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Polarity Orientation and Assembly Process of Microtubule Bundles in Nocodazole-treated, MAP2c-transfected COS Cells

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Microtubule bundles reminiscent of those found in neuronal processes are formed in fibroblasts and Sf9 cells that are transfected with the microtubule-associated proteins tau, MAP2, or MAP2c. To analyze the assembly process of these bundles and its relation to the microtubule polarity, we depolymerized the bundles formed in MAP2c-transfected COS cells using nocodazole, and observed the process of assembly of microtubule bundles after removal of the drug in cells microinjected with rhodamine-labeled tubulin. Within minutes of its removal, numerous short microtubule fragments were observed throughout the cytoplasm. These short fragments were randomly oriented and were already bundled. Somewhat longer, but still short bundles, were then found in the peripheral cytoplasm. These bundles became the primordium of the larger bundles, and gradually grew in length and width. The polarity orientation of microtubules in the reformed bundle as determined by "hook" procedure using electron microscope was uniform with the plus end distal to the cell nucleus. The results suggest that some mechanism(s) exists to orient the polarity of microtubules, which are not in direct continuity with the centrosome, during the formation of large bundles. The observed process presents a useful model system for studying the organization of microtubules that are not directly associated with the centrosomes, such as those observed in axons.

INTRODUCTION

Microtubules are one of the main cytoskeletal elements in eukaryotic cells. They are particularly abundant in nerve cells, in which they appear in parallel arrays in axons and dendrites. They are composed of microtubules, associated crossbridges, and granular materials (Hirokawa, 1982; Schnapp and Reese, 1982). High molecular weight microtubule-associated proteins (MAPs)[1] (MAP1A, MAP1B, and MAP2) and tau, which copurify with brain tubulin during repetitive cycles of temperature-dependent assembly and disassembly, have been shown to be components of these crossbridges (reviewed in Hirokawa, 1991).

When fibroblasts or Sf9 cells are transfected with tau, MAP2, or MAP2c, microtubule bundles are formed (Kanai *et al.*, 1989, 1992; Lewis *et al.*, 1989; Lewis and Cowan, 1990; Knops *et al.*, 1991; Chen *et al.*, 1992; Weisshaar *et al.*, 1992; Edson *et al.*, 1993; Umeyama *et al.*, 1993). These microtubule bundles mimic the characteristics of parallel long microtubule arrays in the neuronal axon in several important aspects (Kanai *et al.*, 1989; Lewis *et al.*, 1989; Baas *et al.*, 1991; Chen *et al.*, 1992). As shown in Sf9 cells, the bundles are composed of long parallel arrays of microtubules and associated crossbridges (Chen *et al.*, 1992). These mi-

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[1] Abbreviations used: DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; MAP, microtubule-associated proteins; PBS, phosphate-buffered saline; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid).

crotubule bundles resemble those found in axons in terms of increased stability and posttranslational modifications of microtubules (Lewis *et al.*, 1989; Takemura *et al.*, 1992; Umeyama *et al.*, 1993). Another important aspect is that these microtubule bundles are not associated with the traditional microtubule-organizing center, the centrosome, as in the axon (Lewis *et al.*, 1989; Weishaar *et al.*, 1992; Umeyama *et al.*, 1993). In undifferentiated cells such as fibroblasts, on which most studies on the organization and assembly of microtubules have been performed, microtubule nucleation is spatially regulated by the centrosome (reviewed in Brinkley, 1985). Microtubules are anchored to the centrosome at the minus end. In nerve cells, microtubules in the axon and dendrite are not attached to any observable nucleating structure, nor to any other cytological structures that might regulate their polarity orientation (Okabe and Hirokawa, 1988; Baas and Black, 1990; Baas and Joshi, 1992). In Sf9 cell processes, microtubules have a uniform polarity, the plus end distal to the cell body, the same as the axon. It is not known how the organization and polarity of microtubule bundles is formed and maintained, and a good model system for studying the process of the formation of the bundles has until now not been available.

In the present investigation, we show that the assembly of microtubule bundles of uniform polarity could be studied in living cells by using COS cells transfected with MAP2c. When MAP2c is transfected into COS cells, microtubule bundles are formed with particularly high efficiency. We have previously studied the turnover rates and dynamics of microtubules in the microtubule bundles formed in MAP2c-transfected COS cells by photoactivation study (Umeyama *et al.*, 1993). In the present report, the microtubule bundles formed in the MAP2c-transfected cells were depolymerized with nocodazole, and upon its removal the reformation of microtubule bundles was directly observed in living cells by microinjecting rhodamine-labeled tubulin. Thus, this system could be useful in studying the microtubule polymerization not associated with the centrosomes, such as is found in axonal cytoplasm.

MATERIALS AND METHODS

The Observation of the Reassembly of Microtubule Bundles Using Immunofluorescence

COS cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) (Life Technologies, Grand Island, NY). Nocodazole (Janssen Pharmaceutica, Beerse, Belgium) was kept as stock solutions of 2 mg/ml in dimethyl sulfoxide (DMSO). Transfection of MAP2c to COS cells was performed by a calcium phosphate-mediated method using pβact2c, which contains a full length mouse MAP2c cDNA, as described previously (Umeyama *et al.*, 1993). Forty-eight hours post-transfection, cells were treated with 10 μM nocodazole in RPMI 1640 medium supplemented with 10% FBS for 2 h to disassemble microtubule bundles. Then, nocodazole was washed out, and cells were incubated in RPMI 1640 medium supplemented with 10% FBS to allow the reassembly of microtubule bundles.

After the experiments, cells were fixed with methanol at −20°C for 6 min, double labeled with rabbit polyclonal anti-MAP2 and mouse monoclonal anti-tubulin (Amersham, Arlington Heights, IL) followed by FITC- or rhodamine-labeled secondary antibodies as described previously (Takemura *et al.*, 1992), and observed using an Axiovert microscope (Carl Zeiss, Oberkochen, Germany). For staining of the centrosome, human autoantibody, a gift from Drs. Oota and Masuda, the Institute of Physical and Chemical Research (RIKEN), Japan, was used (Moroi *et al.*, 1983)

Microinjection and Observation of the Reassembly of the Microtubule Bundles Using Rhodamine-labeled Tubulin

To observe the reassembly of microtubule bundles continuously in living cells, the cells were first co-transfected with pβact2c and pCDM-L3T4 to identify the transfected cells (Umeyama *et al.*, 1993). Forty-eight hours post-transfection, the living cells were incubated with GK1.5 hybridoma supernatant, and those stained positively were microinjected with rhodamine-labeled tubulin for observation with an Axiovert microscope equipped with a cooled CCD camera (C3640, Hamamatsu Photonics, Hamamatsu, Japan) and an optical disk recorder (RS-92000, Ricoh, Tokyo, Japan) as described previously (Umeyama *et al.*, 1993). Four hours after the microinjection, when microtubule bundles were uniformly labeled with rhodamine-tubulin, the cells were transferred to MEM (Life Technologies) containing 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) and 10% FBS, and the bundles were recorded using the cooled CCD camera. Then the cells were treated with 10 μM nocodazole for 2 h, washed, and observed in MEM containing 15 mM HEPES and 10% FBS on a stage warmed to 37°C.

Transmission Electron Microscopic Observation of Microtubule Bundles

For observation of microtubule bundles by thin-section transmission electron microscopy, cells were permeabilized with 0.2% Triton X-100 in PHEM buffer (60 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES), 25 mM HEPES, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9; Schliwa, 1982) containing 10 μM taxol for 2 min at 37°C, and fixed with 1% glutaraldehyde in PHEM buffer. Bundles were then observed by phase contrast microscopy, recorded, and the cells were marked. After postfixation with 0.15% tannic acid, 1% OsO_4, and 1% uranyl acetate, the cells were dehydrated and embedded in Epon. Silver-gold sections were collected, stained with uranyl acetate and lead citrate, and observed by JEOL1200EX microscope.

To see the relation of the position of the centrosome and the microtubule arrays at the electron microscopic level, sections were cut horizontal to the substrate. The microtubule bundles, the contour of the nuclei, and the boundary of the cell were traced on transparent sheets at a magnification of 2400×. Microtubule arrays were sometimes confirmed with the aid of a 10× magnifying glass. The tracings were overlaid, and the outermost contours of the nuclei, the boundary of the cell, and the recombined arrays of microtubule bundles were drawn on another transparent sheet. Because the COS cell is a large cell, and because not all sections were perfectly extended to the same degree, it was difficult to align all the markers perfectly throughout the cell. Therefore, we limited the purpose to see the approximate relation of the centrosome and the microtubule arrays (mostly to see what fraction of microtubules was directly associated with the centrosome). The overlaying of the sheets was compromised to obtain the maximum alignment of the major markers such as the nuclei, the position of the centrosome,

and the arrays of the microtubule bundles. The recombined tracing was reduced in size and shown in Figure 6.

