Microinjection of Fluorescent Tubulin into Dividing Sea Urchin Cells

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ABSTRACT To follow the dynamics of microtubule (MT) assembly and disassembly during mitosis in living cells, tubulin has been covalently modified with the fluorochrome 5-(4,6-dichlorotriazin-2-yl)aminofluorescein and microinjected into fertilized eggs of the sea urchin *Lytechinus variegatus*. The changing distribution of the fluorescent protein probe is visualized in a fluorescence microscope coupled to an image intensification video system. Cells that have been injected with fluorescent tubulin show fluorescent linear polymers that assemble very rapidly and radiate from the spindle poles, coincident with the position of the astral fibers. No fluorescent polymer is apparent in other areas of the cytoplasm. When fluorescent tubulin is injected near the completion of anaphase, little incorporation of fluorescent tubulin into polymer is apparent, suggesting that new polymerization does not occur past a critical point in anaphase. These results demonstrate that MT polymerization is very rapid in vivo and that the assembly is both temporally and spatially regulated within the injected cells. Furthermore, the microinjected tubulin is stable within the sea urchin cytoplasm for at least 1 h since it can be reutilized in successive daughter cell spindles. Control experiments indicate that the observed fluorescence is dependent on MT assembly. The fluorescence is greatly diminished upon treatment of the cells with cold or colchicine agents known to cause the depolymerization of assembled MT. In addition, cells injected with fluorescent bovine serum albumin or assembly-incompetent fluorescent tubulin do not exhibit fluorescence localized in the spindle but rather appear diffusely fluorescent throughout the cytoplasm.

A variety of cell motility phenomena depend upon the proper assembly and spatial distribution of microtubules (MT)[1] within the cytoplasm. The arrangement of the MT and the changes in their distribution during the cell cycle have been elegantly demonstrated immunocytochemically by indirect immunofluorescence with tubulin antibody (5, 29, 41) as well as conventional electron microscopy (25, 36). However, our understanding of the dynamic nature of these events has been limited by the fact that these techniques do not permit direct observation of living cells.

An alternative approach has been to use sensitive optical techniques to observe motility in living cells. For example, differential interference-contrast microscopy has revealed waves of particle movements within spindles (12) and polarized light microscopy has documented the fluctuations and changes in spindle fiber birefringence in living cells under a variety of experimental conditions (14). While these elegant techniques have described the dynamics of various motile events, the macromolecules responsible for the phenomena are not identified directly.

Recently, several groups have used native, fluorescent derivatives of various cytoskeletal proteins to observe their distribution and rearrangement in living cells (see reference 39). To date, fluorescent derivatives of actin (10, 11, 21, 35), α-actinin (8,000-mol-wt), 130,000-mol-wt protein (4), and tubulin (17) have been prepared, microinjected into suitable cells, and observed in a fluorescence microscope coupled to an image intensification video system (27, 44). The results of such experiments demonstrate the utility of the technique to examine the pattern and time course of incorporation of the labeled protein into cytoskeletal structures in vivo. In the experiments described here, 5-(4,6-dichlorotriazin-2-yl)aminofluorescein (DTAF)-labeled tubulin (17) has been prepared and microinjected into dividing eggs of the sea urchin, *Lytechinus variegatus*. The results demonstrate that microinjected fluorescent tubulin is capable of repeated assembly and disassembly in association with the spindles of living cells and show the spatial and temporal dependence of this assembly. Portions of this work have appeared previously in abstract form (38).

[1] *Abbreviations used in this paper:* DTAF, fluorochrome 5-(4,6-dichlorotriazin-2-yl)aminofluorescein; and MT, microtubule.

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MATERIALS AND METHODS

Protein Purification: Microtubule proteins were purified from pig brains using cycles of temperature-dependent assembly and disassembly as described by Sloboda et al. (31). Polymerization buffer consisting of 0.1 M PIPES, pH 6.9, 1 mM $MgSO_4$, 2 mM EGTA, and 1 mM guanosine triphosphate was used throughout the procedure. The supernatant obtained from centrifugation of a brain homogenate for 1 h at 100,000 *g* was mixed with an equal volume of polymerization buffer containing 8 M glycerol. MT, assembled during incubation at 37°C for 40 min, were collected by centrifugation at 100,000 *g* for 40 min, resuspended in fresh polymerization buffer, and depolymerized on ice for 30 min. The depolymerized MT were clarified by centrifugation at 100,000 *g* for 30 min. The supernatant was again mixed with an equal volume of polymerization buffer containing 8 M glycerol, polymerized, and spun as before. The sedimented MT were overlaid with polymerization buffer containing 4 M glycerol and stored at −70°C.

