Microtubule Assembly in Cytoplasmic Extracts of *Xenopus* Oocytes and Eggs

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Abstract. We have investigated the differences in microtubule assembly in cytoplasm from *Xenopus* oocytes and eggs in vitro. Extracts of activated eggs could be prepared that assembled extensive microtubule networks in vitro using *Tetrahymena axonemes* or mammalian centrosomes as nucleation centers. Assembly occurred predominantly from the plus-end of the microtubule with a rate constant of 2 $\mu m \cdot min^{-1} \cdot \mu M^{-1}$ (57 $s^{-1} \cdot \mu M^{-1}$). At the in vivo tubulin concentration, this corresponds to the extraordinarily high rate of 40–50 $\mu m \cdot min^{-1}$. Microtubule disassembly rates in these extracts were -4.5 $\mu m \cdot min^{-1}$ (128 s^{-1}) at the plus-end and -6.9 $\mu m \cdot min^{-1}$ (196 s^{-1}) at the minus-end. The critical concentration for plus-end microtubule assembly was 0.4 μM. These extracts also promoted the plus-end assembly of microtubules from bovine brain tubulin, suggesting the presence of an assembly promoting factor in the egg.

In contrast to activated eggs, assembly was never observed in extracts prepared from oocytes, even at tubulin concentrations as high as 20 μM. Addition of oocyte extract to egg extracts or to purified brain tubulin inhibited microtubule assembly. These results suggest that there is a plus-end–specific inhibitor of microtubule assembly in the oocyte and a plus-end–specific promoter of assembly in the eggs. These factors may serve to regulate microtubule assembly during early development in *Xenopus*.

THE dynamic nature of the cytoskeleton is perhaps most evident in the dramatic rearrangement of cellular microtubules during the cell cycle. During interphase most eukaryotic cells possess a radial array of cytoplasmic microtubules emanating from the centrosome. At the onset of mitosis this array is replaced by the characteristic bipolar spindle responsible for chromosomal segregation (Brinkley et al., 1976).

Although these abrupt transitions in microtubule assembly have been extensively studied at the structural level, we still have little idea of the biochemical mechanisms that regulate microtubule assembly through the cell cycle in vivo. Previous studies in eggs of marine invertebrates (Keller and Rebhun, 1982; Suprenant and Rebhun, 1984*a*, *b*) have indicated that purified spindle and cytoplasmic (interphase) tubulins are identical in their biochemical and assembly properties. It has been suggested that other factors such as Ca^{2+}, pH, or accessory proteins may serve to regulate microtubule assembly during the cell cycle (Weisenberg and Rosenfeld, 1975).

Studies of *Xenopus* eggs have recently provided insight into the regulation of microtubule assembly during the eukaryotic cell cycle. The *Xenopus* egg and oocyte are naturally arrested at distinct points of the cell cycle (Gerhart, 1980) that differ in microtubule assembly and organization. Full-grown oocytes are arrested at meiotic prophase, where few microtubules can be observed by electron microscopy (Heidemann et al., 1985). After meiosis begins, unfertilized eggs arrest at the second meiotic metaphase with an intact spindle (Gerhart, 1980). Eggs and oocytes differ not only in their organization of microtubules, but also in their response to agents that affect microtubule polymerization. For example, D_2O and taxol, compounds that promote microtubule assembly in vitro, induce the assembly of numerous cytoplasmic microtubule asters in eggs, but not oocytes (Heidemann and Gallas, 1980; Heidemann et al., 1985; Karsenti et al., 1984). In addition, exogenous microtubules polymerized from mammalian brain tubulin are less stable when microinjected into oocytes than those microinjected into unfertilized eggs (Heidemann et al., 1985).

