

Immunofluorescence Microscopy of Tubulin and Microtubule Arrays in Plant Cells.

I. Preprophase Band Development and Concomitant Appearance of Nuclear Envelope-associated Tubulin

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ABSTRACT The development of the preprophase band (PPB) of microtubules (MT) in meristematic plant cells was studied by using antibodies to pig brain tubulin and indirect immunofluorescence microscopy. The PPB is first visible as a wide band of MT that are arranged only slightly more densely than flanking MT of the cortical interphase array. MT progressively become more tightly packed together, and other cortical MT are no longer seen as the PPB matures. The surface of the nuclear envelope (NE) displays no tubulin fluorescence during interphase but begins to fluoresce in the early stages of PPB development, and its intensity progressively increases thereafter. The pattern at the NE is usually diffuse at first, suggesting the presence of nonpolymerized tubulin, but fibers along the NE can be resolved at later stages.

MT, arranged either radially or as a meshwork, can occur between the nucleus and cell cortex, and sometimes appear to connect the PPB and NE directly. Isolated preprophase nuclei from cells ruptured during processing often retain the PPB in its normal orientation, indicating stable linkages between the nucleus and PPB. Fluorescent cross-bars perpendicular to the axis of the MT were resolved in some PPB, suggesting lateral linkages. This suggestion is reinforced by the presence of PPB that hold together as a ribbon of MT in certain preparations, allowing PPB to be isolated from the rest of the cytoplasm and the nucleus.

In the 17 years since it was first described by Pickett-Heaps and Northcote (27, 28), the preprophase band (PPB) of microtubules (MT) has been seen in a wide variety of plant cells using electron microscopy. Although its precise role remains conjectural, it is apparent that the PPB is linked to a morphogenetic control mechanism. It is found in dividing cells of complex three-dimensional tissues and organs, and also in simpler (yet still organized) one- and two-dimensional cellular ensembles (summarized in 6, 13, 14). In all cases, the PPB accurately defines the plane in which will lie the cell plate formed at the conclusion of the following round of mitosis and cytokinesis (6, 12–15, 22, 25–28). This rule holds even when the partitioning of cytoplasm at that division is highly asymmetrical or when curved or unusually-shaped cell plates are laid down. Thus the PPB is one of the first visual heralds

of the onset of division and its position and orientation within a cell indicate how the cell will develop. For a structure that occupies such a key position, we know few details about how it develops and changes through time, and what its spatial relationships are with other MT arrays that precede and follow it (namely interphase MT and the mitotic spindle). The paucity of information results in part from the difficulty of following the dynamics of PPB MT. Birefringent cell walls enclose all cells that have a PPB and preclude effective use of polarized light microscopy, and serial section reconstruction of three-dimensional features of the PPB through many developmental stages is a formidable task.

Immunocytochemical procedures to localize tubulin in plant cells that have been fixed to retain their *in vivo* internal organization have recently been developed (32), providing a

means of assessing PPB development. The procedures allow fairly rapid assessment of large populations of cells that have not been distorted through protoplast formation or suspension culture. In this paper we trace changes in the PPB from the time it is first distinguishable from the interphase MT array until it reaches the mid-stage of its development. We correlate its various stages with the premitotic changes in nuclear organization and document the corresponding changes in microtubule fluorescence patterns along the nuclear envelope (NE).

MATERIALS AND METHODS

Allium cepa L. root tips were handled according to the procedures described in Wick et al. (32), with the following modifications and additions: 50 mM potassium phosphate containing 5 mM EGTA (EGTA-phosphate) was the buffer used throughout fixation and presquashing rinses. Root tips were exposed to EGTA-phosphate in the rinse steps after fixation and wall digestion for a combined total of at least 1.5 h before squashing was attempted. Partial cell wall digestion was routinely performed with 1% cellulysin in 0.4 M mannitol plus 5 mM EGTA for 13–16 min. Poly-lysine coating of coverslips was eliminated; after squashing the root tips, cells were simply air-dried onto the acetone-cleaned glass surface. We found a cold methanol step unnecessary and eliminated it. For experiments in which the effects of prolonged wall digestion were examined, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 1 μ g/ml leupeptin, and 0.1% chloramphenicol were added to the cellulysin solution.

Affinity column-purified rabbit antitubulin was the generous gift of Professor Klaus Weber, Max-Planck-Institute, (Göttingen, Federal Republic of Germany). Either fluorescein- or rhodamine-labeled goat anti-rabbit IgG was used as the second antibody. Mounting medium pH was raised to ~8 to reduce fluorescence fading. For viewing, we used either a Zeiss photomicroscope (Carl Zeiss, Inc., New York, NY) fitted with a CSI 250W lamp or a Leitz orthoplan (E. Leitz Inc. Industrial Div., Rockleigh, NJ) with a HBO 100W lamp, both in the epifluorescence mode. Filters were standard fluorescein and rhodamine combinations. Kodak Tri-X was exposed at DIN 33 for fluorescein-labeled material and DIN 34 for rhodamine, and developed in Dialfine (Acufine, Inc., Chicago IL). Kodak 2475 was used at DIN 31 and developed in Acufine (Acufine, Inc.).

RESULTS

Cell Preparation

Even the very short wall digestion times used in this study yielded wall-less cells when fixed tissues were squashed, as evidenced by lack of fluorescence in the presence of the cellulose indicator Calcofluor M2R New (Cyanamid of Canada) (data not shown). Success in obtaining intact cells during squashing appeared to be more dependent on the total length of time that tissues were exposed to the Ca^{2+} -chelating action of EGTA prior to the squash than on length of the digestion step. After digestion, specimens were permeable to antibody molecules without further treatment. Immunofluorescence was equally bright with or without a cold organic solvent step.