Fluorescent Modification of Tubulin: For fluorescent labeling of tubulin, a modification of the procedure of Keith et al. (17) was used. Briefly, three-times-cycled microtubule proteins were polymerized at 37°C and mixed with the DTAF (Sigma Chemical Co., St. Louis, Mo) from a 5 mM stock solution, made fresh daily in dimethyl sulfoxide. The fluorochrome:protein ratio varied from 0.5:1.0 to 4.0:1.0, depending on the experiment. Intact, assembled MT were labeled for 5 min at 37°C, and the fluorescent polymer was collected by centrifugation. Assembled MT were labeled so that sites on the tubulin which are essential for tubulin-tubulin interaction during MT polymerization would be blocked from reaction with the fluorochrome. The sedimented fluorescent MT were resuspended in buffer in a Dounce homogenizer, and depolymerized on ice. The depolymerized, fluorescent MT proteins were desalted on Sephadex G-25 to remove any unbound fluorochrome, using column buffer containing 50 mM PIPES, pH 6.9, 0.5 mM $MgSO_4$, 1 mM EGTA, and 0.1 mM guanosine triphosphate. The fluorescent protein was then subjected to a further round of temperature-dependent assembly and disassembly to select for assembly-competent protein and then either applied to a phosphocellulose column to prepare purified tubulin dimers (42, 43) or centrifuged at 140,000 *g* for 90 min to prepare a high-speed supernatant which was greatly attenuated in its ability to self-nucleate MT. Labeling stoichiometries, calculated using an extinction coefficient of 5.4×10^5 for DTAF (17) and a molecular weight of 110,000 for the tubulin dimer, ranged from 0.01 to 0.1 mol of fluorochrome per mole of tubulin dimer. At these stoichiometries the MT protein and purified tubulin dimers remained competent to assemble, with kinetics comparable to those of control proteins (17; Wadsworth and Sloboda, manuscript in preparation). In addition, this low stoichiometry ensured that any deleterious effects of the fluorochrome would be minimized. Using the optics and recording equipment described, fluorescent polymer labeled with a stoichiometry as low as 0.004 mol DTAF/mol of tubulin dimer could be detected.

Assembly-incompetent, denatured tubulin was recovered from the supernatant remaining after centrifugation of the fluorescent, polymerized protein and was dialyzed exhaustively to remove any unbound fluorochrome. This protein fraction typically had a dye-to-protein ratio greater than that of the assembly-competent fraction and was present in the supernatant at a concentration greater than predicted from the critical concentration alone. Thus it was inferred that a portion of the protein was denatured as a result of fluorescent modification of sites on the molecule that are necessary to maintain the normal protein conformation. When analyzed by SDS PAGE, we found this fraction to contain predominantly tubulin. Fluorescein isothiocyanate-labeled bovine serum albumin, which was also used for control injections, was obtained from Sigma Chemical Co.

Microscopy: Phase-contrast and fluorescence observations were made with a Nikon Optiphot microscope equipped with a 50-W Hg lamp for epi-illumination and a 40X, 1.3 N.A. glycerin immersion objective lens. Images were recorded either on Kodak Tri-X film or on video tape using a Hamamatsu Silicon Intensified Target video camera coupled to a SONY TVO 9000 time-lapse video recorder. For reproduction of images from the video tape, 35-mm photographs were taken from the TV monitor with Kodak Plus-X film.

Microinjection: Microinjection was performed as described by Kiehart (19), with chambers constructed from mylar film or double-stick scotch tape. Micropipettes were pulled from Drummond capillary tubing using a WPI pipette puller (W-P Instruments, Inc., New Haven, CT). The diameter of the pipette tip, initially too fine to measure accurately, was broken to obtain an outer diameter of 1–5 μm. Ice-cold fluorescent protein in a capillary reservior was placed on the microscope stage and immediately used to fill the injecting pipette. The volume injected was determined as described by Kiehart (19) and ranged from 10 to 150 pl/cell, a volume equal to ~1–15% of the cell volume.