A major transition in microtubule assembly and organization also occurs after activation or fertilization. Activation, either artificially or by sperm, results in rapid completion of meiosis and return to the interphase state. Subsequently interphase rapidly alternates with mitosis. The first interphase state after fertilization is characterized by the rapid assembly of a microtubule aster from the sperm centriole. This sperm aster enlarges to fill the animal hemisphere of the egg during the period from 20 to 45 min after fertilization (Stewart-Savage and Grey, 1982). Given the large size of most amphibian eggs (1.2–1.4 mm in the case of *Xenopus laevis*), this process requires microtubule assembly rates of 30–50 μm/min.

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This rate is 10-fold higher than the rate typically observed in cultured cells, and is 20-fold greater than would be expected for the tubulin concentration found in eggs.

The *Xenopus* egg thus defines cytoplasmic states in the cell cycle, that differ in their ability to assemble microtubules. However, the mechanisms underlying the regulation of microtubule assembly in the egg are not understood. Changes in tubulin content cannot be responsible, in that the tubulin content of eggs and oocytes is comparable (Heidemann and Kirschner, 1975). Tubulin from these two stages of oogenesis (which differ greatly in microtubule content and organization in vivo) have also been found to be equally competent to coassemble with bovine brain tubulin in vitro (Heidemann et al., 1985).

In this article we address two issues: (*a*) the biochemical basis for the observed differences in microtubule assembly in oocytes and eggs and (*b*) the basis for the unprecedented rapid rate of assembly required to explain the growth of the sperm aster in the interphase egg. To bridge the gap between studies of microtubule assembly in vivo and the use of purified components in vitro, we have examined the assembly of microtubules from concentrated cytoplasmic extracts. Assembly was nucleated from either centrosomes or axonemes, and the microtubules were visualized by immunofluorescence with tubulin antisera. Because assembly in such assays is dependent upon both the endogenous tubulin and any accessory proteins which might modulate assembly, the in vitro assembly should reflect the assembly properties of intact eggs or oocytes. We found that, as expected from in vivo observations (Heidemann and Gallas, 1980; Heidemann et al., 1985; Heidemann and Kirschner, 1975; Karsenti et al., 1984), extracts of oocytes and activated eggs differ dramatically in their ability to assemble microtubules. Oocyte extracts were unable to assemble microtubules, whereas extracts of activated eggs assembled extensive microtubule arrays at rates which were sufficient to account for the rate of sperm aster enlargement. Oocyte extracts were found to contain a factor that inhibits assembly from either egg extract or purified bovine brain tubulin, whereas egg extracts were found to contain an activity that promotes assembly of bovine brain tubulin. Both the inhibitory factor in the oocyte and the assembly promoting factor in the egg exhibit the novel property of specificity for the plus- (or fast growing) end of microtubules.

Materials and Methods

Preparation of Cytoplasmic Extracts

Eggs were obtained from *Xenopus laevis* females as previously described (Newport and Kirschner, 1982). Eggs were dejellied in modified Ringer's solution (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, 0.1 mM EDTA, 5 mM Hepes, pH 7.5) with 5 mM dithiothreitol (DTT) and 30 mM Tris HCl (pH 8.5). After removal of the jelly coat eggs were rinsed into modified Ringer's solution/5, and activated by application of an alternating current electric field (12 V AC with an electrode spacing of 3 cm) for 7 s. Activated eggs were incubated 15 min at room temperature prior to preparation of extracts.

Oocytes were obtained by dissection and freed of follicle cells by incubation for 45 min with collagenase (10 mg/ml type 1A, Sigma Chemical Co., St. Louis, MO) at room temperature with agitation.