Microtubule Polarity Analysis

Microtubule polarity orientation was determined using the hook method (Heidemann, 1991). After rinsing briefly with phosphate-buffered saline (PBS), the cells were incubated for 20 min at 37°C with 1% Triton X-100, 0.5% deoxycholate, and 0.2% sodium dodecyl sulfate in a microtubule assembly buffer (0.5 M PIPES, 1 mM $MgCl_2$, 1 mM EGTA, 1 mM GTP, 2.5% DMSO) containing 1.5 mg/ml porcine brain microtubule protein. The cells were then fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.3), postfixed with 0.15% tannic acid in 0.1 M cacodylate buffer for 5 min, with 1% OsO_4 in 0.1 M cacodylate buffer for 10 min, dehydrated through a graded series of ethanol, and embedded in Epon.

For microtubule polarity determination of bundles in cells without nocodazole treatment, the microtubule bundles for sectioning chosen by phase-contrast microscopy after detergent treatment for the hook formation were sketched, marked, and then sectioned perpendicular to their axis. To section the bundles formed after nocodazole treatment, cells were transfected either with pβact2c alone or together with pCDM-L3T4 and microinjected with rhodamine-labeled tubulin 48 h after transfection. After 2–4 h, the pattern of the microtubule bundles was recorded and then the cells were treated with nocodazole as described above. After 2 h, the remaining bundles were recorded, and the cells were washed and incubated in the medium without nocodazole for 1–2 h before the hook formation. After the cells were fixed, the bundles newly formed after the nocodazole treatment, which were not present before the treatment, were chosen and marked for sectioning.

Because consecutive sections showed identical hooking patterns, sections short distances apart were used to show different hooking patterns (usually a few micrometers away). Only the hooking patterns of microtubules that were cut almost perfectly perpendicular to the long axis and those with immediately clear, distinct hooking pattern were scored. The sections were mounted on a tilting holder, and photographs were taken at several different angles for minor corrections of orientation to ensure that the scoring was correct.

Other Methods

To observe the effect of cytochalasin D on the reassembly of microtubule bundles, after 2 h treatment with nocodazole, cells were first treated with 0.5 μg/ml cytochalasin D in RPMI 1640 medium supplemented with 10% FBS in the presence of 10 μM nocodazole for 30 min, washed, and incubated in 0.5 μg/ml cytochalasin D in RPMI 1640 medium supplemented with 10% FBS for 1 h. Cytochalasin D (Sigma Chemical, St. Louis, MO) was kept as a stock solution of 1 mg/ml in DMSO.

For immunofluorescent staining of vimentin, mouse monoclonal anti-vimentin (Amersham) was used. Rhodamine-phalloidin (Molecular Probes, Junction City, OR) was used at 0.2 μM diluted in PBS, after the cells were fixed with 2% paraformaldehyde and 0.1% glutaraldehyde in PBS, permeabilized with 0.1% Triton-X 100 in PBS, and treated with sodium borohydride in PBS and 5% skim milk in PBS.

RESULTS

Reformation of Microtubule Bundles in Nocodazole-treated, MAP2c-transfected COS Cells

As we have previously shown, when COS cells were transfected with pβact2c, which contains a full length mouse MAP2c cDNA, microtubule bundles were formed (Umeyama *et al.*, 1993). Their formation appeared to occur in those cells that expressed an above the threshold level of MAP2c (Lewis *et al.*, 1989) (60–70% of the transfected cells), and the number of bundles observed in these bundle-forming cells usually ranged from 2–18, although the range of the number of bundles per cell varied somewhat from experiment to experiment. When the cells that developed microtubule bundles were treated with 10 μM nocodazole for 2 h, many microtubule bundles disintegrated. When some microtubule bundles still remained, they were usually much more slender and shorter than those observed before the nocodazole treatment. Because it is known that the microtubule arrays reestablish their organization when microtubule-depolymerizing drugs were removed (Osborn, 1976; Gundersen *et al.*, 1987), we thought that we might be able to observe the process of the reassembly of microtubule bundles in these nocodazole-treated, MAP2c-transfected COS cells upon the removal of nocodazole.

When nocodazole was washed away and the cells were incubated in the absence of nocodazole, the reformation of bundles occurred (Figure 1). Their complete reformation was observed after about 1 h of incubation at 37°C, in approximately the same proportion of transfected cells as that possessing microtubule bundles before the nocodazole treatment. There was no significant difference in the number of bundles per cell before the treatment and after the reformation was completed when the same set of experiments were compared. Further, there was no significant difference in the general appearance of the post-reformation bundles.

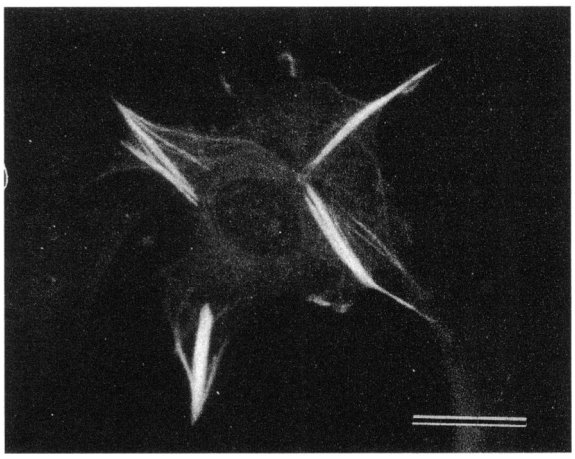

Figure 1. Microtubule bundles reformed in MAP2c-transfected COS cells after nocodazole-treatment. Forty-eight hours post-transfection with MAP2c cDNA, COS cells were treated with nocodazole for 2 h. Nocodazole was then washed away and the cells were incubated for 60 min, fixed with cold methanol, and labeled with anti-MAP2 by indirect immunofluorescence. Bar, 20 μm.