RESULTS

When a high-speed supernatant of fluorescently labeled tubulin is pressure-microinjected into one cell of a *L. variegatus* embryo at the two-cell stage, the results shown in Fig. 1 are obtained. In the phase-contrast image (Fig. 1 *b*), the nucleus is clearly visible, as are the astral MT, indicating that the cell is in prophase. The oil drop confirms that this is the injected cell and marks the approximate location of the pipette tip during the injection. The fluorescent image (Fig. 1 *a*) illustrates linear fluorescent MT within the cytoplasm of this blastomere. The fluorescence is not distributed evenly on either side of the nucleus, as would be expected if the fluorescent proteins had diffused throughout the cytoplasm. Instead, it appears that polymerization is more rapid than diffusion because linear structures become visible almost immediately in the injected cell. This apparently occurs when fluorescent proteins are microinjected into a region of the cytoplasm that is competent to polymerize MT, as in the astral region of this prophase cell. When cells are injected with tubulin at a time when the cytoplasm is not competent to support MT polymerization, such as before prophase of mitosis, tubulin diffuses throughout the cell as do control fluorescent proteins such as bovine serum albumin (see below).

When colchicine is added to the sea water surrounding the cell, the linear fluorescence pattern (Fig. 1 *a*) appears instead as diffuse fluorescence throughout the cytoplasm. Fig. 2 shows the video image of the cell in Fig. 1 before (Fig. 2, *a* and *b*) and after (Fig. 2, *c* and *d*) treatment with 5×10^{-4} M colchicine. The fluorescence is reduced within minutes to dim punctate fluorescence that is visible only after the electronic gain of the video camera is increased several-fold. MT in injected cells are also sensitive to cold temperature, and when the cells were rewarmed a fluorescent spindle reforms (data not shown).

In the experiments shown in Figs. 1 and 2, the same cell has been either photographed directly with 35-mm film (Fig. 1) or recorded on video tape with a Silicon Intensified Target camera and subsequently photographed off the TV monitor (Fig. 2). While the decrease in resolution in the video image is readily apparent (compare Figs. 1 and 2), this method of data collection has been used because of the greater sensitivity of the video camera to low light levels and the convenience of video recording.

The rearrangement of the fluorescent polymers can be followed as the cells progress through mitosis. As seen in Fig. 3, fluorescent protein is rapidly incorporated into MT in an anaphase cell. Intense fluorescence is visible in the region of the midbody as well as within the astral region (Fig. 3, *a* and *b*). As the cell completes division the fluorescent MT are depolymerized (not shown), and as the cell prepares to divide again (Fig. 3, *c* and *d*) fluorescence is seen in association with the asters that form at right angles to the axis of the previous spindle. These changes in fluorescent MT distribution demonstrate that the injected fluorescent proteins can assemble, disassemble, and reassemble into different MT arrays within the sea urchin cytoplasm.

The temporal regulation of MT assembly during the mitotic cycle is also apparent from observation of cells injected with fluorescent tubulin. For example, when fluorescent protein is injected during late anaphase, incorporation of fluorescent material into the spindle is not readily detected. When the cell is observed just after cytokinesis (Fig. 4), the only fluores-