Oocytes or activated eggs (3–5 ml) were rinsed five times with 3 vol of cold (5°C) reassembly buffer (BRB[1]: 80 mM KPipes, pH 6.8, 1 mM $MgCl_2$, 1 mM EGTA) containing 1 mM DTT, 5 mM NaF, and protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride (PMSF) and benzamidine HCl, 2 μg/ml *O*-phenanthroline and pepstatin A). Cells were then packed by centrifugation (1,000 *g* for 15 s), and all excess buffer was removed. Activated eggs were lysed by repeated pipetting five times with a Pasteur pipette. Oocytes were lysed by hand homogenization with a glass/teflon homogenizer. High-speed supernatants were prepared by centrifugation at 50,000 rpm for 90 min at 4°C in an SW50.1 rotor (Beckman Instruments, Inc., Palo Alto, CA), in 5 × 41-mm tubes. The cytoplasmic extract was recovered by cutting the centrifuge tube below the lipid layer, and extracting the clear cytoplasm with a pipette. Cytochalasin B (Sigma Chemical Co.) was added to 10 μg/ml to prevent subsequent polymerization and gelation of actin (if omitted, actin polymerization and gelation interfered greatly during fixation and immunofluorescence). Extracts could be stored on ice and retained their respective assembly properties for several hours. Freezing destroyed the ability of egg extracts to assemble microtubules in the absence of added tubulin, a characteristic that was used in later experiments assaying assembly promoting activity.

Protein concentrations were measured according to the method of Bradford (1976), using bovine serum albumin (BSA) as a standard.

Gel Electrophoresis and Quantitative Immunoblotting of Extract Tubulin

Electrophoretic analysis of proteins (SDS-PAGE) was according to Laemmli (1970), on 7% polyacrylamide gels with a pH of 9.2 in the resolving gel to facilitate resolution of tubulins. For determination of tubulin concentrations in the extracts 30 μg of oocyte or egg proteins were separated by SDS-PAGE and transferred to nitrocellulose by electroblotting in 150 mM glycine, 0.02% SDS, and 20% methanol for 20 hours at 25 mA (yielding ~20 V). Nitrocellulose blots were probed with a mixture of monoclonal antisera to α- and β-tubulin (DMα1 and DMβ1, generously provided by Dr. S. Blose, Protein Databases, Inc., East Northport, NY, 1:1,000 dilution), followed by ^{125}I-conjugated rabbit anti-mouse IgG (10^6 cpm/ml). For determination of the total egg or oocyte tubulin extracts were prepared from 30 oocytes or eggs, and one egg equivalent of protein was loaded per lane, followed by immunoblotting. Immunoblots were exposed on XAR film (Eastman Kodak Co., Rochester, NY) using Cronex Lightning-Plus intensifying screens (DuPont Instruments, Wilmington, DE). Quantitation of autoradiographs was by scanning densitometry, using known amounts of bovine brain tubulin included on the blots as a standard.

In Vitro Assays of Microtubule Assembly

Centrosomes (prepared from N115 neuroblastoma cells), *Tetrahymena axonemes*, and phosphocellulose-purified bovine brain tubulin were prepared as described by Mitchison and Kirschner (1984*a*, *b*).

For assembly assays using centrosomes adsorbed to glass coverslips, centrosomes were first diluted into 10 mM KPipes, 1 mM EGTA, pH 7.0, and sedimented onto 12-mm clean glass coverslips (Evans et al., 1985). Coverslips were then placed centrosome side up on a sheet of parafilm. Excess buffer was gently aspirated, and 40 μl of diluted extract (either oocyte or egg) was added to each coverslip. Coverslips were incubated for 5–15 min at 22–23°C as indicated. Excess extract was gently aspirated and 100 μl of 1% gluteraldehyde in BRB plus 0.2% Triton X-100 was added. Coverslips were fixed for 1 min at room temperature, rinsed briefly with Tris-buffered saline (TBS: 0.15 M NaCl, 10 mM Tris HCl, pH 7.4), and fixed for 1 min in methanol at −20°C. Coverslips were then rehydrated by three rinses in TBS, and processed for immunofluorescence.

