

Biotin-Tubulin Incorporates into Kinetochore Fiber Microtubules during Early but Not Late Anaphase

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Abstract. The dynamic behavior of kinetochore fiber microtubules has been examined in PtK₁ cells during anaphase of mitosis. Cells in anaphase were injected with biotin-tubulin and, at various intervals after injection, fixed for light or electron microscopic immunolocalization of biotin-tubulin-containing microtubules. When cells in early to mid anaphase were injected with biotin-tubulin and fixed 1–2 min later, fluorescence was observed throughout the spindle, including the region of the kinetochore fibers. Electron microscopy of early to mid anaphase cells, after processing with immunogold methods, revealed both labeled and unlabeled microtubules in the kinetochore fibers; some labeled microtubules contacted the

kinetochores. When late anaphase cells were injected with biotin-tubulin, and fixed a few minutes later, little fluorescence was observed in the kinetochore fibers. Electron microscopy confirmed that kinetochore fibers in late anaphase cells were refractory to tubulin incorporation. The results of these experiments demonstrate that the kinetochore fiber incorporates new microtubules during early anaphase but that this incorporation ceases in mid to late anaphase. Thus, microtubule turnover within the kinetochore fiber does not abruptly cease at the onset of anaphase and anaphase kinetochore fiber microtubules are more dynamic than previously suspected.

RECENT experiments clearly demonstrate that spindle microtubules are dynamic polymers which continually undergo assembly/disassembly reactions in living metaphase cells (Inoue, 1981; Salmon et al., 1984a,b; Saxton et al., 1984; Wadsworth and Salmon, 1986a,b). This dynamic behavior has been observed using fluorescent analogues of tubulin which rapidly incorporate into spindle microtubules after microinjection into living cells (Keith et al., 1981; Wadsworth and Sloboda, 1983; Salmon et al., 1984a; Saxton et al., 1984). In addition, the rapid recovery of tubulin fluorescence after photobleaching demonstrates that the majority of microtubules undergo rapid turnover in metaphase cells (Salmon et al., 1984a; Wadsworth and Salmon, 1986a,b). However, these photobleaching studies also reveal that a subset of spindle microtubules, most likely those comprising the kinetochore fiber (Rieder, 1982), turn over more slowly (Wadsworth and Salmon, 1986a).

Immunogold electron microscopic localization techniques have also been used to examine the assembly of individual microtubules in metaphase and prometaphase cells. In these experiments, cells are injected with biotin-tubulin, fixed, and processed at various intervals after injection to reveal biotin-containing microtubules (Mitchison et al., 1986;

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Geuens et al., 1989). Again, rapid turnover of spindle microtubules is clearly demonstrated. In addition, incorporation of labeled tubulin proximal to the kinetochore, at the plus ends of the microtubules, can be demonstrated (Mitchison et al., 1986; Mitchison, 1988). Together these experimental approaches support a model for metaphase spindle microtubule behavior in which the majority of microtubules continually assemble and a subset catastrophically disassembles (Mitchison and Kirschner, 1984a,b). These experiments also indicate, however, that turnover of microtubules in the kinetochore fiber occurs more slowly than nonkinetochore microtubules (Mitchison et al., 1986; Wadsworth and Salmon, 1986a,b; Geuens et al., 1989), presumably due to the stabilizing effect of the kinetochore on the microtubules (Mitchison and Kirschner, 1985b; Huitorel and Kirschner, 1988).

During anaphase, a major reorganization of the spindle occurs (Bajer and Mole-Bajer, 1975; McIntosh et al., 1975; Jensen, 1982; McIntosh, 1985). The kinetochore fibers (composed of microtubules which contact the kinetochore and extend part or all of the distance to the pole, microtubules which originate at the pole and end before contacting the kinetochore, and microtubules with two free ends; Rieder, 1981a, 1982) shorten as the chromosomes move poleward. Concurrently, interzonal and astral microtubules lengthen (Saxton and McIntosh, 1987). Despite these dramatic changes,

the dynamic assembly/disassembly behavior of microtubules during anaphase remains incompletely characterized. In photobleaching experiments on anaphase cells, bleached regions on the kinetochore fiber remain stationary with respect to the spindle pole while the distance between the kinetochores and the bleached region shortens (Gorbsky et al., 1988). Thus, kinetochore microtubules disassemble from their plus ends (Euteneuer and McIntosh, 1981) during anaphase. Microinjection of labeled tubulin into living cells during late anaphase indicates that the late anaphase spindle continues to incorporate new microtubules, in the asters, interzonal region, and half-spindle (Saxton and McIntosh, 1987). In these experiments, however, kinetochore and non-kinetochore microtubules could not be distinguished.

In the experiments reported here, we have examined the incorporation of new microtubules into the anaphase kinetochore fiber by microinjecting individual cells with biotin-tubulin during anaphase, fixing the cells, and examining the pattern of incorporation using conventional and confocal fluorescence light microscopy and immunogold electron microscopy. Our results clearly demonstrate that incorporation of newly assembled microtubules into the kinetochore fiber occurs during anaphase chromosome motion, but that this incorporation halts as the cells progress through anaphase. These data suggest that microtubule recruitment into the kinetochore fiber occurs by capture and release of individual microtubules and demonstrate that the kinetochore fiber of anaphase cells is more dynamic than previously recognized.

Materials and Methods

Preparation of Biotin-Tubulin

Biotin-tubulin was prepared as described (Mitchison et al., 1986). The assembly characteristics of the biotin-tubulin were tested by measuring the change in optical density of a mixture of unlabeled tubulin, microtubule-associated proteins, and biotin-labeled tubulin in 0.1 M Pipes, 1 mM MgSO₄, 2 mM EGTA, and 1 mM GTP, after warming to 37°C. At a final ratio of 1:7, biotin-labeled to unlabeled tubulin, assembly was indistinguishable from reaction mixtures containing no biotin-tubulin. Small aliquots of biotin-tubulin were stored at -70°C in injection buffer (20 mM sodium glutamate, 1 mM EGTA, and 0.5 mM MgSO₄, pH 7.2). Biotin-tubulin was adjusted to 1 mM GTP and centrifuged for 10 min at maximum speed in an Eppendorf microcentrifuge (Brinkmann Instruments Co., Westbury, NY) immediately before microinjection. The concentration of biotin-tubulin in the microinjection pipette was 3-4 mg/ml as determined by a modification (Schacterle and Pollack, 1973) of the method of Lowry (Lowry et al., 1951).