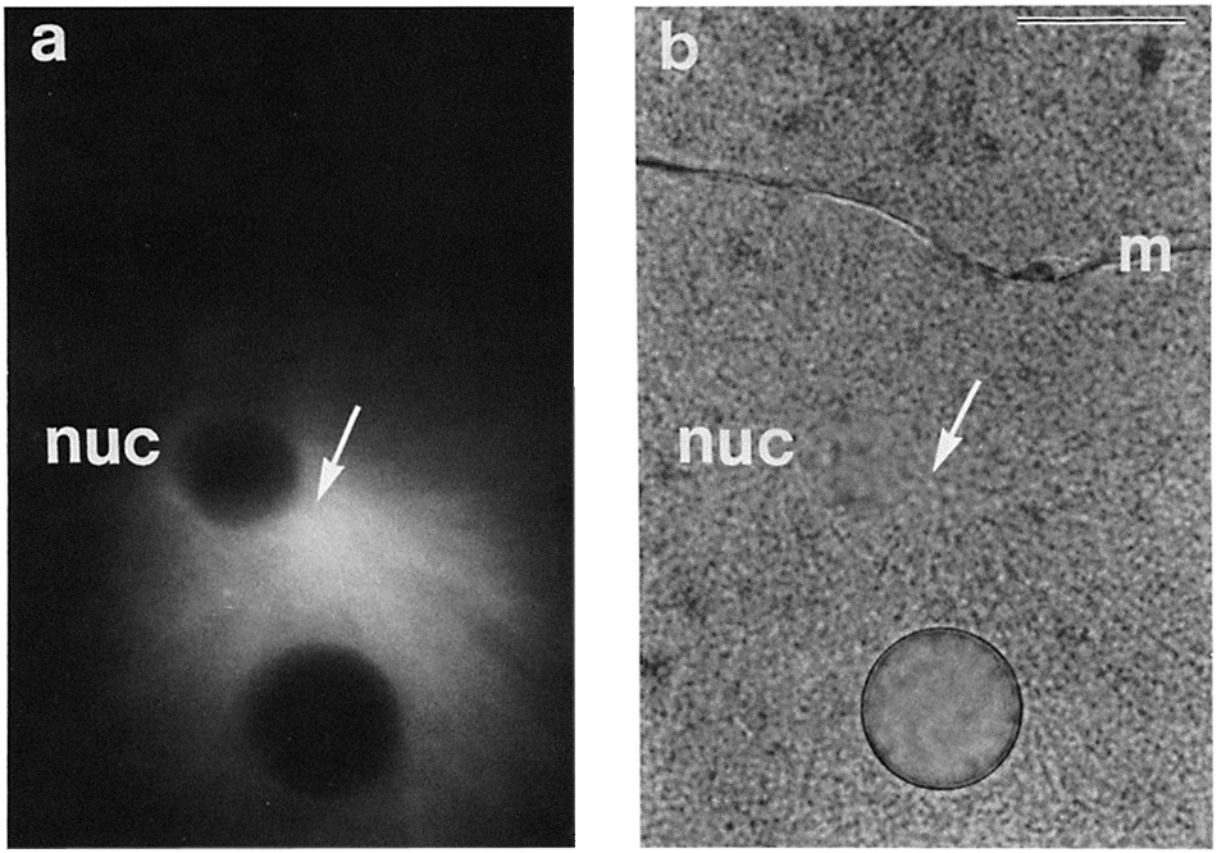

FIGURE 1 Corresponding fluorescence (*a*) and phase-contrast (*b*) micrographs of a *L. variegatus* embryo at the 2-cell stage.The lower cell has been injected with 137 pl of a 5.5 mg/ml solution of a fluorescently labeled, high-speed supernatant (see Materials and Methods). The oil drop marks the approximate position of the pipette tip during injection. Note the coincidence of fibers in the phase-contrast and fluorescent images. *Nuc*, the nucleus. *m*, the membrane separating the two blastomeres. The arrows mark the approximate position of the centrosomes. Bar, 20 μm.

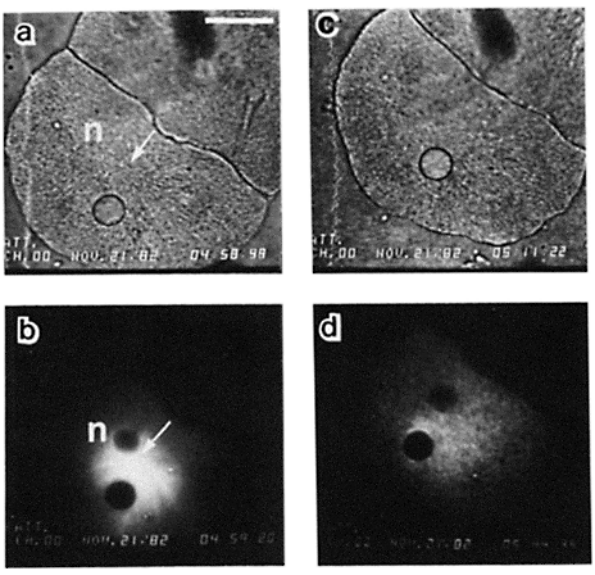

FIGURE 2 Phase-contrast (*a* and *c*) and fluorescence (*b* and *d*) micrographs photographed off the video monitor of the cells shown in Fig. 1. The cells are illustrated before (*a* and *b*) and after (*c* and *d*) treatment with 5×10^{-4} M colchicine. The electronic gain of the video camera has been increased in *d* to detect the dim fluorescence. In *a* and *b*, *n* marks the position of the nucleus and the arrows mark the approximate position of the centrosome. Bar, 50 μm.