For assembly reactions in solution, extracts, purified brain tubulin, and BRB were mixed at 4°C (as indicated) to yield a final volume of 60 μl. To these mixtures was added 5 μl of centrosomes diluted in BRB. Samples were mixed well and placed at 22–23°C (for extract experiments) or 37°C (for assays using brain tubulin) for 5–15 min. Samples were fixed by adding 400 μl of 1% gluteraldehyde in BRB plus 0.2% Triton X-100. After 1 min of incubation, an additional 400 μl of cold BRB was added, and samples were set on ice for 5–15 min. Samples were layered over a 5-ml cushion of 15% (wt/vol) glycerol in BRB plus 0.1% Triton X-100, and sedimented onto polylysine-coated coverslips (10,000 rpm for 25 min at 4°C). Samples of sheared microtubules were sedimented onto coverslips from 0.5 ml of BRB, without any sucrose cushion. Coverslips were rinsed with TBS and fixed in methanol as above.

Coverslips were incubated for 10 min with 25 μl DMα1 monoclonal antisera to α-tubulin (1:250 dilution in TBS plus 1 mg/ml BSA and 0.1% Triton X-100) washed three times in TBS and incubated 10 min in a 1:50 dilution of rhodamine-conjugated rabbit anti-mouse IgG (Cappel Laboratories, Cochranville, PA). Coverslips were rinsed three times with TBS, once with distilled water, and mounted on slides for microscopy.

1. *Abbreviations used in this paper*: BRB, reassembly buffer; MAP, microtubule-associated protein; TBS, Tris-buffered saline.