We suspected that cultured cells and cells from certain plant tissues would be less amenable than meristematic cells to release from their walls as intact cells, and would require more prolonged exposure to enzymes to weaken or totally digest the wall. To make our preparative techniques more generally useful, longer digestion times were tested. Tubulin antigenicity was very labile under the conditions of the original digestion regime. Immunofluorescence decreased dramatically when the incubation in cellulysin was extended even briefly beyond the usual 13–16 min duration. Addition of bovine serum albumin to cellulysin solutions to saturate contaminating proteases appeared to make no difference. However, adding the protease inhibitors PMSF and leupeptin markedly inhibited tubulin degradation, and digestion periods as long as 16 h resulted in immunofluorescence intensities equal to

those attained with a 15-min digestion. Furthermore, whereas MT were well preserved throughout this treatment, other cytoplasmic constituents were less resistant to degradation. Severe disruption of and loss of cytoplasm ensued, enhancing resolution of details of MT arrays (Fig. 1 *a* and *b*).

Optics and Photography

Since root cells have no interfering red autofluorescence from chloroplasts, and since *Allium* cells have no other components that fluoresce in the red region of the spectrum, rhodamine-conjugated second antibodies could be used. This was highly desirable because rhodamine is much less prone to fading upon exposure to light than is fluorescein. When fluorescein was used, observation and fluorescence photography was improved by increasing the pH of the medium in which cells were mounted. The rate of fading was reduced, allowing for shorter exposure times and negatives with higher contrast and greater detail. The best balance of film speed and grain size was obtained with the Tri-X and Dialfine combination.

Further improvement of optical aspects was achieved with a CSI lamp. It has strong emission peaks in the regions of fluorescein and rhodamine absorption (3) and gave excellent results with both fluorochromes. A Leitz $\times 50$ sea water-immersion lens, (NA = 1.0; E. Leitz, Inc.) also proved convenient for certain applications. This lens is designed to be used for viewing slides without a coverslip; we found it equally useful with conventionally mounted coverslips and on coverslips viewed cell-side up, with phosphate-buffered saline for immersion. Whereas phase observation was not possible with this lens, fluorescence was considerably brighter than with a planachromat lens giving the same depth of field.

Where MT occupied a plane of the cell somewhat distant from other fluorescent objects, such as the near and far surfaces of the PPB, which are several micrometers apart, fine details were best observed with a planapochromat $\times 63$ lens (NA = 1.4; Carl Zeiss, Inc.). The shallow depth of field for this lens, calculated to be $\sim 0.27 \mu\text{m}$ at fluorescein emission wavelengths, made it possible to focus on a particular plane of interest, rendering fluorescence of MT in other optical planes indistinct. By successively focusing on different levels, we were able to optically "section" through cells (e.g., Fig. 1, *a* and *b*; *g* and *h*; *j* and *k*; Fig. 2, *e* and *f*; Fig. 3, *c* and *d*). The depth of field obtained with a planapochromat $\times 40$ lens (Carl Zeiss, Inc.) or the sea water-immersion lens, both with NA = 1.0, is $\sim 0.53 \mu\text{m}$ with fluorescein emission. These lenses were less useful for optical sectioning but more useful for three-dimensional imaging.

Early PPB Development

The PPB stage was marked by at least two tubulin immunofluorescence patterns that were not seen in interphase cells. The most obvious was the PPB itself, comprising MT that were aligned parallel to each other to form a band that lay immediately interior to the plasmalemma. In onion root tip cells, the most common PPB orientation was transverse to the root axis. Consequently, the PPB was approximately circular, since it described the periphery of a cell that was polygonal in this view. When a cell became attached to the coverslip at a slight angle relative to the plane perpendicular to the PPB, the roughly hooplike band could be discerned readily by focusing through the cell (Fig. 1, *a* and *b*).