Cell Culture

PtK₁ cells were grown at 37°C in Ham's F-12 medium supplemented with 10% fetal bovine serum, 10 mM Hepes, pH 7.2, and antibiotics. For use in experiments, cells were plated on glass coverslips and allowed to grow for 36-48 h.

Microinjection

A laboratory-built microinjection chamber was used for these experiments. The chamber was constructed by drilling a 19-mm-diam hole in a 50 × 76-mm glass slide; a 5-mm layer of Sylgard 184 silicone elastomer (Dow Corning Corp., Midland, MI) was then added to the top surface of the slide, surrounding the hole, and allowed to polymerize. A 22 × 22-mm well was then cut in the Sylgard centered over the hole. The coverslip of cells was held in place in the bottom of this well using vaseline. Two notches were cut in the Sylgard to accommodate a temperature probe and the microinjection needle and the chamber was mounted on the stage of an IM 35 microscope (Carl Zeiss, Inc., Thornwood, NY). Individual microinjected cells could be

followed before microinjection and readily relocated after microinjection and processing by replacing the coverslip in the chamber and by using the stage position markers. For room temperature studies (22°C), cells were equilibrated in the injection chamber for 5 min before injection. For experiments at 30°C, temperature was maintained using an Opti-Quip red beam incubator calibrated with a YSI telethermister. Incubation at these temperatures slows, but does not inhibit, the progression of PtK₁ cells through mitosis (Rieder, 1981b) and reduces the number of nonkinetochore microtubules in the cells. As a result, kinetochore fiber dynamics are easier to study since, at these temperatures, kinetochore fibers are easier to delineate from the remaining spindle microtubules. Microinjection was performed using a Narishige micromanipulator and an Eppendorf 5242 microinjector. Needles were pulled on a Sutter Instruments (San Rafael, CA) P-80 Brown-Flaming micropipette puller using Microdot (Friedrich and Dimmock, Millville, NJ) capillaries. Pipettes were back loaded using a 10- μ l Hamilton Co. (Reno, NV) syringe.

Immunofluorescence

After microinjection, cells were rinsed in saline and lysed in buffer containing 80 mM Pipes, 5 mM EGTA, 1 mM MgCl₂, and 0.5% Triton X-100, pH 6.8 (Cassimeris et al., 1986). This lysis procedure removes soluble tubulin from the cells and reduces background fluorescence. Cells were then fixed for 15 min in 2% paraformaldehyde, 0.1% glutaraldehyde in PBS, pH 7.3, and rinsed in PBS containing 0.1% Tween and 0.02% azide (PBS-Tw-Az). Antibody staining was performed in humid chambers at room temperature. Cells were incubated with rabbit anti-biotin antibodies (1:50 dilution) (Enzo Biochem Inc., New York, NY) for 30 min, rinsed in PBS-Tw-Az, and incubated first with fluorescein-goat anti-rabbit and then fluorescein-rabbit anti-goat antibodies, each for 30 min. Cells were then incubated in a mouse monoclonal anti-tubulin antibody (generous gift of Dr. J.R. McIntosh, University of Colorado, Boulder, CO) for 1 h followed by rhodamine-goat anti-mouse antibodies for 30 min. All fluorescent secondary antibodies were purchased from Organon Teknica (West Chester, PA); both primary and secondary antibodies were diluted in PBS-Tw-Az containing 1% BSA before use. Cells were rinsed thoroughly with PBS-Tw-Az between successive antibodies. Stained cells were mounted in *n*-phenylenediamine (Valnes and Brandtzaeg, 1985) and sealed with nail polish.

For standard immunofluorescence observations, a Zeiss IM-35 microscope with a 63× 1.4 NA lens and filters for rhodamine and fluorescein excitation/emission was used. Cells were photographed on Kodak TMAX 400 film (Eastman Kodak Co., Rochester, NY) developed in HC 110 dilution B. For confocal microscopy, a Bio-Rad Laboratories laser-scanning confocal microscope (Richmond, CA) was used. The scan head was mounted on a Nikon Optiphot microscope equipped with a 60× 1.4 NA objective lens. The pinhole was set so that section thickness was \sim 1.0 μ m.

Electron Microscopy

For electron microscopic immunocytochemistry, injected cells were rinsed in saline, lysed in buffer containing 80 mM Pipes, 5 mM EGTA, 1 mM MgCl₂, and 0.5% Triton X-100, pH 6.8, and then fixed in 1% glutaraldehyde, 2% paraformaldehyde in PBS, pH 7.4, for 5 min (EM fix), and finally fixed as described above for an additional 15 min. Fixed cells were incubated in anti-biotin antibodies for 30 min followed by 5-nm gold-labeled goat anti-rabbit antibodies (Janssen Pharmaceutica, Piscataway, NJ) at a 1:2 dilution in Tris buffer, pH 8.2, for 3 h at room temperature. Immunolabeled cells were rinsed briefly in PBS-Tw-Az and rinsed again in 0.1 M Nacacodylate buffer for 10 min on a shaker, then refixed in EM fix for 10 min. Cells were then osmicated (0.5%) for 10 min, dehydrated in an ethanol series, and embedded in Epon (Rieder et al., 1985). Cells followed in vivo were relocated within the Epon, excised, and mounted on Epon pegs for serial sectioning with a diamond knife. Ribbons of sections were mounted on slot grids and stained with uranyl acetate and lead citrate. Sections were examined and photographed in a Philips Electronic Instruments, Inc. (Mahwah, NJ) EM 301 electron microscope operated at 80 kV using a 70-nm objective aperture (Rieder et al., 1985).