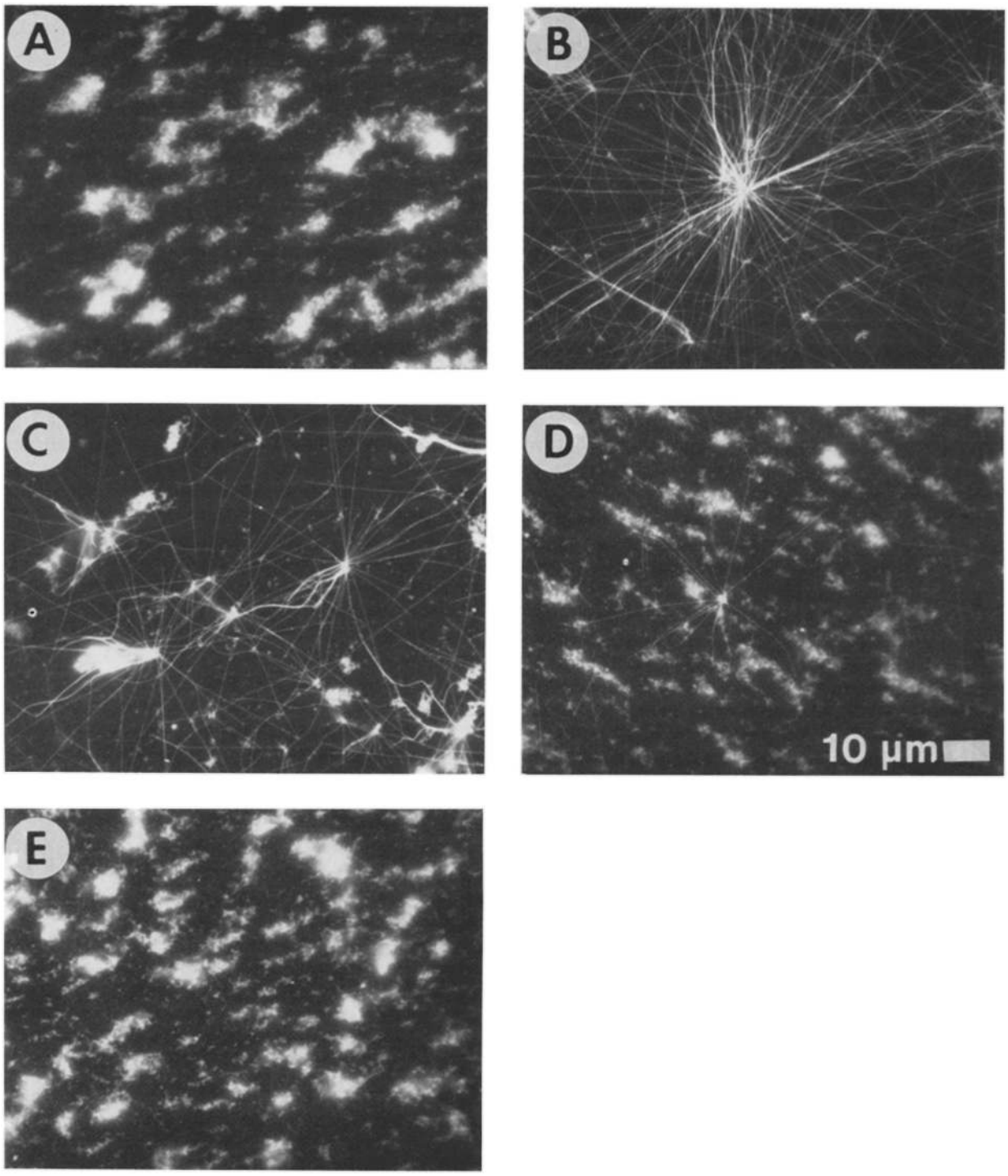

Figure 1. Cytoplasmic extracts from *Xenopus* oocytes and eggs differ in their ability to assemble microtubules in vitro. Centrosomes adsorbed to coverslips were incubated for 10 min at 23°C with (*A*) oocyte extract or (*B*) activated egg extract at 15 mg/ml protein. An extensive radial array of microtubules is assembled from centrosomes in the egg extract. Addition of (*C*) 2.5, (*D*) 5.0, or (*E*) 7.5 mg/ml of oocyte protein (final concentration) to a constant 15 mg/ml egg protein results in a progressive inhibition of microtubule assembly. Bar, 10 μm.

Microtubules were observed on a Zeiss photomicroscope III equipped with a Leitz 63× planapo neofluor objective. Photographs were taken on Kodak technical pan 2415 film with ~30-s exposures. Film was developed in HC110 dilution D for 6.5 min. Microtubule lengths were quantitated by projection of the 35-mm negatives onto a digitizing board interfaced with an IBM PCXT (IBM Instruments, Inc., Danbury, CT). Assembly rates were determined by dividing the mean length of microtubules (at least 100 microtubules per point) by the time of assembly. Values in parentheses are standard errors of the mean.

Results

Cytoplasmic Extracts from Oocytes and Activated Eggs Differ in Their Ability to Assemble Microtubules In Vitro

We investigated the ability of concentrated cytoplasmic extracts from oocytes and activated eggs to assemble microtu-

bules in vitro. Extracts were prepared 15 min after eggs were activated electrically. This corresponds in time to entry into interphase in vivo, which is characterized by the rapid assembly of microtubules (the sperm aster in fertilized eggs; Gerhart, 1980). Preliminary experiments indicated that extracts prepared at this time after activation gave the most consistent microtubule assembly (not shown). Extracts were prepared by lysing cells in a minimal volume of BRB and removing yolk and pigment by high-speed centrifugation. Extracts typically contained 30–40 mg/ml soluble protein. Microtubule assembly was assayed by growth from centrosomes either adsorbed to glass coverslips, or in solution. Assembly of microtubules from centrosomes in these extracts relies on the endogenous cytoplasmic pools of tubulin and any required cofactors or accessory proteins, and thus should reflect the microtubule assembly competence of the intact cells. Because nucleation sites are provided by centrosomes (or in later experiments, by axonemes), this assay measures microtubule elongation, and does not depend on the nucleation capacity of the extracts.