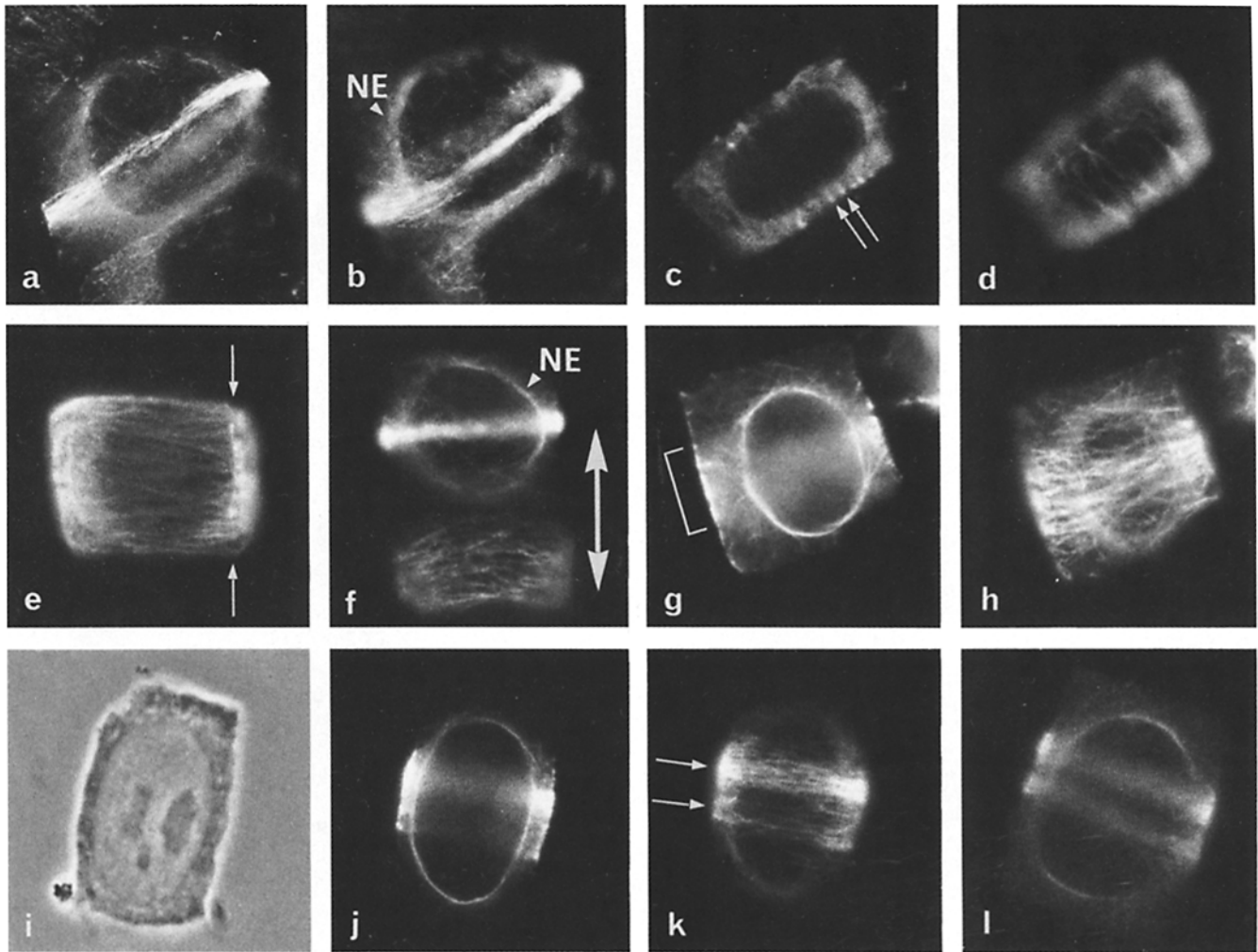


FIGURE 1 (a and b) Two levels of focus of a preprophase cell (top) and a neighboring interphase cell (bottom). The root tips were digested for 16 h prior to squashing, and much of the cytoplasm has been extracted. The fibrillar nature of the PPB is evident on the PPB surface attached to the coverslip (a) and on the segment passing over the opposite face of the cell (b). Grazing views of the nucleus also reveal some NE fluorescence. $\times 1,060$. (c and d) Interphase cell at two levels of focus. Cortical interphase MT are viewed "end-on" (c, arrows) and near the cell surface (d). The nucleus is clearly outlined in c, and shows no evidence of NE-associated fluorescence. $\times 1,450$. (e) Interphase cell focused between the plasmalemma and NE, with no indication of NE fluorescence. Cortical MT going around cell corners produce an edge effect (arrows). $\times 1,300$. (f) Preprophase cell (top) and interphase cell (bottom) isolated as a pair. The former exhibits fluorescence of the PPB, the NE, and fibrillar fluorescence extending away from the nuclear surface. MT in both cells are transverse to the longitudinal axis of the root (double-headed arrow). $\times 1,150$. (g and h) Two levels of focus of an early PPB. The PPB is seen in cross-section (g, brackets), as are strong NE fluorescence and a meshwork of MT between the nucleus and cell periphery. The relatively dispersed MT arrangement is visible in tangential section (h). $\times 1,080$. (i-k) Early preprophase cell. Phase contrast microscopy (i) indicates that nucleoli are becoming irregular in shape, but that chromatin is not yet condensing. In medial view (j) NE fluorescence is also visible, whereas in surface view (k) wide spacing of PPB MT allows individual fibers to be resolved. The PPB seems to include two fluorescent sub-bands, including one narrow bright band and a second less intense band (k, arrows). $\times 1,210$. (l) Early preprophase cell with double PPB. The overall width of both bands relative to other cellular dimensions is similar to that seen in j and k, but here the two component bands are clearly separated. $\times 1,500$.

The second tubulin fluorescence pattern unique to the PPB stage was that of the outer surface of the NE or the cytoplasm immediately adjacent to it. The nucleus was usually distant enough from the plasmalemma so that when we focused on the extreme periphery of the cortical cytoplasm, it was not possible to see nuclear fluorescence (compare Fig. 1, g with h and j with k; Fig. 2, e with f; and the upper right vs. lower left of the upper cell in Fig. 1f.) Because interphase MT arrays occupied a cortical position in the cytoplasm, a picture including interphase MT was not expected to reveal any fluorescence associated with the NE (Fig. 1, d and e, lower cell in

f). However, optical sections through the nucleus showed the NE outline, and should have revealed whether or not there was fluorescence at the NE surface. An example of such observations on interphase cells is seen in Fig. 1c. Here the nuclear boundary is clearly delimited in most of the cell, but nowhere is tubulin fluorescence associated with the NE.

The earliest stage of PPB formation was recognized by the simultaneous appearance of both NE and PPB fluorescence. Fig. 1h shows a cell that contained a wide band of peripheral MT that were packed together only slightly more densely than MT in the rest of the cortex. The nucleus, which is not in