Analysis of Chromosome Motion

Recordings of chromosome motion were made using a Dage Newvicon Inc. (Wabash, MI) camera (model 67M) and a Gyr Products (Anaheim, CA) one-half inch time-lapse tape recorder (TLC 1400). The rate of chromosome motion was determined from traces of chromosome position made onto sheets of clear acetate. Alternatively, for cells injected at room temper-

ature, the position of the chromosomes was measured from phase-contrast micrographs taken at successive intervals. The rate of chromosome-to-pole motion was measured from the slope of a plot of chromosome-to-pole distance vs. time. The initial slope was used, so that the contribution of pole-pole separation would be minimized.

Determination of the Extent of Anaphase Motion

To determine the extent of chromosome-to-pole motion which had occurred in each anaphase cell, the following measurements were made. Using a survey electron micrograph, the pole-to-pole spindle length and the kinetochore-to-pole distance were measured for each fixed, injected cell. The ratio of chromosome-to-pole length, X , to one-half the pole-to-pole distance, Y , was used as an index for the extent of chromosome-to-pole motion which had occurred for each cell by the time of fixation for electron microscopy. Since electron micrographs were used for these measurements, it was not possible to calculate the contribution of pole-pole separation to the Y value that was measured. Thus, these measurements are only an estimate of the extent of chromosome motion which occurred in these cells. Low X/Y values indicate that most of anaphase A had already occurred and high values indicate that cells were in early anaphase at the time of fixation for electron microscopy. Note that in some cases cells were injected during early anaphase, but had progressed to later anaphase by the time of fixation.

Results

Kinetochore Fiber Microtubule Dynamics during Early Anaphase

To determine whether microtubule incorporation into kinetochore fibers ceases during anaphase, we microinjected biotin-tubulin into cells after chromatid separation had occurred. These cells were then fixed at various intervals after injection and the location of biotin-tubulin-containing microtubules determined using light and electron microscopy. Phase-contrast micrographs or video records of each injected cell were used to record changes in chromatid position during the experiment. The rate of chromosome motion for control, uninjected cells, and for cells injected with biotin-tubulin during anaphase chromosome motion at 22 and 30°C was also measured (see Materials and Methods). Microinjection

Table I. Rate of Anaphase Chromosome Motion in PtK₁ Cells

	Rate
	$\mu\text{m}/\text{min}$
Uninjected, 30°C	$1.6 \pm 0.7^*$
Injected during anaphase, 30°C	$1.5 \pm 0.2^*$
Uninjected, 22°C	$0.4 \pm 0.2^*$
Injected during anaphase, 22°C	$0.4 \pm 0.1^\ddagger$

* $n = 4$.

‡ $n = 3$.

did not significantly alter the rate of chromosome-to-pole motion (Table I).

When cells were observed 1–2 min after injection, while still in early to mid anaphase, biotin-tubulin was detected in the spindle fibers, asters, and interzonal region (Fig. 1). Spindle microtubule assembly, therefore, continues in early anaphase cells. However, these light microscopic observations lacked sufficient resolution to reveal whether individual kinetochore microtubules were labeled in these cells. In addition, the data collected using confocal microscopy were digitally processed to provide the cleanest image, so the resulting fluorescence was not a quantitative measurement of the number of biotin-labeled microtubules. Our immunofluorescence observations indicate that cells injected at 30°C incorporate more biotin-tubulin than cells injected at 22°C in a similar stage of mitosis (data not shown).

To examine the dynamic behavior of individual microtubules within the anaphase kinetochore fiber, early and mid anaphase cells at 22 or 30°C were injected with biotin-tubulin and processed for electron microscopic immunocytochemistry at various intervals after injection. The extent of anaphase chromosome motion which had occurred in each cell at the time of fixation was estimated from survey electron

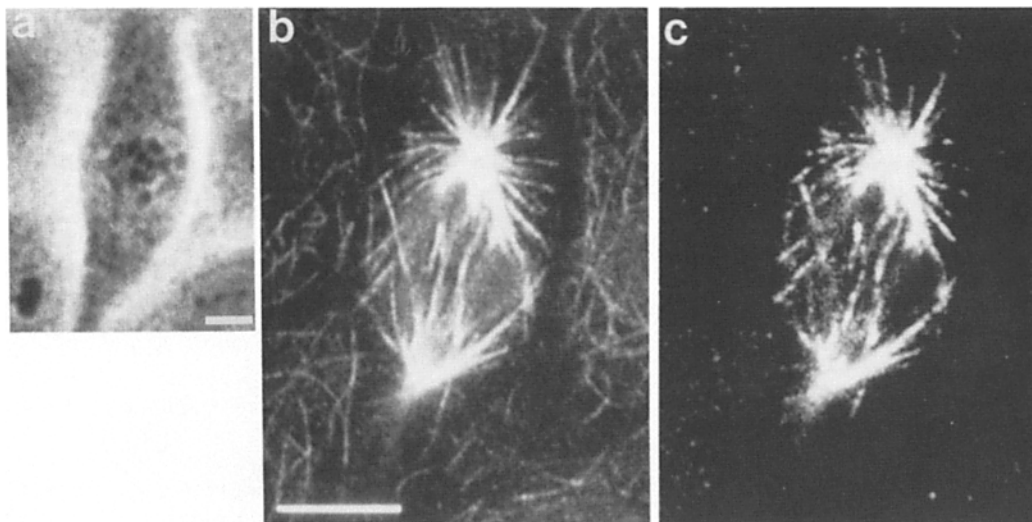


Figure 1. Immunolocalization of biotin-tubulin in an anaphase cell injected at 30°C and lysed 65 s after injection. (a) Phase-contrast photograph of the cell just after injection; (b) after anti-tubulin staining; and (c) after anti-biotin staining. Biotin fluorescence is observed in the spindle fibers, astral, and interzonal regions. This cell was imaged using confocal fluorescence microscopy, as described in Materials and Methods. Bar, 10 μm .