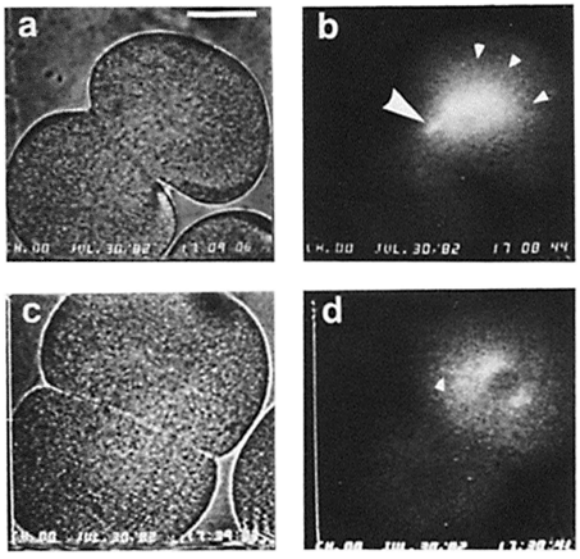

FIGURE 3 Corresponding phase-contrast (*a* and *c*) and fluorescence (*b* and *d*) micrographs of a *L. variegatus* embryo. The cell was injected with a fluorescently labeled, high-speed supernatant at 6.6 mg/ml; an injection volume equal to ~5% of the cell volume was used. Less than 1 min after injection (*a* and *b*), linear fluorescent fibers are seen in the astral region (small arrowheads) and midbody (large arrowhead) (*b*). After 22 min (*c* and *d*), fluorescence is located in the asters of the spindle forming in the upper blastomere. Arrowhead marks a linear fluorescent fiber. Bar, 50 μm.

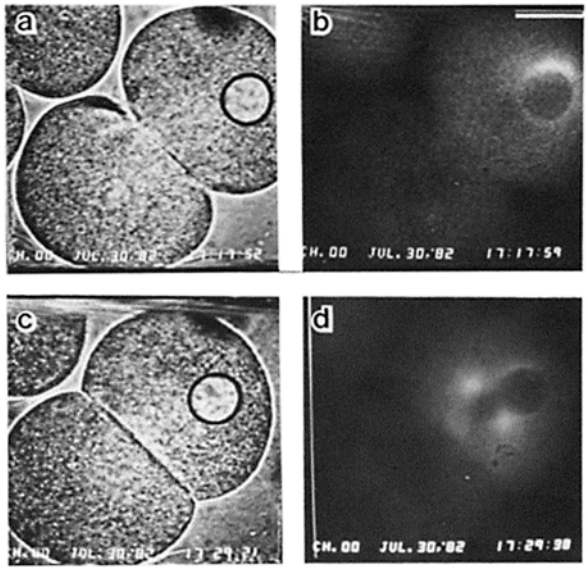

FIGURE 4 Corresponding phase-contrast (*a* and *c*) and fluorescence (*b* and *d*) micrographs of a cell injected with a fluorescently labeled, high-speed supernatant at 6.6 mg/ml; an injection volume equal to ~5% of the cell volume was used. The cell was injected at late anaphase and is shown after cytokinesis, (*a* and *b*). Fluorescence is observed surrounding the oil drop (compare Fig. 3*b* with panel *b*). 12 min later (*c* and *d*), the injected protein has incorporated into the asters of the forming spindle. The level of fluorescence in this cell is comparable to that seen in Fig. 3 and indicates that the injected protein was not degraded but remains competent to assemble. Bar, 50 μm.

cence that is visible is a rim of fluorescence surrounding the oil drop; no fluorescence is seen in the region of the nucleus or remaining astral fibers. Within 12 min postinjection, however, assembly of the new asters has begun in the daughter cell and these newly assembled MT are distinctly fluorescent (Fig. 4*d*). These results indicate that the fluorescent protein is not incorporated into MT if injected after a certain time in anaphase, yet remains competent to assemble since it subsequently incorporates into the MT of the daughter cell's mitotic apparatus.