An extensive array of microtubules can be seen to radiate from the centrosomes after incubation of an activated egg extract (diluted to 15 mg/ml protein with BRB) with centrosomes (**Fig. 1 *B***). The identity of these structures as microtubules was confirmed by thin-section electron microscopy (not shown). Microtubule assembly from the egg extract was dependent upon the presence of added centrosomes or flagellar axonemes, and was not observed in the absence of such nucleating centers (not shown). No microtubule assembly was observed from centrosomes incubated in extracts similarly prepared from oocytes (Fig. 1 *A*). These in vitro results are consistent with observations previously made in vivo. Very few microtubules are observed in intact oocytes (Heidemann et al., 1985), and oocyte extracts are deficient in assembly in vitro; activated eggs, however, are capable of extensive assembly in vivo (Karsenti et al., 1984), and extracts prepared from these eggs also exhibit extensive microtubule assembly in vitro.

Oocyte and Egg Extracts Contain Comparable Tubulin Concentrations

To further characterize microtubule assembly in vitro we measured the tubulin concentrations and rate of assembly in the extracts. The tubulin content of egg and oocyte extracts was measured by quantitative immunoblotting using monoclonal antibodies to α- and β-tubulins. Tubulin (α and β dimer) was found to represent 2.8% (±0.8%, n = 16) of the total soluble protein in egg extracts. Oocyte extracts contained 2.2% (±1.1%, n = 16) tubulin. The slightly lower tubulin concentration in oocyte extracts is due to a greater loss of tubulin in the yolk pellet during preparation. The highest concentration of tubulin observed in any extract was 2.4 mg/ml (corresponding to 24 μM tubulin dimer) representing 4.1% of the soluble protein in an oocyte extract containing 59 mg/ml total protein. This figure thus represents a lower limit for the actual tubulin concentration in the oocyte and egg.

Quantitative immunoblotting was also used to estimate the total tubulin content of eggs and oocytes. Eggs were found to contain 0.44 (±0.04, n = 13) μg tubulin per egg as compared to 0.43 (±0.04, n = 16) μg per oocyte. These estimates compare roughly with those of Heidemann (0.1 μg per egg, by colchicine binding, Heidemann and Kirschner, 1975) and Elinson (1985) (1 μg per egg by immunoblotting). Eggs and oocytes thus contain equivalent pools of tubulin.

Microtubule Assembly Rate Is Dependent on Extract Concentration

Assembly rates at different egg tubulin concentrations were determined by diluting extracts of known tubulin concentration with BRB (with 1 mM GTP added), and measuring the microtubule assembly rate of the diluted extract (Fig. 2, see Materials and Methods). Note that dilution of the egg extract would dilute any assembly factors in addition to tubulin. However, these factors would remain in constant stoichiometry to tubulin. The assembly rate of activated egg extract using centrosomes as nucleation centers is proportional to tubulin concentration with an observed slope of ~2 $\mu m \cdot min^{-1} \cdot \mu M^{-1}$ (57 $s^{-1} \cdot \mu M^{-1}$), and an x-intercept of ~0.4 μM. Some scatter is observed at high tubulin concentrations presumably due to shearing of the long microtubules observed. Shearing and tangling of microtubules prevented the determination of growth rates at tubulin concentrations greater than ~8 μM. Assembly rates from assays from adsorbed centrosomes and centrosomes in solution were similar. No microtubule assembly was observed in oocyte extracts at even the highest tubulin concentrations assayed (as high as 20 μM).

Microtubule Assembly in Egg Extracts Occurs Predominantly at the Plus-End

Tetrahymena axonemes were used as nucleation sites to assess the polarity of microtubule assembly of extract microtubules (Fig. 3). These assays were performed by incubating axonemes adsorbed to glass coverslips with dilutions of egg extracts containing 5 or 10 mg/ml protein (corresponding to 1.4 and 2.8 μM egg tubulin, respectively) for 5 min at 22°C. Under these conditions microtubule elongation was only observed from the distal (frayed) end of axonemes (Fig. 3, *A*