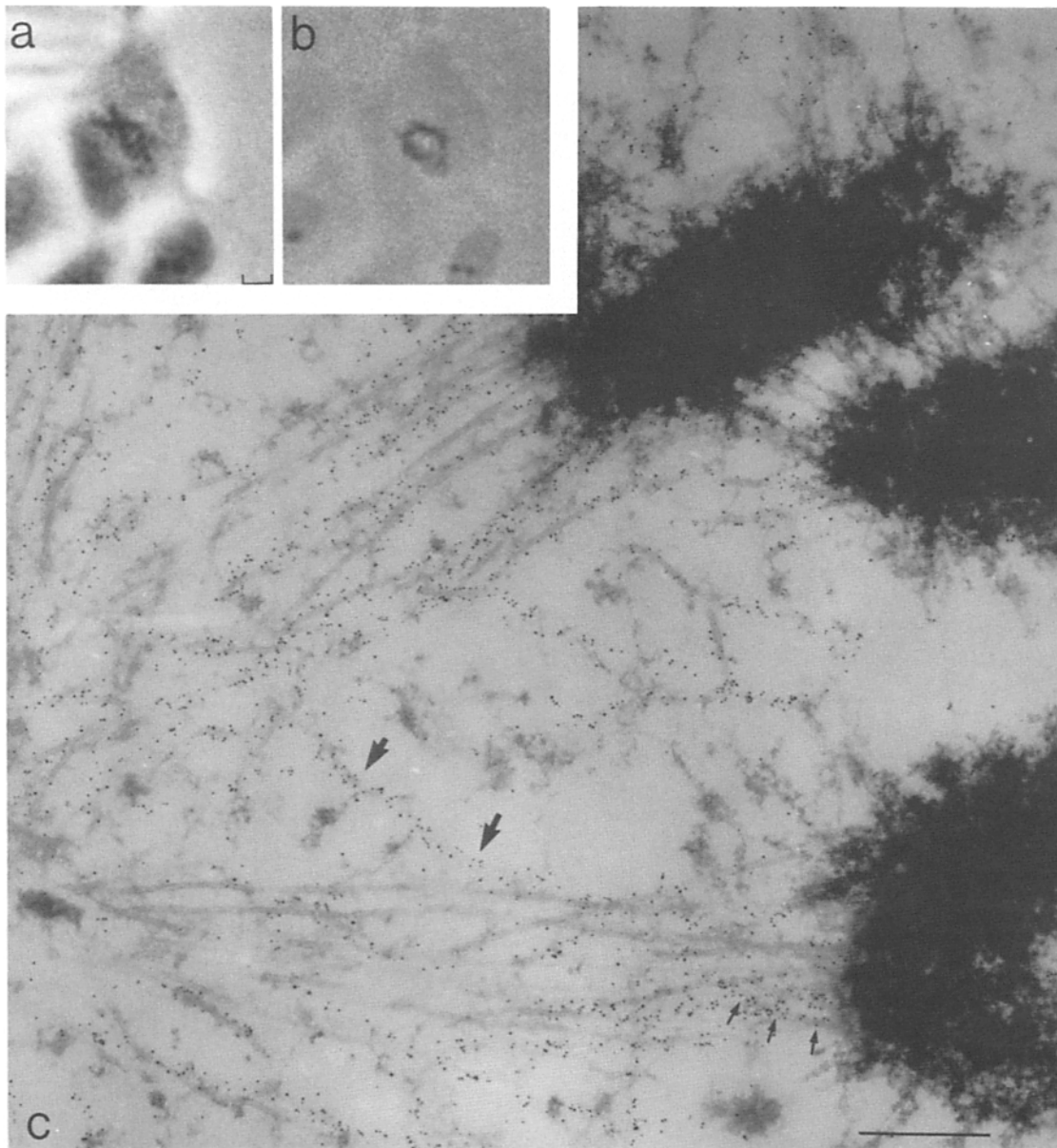


Figure 2. Electron microscopic immunolocalization of biotin-tubulin in an early anaphase cell. Phase-contrast light micrographs of cells just after microinjection (*a*) and after lysis, fixation, and embedding (*b*). The cell was injected at 30°C and lysed 150 s after injection. Note that the chromosomes have moved poleward after injection. (*c*) Electron micrograph of the region between the chromosomes and pole; both labeled and unlabeled microtubules are detected. *Large arrows*, a labeled microtubule peripheral to the fiber; *small arrows*, a labeled microtubule within the fiber. Bars: (*a* and *b*) 5 μm ; (*c*) 0.5 μm .

micrographs as described in Materials and Methods. Briefly, the ratio of the kinetochore-to-pole distance (X) and one-half the pole-to-pole distance (Y) was used to estimate the extent of chromosome motion which had occurred in each cell. Early to mid anaphase cells are defined here as those cells with an X/Y ratio of 0.4 or greater.

The chromosome-to-pole region of a cell injected in early anaphase at 30°C and fixed 2.5 min after injection is shown in Fig. 2. A labeled microtubule at the periphery of the kinetochore fiber is marked with large arrows. Labeled microtubules are also observed within the kinetochore fiber and some of these labeled microtubules contact the kinetochore (Fig. 2, *small arrows*). A mid anaphase cell which has been injected with biotin-tubulin at 22°C, fixed at 2 min after in-

jection, and prepared for electron microscopic immunolocalization is shown in Fig. 3. Phase-contrast micrographs (not shown) reveal that the chromosomes were approximately midway to the poles at the time of fixation. Again, both labeled and unlabeled microtubules are present in the region between the chromosomes and the pole (Fig. 3). Labeled microtubules are not restricted to the region proximal to the kinetochores, but extend from the polar region toward the kinetochores. As in early anaphase cells (Fig. 2), some labeled microtubules contact the kinetochores.