The reutilization of the fluorescent protein during subsequent rounds of division is more clearly illustrated in Fig. 5. In this example, the cell was injected with purified fluorescent tubulin dimers at late anaphase of the first division. Since cytokinesis was not complete at the time of injection, the fluorescent protein entered both cells, although the cell on the right received a greater proportion of the injected protein. The spindles which formed in each of the two blastomeres were distinctly fluorescent (Fig. 5, *a* and *b*) and division proceeded normally. A fluorescent spindle then formed in each of the four resulting daughter cells (Fig. 5, *c* and *d* and *e* and *f*), although the cells on the right were slightly delayed with respect to those on the left. Tubulin fluorescence could not be followed in further rounds of division (Fig. 5, *g* and *h*) because of two compounding factors: the quantity of the fluorescent protein was reduced with each division and the fluorochrome was bleached by repeated exposure to the excitation wavelength from the epiilluminator.

A series of control injections were performed and compared with the pattern obtained with fluorescent tubulin. When cells were injected with denatured, assembly-incompetent fluorescent tubulin no localization of the fluorescent protein was evident; rather, the cells were diffusely fluorescent throughout

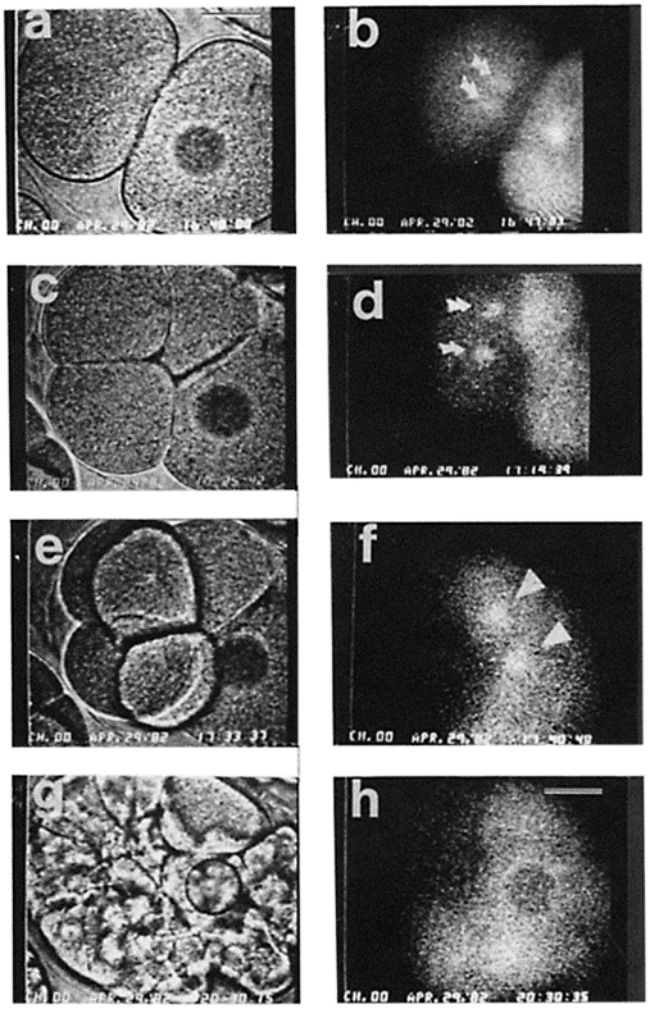

FIGURE 5 Corresponding phase (a, c, e, and g) and fluorescence (*b*, *d*, *f*, and *h*) micrographs of a *L. variegatus* embryo that was injected during late anaphase with 15 pl of a 5.6 mg/ml solution of fluorescent tubulin dimers. Arrows in *b*, *d*, and e mark the fluorescence which was observed in each cell during mitosis. The cells continued to cleave (g and *h*), although the remaining fluorescence was too dim to detect. Bar, 50 μm.

the cytoplasm (Fig. 6, *a* and *b*). Similarly, no localized fluorescence was detected in cells injected with fluorescein isothiocyanate-labeled bovine serum albumin (Fig. 6, *c* and *d*). It is important to note that control injections were performed under conditions comparable to those used in experimental injections. When injected at a higher dye-to-protein ratio, the fluorescein isothiocyanate-labeled bovine serum albumin appears to be concentrated slightly in the spindle region, an observation which is apparently the result of the greater accessible volume in the spindle region of the cell. This effect is more pronounced when the cell is viewed with an objective lens of lower magnification and numerical apperture (data not shown). When tested under similar conditions, fluorescent bovine serum albumin or denatured tubulin shows diffuse fluorescence. Only fluorescent, assembly-competent tubulin has shown linear structures coincident with the location of astral microtubules.