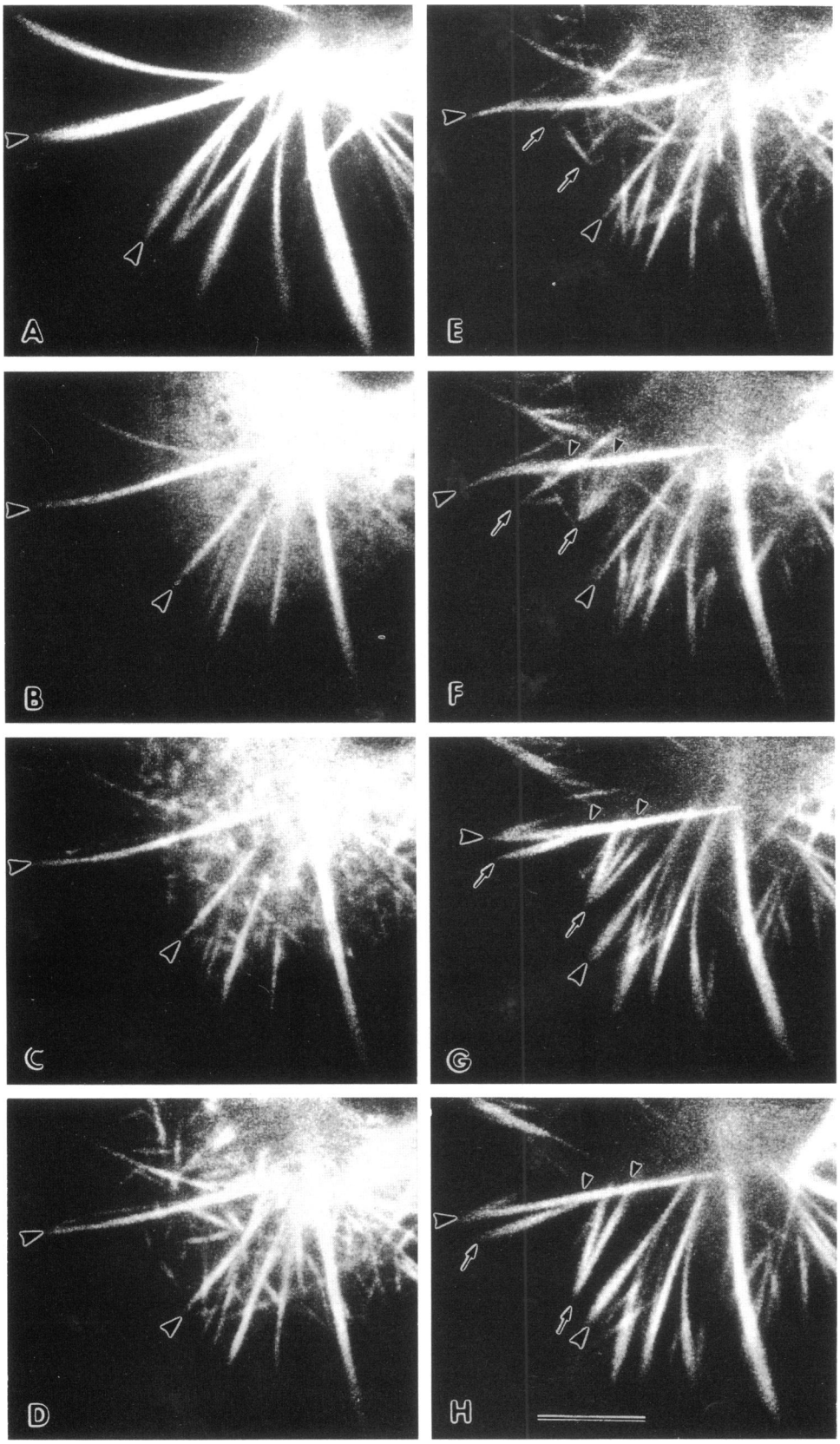

Figure 2. The process of reformation of microtubule bundles observed in living cells. MAP2c-transfected COS cells were microinjected with rhodamine-labeled tubulin, and observed with a cooled CCD camera. A part of a cell, corresponding approximately to the lower left quadrant, is shown. (A) Before nocodazole treatment, 2 h after microinjection, rhodamine-tubulin was evenly incorporated into the preexisting bundles; (B) after nocodazole treatment for 2 h, the microtubules were depolymerized. The cytoplasm was diffusely stained with monomer tubulin. The remaining microtubule bundles were thin and short. (C) Immediately after washing out the nocodazole, the nucleation of tubulin was already apparent in the cytoplasm; (D) 10 min after washing out the nocodazole, short fragments of microtubules were seen in the cytoplasm. Judging from the intensity, in comparison with the neighboring nontransfected cells, the microtubules were already bundled at this stage; (E) 23 min after washing away the nocodazole, the first primordia of the bundles were in place (arrows); (F) 46 min after washing out the nocodazole, the formation of bundles was more evident. Often, two to three short bundles converged at the distal end, later to fuse and form a bundle of larger diameter; (G) 104 min after washing out the nocodazole, the bundles grew in size. Note that the proximal end (arrowheads) did not change position appreciably, while the distal end of the bundles (arrows) was pushed outward. The bundle indicated by the left arrow fused from two to one; (H) 161 min after washing out nocodazole, the bundles had grown further. The bundle indicated by the right arrow fused from three to two. The nucleus of this cell is situated above the upper right hand corner and part of its lower edge is seen as a dark line in the upper right hand corner. Bar, 10 μm.

Process of Reassembly of Microtubule Bundles Observed in Living Cells

We performed video microscopic observation of living cells during the reformation of microtubule bundles in transfected cells. For this purpose, COS cells were co-transfected with pβact2c and pCDM-L3T4, a cDNA of murine T-cell surface glycoprotein (Umeyama *et al.*, 1993). As we have shown previously, the MAP2c-expressing cells can be identified by staining the living co-transfected cells with anti-L3T4 antibody. The cells positively stained with anti-L3T4 antibody were microinjected with rhodamine-labeled tubulin, and the cells were incubated until rhodamine-tubulin was evenly incorporated into the microtubule bundles. Then the cells were treated with 10 μM nocodazole for 2 h, and the nocodazole was washed out. The reformation process of the bundles was recorded using a cooled CCD camera.

Figure 2 shows an example in which the reformation process was followed in several frames until 161 min after washing out the nocodazole. Figure 2A shows the microtubule bundles before the nocodazole treatment. At the end of the 2-h treatment with nocodazole, a lot of free tubulin was observed diffusely distributed in the cytoplasm (Figure 2B). Due to the extremely high efficiency of bundle formation in this particular cell, slender and short microtubule bundles remained. However, the formation of new bundles can be clearly observed in the area between two bundles marked by large arrowheads. Immediately after washing away the nocodazole, the cytoplasm appeared to be different from that observed after the end of the nocodazole treatment, indicating that polymerization had begun at multiple sites (Figure 2C). Interestingly, the initially formed microtubules appeared to be already bundled, judging from the intensity of the filamentous structures in comparison with that of single microtubules in nontransfected cells. Ten minutes after washing away the nocodazole, the thickness of the short fragments of microtubule bundles increased, and the length of the fragments also increased to a few micrometers (Figure 2D). The fragments were apparently of random orientation and were distributed throughout the cytoplasm.

Between 23 and 46 min after washing away the nocodazole, some fragments several micrometers in length became more or less radially oriented in the peripheral cytoplasm at the sites where larger bundles were established at later time points (note arrows in Figure 2, E and F). Therefore, this appeared to be the first step in the formation of stable bundles. It was common to observe that two or three short fragments converged at the distal end.

Once these short bundles were established, they gradually became longer. This elongation appeared to occur primarily in an outward direction. When the

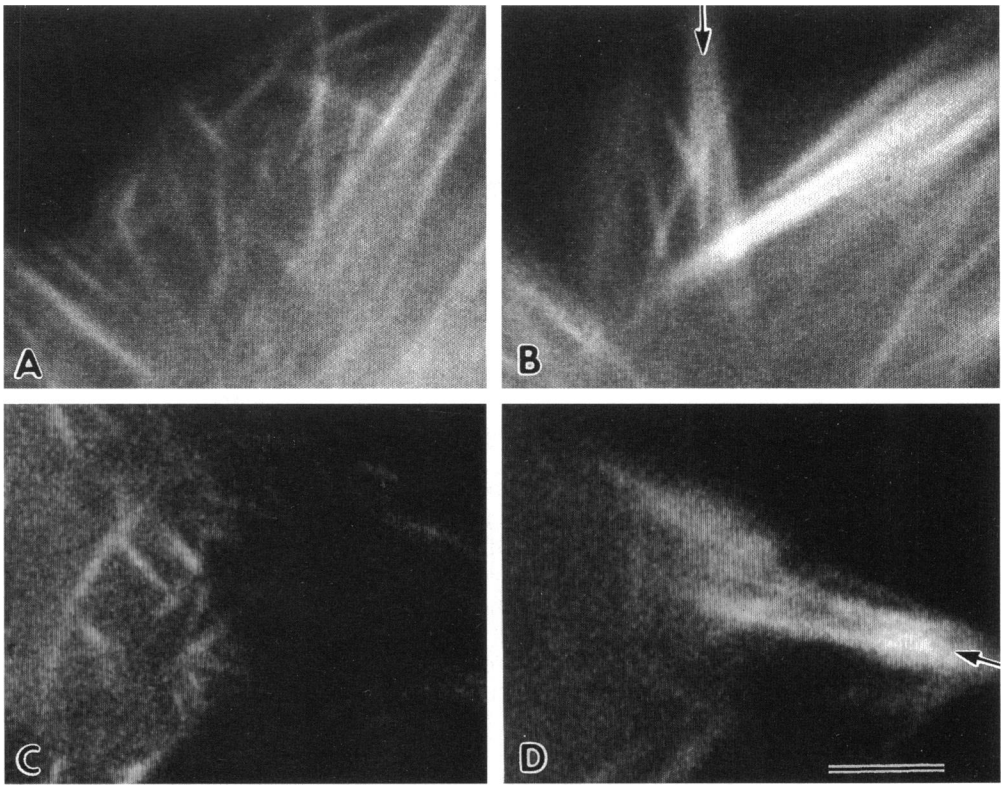

Figure 3. Orientation of bundles at early and later time points during the reformation of microtubules observed in living cells. MAP2c-transfected COS cells were microinjected with rhodamine-labeled tubulin, and observed with a cooled CCD camera as in Figure 2. A peripheral part of a cell from two representative time points are shown. Panels A and B are from a cell different from that shown in Figure 2, at 12 and 193 min after washing out nocodazole. Panels C and D are from another cell at 32 and 121 min after washing out nocodazole. In panels B and D, a large bundle was formed (marked by arrows) in the area where the short randomly oriented bundles were observed at earlier time points in panels A and C. Bar, 5 μm.