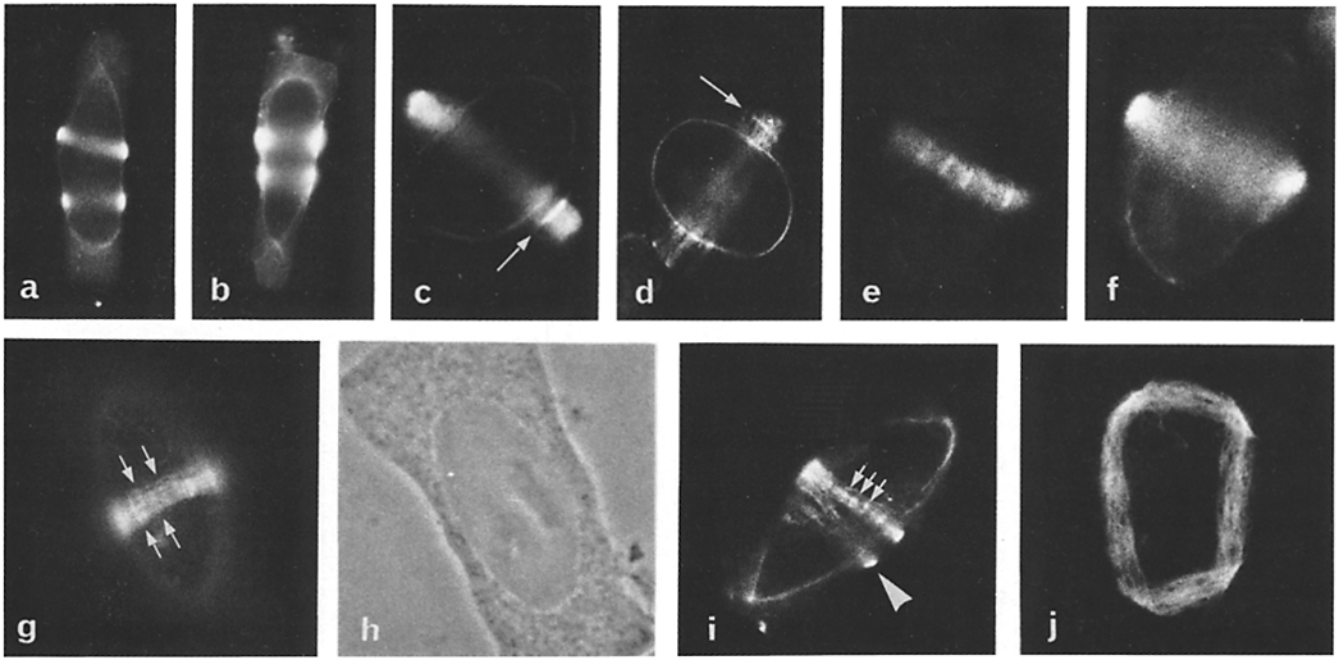


FIGURE 2 (a and b) Elongate cells with double PPB. Various degrees of separation between the two bands are shown. (a) $\times 770$; (b) $\times 700$. (c and d) Enhancement of fluorescence seen when PPB MT pass around a cell edge. Arrows mark the edge that has been brought into focus. c is an isolated nucleus/PPB complex. (c) $\times 1,440$; (d) $\times 880$. (e and f) Two views of a PPB. Periodic striations can be discerned in tangential section (e). This nucleus and PPB have been isolated as a unit after a 16 h incubation in cell wall-digesting enzymes. $\times 1,640$. (g and h) Cell with faint fluorescent striations crossing the PPB (arrows, g), judged by phase contrast microscopy to be in early preprophase (h). If printed so that the PPB is brighter, striations are no longer discernible, but NE fluorescence, just out of the plane of focus, becomes more visible. $\times 1,200$. (i) Wide, forked PPB with periodic striations visible on the upper fork of the band (arrows). The surface of the lower fork is not in focus, but its MT can be seen in cross-section at the cell edge (arrowhead). This is a partially isolated nucleus and PPB obtained after 16 h digestion. $\times 1,230$. (j) Isolated PPB after prolonged enzymatic digestion. Constituent MT of the band remain associated as a ribbon, which has folded over on itself while flattening onto the coverslip. $\times 1,270$.

focus here and which is visible as a nonfluorescent ovoid area, provides a good backdrop for visualizing PPB MT. A medial section that provides an end-on view of MT along edges of the same cell allows an easier assessment of the band width (Fig. 1g). Because surfaces of the PPB that are parallel to the optical plane are not in focus the PPB is seen as a blurred band of fluorescence across the nucleus. The nucleus, however, is clearly outlined by fluorescence at this plane, indicating that changes in tubulin distribution at and near the nuclear surface occurred very early in the process of PPB formation, when the arrangement of cortical MT was barely distinguishable from that of interphase.

A band that was forked or was composed of two more-or-less distinct component bands was frequently observed early in PPB formation (Fig. 1, j-l and Fig. 2, a, b and i). In cells that were much longer than wide, the double nature of such PPB could be pronounced, indicating the existence of two totally separate bands, each consisting of a tightly-packed array of MT (Fig. 2, a and b). Occasionally double PPB were found in cells that were not highly elongate (Fig. 1l). Phase contrast views of such cells indicated that nuclei were in very early stages of preparation for mitosis: nucleoli were irregular in shape and little or no chromosome condensation had begun (Fig. 1i).

PPB widths in Fig. 1, g-l were calculated to be between 5.6 μm (Fig. 1l, total width occupied by both bands) and 8.3 μm (Fig. 1, g and h). These figures equal $\sim 30\%$ of total cell length in Fig. 1, i-l, and $\sim 40\%$ in Fig. 1, g and h. Other PPB in transition to the mature PPB stage (Fig. 3, a-i, k, and l) had

values of 3.5–5.8 μm ; these range from 13 to 22% of cell length, averaging $\sim 20\%$. Although measurements of this type contain inherent inaccuracies due to spreading of light from a fluorescent object, rough comparisons between PPB developmental stages are still possible. On the basis of these measurements, we concluded that the PPB became narrower as it progressed from its earliest to mid-stages. The band occasionally assumed a fan shape (i.e., was slightly wider on one side than on the other), but PPB width and fluorescence intensity were normally uniform across a cell face, and we saw no evidence in onion of one-sided or lopsided PPB such as those found in studies of *Azolla* roots with electron microscopy (15).

NE fluorescence could be discerned throughout early PPB development, and usually remained associated with the NE, even after prolonged (16 h) digestion periods that severely disrupted cytoplasmic integrity. NE fluorescence likewise remained with the partially isolated nucleus/PPB complex of Fig. 2i.

Distinct fibers were rarely resolved at the NE surface during the earliest stages of PPB development (although some can be discerned approaching the nucleus in Fig. 1g). This cell contained a meshwork of MT that traversed the cytoplasm between nucleus and cell cortex and were most easily seen when the nucleus-to-plasmalemma distance was large.

