribution in cells and cytoplasts. The steady state properties of microtubules, particularly the critical concentration difference between the two ends, is invoked to account for the preferential stabilization of centrosomal microtubules. However, an alternative view of the microtubule network which relies more on kinetic than thermodynamic arguments can also explain the experimental observations. In this view, microtubules are very dynamic structures, continually undergoing polymerization and depolymerization, and immunofluorescence gives a frozen "snapshot" of the situation. The centrosome may act mainly as a catalyst of nucleated polymerization, thus increasing the number of microtubules seen in sparse cytoplasts. The stability of centrosomal and noncentrosomal microtubules might not be differentially nocodazole sensitive, but rather their respective nucleation rates. Nucleation events catalyzed by the centriole might be less concentration dependent than those occurring spontaneously in the cytoplasm, leading to a preponderance of centrosome-derived microtubules under any conditions that lower the effective tubulin concentration.

In summary, the removal of the nucleus from the cell makes it flatter and the microtubule array more easily visualized. Under these conditions, when the centrosome is removed from the cell, its role in microtubule assembly can be clearly tested. However, the data is still insufficient to distinguish between alternate models for the role of the centrosome in nucleating microtubules. Answers to some of the outstanding questions may only come from experiments on nucleated polymerization in vitro in concert with in vivo studies such

as this one. This study does, however, show that under certain conditions the centrosome can affect the extent of microtubule assembly in the cell. It also shows that centrosomal and noncentrosomal microtubules can co-exist in the same cell. The respective biological roles of these different microtubules warrants further investigation.

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