DISCUSSION

The results of these experiments document the utility of fluorescent tubulin as a probe to investigate MT assembly and disassembly in vivo. The microinjected fluorescent pro-

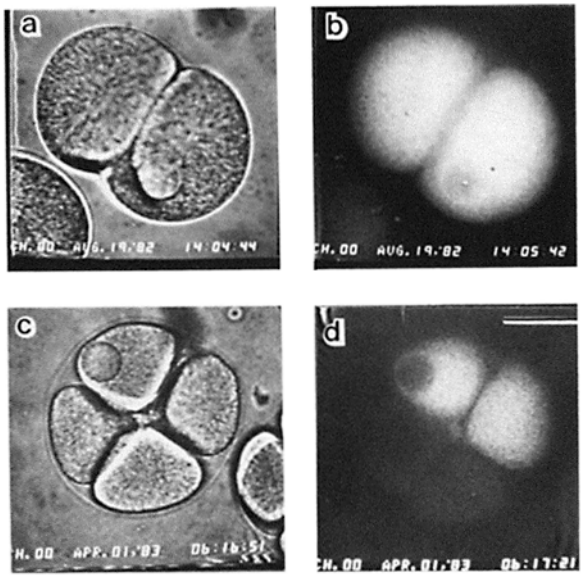

FIGURE 6 Corresponding phase-contrast (*a* and *c*) and fluorescence (*b* and *d*) micrographs of control injections. The cell in (*a* and *b*) was injected with 30 pl of 2.6 mg/ml solution of denatured fluorescent tubulin at the one-cell stage. Mitosis continues normally, and diffuse, uniform fluorescence is observed in both daughter cells. In *c* and *d*, one blastomere at the two-cell stage was injected with 35 pl of a 5 mg/ml solution of FITC-BSA. Again, only diffuse fluorescence is observed and development continues normally. Bar, 50 μm.

tein probe assembles into highly fluorescent linear structures within the cytoplasm; furthermore, the organization of the observed fluorescence into linear structures is cold- and colchicine-sensitive as would be expected if normal MT were formed. Control injections of fluorescent denatured tubulin or fluorescent bovine serum albumin showed diffuse fluorescence throughout the injected cell.

The distribution of fluorescence in injected cells was not uniform; rather, we observed the majority of the fluorescence in the asters of the spindle. This apparent lack of fluorescence within the spindle could be due to several factors. (*a*) Fluorescent tubulin may be present within the spindle, but at a concentration that is not easily detected. (*b*) Fluorescent subunits may not be used by the cell to form the MT of the spindle because these MT may represent a different subclass of spindle MT. (*c*) The fluorescent subunits are distinctly different from the sea urchin subunits, by virtue of both the fluorescent modification and the variation in tubulins isolated from different sources (18, 34). Such variation may not be tolerated in the kinetochore fibers to the same extent as in the astral MT. (*d*) In cells microinjected after spindle formation had occurred, equilibration of the fluorescent tubulin with the endogenous tubulin pool may not have occurred, thus limiting incorporation into the spindle. (*e*) Neither the kinetochore ends nor the polar ends of the spindle MTs may be free to exchange subunits, thus preventing incorporation.

The rapid assembly of fluorescent MT in injected cells precluded growth-zone studies. For example, within 30 s after injection into "assembly-competent" cytoplasm, linear fluorescent arrays extending many micrometers in length were observed (see Fig. 1). Incorporation of the fluorescent subunits always occurred at a rapid rate throughout the MT-containing region nearest the site of injection (see Fig. 1 *a* and Fig. 3 *b*). Even in cells injected with less than the average quantity of fluorescent tubulin,[2] fluorescence was rapidly detected as linear arrays within the injected cell. This observation suggests that the observed flux of fluorescent tubulin into MT is not solely the result of perturbation of the tubulin subunit concentration, but may be a continual process within MT in vivo.