A single mid anaphase cell, injected with biotin-tubulin, was also examined in a complete series of serial sections (Fig. 4). Although the microtubule distribution has not been reconstructed from these micrographs, gold-labeled micro-

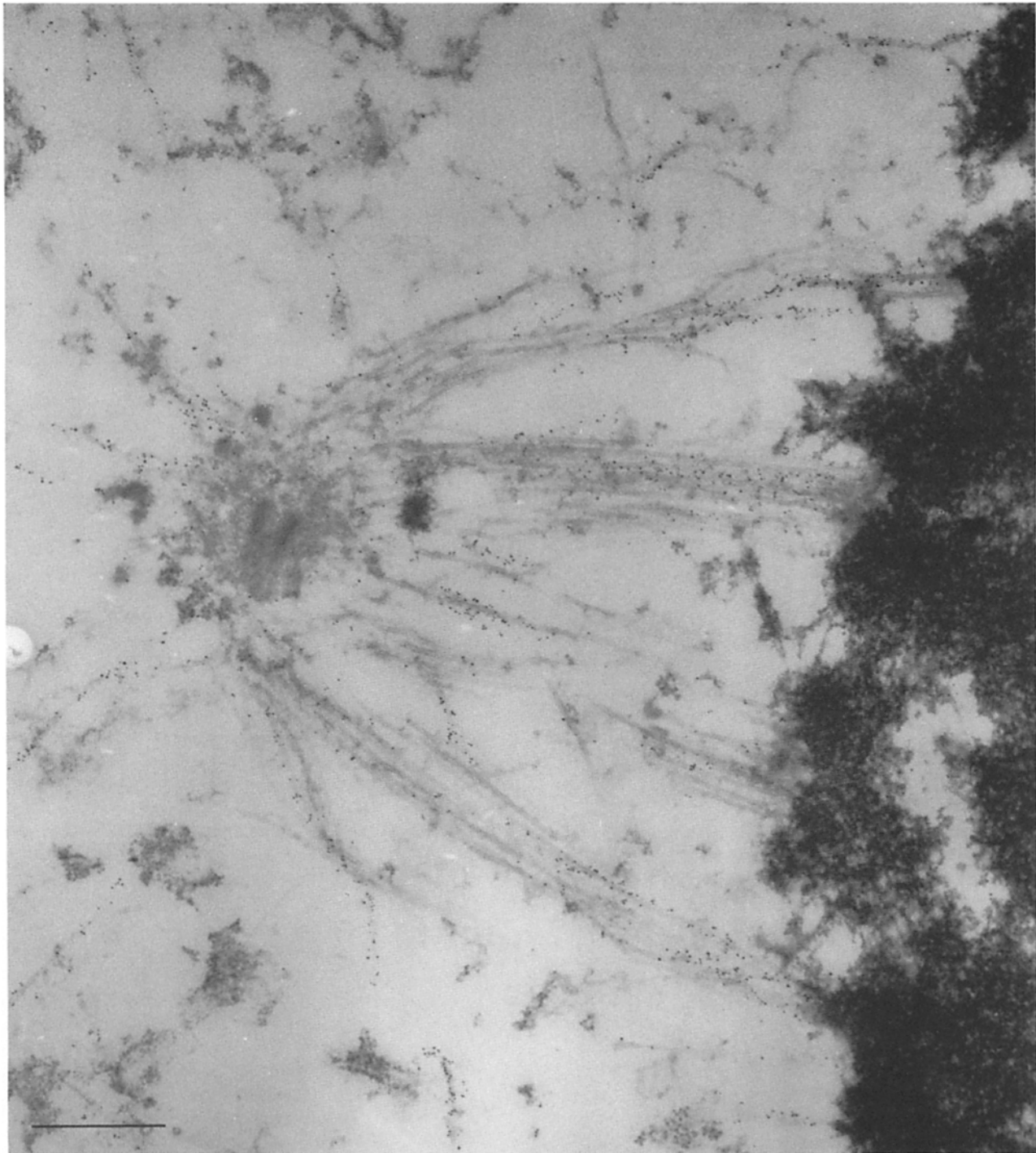


Figure 3. Electron microscopic immunolocalization of biotin-tubulin in a midanaphase cell injected at 20°C. The cell was lysed at 120 s after injection. Labeled and unlabeled microtubules are present within the kinetochore fibers. Bar, 0.5 μm .

tubules, which continue for much of the distance from the kinetochores to the poles, are clearly observed. Our electron microscopic data are therefore consistent with our light microscopic observations and further reveal that both labeled and unlabeled microtubules are present in the kinetochore fiber of early to mid anaphase cells.

Kinetochore Fiber Microtubule Dynamics in Late Anaphase Cells

Cells were also injected with biotin-tubulin after the initial

separation of the chromosomes and examined using these techniques. At the light microscope level, dim fluorescence was observed in the region between the chromosomes and the poles in these late anaphase cells (Fig. 5). It was difficult to determine whether this labeling was due to kinetochore fiber microtubules, remaining nonkinetochore microtubules, or merely due to background fluorescence within the spindle region of the cell. Small bundles of interzonal fibers, which ran between the poles, were labeled with both total tubulin and biotin-tubulin antibodies, illustrating that interzonal mi-