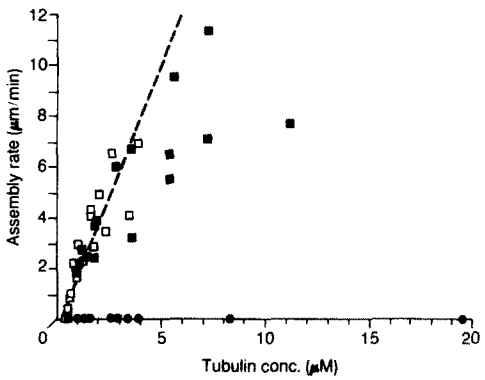

Figure 2. Microtubule assembly was assayed for 5 min at 23°C from diluted extracts of oocytes (●) or activated eggs (■) assayed by solid phase, (□) or in solution. Microtubules were photographed and measured (at least 200/point). Tubulin content of the extracts was determined by immunoblotting. Results from five experiments were plotted as the microtubule assembly rate vs. tubulin concentration. Egg extracts exhibit a dependence of assembly rate on tubulin concentration of 2 $\mu m \cdot min^{-1} \cdot \mu M^{-1}$ corresponding to a tubulin on rate of 57 $s^{-1} \cdot \mu M^{-1}$. The apparent critical concentration (x-intercept) was ~0.4 μM. No assembly of microtubules was observed from oocyte extracts at any tubulin concentration.