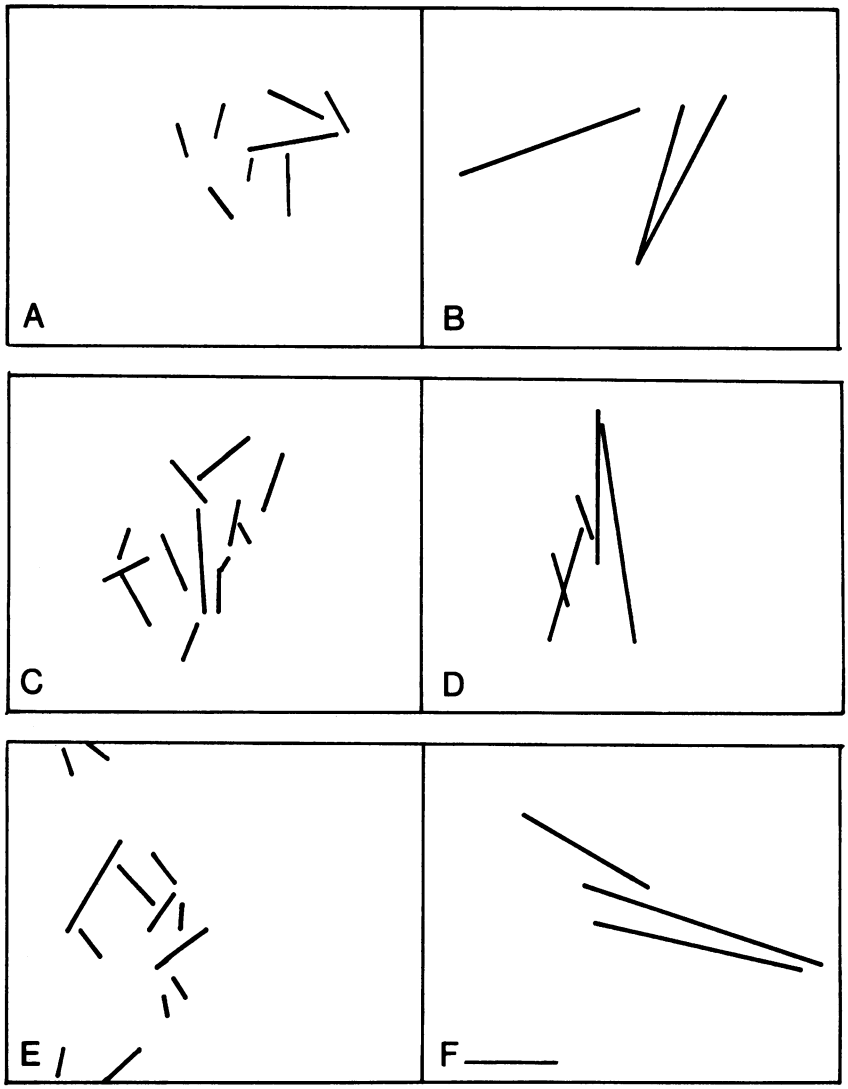

Figure 4. Tracings of orientation of bundles at early and later time points in the reformation process of bundles after nocodazole treatment. Panels A and B are a part of Figure 2D (10 min) and Figure 2H (161 min). The bundles marked by arrows in Figure 2H and the corresponding area in Figure 2D were traced. Panels C and D are a part of Figure 3A (12 min) and Figure 3B (193 min). The bundle marked by an arrow in Figure 3B and the corresponding area in Figure 3A were traced. Panels E and F are a part of Figure 3C (32 min) and Figure 3D (121 min). The bundle marked by an arrow in Figure 3D and the corresponding area in Figure 3C were traced. In panels A, C, and D, the short bundles are apparently randomly oriented. In contrast, in panels B, D, and F, the orientation of the bundles had occurred. Bar next to the letter F, 5 μm.

position could be judged against some cytological features, the location of the proximal end (e.g., small arrowheads in Figure 2, F–H) did not change appreciably, but that of the distal end (e.g., arrows in Figure 2, F–H) was pushed forward. However, during this elongation, the bird's claw–like shape at the distal end was retained.

By 104 min after washing out the nocodazole, the bundles grew considerably in length (in many cases exceeding 10 μm) and to some extent in width (Figure 2G). Most of the microtubules were incorporated into one of the clearly established bundles, and the short fragments, which were randomly oriented and free in the cytoplasm, had become rare by this time point. At 161 min after removing the nocodazole, the bundles grew further in length and width (Figure 2H).

In addition to the gradual, relatively slow growth in width, it was often observed that some bundles, which were clearly separate entities at earlier time points, converging at the distal end, but separated by a few micrometers at the proximal end, had fused to form a large-diameter bundle. This activity appeared to contribute significantly to the increase of the width of the bundles, and was seen to occur at various stages during the elongation of the bundle (e.g., two bundles converging at the distal end marked by the left arrow in Figure 2F fused into one in Figure 2G; three bundles converged at the distal end marked by the right arrow in Figure 2G fused into two bundles in Figure 2H).

We examined the reformation process of microtubule bundles in real time in six different cells in three distinct experimental runs, and a similar time course of microtubule bundle formation was observed each time. When studied by immunofluorescence, some microtubule bundles were as long as 30 μm by 30 min,

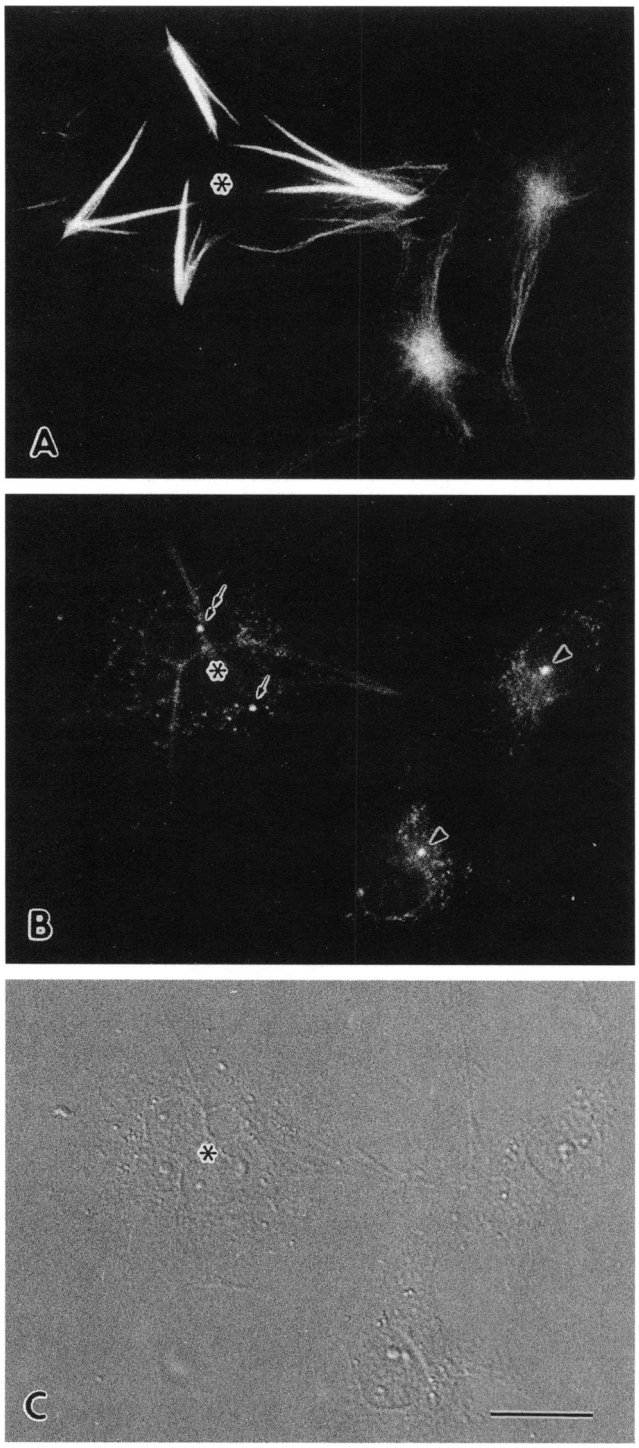

Figure 5. Relationship of microtubule bundles and the centrosomes. Cells, which are transfected with MAP2c, treated with nocodazole, and allowed to reform bundles after washing out nocodazole were double-stained with anti-tubulin antibody (A) and human autoantibody directed to the centrosome (B). A cell, marked by the asterisk, was transfected with MAP2c and two centrioles (a double-headed arrow and a single-headed arrow) were stained. Although one of the bundles appears to be closely associated with the centrosome (the double-headed arrow), most of them do not appear to be

and as described in the previous section, thick bundles were observed in many cells by 60 min. It seemed that the process was slower by the observation of living cells than by the immunofluorescence experiments. Although a heated stage set at 37°C was used with the microscope, the atmosphere of the cells did not appear to be maintained at 37°C.

To show the relation of orientation of short bundles formed at early stage and the large bundles formed at later stage, the examples from two other cells are shown in Figure 3. Figure 3, A and B, is the peripheral part of a cell different from that shown in Figure 2 at 12 min and 193 min after washing out nocodazole. Figure 3, C and D, is also the peripheral part of another cell at 32 min and 121 min. To show the relative orientation of bundles more clearly, the orientations of the bundles were traced in Figure 4. Figure 4B is a trace of the bundles marked by arrowheads in Figure 2H (161 min). Figure 4A is a trace of the corresponding area in Figure 2D (10 min). Bundles marked by arrowheads in Figure 3, B and D, and the corresponding area in Figure 3, A and C, are traced in Figure 4, C–F. As can be seen, a large bundle was formed in the area where the short bundles of random orientation were formed at early time points.