Another phenomenon associated with some early PPB was a pattern of fluorescence perpendicular to the PPB axis (Fig. 2, e, g, and i). The striations were only seen when the PPB surface was carefully focused. Care also must be taken in

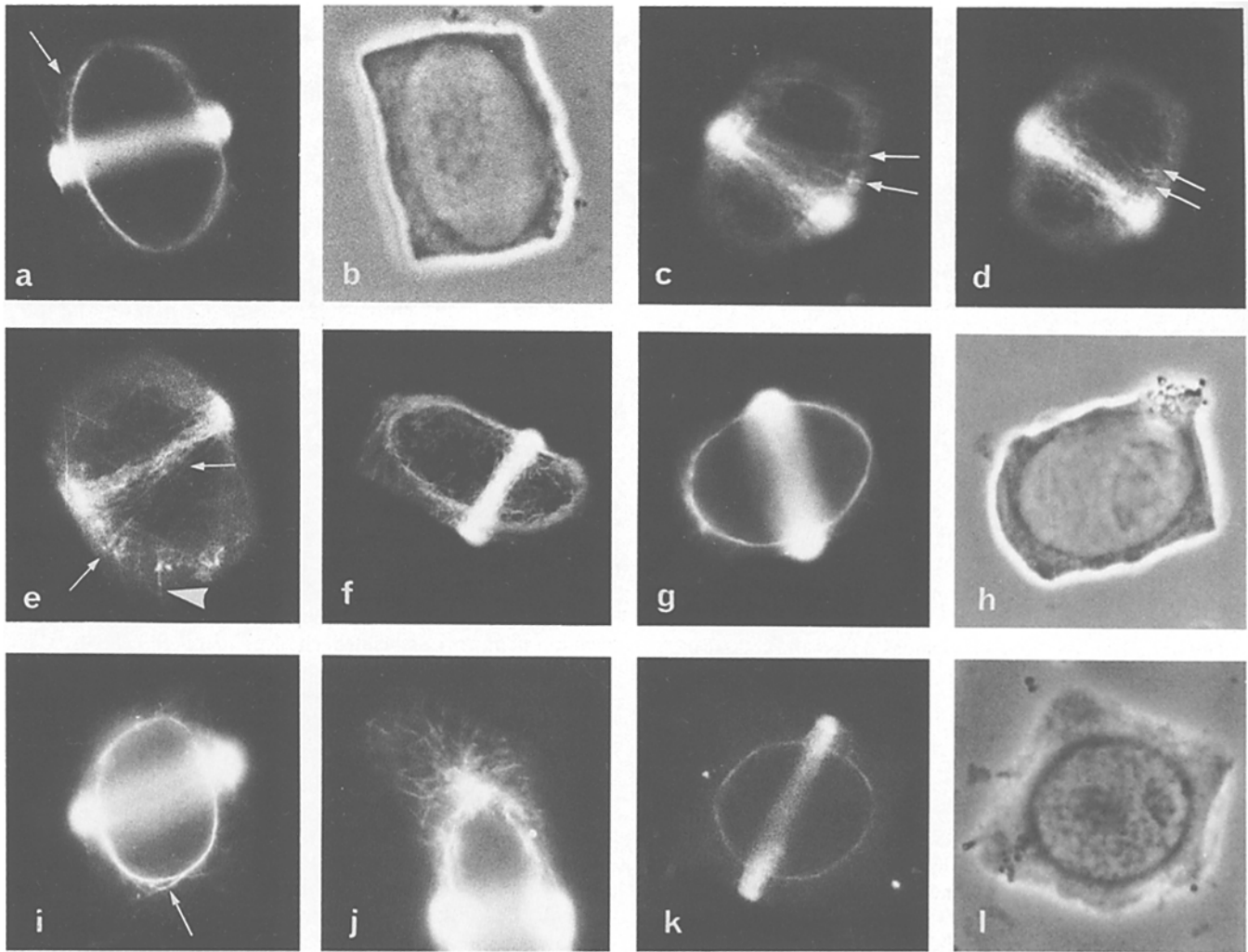


FIGURE 3 (a and b) Mid-stage PPB, focused on cell interior to show PPB in cross section, NE fluorescence, and indistinct fluorescence directly adjoining the nucleus (a, arrow). Nucleoli are indistinct, and little chromatin condensation has occurred (b). $\times 1,550$. (c and d) Two levels of focus to show the spatial relationship between PPB fibers and those near the NE. Focused a slight distance into the cell (c), parts of the PPB to either side of the cell center are in focus, as are a few other fibers that fan away from the parallel arrangement of PPB MT (arrows). These latter MT appear to angle between the PPB and NE. Exterior to this, at the juncture of the PPB's inner surface with the thin layer of cortical cytoplasm overlying the nucleus (d), band MT as well as other fluorescent fibers are visible in the peripheral cytoplasm (arrows). $\times 1,390$. (e) Cell with a mid-stage PPB, focused just inside the inner (cytoplasm-facing) PPB surface. Whereas MT of the PPB are themselves not clearly in focus, other MT underlying the PPB have become distinguishable. Some of these splay away from the PPB MT (arrows below PPB), whereas others assume a random orientation across the NE (area above the PPB). That some MT extend between cortical and perinuclear regions is suggested by MT profiles projecting from areas of NE seen in grazing view (arrowhead). $\times 1,280$. (f) Mid-stage PPB with many MT visible near the NE and in the cytoplasm between NE and plasmalemma. $\times 960$. (g and h) Cell with a mid-stage PPB. Wisps of fluorescence deviate from the fluorescence outlining the nucleus. $\times 1,440$. (i) Mid-stage PPB. Wispy fluorescence surrounding the nucleus and a thicker fiber, probably an MT bundle (arrow) are shown. $\times 1,200$. (j) An elongate preprophase cell with prominent fibers between the nucleus and the end of the cell. $\times 1,420$. (k and l) Mid-stage PPB (k), and the most advanced state of chromatin condensation found in cells of this stage (l). $\times 1,120$.

printing these pictures to achieve the necessary contrast, since the fluorescence intensity of the PPB MT, if printed to a normal level of brightness, masks the pattern. The distance between cross-bars varied from one cell to another, but it appeared to be consistent within a given cell (periodicity is $2 \mu\text{m}$ in Fig. 2e and $1.2 \mu\text{m}$ in Fig. 2i). This periodicity should not be confused with the fluorescent bars that were seen on the PPB near the edges of a cell (Fig. 2, c and d), which were in focus only when the PPB itself was not in focus across the cell face. The latter were simply the results of an "edge effect," an optical reinforcement of fluorescence that occurred as the PPB followed the polyhedral cell contour. In certain cells,

various bright edges such as these could be visualized one at a time by focusing through the entire cell depth. Since the spacing of fluorescent bars in Fig. 2, e g and i was less than cell edge-to-edge distance and since the bars came into focus simultaneously, they could not represent an edge effect. Rather, they may have reflected a particular structural organization of PPB MT. Whether this was a feature restricted to early PPB stages was not clear: it may be that the intense MT fluorescence of mid-stage and mature PPB had prevented us from visualizing these delicate striations at later stages. The PPB apparently has some means of maintaining strong affiliations among its constituent MT because with vigorous

squashing it was possible to sever the nucleus-PPB connections, yet retain the PPB as an intact, isolated unit which folded up onto itself as it settled on the coverslip (Fig. 2j). Unless excessive shear forces were applied during the squash, MT did not splay outward from these isolated PPB.