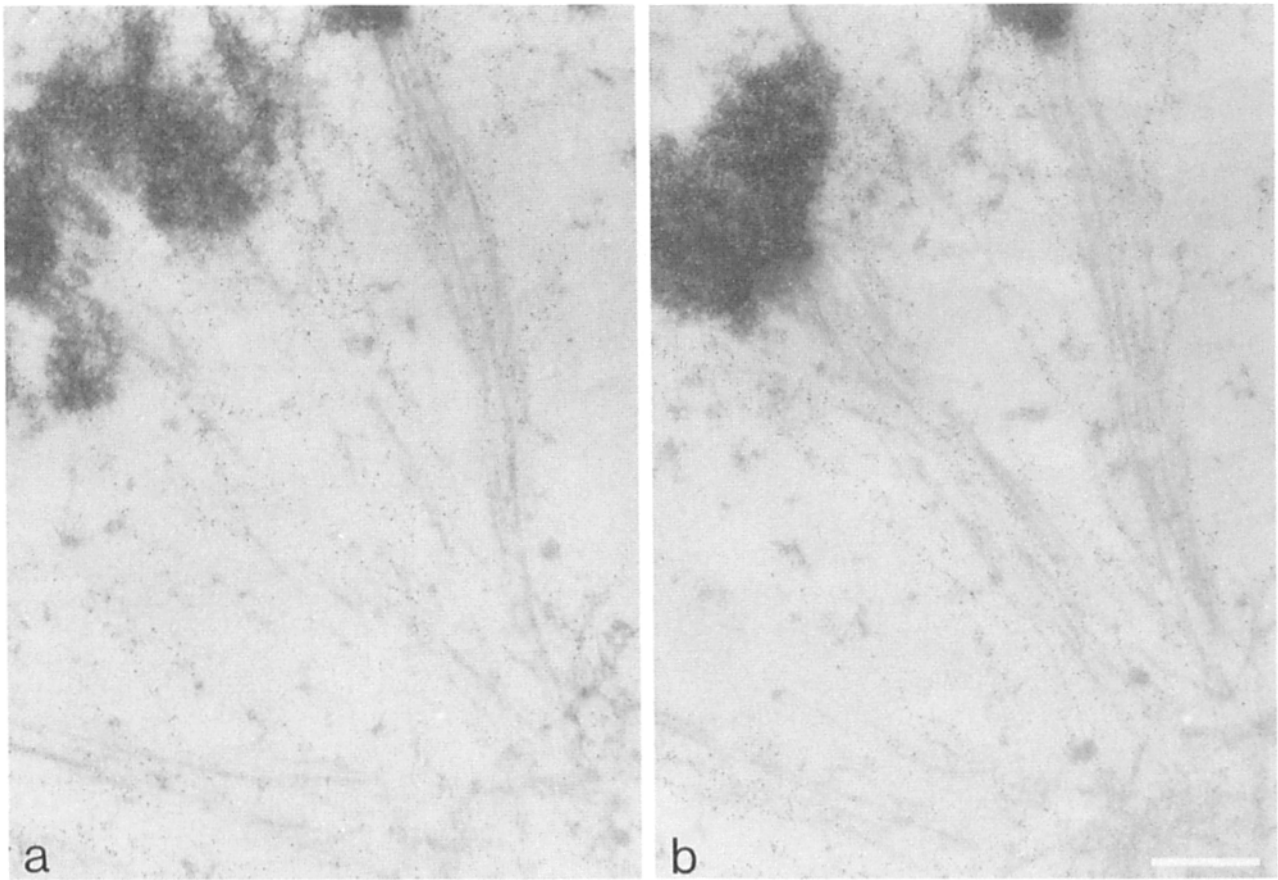


Figure 4. Adjacent serial section electron micrographs of an anaphase cell which was injected with biotin-tubulin at 30°C and lysed 120 s after injection. Numerous gold-labeled microtubules which extend from the kinetochore region to the pole are detected. Bar, 0.5 μ m.

microtubule assembly occurs in these late anaphase cells (Fig. 5; Saxton and McIntosh, 1987). Astral fibers were also labeled, but were reduced in number by the lysis and incubation conditions.

Late anaphase cells injected with biotin-tubulin were also processed for electron microscopic immunocytochemistry at various intervals after injection. Late anaphase cells were defined as cells with an X/Y ratio less than 0.4. A late anaphase cell which was injected at 22°C and fixed 3 min after injection is shown in Fig. 6. No labeled kinetochore fiber microtubules were observed. Scattered gold label was seen in most late anaphase cells, but clear examples of linear gold labeling along microtubules were absent. Again, interzonal

microtubules were labeled (Shelden, E., and P. Wadsworth, manuscript in preparation). In cells injected during late anaphase at room temperature, anaphase was completed much more slowly, and longer incubations after injection could be carried out. Despite these longer incubations, no specific labeling of kinetochore fiber microtubules was detected.

The number of gold-labeled microtubules was estimated from electron micrographs of the chromosome-to-pole region of each injected cell. Approximately 10–40% of the microtubules in early anaphase cells incorporated some label during a 2–5-min incubation after injection. In agreement with our observations at the light microscopic level, the stage

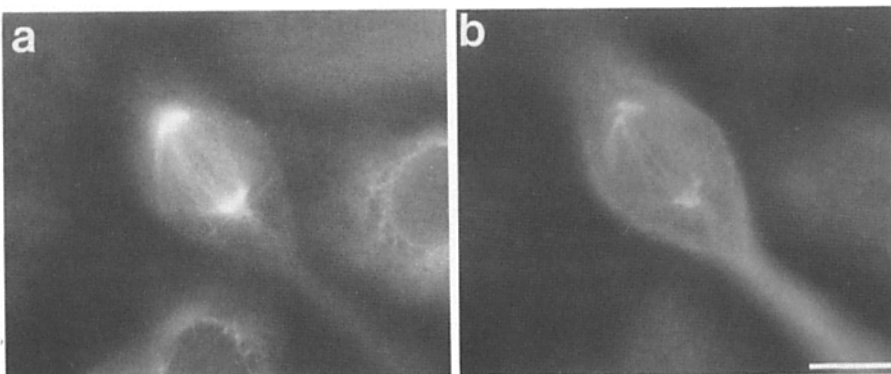


Figure 5. Immunolocalization of biotin-tubulin in a late anaphase cell. The cell was injected at 30°C and lysed 60 s after injection. Total tubulin staining (a) and biotin-tubulin staining (b) are shown. Biotin-tubulin fluorescence in the region of the shortening kinetochore fibers is much less intense than the corresponding anti-tubulin staining. Bar, 10 μ m.