It was difficult to determine whether any cytological features could be consistently identified at the site of bundle growth. Centrosomes did not appear to play major roles in the formation of bundles after the nocodazole treatment. Most bundles were not associated with the centrioles shown by immunofluorescent double-label study using anti-tubulin and anti-centrosome antibody (Figure 5). To quantify the results, the number of bundles for each cell was counted and each bundle was scored for whether they were associated with the centrosome. The average number of bundles was 10.6 ± 5.4 (the mean ± standard deviation, n = 24), and that associated with the centrosome was 1.5 ± 1.0 per cell. In the same experiment, the average number of bundles observed at the end of the nocodazole treatment was 1.8 ± 1.5 (n = 49). Therefore, on the average, ~9 bundles were formed after the nocodazole treatment, and most of them were not associated with the centrosome. That most of the bundles were not directly associated with the centrosome was also observed by transmission electron microscopy (Figure 6). As shown, only a very minor fraction of

Figure 5 cont. associated with the centrosome. One of the centrioles (the single-headed arrow) does not appear to be associated with the bundles. (Panel B is overexposed to facilitate the comparison of the location of the centrioles and the bundles). Corresponding Nomarski optics are shown in panel C. Centrosomes of nontransfected cells are marked by arrowheads in panel B. Bar, 20 μm.

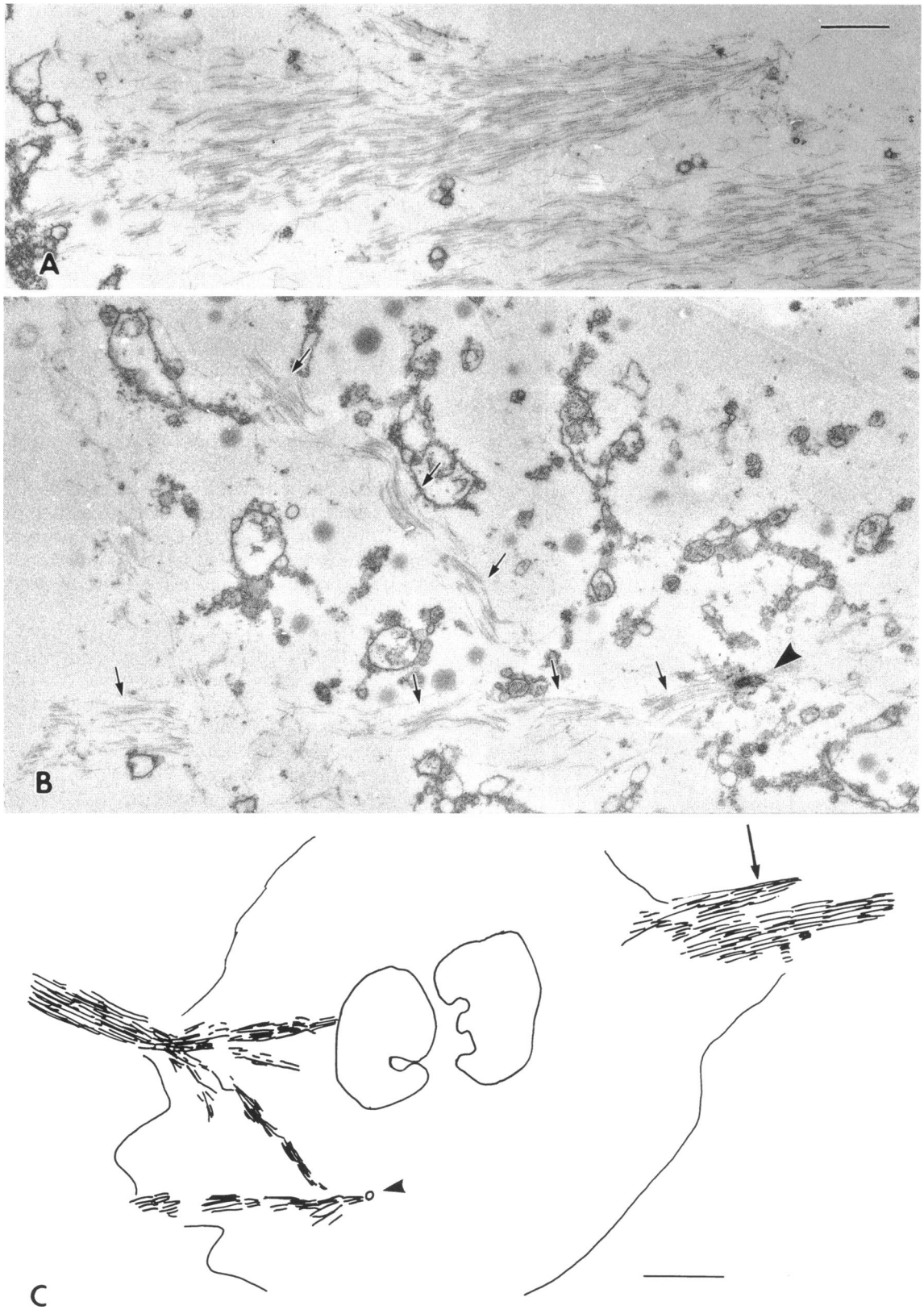

Figure 6.

microtubules (probably much less than 5%) was associated with the centrosome.

After the cells were treated with nocodazole, the central area was rich in vesicles as observed by Nomarski optics, but the peripheral area was organelle poor, as in nontreated cells (Figure 7). Therefore, the site of the first establishment of stable bundles was in the organelle-poor region. Actin filaments appeared to form a fine filamentous network in this region, as this region was stained diffusely by rhodamine-phalloidin. Stress fibers were poorly developed in COS cells. Thin actin cables, if present, did not appear to have any relationship to the distribution of bundles. Vimentin tended to be clumped near the nucleus in the transfected, bundle-forming cells, and this tendency did not change after nocodazole treatment (Figure 8). Therefore, the peripheral cytoplasm was mostly devoid of intermediate filaments, and it was unlikely that intermediate filaments had any effect on the establishment of microtubule bundles.

The above results suggested that the initial step of the reassembly of microtubule bundles was the formation of short microtubule bundles of random orientation at multiple sites, which were not associated with the centrosome, and that large bundles were then formed by gradual growth in radial and longitudinal direction.

Polarity Orientation of Microtubules in the Reformed Bundles

We next determined the polarity orientation of microtubule bundles both formed without nocodazole exposure and reformed after nocodazole treatment, using the standard hook procedure for microtubule polarity determination (Heidemann and McIntosh, 1980; Baas *et al.*, 1991; Heidemann, 1991; Chen *et al.*, 1992). Low magnification photographs of the typical appearance of microtubule bundles in MAP2c-transfected cells both without nocodazole exposure and those reformed after nocodazole treatment observed by conventional thin-section transmission electron microscopy without hook formation are shown in Figure 9. Exogenous tubulin was added to the bundles such as shown in Figure 9, and the orientation of hooks formed on the wall of microtubules was analyzed for the determination of microtubule polarity. To be certain of the polarity orientation of microtubules within the cell, the shape of the bundles was recorded by light microscopy (see MATERIALS AND METHODS for details) before embedding in Epon, and the bundles were sectioned from the known direction. Only the bundles that ran straight from the cell center to the periphery were chosen for analysis.

First, the orientation of the hooks formed on the bundles without nocodazole exposure was determined (Figure 10A and Table 1). Five bundles were analyzed, and in all of these bundles more than 90% of the microtubules had clockwise hooks as viewed from the distal end of the cell. Due to some particularity of nature in this assay, microtubule arrays of uniform polarity orientation may show the proportion of hooks of the same curvature ranging from 90–100%, and a proportion greater than 90% is considered an accurate indicator of uniform polarity orientation (Heidemann and McIntosh, 1980; Baas *et al.*, 1991). Therefore, the result indicated that microtubule bundles formed in MAP2c-transfected cells had a uniform polarity orientation, with the plus end distal to the cell nucleus.

Next, the orientation of hooks formed on the bundles reformed after nocodazole treatment was determined. In this analysis, it was important to avoid sectioning the bundles that withstood nocodazole treatment. Therefore, the transfected COS cells were microinjected with rhodamine-labeled tubulin (Figure 11). The bundles present at the completion of the nocodazole treatment were recorded, and only the newly formed bundles (arrows in Figure 11C) were sectioned after the hook formation. Four such bundles were analyzed (Figure 10B and Table 2), and it was found that more than 90% of the microtubules had clockwise hooks as viewed from the distal end of the cell in all the bundles. Therefore, the bundles reformed after nocodazole treatment also possessed the uniform polarity, with the plus end distal to the cell nucleus.

The results indicated that the polarity orientations of microtubules in bundles formed without nocodazole exposure and in those reformed after nocodazole treatment were both uniform, with the plus end distal to the cell nucleus. Therefore, although the bundles were formed within the cell body, they were oriented in the same direction as in the native axon or in the processes formed in Sf9 cells transfected with tau, MAP2c, or MAP2 (Burton and Paige, 1981; Heidemann *et al.*, 1981; Baas *et al.*, 1991; Chen *et al.*, 1992). In

Figure 6. cont. Transmission electron microscopic study of the relation between centrosome and the bundles. The cells that had bundles reformed after the nocodazole treatment were extracted with the detergent, fixed, and embedded in Epon. The sections were cut horizontal to the substrate. The microtubule bundles, the contours of the nuclei, and the boundaries of the cell from 10 consecutive sections around the centrosome were traced on transparent sheets. The recombined tracing was reduced in size and shown in panel C (see MATERIALS AND METHODS for detail). (A) The microtubule bundles, marked by an arrow in panel C, are shown. (B) The area around the centrosome. Only a small number of microtubules were associated with the centrosome (an arrowhead). Microtubule arrays are marked by arrows. (C) The position of the centrosome is marked by an arrowhead. Bar, 2 μm (A and B), 10 μm (C).