The Mid-stage PPB

The mid-stage PPB, as visualized with immunofluorescence microscopy, was an intensely fluorescent ring of tightly bundled MT. Assessing relative amounts of fluorescence during PPB development was very difficult, given the heterogeneity of cell size, shape, and tissue origin within the root, and the variations in staining intensity on any single coverslip, as well as the gradual fading of the fluorescein tag upon illumination. Therefore, it was not clear whether or not a narrow PPB was made up of more MT than were present in a wider, less compact, early PPB or in the interphase array. It was clear, however, that fluorescence associated with or adjacent to the cytoplasmic side of the NE was characteristic of the mid-stage PPB (Fig. 3, c-g, and i-k), and that at least part of this could be attributed to fibers (Fig. 3, c-g, and i).

Focused at a level partly into the cell, the narrow band was seen in cross-section, and the NE was outlined with fluorescence (Fig. 3, a, g, i, and k), whereas nearer the cell surface, PPB fibers were noticeable (Fig. 3, c, d, and e). In the latter cells, surfaces of the PPB and NE were close enough to each other that parts of each could be seen simultaneously. Although the nucleus is grazed in these views and therefore does not present a sharp outline, thin fluorescent fibers stand out against its indistinct silhouette. Where PPB fibers depart from focus in the center of Fig. 3c, some appear to bevel towards the NE and splay out from the co-parallel alignment they demonstrate in the PPB. A similar situation can be seen at the lower left side of the PPB in Fig. 3e. During this stage, such NE-associated fibers usually assumed an orientation that was at a slight angle to those of the PPB, or else appeared randomly oriented.

As in the earlier stages of PPB formation, fibers crossing between the nucleus and parts of the cell periphery other than that containing the PPB also existed when the PPB was mid-stage (Fig. 3, e-g, i, and j). In most instances, these were discerned as faint threads, suggesting that they were single MT, although occasionally a thicker bundle-like image was found (Fig. 3i). We measured high magnification prints of the thinnest fluorescent fibers seen in our preparations and obtained an apparent diameter of 220–280 nm, which closely agrees with the value, 200–250 nm, calculated by Weber and Osborn (31) for fibers established as individual MT using electron microscopy. In some instances, the fibres tangentially contacted the nucleus, giving the impression of continuity with the MT that lay along the nuclear surface (Fig. 3, g, and i). In other instances, especially in elongate cells, the fibers seemed to form a much larger angle with the nucleus (Fig. 3, e, f, and j). In Fig. 3j, cytoplasmic MT converge at the NE region that later would have served as a spindle pole, i.e., a focal point for spindle MT. Only a small fraction of the preprophase onion cell's total MT complement was found traversing the cytoplasm. This contrasts with the situation seen at late interphase and early prophase in *Haemaphysalis*, in which all MT were arranged in an extensive radial network (7).

The state of the nucleus at mid-preprophase was not markedly different from that seen at early preprophase. Although

there was some variability, nucleoli usually were more indistinct than those in cells with a very wide or double PPB. The nucleoplasm usually had begun to look less homogeneous, with indications of incipient chromatin condensation (Fig. 3, b and h). Occasionally a cell with chromatin in a more advanced stage of condensation could be found (Fig. 3l), but distinct chromosomes were not yet present.

Up to and through this point in PPB development, the fluorescence intensity of the PPB had exceeded that of the NE and the fibers that extended from it. However, as chromatin condensation increased, fluorescence at the NE dominated. As the PPB diminished, fluorescent fibers along the NE were no longer parallel to those of the PPB nor randomly-oriented, but rather assumed the orientation of mitotic spindle MT. This transition in MT distribution from the mature PPB to spindle will be detailed in a future paper, and will be compared with the interphase-to-spindle transition of cells without a PPB, as described by De Mey et al. (7).

DISCUSSION

Characteristics of the Earliest PPB Stages

Comparing immunofluorescence patterns with nuclear morphology has allowed us to follow successive steps of PPB development and associated changes in other cellular MT throughout early to mid-preprophase. In onion root tip cells, two identifying characteristics of preprophase cells, nucleolar breakdown (5, 22, 23, 26, 27, 30) and perinuclear fluorescence, were present from the beginning of PPB development. Another feature of preprophase, one that was less immediately obvious than changes in nucleoli or NE-associated fluorescence, was a system of MT traversing the cytoplasm between nucleus and cell cortex. The traversing MT have been described in several cases in which a well-developed PPB is present (4, 9, 12, 15, 23, 26). Using immunofluorescence microscopy, these MT could be detected even at the earliest stages of PPB formation. They were more visible in cell regions where the PPB fluorescence did not obscure them (Fig. 1g).

Of the various preprophase characteristics, the distinctive fluorescence associated with the NE was especially helpful in detecting the earliest PPB stage. At this time the band was barely distinguishable from an interphase MT array, and might not have been recognized if presence of a discrete MT band had been the sole criterion employed. The bipartite appearance of some of these earliest bands and the more pronounced double bands in elongated cells at slightly later stages were an unexpected discovery, since double PPB have been described previously only in caffeine-treated cells (24). Examples of bipartite and double bands shown herein occurred throughout numerous samples. They were only a small proportion (<5%) of all PBS observed, but very early PPB stages in general were seen much less frequently than later stages. Within the subpopulation of very early preprophase cells, bipartite and double bands were fairly common. No indications of aberrant division patterns (e.g., phragmoplasts that were double or forked between two planes) were observed in cells from normally grown plants, so there was no reason to suspect that these double PPB represented a mistake in the typical MT cycle.