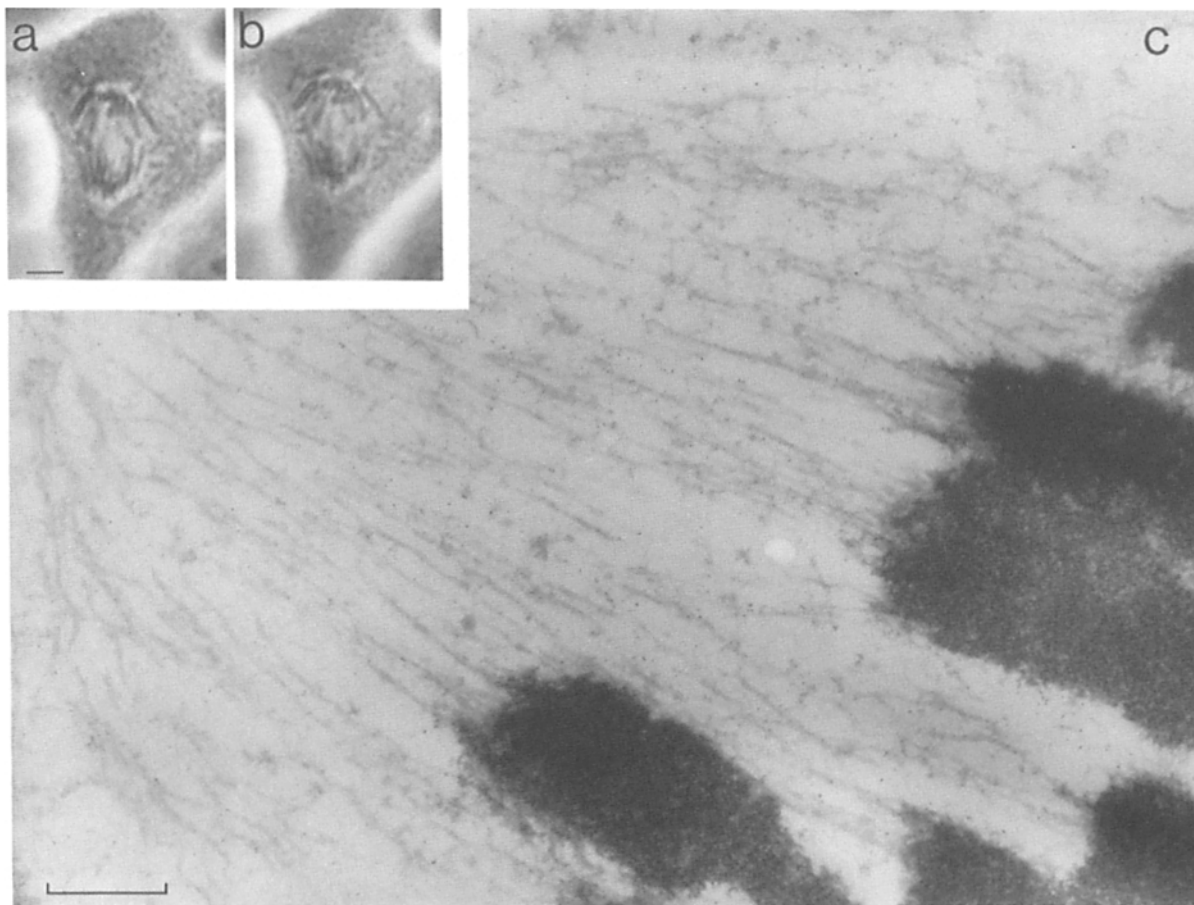


Figure 6. Electron microscopic immunolocalization of biotin-tubulin in a late anaphase cell. The cell was injected at 22°C and lysed 3 min after injection. Phase-contrast micrographs of the cell just after microinjection (a) and just before lysis (b) are given. (c) Electron micrograph of the region between the chromosomes and pole. No specific labeling is detected. Bars: (a and b) 5 μm ; (c) 0.5 μm .

of anaphase had a significant effect on incorporation into the kinetochore fiber. In late anaphase cells, X/Y ratio less than 0.4, incorporation was not detected in cells incubated for 2–6 min. Some variability in the extent of labeling of early and mid anaphase cells was detected; this may be related to the incubation time and temperature, or to the inability to precisely determine the extent of chromosome-to-pole motion which had occurred in each individual cell. Although the number of cells examined by electron microscopy is necessarily restricted, our major finding that incorporation into kinetochore fiber microtubules is reduced as cells progress through anaphase is consistent with results from numerous experiments performed at the light microscope level.

Discussion

Our immunofluorescence observations reveal that cells injected during early to mid anaphase incorporate biotin-tubulin into spindle microtubules, as previously reported for metaphase cells (Mitchison et al., 1986; Mitchison, 1988). Immunogold electron microscopy further reveals that labeled microtubules are present within the kinetochore fiber. Thus, our data reveal that kinetochore fibers incorporated newly assembled microtubules throughout early and mid anaphase. The number of labeled kinetochore fiber microtubules varied with the experimental conditions, and was

difficult to accurately determine from thin sections of injected cells. On average, however, we estimate that $\sim 25\%$ of the kinetochore fiber microtubules incorporated some label in these experiments. This suggests that the average half-time for microtubule turnover within the kinetochore fiber is significantly longer than the 2–5-min incubation period (see Huitorel and Kirschner, 1988).

Incorporation of new microtubules into the kinetochore fiber during anaphase is stage specific. In late anaphase spindles, no incorporation of labeled microtubules into the kinetochore fiber could be detected in thin sections, even with relatively long incubations after injection. At the light microscope level, dim fluorescence was detected in the region of the kinetochore fibers. This discrepancy in our electron and light microscopic results may be due to a small number of labeled microtubules in the chromosome-to-pole region of late anaphase cells, which would be difficult to detect in individual thin sections. However, the cumulative effect of a few labeled microtubules throughout this region would be detected as dim fluorescence in the light microscope.

The decrease in incorporation of labeled microtubules into the kinetochore fiber is most likely a gradual one, with incorporation occurring in the early stages of anaphase and diminishing as anaphase progresses. Variability in the extent of labeling during mid anaphase may result from inaccuracies in estimating the stage of anaphase and the difficulties in

counting labeled microtubules in thin sections. A comparison of these results with similar studies of metaphase (Mitchison et al., 1986; Mitchison, 1988) and prometaphase cells (Geuens et al., 1989) suggests that microtubule turnover is diminished in anaphase cells. However, additional quantitative experiments, performed under similar temperature and incubation conditions, are needed to determine the relative rates of microtubule turnover in cells at various stages of mitosis.