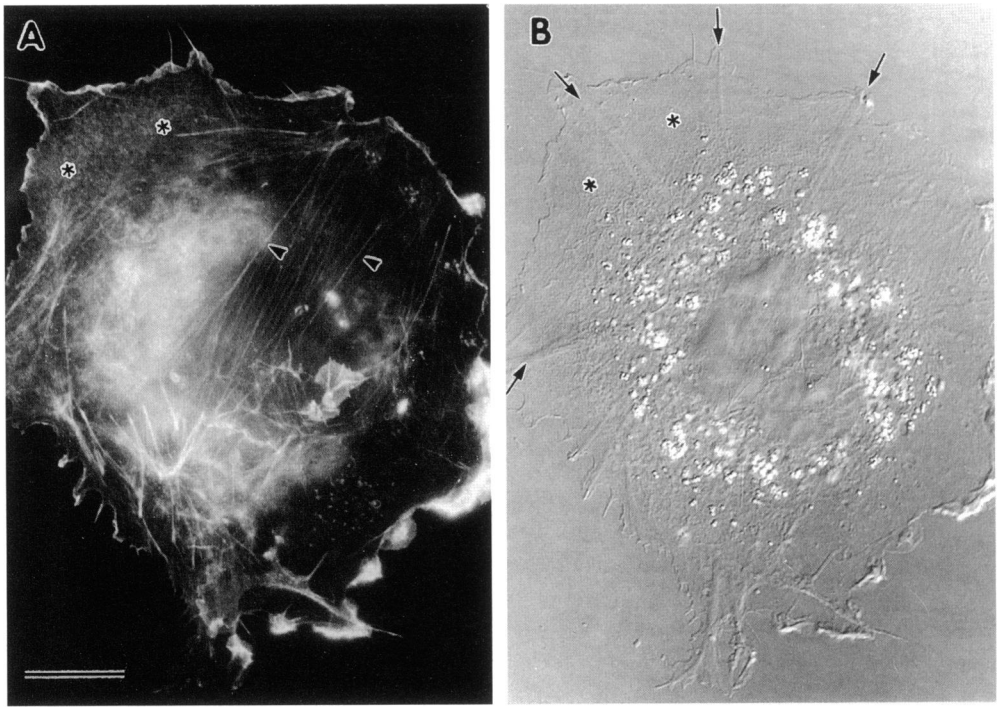

Figure 7. Relation of actin organization and reformed microtubule bundles. Cells transfected with MAP2c were treated with nocodazole, and nocodazole was washed out. After the microtubule bundles were reformed, cells were stained with rhodamine phalloidin (A). Microtubule bundles (as observed by Nomarski optics and marked by arrows in panel B) can be seen in the peripheral part of the cell, where the fine filamentous actin (marked by asterisks) is abundant. Thin actin cables (marked by arrowheads) do not seem to be specifically associated with most of the microtubule bundles. Bar, 20 μm.

addition, because the short microtubule bundles of random orientation, which were not directly associated with the centrosome, were initially formed during the reassembly process of microtubule bundles after nocodazole treatment, the results indicated that there must be some mechanism(s) to orient the polarity of these short bundles during the reformation of large bundles.

The Effect of Cytochalasin D on the Formation of Large Bundles

The reformation process of bundles was rather complex. Once the short microtubule bundles were established in the peripheral cytoplasm, in addition to elongation, stepwise fusion of neighboring bundles was observed. The association of MAP2c to microtubules and the cross-linking ability of this molecule were likely to have driven the initial formation of short microtubule bundles. However, it was difficult to presume that the fusion of neighboring bundles that were still separated by a few micrometers occurred by the association of MAP2c and microtubules alone. In this regard, because the peripheral cytoplasm where the formation of microtubule bundles occurred appeared to be enmeshed in a network of filamentous actin, the effect of cytochalasin on the formation of microtubule bundles was studied.

In COS cells, cytochalasin D, when used at 0.5 μg/ml, caused the formation of actin foci in the cell periphery, as seen in other cells (Schliwa, 1982), judged by the staining with rhodamine phalloidin, but did not produce cell arborization in more than 90% of the cells for at least 2 h. Because extensive cell arborization was thought to complicate the interpretation of the results, the effect of cytochalasin D on microtubule reformation was studied at 0.5 μg/ml. As shown in Figure 12, the microtubule bundles reformed in the presence of cytochalasin D were thin, and the number of bundles per cell was large. Although there was some variation in the number of bundles per cell formed in the control cells from experiment to experiment, there was a clear difference in the number formed in the presence of cytochalasin D. In one typical experiment, the mean $\pm$ standard deviation for the number of bundles per cell was 24.8 $\pm$ 16.4 (n = 26) for cytochalasin D, and 7.2 $\pm$ 3.7 (n = 33) for control. The numbers were statistically different at $p < 0.05$, based on a two-sample t-test.

The results suggested that the fusion of neighboring microtubule bundles may require intact microfilament organization.

DISCUSSION

Role of MAP2c in Microtubule Bundling

We have previously shown that MAP2 and tau form crossbridges between microtubules in vitro, in vivo, and also in cells transfected with these molecules (Hirokawa, 1991; Chen *et al.*, 1992). Our present observa-

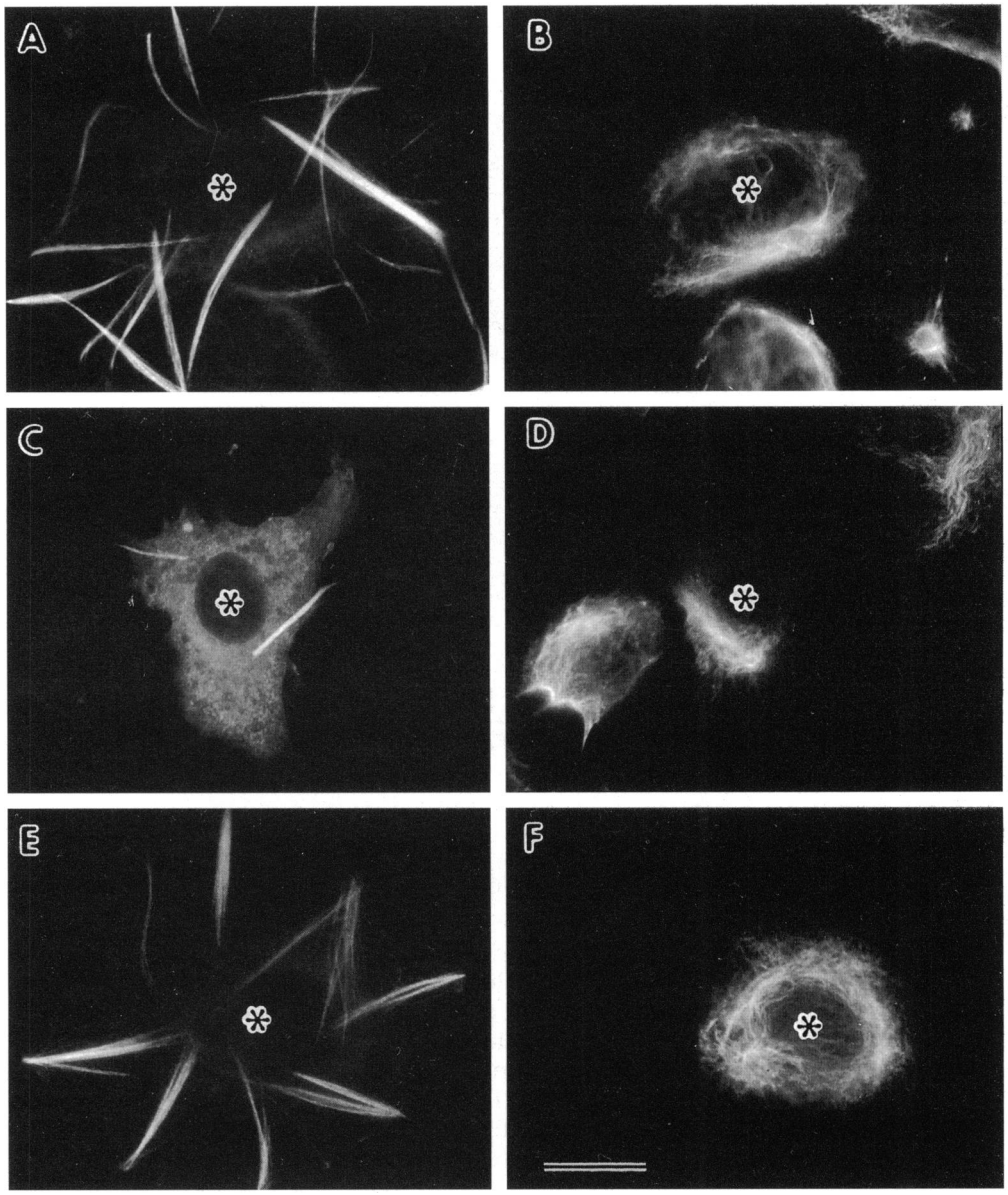

Figure 8. Relation of vimentin organization and the reformation of microtubule bundles. COS cells were transfected with MAP2c (A and B), treated with nocodazole for 2 h (C and D), washed out of nocodazole, allowed to reform microtubule bundles (E and F), and double-stained with rabbit polyclonal anti-MAP2 (A, C, and E) and mouse monoclonal anti-vimentin (B, D, and F). Cells transfected with MAP2c are marked by asterisks. Vimentin was mostly concentrated around the nuclei in these cells in all stages examined. Bar, 20 μm.