Establishment of the PPB

Pictures of wide PPB with low MT densities at early preprophase vs. narrow PPB with densely-packed MT at later

preprophase suggest that a cell gradually approximated the correct site for its PPB. Two general suggestions have been put forward to explain how MT become organized to form a mature PPB. If a particular location in the cell cortex contains a microtubule organizing center or centers (MTOC), intact interphase cortical MT might move to form the band (23), with the MTOC specifying the correct orientation of constituent MT. It is conceivable that double PPB represent intermediate steps in which the pair of bands flank the ultimate PPB plane and progressively merge into a single band. Such a consolidation of existing MT was easily conceptualized for the most common situation found in dividing *Allium* root cells, in which interphase and PPB arrays were parallel to each other, both being transverse to the long axis of the root, e.g., Fig. 1*f*. However, in cells dividing to produce new longitudinal files (6, 15) and in guard mother cell divisions of certain stomatal complexes (6, 11), PPB MT assume a perpendicular orientation relative to the MT they eventually replace, and a simple "bunching up" of interphase MT that are already in the necessary orientation obviously cannot be operative. The two perpendicular arrays temporarily co-exist, and individual MT at an oblique angle to them are present in some cases (11, 15), but it is not clear whether or not they are a constant feature of the transition. Immunofluorescence studies of cells preparing for longitudinal divisions should provide valuable information on the manner in which the shift between orientations is made. Any instance demonstrating a clear absence of oblique MT would strongly argue against movement and reorientation of intact MT.

The second suggested mechanism of PPB formation is that some interphase MT depolymerize and that others polymerize at the PPB site (5, 8, 11, 16). Observations of structures interpreted as cortical nucleating sites (NS) (16) and MTOC (11) found in the PPB plane in certain cells, toward which are focused short MT segments, lend support to this proposal, but it is not clear whether or not this is a universal mechanism for establishing a PPB. Gunning et al. (16) found NS associated with developing PPB exclusively at cell edges, rather than along cell faces. Were NS present in *Allium* cell edges, we could expect to see indications of punctate fluorescence along edges brought into focus as in Fig. 2, *c* and *d*.

Convergence points for short MT in the cell cortex have not been noted in *Allium*, but difficulties in focusing and recognizing these structures in cell edges that are not lying flat with respect to the optical path may have precluded detection of them. That the dense matrix that characterizes plant cortical MTOC and NS may be preventing antibody access to MT, as it does in phragmoplasts (Fig. 5, *n-r* in reference 32, and Fig. 2, *a*, *b*, and *d* in reference 7), is another possible explanation for their absence in *Allium*. Electron microscopy should be able to settle the issue of whether or not NS generally are present at the time of PPB formation.

As a point of clarification, it should be stressed that any lack of cortical NS does not preclude the presence of MTOC function. By definition, an organizing capacity must exist at the location of the mature PPB to ensure specific MT alignment, whether MT comprising the PPB originate at NS within the PPB site or whether they are nucleated originally at interphase NS and subsequently relocated. An entity to which a PPB organizing function can be ascribed remains undescribed. Conversely, finding NS among PPB MT is not proof of their involvement in PPB formation. Such NS could be indicative of an independent nucleating or organizing capacity

of this particular region of the cortex without themselves being the effective agent in establishment or alignment of PPB MT.

Structural Stability of the PPB

Hardham and Gunning (17) presented examples of cross-bridging among PPB MT as seen with electron microscopy. The bridge periodicity was reported to be ~20–25 nm. Resolution to that level of detail obviously is not possible with immunofluorescence microscopy, but we have found cross-bars on some PPB with periodicity of roughly 1–2 μm . We have never seen, nor do we know of any electron microscopy evidence of short MT crossing the PPB at a right angle to its axis in a periodic fashion, although individual MT may do so (15). Further, there is evidence that areas of MT overlap in the band do not lie in register (17). It is possible that fluorescent striations represent regions along the PPB that are more accessible to antitubulin. This situation could occur if there were components bound to MT in a periodic fashion. The reaction with antitubulin indicates that the striations reported herein did contain tubulin. It would be interesting to see if anti-MT associated proteins (MAP) or antidynein react with PPB to reveal complementary cross-banded patterns.

Whether or not the fluorescent bars represent a large scale cross-bridging of MT across the entire band width, there can be no doubt that effective MT cross-linking operates at some level. Although very few MT are interwoven through the PPB array (17), the band retains its parallel alignment of MT in the face of treatments that damage or remove other cytoplasmic components. Its stability was evinced most dramatically when prolonged digestion and squashing resulted in isolated bands such as that of Fig. 2*j*.

Structural Stability of the Nucleus-Cortex Linkage

Cytoplasmic MT visible at early PPB stages have already been mentioned briefly. Often MT approach the nucleus from all sides and at varying angles, but occasionally MT appear to focus on NE regions that subsequently will be replaced by spindle poles. It is not possible yet to say whether an individual MT or bundle covers the entire distance from nucleus to cortical cytoplasm or whether there are shorter, overlapping MT segments. The end effect of a structural link between nucleus and cortex that possesses considerable mechanical strength is apparent, however. This was first demonstrated by the centrifugation experiments of Pickett-Heaps (23), in which a nucleus was found to more strongly resist displacement at preprophase than at other cell cycle stages, and to a greater degree as preprophase progressed. The particular link between nucleus and PPB site in the cortex is borne out by the isolation of nuclei and their associated PPB as a single unit. In cells in which the nucleus migrates after PPB formation, MT may lie preferentially between the nucleus and the area of cortex occupied by the PPB (15). In the more usual case in which the PPB girdles the nucleus, sometimes MT appear to lie at an angle specifically between the PPB and nucleus, with roughly the same orientation as PPB MT (4, 23). MT parallel to PPB MT are also found at the NE surface in these cells, in accord with our pictures suggesting that PPB MT deviate through the cortical cytoplasm toward the underlying nucleus at the mature PPB stage. Galatis (11), however, thinks that MT along the NE, although aligned with the PPB, do not diverge from it but instead are an independent system. Direct

MT links between nucleus and PPB would provide a logical explanation for the observed stability of their association, but other factors such as gelation of the intervening cytoplasm could produce the same result.