Previous ultrastructural observations, which have documented the changing distribution of spindle microtubules during mitosis, reveal that kinetochore fiber microtubules are gradually lost during anaphase (McIntosh et al., 1975; Jensen, 1982; McIntosh, 1985). In cultured mammalian cells, the ratio of the number of microtubules between the chromosomes and pole and within the interzone remains relatively constant during metaphase and early anaphase but decreases in later anaphase (McIntosh and Landis, 1971). In *Haemaphysalis endosperm*, the number of kinetochore microtubules decreases and the number of short microtubules in the vicinity of the kinetochore rises as anaphase progresses (Jensen, 1982). These ultrastructural observations are consistent with our major finding that the kinetochore fiber incorporates newly assembled microtubules during the initial stages of anaphase, and that this incorporation halts as anaphase progresses.

Incorporation of microtubules into the anaphase kinetochore fiber may occur as individual microtubules, nucleated from the spindle pole, become laterally associated with the existing fiber (Bajer and Mole-Bajer, 1985; Cassimeris et al., 1988). Some of these microtubules may then be captured by the kinetochore (Mitchison and Kirschner, 1985*a,b*; Huitorel and Kirschner, 1988). The fact that labeled microtubules are present in the polar regions of the injected cells supports this possibility. An additional possibility is that the free microtubule ends within the kinetochore fiber (Rieder, 1981*a*, 1982) elongate and contribute to the labeling detected here. The decline in incorporation as anaphase progresses may result from changes in spindle structure. For example, kinetochore fiber microtubules shorten and splay during anaphase (Bajer and Mole-Bajer, 1975; McIntosh et al., 1975; Jensen, 1982). This disorganization of the fiber may effectively prevent lateral interactions of microtubules nucleated from the centrosome with the late anaphase fiber. In addition, modifications to the kinetochore, which could influence microtubule capture and/or binding reactions, and changes in the activity of the centrosome may occur during anaphase and contribute to decline in labeled microtubules within the fiber (Mitchison and Kirschner, 1985*b*; Huitorel and Kirschner, 1988).

Microtubule dynamics in anaphase cells has been examined in two previous studies. Saxton and McIntosh (1987) demonstrated that 5-(4,6-dichlorotriazin-2-yl)aminofluorescein-tubulin incorporates in the chromosome-to-pole region of the late anaphase spindle of living PtK₁ cells. In these experiments, however, nonkinetochore and kinetochore microtubules could not be distinguished. Thus, nonkinetochore microtubules may continue to turn over, even in late anaphase. Under the temperature and lysis conditions used in our experiments, most nonkinetochore microtubules are disassembled (Wise et al., 1989), so incorporation into these microtubules could not be examined accurately. Our data conflict with a more recent study reporting that the kinetochore fiber

does not incorporate new microtubules during anaphase (Gorbisky and Borisy, 1989). In these experiments, cells were photobleached, lysed, and the recovery of fluorescence in the bleached zone quantitated. In metaphase cells, ~70% of the bleached fluorescence recovered, while in anaphase no significant recovery was detected. Because these experiments rely on the ability to resolve single microtubules which have recovered fluorescence, microtubule incorporation into kinetochore fibers during early anaphase may not have been detected. It is also possible that these cells were photobleached in late anaphase, at which time no incorporation would be expected.

The functional significance of microtubule incorporation into the anaphase kinetochore fiber is not known. Since chromosome motion continues in injected cells, incorporation of a relatively small percentage of new microtubules into the kinetochore fiber does not inhibit chromosome motion. Previous observations of chromosome behavior during anaphase reveal oscillations towards and away from the pole (Bajer, 1982; Rieder et al., 1986). Perhaps these oscillations are related to microtubule turnover within the fiber. Incorporation of microtubules into the kinetochore fiber may function during prometaphase and metaphase to align the chromosomes at the metaphase plate and to maintain the fiber (Mitchison, 1988; Geuens et al., 1989). The incorporation observed in anaphase may simply result from a gradual reduction in this metaphase activity. Perhaps a gradual loss of microtubule incorporation into the kinetochore fiber ensures a gradual disassembly of the kinetochore fiber.

The incorporation of labeled tubulin during anaphase could be due to the stimulation of microtubule assembly after injection of tubulin subunits. While this possibility cannot be formally ruled out, several observations suggest that our experiments do not greatly perturb cellular processes and thus may detect endogenous microtubule dynamics. First, the rate of chromosome-to-pole motion was not altered by injection. While slight changes in the rate of chromosome motion just after injection could not be readily measured, no significant difference in the rate of chromosome motion was detected. In cells incubated with taxol, for example, motion of anaphase chromosomes away from the pole has been observed; this abnormal motion is likely due to a stimulation of microtubule assembly (Bajer et al., 1982). Second, relatively low concentrations of biotin-tubulin were used in these experiments to minimize the perturbation of the cell's endogenous tubulin pool (Mitchison et al., 1986). In addition, we estimate that 10% or less of the cell volume was injected. This lower concentration of biotin-tubulin subunits decreased the intensity of the biotin-tubulin staining compared with previous experiments (Mitchison et al., 1986), but labeled and unlabeled microtubules could be readily distinguished. Finally, and perhaps most importantly, these experiments document time-dependent changes in the assembly behavior of the injected tubulin. In the kinetochore fibers, only early to mid anaphase cells incorporated the injected tubulin. Kinetochore fibers in late anaphase cells were somehow refractory to further microtubule polymerization. Additional observations of late anaphase cells, however, document that interzonal and astral microtubules continue to polymerize (Shelden, E., and P. Wadsworth, manuscript in preparation). These observations indicate that spatial regulation of microinjected, biotin-labeled tubulin polymerization occurs in

these anaphase cells and suggests that this microinjection technique reveals the endogenous microtubule dynamics.

In summary, our results reveal several new features of kinetochore fiber behavior during anaphase. First, incorporation of newly assembled microtubules into the kinetochore fiber occurs in early and mid anaphase cells. This suggests that at least some microtubules of the kinetochore fiber are renewed during anaphase. Second, the kinetochore fiber undergoes a time-dependent change in its dynamic properties becoming refractory to further incorporation during mid to late anaphase.

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