tion that reformed microtubules are already bundled at the very early stages of recovery from nocodazole strongly suggests that a purely thermodynamic interaction between tubulin and MAP2c molecules can induce the formation of bundles. In the case of tau protein, the projection domain of the molecule located at the N-terminal half has been shown to be important for microtubule bundling. Although it is not yet determined whether the N-terminal projection domain is involved in microtubule cross-linking, it is possible that the initial formation of thin microtubule bundles is driven by the cross-linking of microtubules generated adjacently in the cytoplasm, one by one, finally forming complexes of microtubules parallel to each other. This idea is supported by the findings that MAP2c and MAP2 form antiparallel dimers in vitro (Wille *et al.*, 1992a,b).

In the later stage of microtubule reformation, we observed the gradual fusion of preformed thin bundles. Because this process was slow and inhibited by the actin-disrupting agent, it is less likely that the gradual formation of thick bundles is driven by pure thermodynamic interaction between microtubules and MAP2c. Rather, the passive movement of microtubule bundles by interaction with actin network (somewhat different aspects of the interaction of microtubule bundles and actin network from that proposed by Edson *et al.*, 1993) seems to be in-

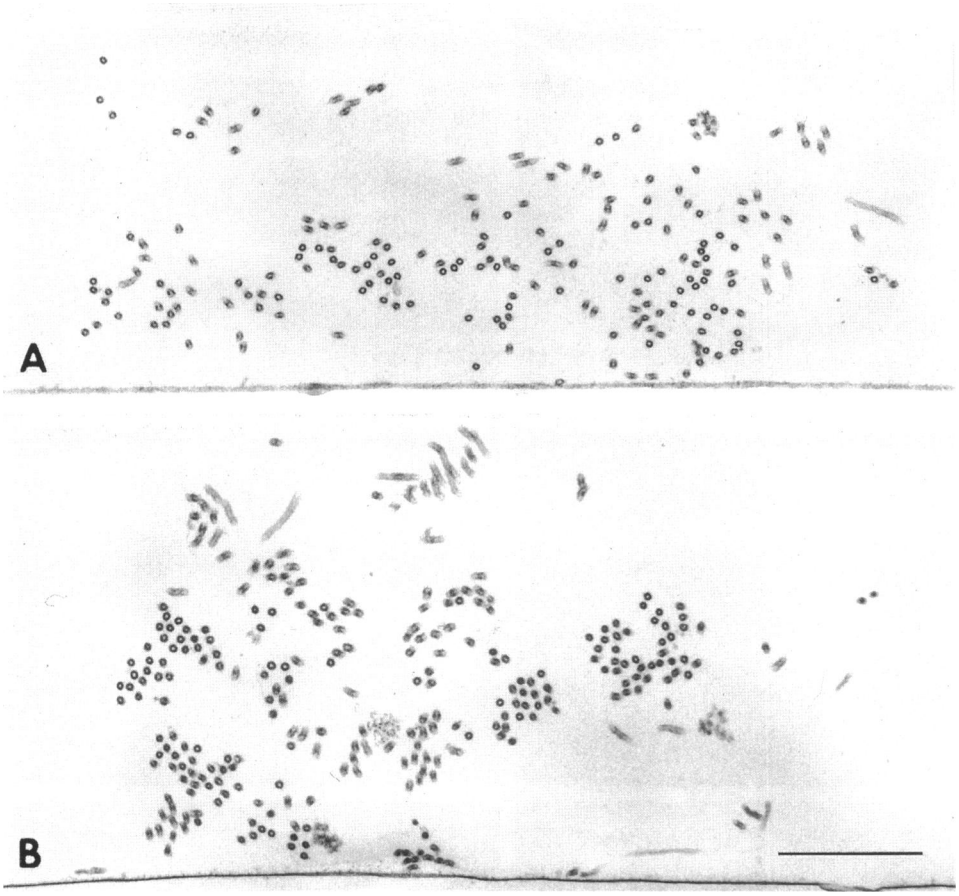

Figure 9. Cross-section of microtubule bundles observed by thin-section transmission electron microscopy. (A) Without exposure to nocodazole; (B) reformed after exposure to nocodazole. Although the spacing between adjacent microtubules was very close, unlike microtubules in the axon, the whole bundles were not tightly packed. There were wide variations in the size and looseness of the packing of microtubules. Both panels A and B represent bundles of an intermediate level as to size and looseness of the packing of microtubules, but the orientation of microtubules was relatively well aligned in these regions. In some other regions, microtubules ran in various directions. In some cases, the whole bundles were more or less tightly packed as in the axon. Bar, 0.5 μm.

volved. It should be stressed that MAP2c is a molecule of <100 nm in length and forms projections <50 nm in length on the microtubule surface. Therefore, the attraction of two thin microtubule bundles that are over a few micrometers apart will logically require some other mechanism(s). However, it should also be emphasized that the cross-linking activity of MAP2c would still play an important role in this later stage of bundle formation. Once microtubule bundles come close enough together, namely within the distance of 50 nm, interaction between MAP2c molecules or between MAP2c and tubulin molecules will tightly fix the two thin microtubule bundles to be reorganized into a single, thick bundle. In this sense, the cross-linking activity of MAP2c presumably plays an important role in both

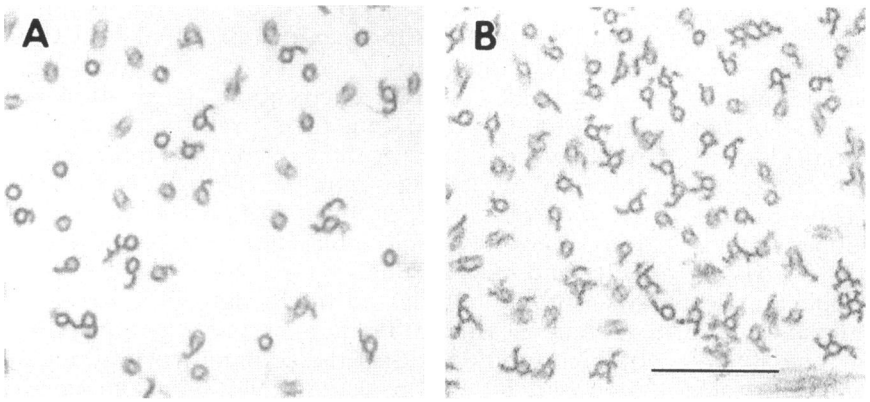

Figure 10. Microtubule polarity orientation in microtubule bundles formed in MAP2c-transfected cells. Microtubule polarity orientation was determined using the hook procedure (A) without exposure to nocodazole, and (B) reformed after exposure to nocodazole. In both panels A and B, the hooks were predominantly clockwise from the vantage point of the distal end, indicating the uniform microtubule polarity orientation of the plus end distal to the cell nucleus. Bar, 0.2 μm.

Table 1. Microtubule polarity orientation of microtubule bundles formed in MAP2c-transfected COS cells

Cell	Bundle	CW (n)	CCW (n)	AMB (n)	UHK (n)	HK (%)	CW (%)
Cell 1	Bundle 1	98	6	19	140	47	94
Cell 2	Bundle 1	78	3	31	72	61	96
Cell 3	Bundle 1	73	6	11	36	71	92
	Bundle 2	121	4	30	148	51	97
	Bundle 3	64	2	7	188	28	97

Microtubule polarity orientation of microtubule bundles formed in MAP2c-transfected COS cells (without nocodazole treatment) was determined by the "hook" procedure. Five microtubule bundles from three cells, which had a clear orientation relative to the cell periphery and cell center, were chosen for analysis. Because >90% of hooks had clockwise orientation, microtubule plarity orientation was uniform, with plus ends distal to the cell nucleus.

CW, microtubules with clockwise hooks as viewed from the end of the bundle distal to the cell nucleus; CCW, microtubules with counter-clockwise hooks; AMB, microtubules with ambiguous hooks; UHK, microtubules with no hooks; HK, microtubules with hooks.

the early and later stages of bundle formation after nocodazole treatment.