As reported here, microinjection of assembly-competent fluorescent tubulin has not revealed a discrete zone of new MT growth at either the spindle poles, the kinetochores or the distal region of the asters. However, MT polymerization in vitro has been described as an end-dependent phenomenon with net assembly occurring at a faster rate at one end of the MT, designated the plus (+) end (2). In addition, the polarity of spindle MT has been determined, identifying the location of the plus ends of the spindle and astral MT as distal to the spindle pole (7, 13). Thus, in vitro information suggests that net assembly would occur exclusively at the plus ends of the MT (22; see also references 2, 20) and furthermore that the predicted site of assembly within the spindle would be distal to the poles (23). Since this predicted result was not obtained, several possible explanations should be considered that might explain the rapid incorporation of fluorescent protein into colchicine-sensitive arrays in the injected region of assembly competent cells. (*a*) MT assembly in vivo may occur specifically at the plus ends of the MT, but the existence of numerous MT ends throughout the spindle and asters may result in the appearance of uniform fluorescence (24). (*b*) Newly assembled, hence fluorescent, MT may form in the MT-containing regions of the cell because the critical concentration for polymerization in this region supports rapid MT polymerization (6). Indeed, an estimate of the rate of subunit addition observed in living cells is within the range expected from in vitro studies[3] suggesting that the formation of new MT may account for the observed fluorescence pattern. (*c*) Fluorescent subunits could incorporate throughout the length of the polymerized MT in living cells (16, 28). While this possibility is not supported by the available in vitro data, the mode of assembly in vivo may differ from that in vitro. For example, MT assembled in vitro are composed of 14 protofilaments, while MT in vivo contain only 13 protofilaments (1, 18). Thus the surface lattice of a MT assembled in vitro differs from a MT in vivo and therefore insertion of subunits along the MT length, rather than exclusively at the MT ends, may occur (16, 28). At present it is not possible to distinguish among these possible patterns of MT polymerization in vivo.

While additional experiments are necessary to resolve the mechanism of in vivo assembly, these observations of rapid MT assembly are consonant with the changes in birefringence observed during spindle formation and when the spindle MT-subunit equilibrium is perturbed (14). For example, spindle

[2] For an average injection volume of 50 pl and a fluorescent tubulin concentration of 7.5 mg/ml, 375 pg of tubulin are injected. The cell, with a volume of ~1,000 pl and a tubulin concentration of 5 mg/ml (9, 26), contains 5,000 pg of tubulin. Thus, on average, the tubulin concentration is increased by 7.5% after microinjection.

[3] Using an average value of 5 mg/ml for the concentration of tubulin in the egg (9, 26) and estimating the rate of elongation of fluorescent linear polymer from the video recordings (20 μm/60 s), a rate of addition of 541 dimers/s can be obtained. In contrast, the in vitro forward rate constant for tubulin dimer addition (2) times the in vivo tubulin concentration yields a predicted rate of elongation of 324 dimers/s. Thus the rate of elongation measured by viewing the process with fluorescent tubulin is within the range expected based on rate constants calculated in vitro.

birefringence is rapidly and reversibly abolished when cells are treated with a variety of MT-depolymerizing agents (see references 16, 28). Such experiments document the labile equilibrium between spindle fibers and their component subunits and demonstrate that MT assembly and disassembly occur at rapid rates in vivo.

Several additional observations concerning the behavior of the fluorescent tubulin in the sea urchin egg should be mentioned. First, these experiments demonstrate that co-assembly of heterologous tubulins can occur in vivo (see also reference 15), a phenomenon which has been well documented in vitro (3, 30, 32, 40). Second, polymerization of the fluorescent, heterologous tubulin is spatially and temporally regulated in the sea urchin cytoplasm. Third, the fluorescent tubulin can be reutilized during successive divisions; it is neither immediately degraded nor sequestered and thus unavailable for polymerization. Fourth, the lack of fluorescence in cells injected with fluorescent tubulin in late anaphase suggests that new polymerization does not occur past a critical time point in mitosis (see reference 33).

Additional experiments performed in vitro using either DTAF-labeled tubulin or tubulin stoichiometrically labeled with another fluorochrome (Bimane, see reference 37; and manuscript in preparation) demonstrate that both the spindle and asters become distinctly fluorescent when isolated mitotic apparatuses are incubated with the labeled tubulin. The basis for this discrepancy between the in vitro and in vivo data is not known, but it suggests that MT assembly in the living cell may differ from the situation in vitro. Given the complexities of the living cell, the need for in vivo data is apparent.

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