Source of NE Fluorescence in Early PPB Stages

Results of the present study lead us to question the nature of the diffuse NE-associated fluorescence of the earliest PPB stages. Even with optimal viewing circumstances and careful focusing, individual fibers lying along the NE surface at very early preprophase were rarely resolved. This indicates that the fluorescence intensity of the NE surface as seen in optical cross-section in these cells was due to either (a) cytoplasmic MT that approached and abutted the NE but were not tangent to it for a long enough distance to be readily discerned in the same focal plane as the NE, (b) a high MT density along the NE, packed so closely that individual resolution was not possible, or (c) nonmicrotubular tubulin.

In *Haemanthus* endosperm cells, which have no PPB, MT arrays radiate from the nucleus from interphase to midprophase (7, 19), and the NE is likewise labeled with antibodies to tubulin (7). However, in cells with PPB no such radial arrays have been described for any stage of the cell cycle. MT at various angles between the nucleus and cell cortex (4, 9, 12, 15, 23, 26, 29, 32) have been reported, but it is not clear from these papers how early in PPB development these cytoplasmic MT appear. Most cells judged on the basis of phase contrast and fluorescence microscopy to be in the earliest PPB stages do not have extensive enough MT arrays approaching the nucleus to account for significant NE fluorescence, thereby eliminating the first possibility. The second possibility seems unlikely because MT that lie along or just exterior to the outer NE can be resolved at later preprophase stages and in a few early preprophase cells with exceptionally extensive networks of non-PPB MT (Fig. 1g). MT at later preprophase stages almost certainly correspond to the well-documented clear zone that generally surrounds the nucleus of mitotic plant cells and contains numerous early prophase perinuclear MT and later the spindle (2, 4, 7, 8, 10, 11, 18, 22, 29). Although a clear zone can be seen during preprophase, ultrastructural studies have not yet determined if it is present at these earliest preprophase stages and if so, whether or not it contains MT. However, it seems very improbable that it would contain a MT density so high as to prevent resolution of individual or bundled MT. This suggests that the lack of fibrillar images on the NE of most very early preprophase cells reflects a real absence of MT and is not simply an artifact.

It should be noted that at least some cellular tubulin not in the form of microtubules is retained and visualized by immunochemical techniques, so that the third option is not an impossible one. Miller et al. (20) report that the nontubular lamellar strata of a spermatid multilayered structure reacts with antitubulin, and comparisons between patterns obtained with immunofluorescence and fluorescently-tagged colchicine led Albertini and Clark (1) to conclude that their antibody binds to both assembled and disassembled tubulin in cells fixed in formaldehyde and subjected to a cold methanol step. Likewise, in cells treated with colcemid, background cytoplasmic fluorescence levels suggest that tubulin from depolymerized MT is capable of reacting with antibody (21). Evidence that membrane-bound, nonfibrillar tubulin is retained after paraformaldehyde fixation and subsequent processing also can be found in the first paper to appear on tubulin

immunofluorescence in plant cells: Franke et al. (10) present views of diffuse staining patterns both in fragmented NE membranes and in the membrane-rich polar cap regions.

If we assume that at least part of the diffuse fluorescence seen at early preprophase represented a pool of nonpolymerized tubulin, then we must conclude that tubulin preferentially bound to the NE rather than to cell membranes in general, or that a pool of soluble tubulin was maintained in the immediate vicinity of the NE. The latter possibility seems less likely, but cannot be disproved by present data. The clear zone is only $\sim 0.1\text{--}0.3\ \mu\text{m}$ wide in onion root cells, and if present by early preprophase, it conceivably could hold tubulin in a nonpolymerized state. Comparisons of matched pairs of fluorescence and phase pictures printed to the same magnification gave no indication that fluorescence extended any appreciable distance from the NE in onion cells. However, even the sharply outlined areas of NE-associated fluorescence were a few tenths of a micrometer wide, or approximately the same width as the clear zone. Conclusive statements as to whether there was nonpolymerized tubulin directly at the NE surface or spread throughout the clear zone awaits the resolution afforded by immunoelectron microscopy with gold or ferritin labels.

The same issue of whether or not there is soluble tubulin near the nucleus prior to prophase arises from the work of De Mey et al. (7) on *Haemanthus* endosperm cells. An immunogold procedure coupled to light microscopy reveals a dense network of MT radiating out from the clear zone into the surrounding cytoplasm before the spindle is established. Staining patterns close to the nucleus become indistinct and individual MT can no longer be resolved. The extensive clear zone here (up to $10\ \mu\text{m}$ wide [2]) and the very large number of MT makes it more apparent that stained material occupied the entire clear zone, but whether diffuse staining immediately adjacent to the nuclear envelope was due solely to overlap of MT meshwork or included a soluble or membrane-bound tubulin component likewise remains to be determined. The data of De Mey et al. (7), showing interphase MT focal centers near the *Haemanthus* NE, and that of Lambert (19), whose higher magnification pictures clearly show prophase MT in *Mnium* and *Haemanthus* abutting end-on to the NE, either singly or in small groups, led both groups to propose that the NE itself functions as a NS in cells with anastral spindles. Examples of extranuclear MT radiating from/focusing onto future pole areas along the NE, in preprophase cells that have not yet set up a mitotic spindle (Fig. 3j), also are in accord with this concept. As an NS, it would be expected that the NE have a nearby source of non-polymerized tubulin to use for assembly. For comparison, it is interesting that a nonmicrotubular tubulin layer associated with a mass spermatid MTOC has been visualized with immunofluorescence microscopy (20).

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