Role of MAP2c in Establishing Microtubule Polarity

The microtubule bundles formed after nocodazole treatment, which were assembled independent of centrosomes, had uniform polarity with the plus end distal to the cell nucleus. The polarity was the same as that found in microtubule bundles before nocodazole treatment, in axons, and in neuronal processes formed in Sf9 cells transfected with MAP2c, tau, or MAP2 (Baas *et al.*, 1991; Chen *et al.*, 1992).

From observation of the reassembly process of the bundles, it can be supposed that the polarity orientation of microtubule bundles is determined when the first primordium of bundles was established at the peripheral cytoplasm. Before that, the microtubule fragments were formed at random orientation. Whether the microtubules were attached to any particular structures at the proximal or distal end was difficult to determine. Neither actin nor vimentin showed any particular pattern that would suggest any involvement in the polarity establishment of microtubule bundles. However, it may be possible to hypothesize on the existence of some mechanism(s) that would transport the short microtubule fragments toward the edge of the cell while aligning the orientation with the plus end distal to the cell nucleus. No particular structures associated with the minus or plus end of microtubules have been identified in the microtubule bundles of the neuronal processes (Okabe and Hirokawa, 1988; Baas and Black, 1990; Baas and Joshi, 1992), but microtubules are transported from the cell body to the axon with the plus end leading (Baas and Ahmad, 1993). Such transport mechanism(s) may be involved.

Regarding the uniform polarity of microtubule bundles induced by MAP2c, it is interesting to note that in cultured hippocampal neurons, the polarity orientation of the microtubules in all of the initial processes is uniform, with the plus ends distal to the cell body, even though most of these processes will become dendrites, which later develop mixed polarity orientation of microtubules, with about equal numbers oriented in each direction (Baas *et al.*, 1989). Therefore, the prototype of the polarity orientation of microtubules may somehow be the plus end distal to the cell nucleus. It may be that to

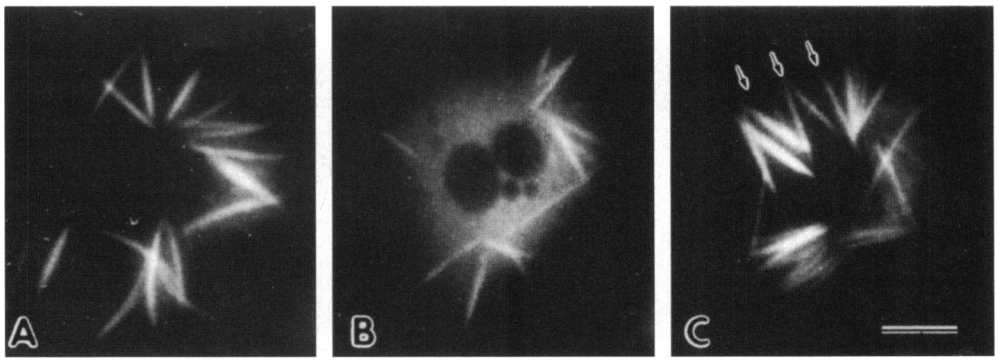

Figure 11. Identification of bundles reformed after nocodazole treatment. MAP2c-transfected COS cells were microinjected with rhodamine-labeled tubulin, and the bundles were recorded before (A) and after (B) nocodazole treatment, and after reformation of bundles (C). Arrows indicate the region where new microtubule bundles were formed after nocodazole treatment. Bar, 10 μm.

Table 2. Microtubule polarity orientation of microtubule bundles reformed after nocodazole treatment in MAP2c-transfected COS cells

Cell	Bundle	CW (n)	CCW (n)	AMB (n)	UHK (n)	HK (%)	CW (%)
Cell 1	Bundle 1	123	7	31	272	37	95
Cell 2	Bundle 1	226	3	14	61	80	99
Cell 3	Bundle 1	153	3	26	16	92	98
	Bundle 2	78	4	8	21	80	95

Microtubule bundles newly formed after nocodazole treatment were identified as in Figure 11 and microtubule polarity orientation of these bundles was determined by the "hook" procedure. Four such bundles from three cells were analyzed. Because >90% of hooks had clockwise orientation, the microtubule polarity orientation was uniform, with plus ends distal to the cell nucleus.

CW, CCW, AMB, UHK, and HK, same as in Table 1.

develop a mixed polarity orientation of microtubules, additional factors are necessary.

Also, regarding the first primordium of bundles of uniform polarity being established at the peripheral cytoplasm, it is interesting to note that when MAP2 is microinjected into fibroblasts, fragments of microtubules tended to accumulate in the peripheral area (Hirokawa, Funakoshi, and Sato-Yoshitake, unpublished observation). In addition, when early mitotic cells are treated with taxol, short bundles of parallel microtubules are formed predominantly at the cell periphery (Maekawa *et al.*, 1991).

Further investigation will be necessary to provide clarification of the mechanisms in orienting the polarity of microtubule bundles, and the presently reported system may be useful for such studies.

Gradual Growth of Bundles

The short bundles that were originally formed, elongated gradually. It has been shown that in TC-7 fibroblasts, tubulin rapidly polymerizes upon nocodazole release, and a plateau level of polymerization is reached by 5–10 min (Gundersen *et al.*, 1987). In our experiments using living cells, the temperature was lower than 37°C, and the plateau level was probably reached by 10–20 min. By this time, the microtubule fragments were about 5 μm in length. Because mature bundles were about 30 μm, the elongation was thought to involve some sort of reorganization of microtubule bundles. We have previously observed sliding between microtubules in the microtubule bundles formed in MAP2c-transfected COS cells (Umeyama *et al.*, 1993). This kind of sliding event may be involved in this elongation.

In addition, some form of annealing might take place. If we assume that the average length of microtubules in the bundle is 5 μm, we should be able to observe approximately 20 proximal and 20 distal ends per 100 microtubules when a length of 1 μm is followed. By following the serial sections cut perpendicular to the long axis of the bundles such as shown in Figure 9B and counting the number of proximal ends,

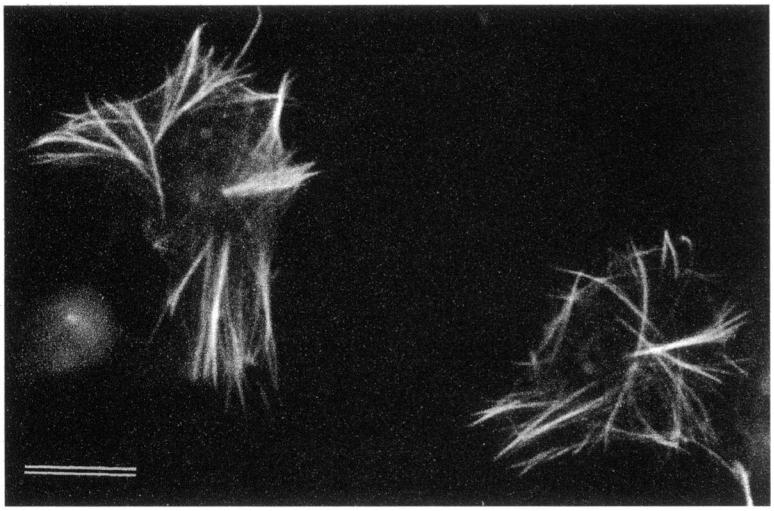

Figure 12. The effect of cytochalasin D on the reformation of microtubule bundles. After 2 h of nocodazole treatment, cells were pretreated with cytochalasin D in the presence of nocodazole for 30 min, and microtubule bundles were reformed in the presence of 0.5 μg/ml cytochalasin D for 60 min. Cells were then fixed with cold methanol and labeled with anti-MAP2 by indirect immunofluorescence. Microtubule bundles formed in the presence of cytochalasin D were thin, and the number of microtubule bundles per cell was large (compare Figure 12 with Figure 1. They were both from the same set of experiments). Bar, 20 μm.

we counted 3.7–15 proximal ends per 100 microtubules per μm. This suggests that annealing might be occurring in our system. Annealing of microtubules has been shown previously in vitro (Rothwell *et al.*, 1986), although it is not known whether it actually occurs in vivo. The length of microtubules in the axon has been reported to be extremely long (100–800 μm) in some instances (Bray and Bunge, 1981; Tsukita and Ishikawa, 1981; Burton, 1987), and it is possible that annealing may be involved in their formation.

Recent studies indicate that in neurons, microtubules are nucleated exclusively at the centrosome, but rapidly released, and transported to the neurites with the plus end leading (Joshi and Baas, 1993; Yu *et al.*, 1993). The short fragments, less than the diameter of the cell body, are reorganized in the axon into the longer microtubules, more than 100 μm by the elongation specifically at the distal end. Although there may be some limitation as to how closely the bundle formation observed in nocodazole-treated, MAP2c-transfected COS cells resembles the bundle formation in the native axon, the system described herein may serve to clarify these aspects as well.

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