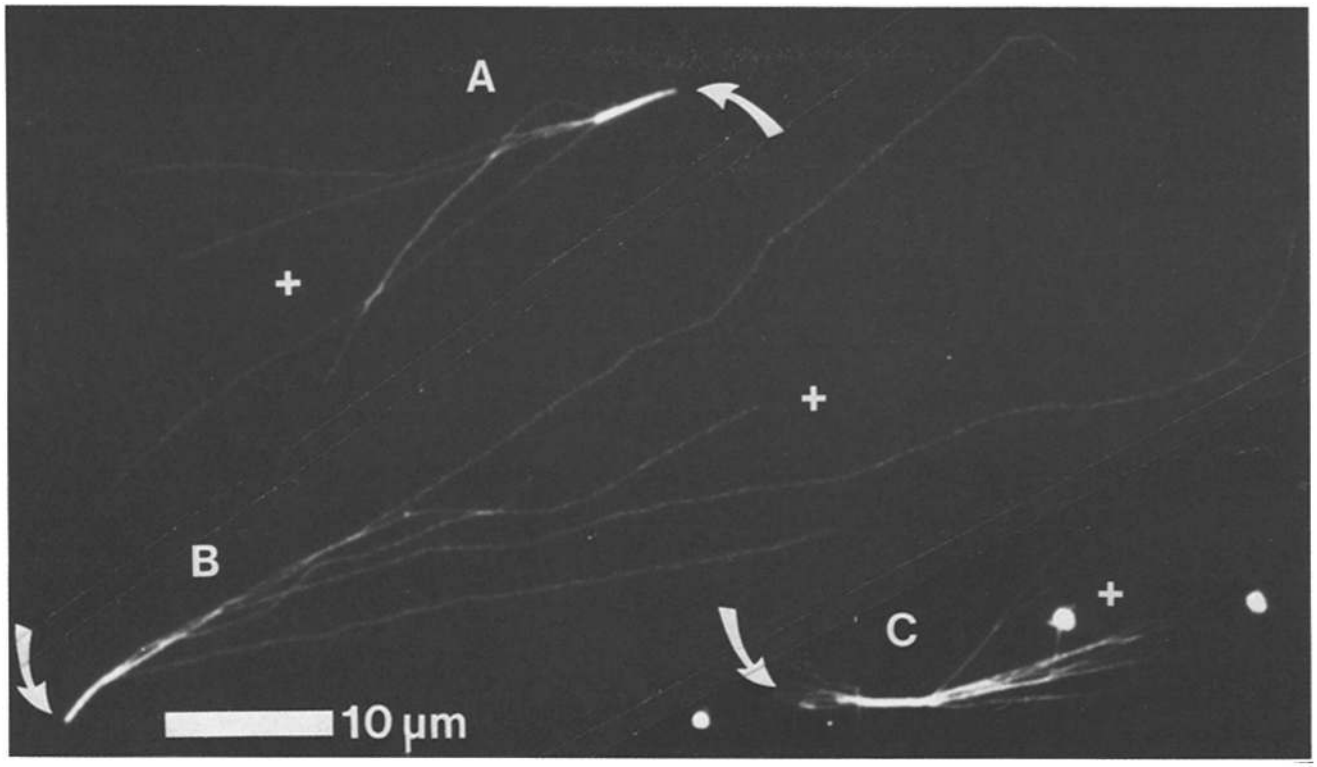

Figure 3. Microtubule assembly from activated egg extracts occurs predominantly at the plus-end. *Tetrahymena* axonemes were adsorbed to glass coverslips and used to nucleate microtubule assembly (5 min at 23°C) from egg extract at 5 mg/ml protein (1.4 μM egg tubulin, *A*) or 10 mg/ml (2.8 μM tubulin, *B*). Minus-end microtubule assembly was undetectable in axonemes elongated in egg extract (*arrows* in *A* and *B*). For comparison, 2.5 mg/ml bovine brain tubulin (25 μM, *C*) exhibits both plus- (+) and minus-end (*arrowed*) assembly. Bar, 10 μm.

and *B*), which corresponds to the fast-growing (or plus) end (Allen and Borisy, 1974). Assembly rates of the plus-end of axonemes were comparable to those observed from centrosomes, which nucleate plus-end microtubule assembly. Examination by immunofluorescence of a large number of axonemes with microtubules assembled at different egg tubulin concentrations (as high as 6 μM) revealed negligible assembly of microtubules at the minus-end (<0.5 μm). Although some minus-end assembly might be detectable with the greater resolution of electron microscopy, the minus-end assembly is certainly much less dramatic than that observed at the plus-end (see Discussion). Axonemes incubated in 2.5 mg/ml (25 μM) purified brain tubulin for 15 min at 37°C exhibit assembly from both plus- and minus-ends, with a length ratio of ~3:1.

Microtubules Assembled from Egg Extract Are More Stable to Dilution-induced Disassembly than Bovine Brain Microtubules

The disassembly rates of microtubules assembled in these egg extracts (assembly was nucleated by centrosomes at 10–12 mg/ml egg protein, corresponding to ~3 μM tubulin) were determined by dilution into assembly buffer (at 37°C). Microtubules were fixed at intervals after dilution, and observed by immunofluorescence, photographed, and measured. The initial rate of disassembly at the plus-end was 4.5 ± 0.3 (n = 3) μm·min^{-1} (128 s^{-1}; Fig. 4). Identical initial rates of disassembly were obtained when the microtubules were diluted 11-fold (to 0.3 μM tubulin), 31-fold, or 51-fold (to 0.06 μM tubulin). The plus-end critical concentration calculated from the observed on-rate and off-rate was 0.45 μM.

The minus-end disassembly rate could not be measured in such a direct manner, in that little or no minus-end assembly occurs in egg extracts (see above). To estimate the minus-end off-rate microtubules were assembled from centrosomes as

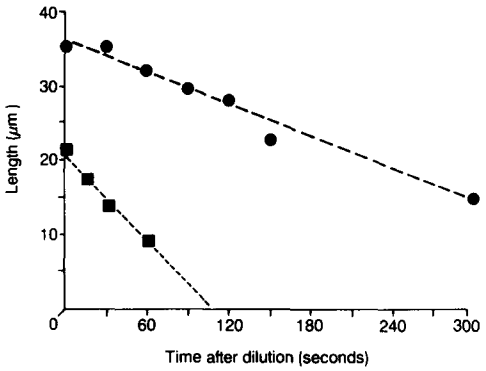

Figure 4. Disassembly rates of centrosomal and sheared microtubules assembled from activated egg extract were determined by dilution (51-fold to 0.6 μM tubulin; see text and Table I). The initial rate of disassembly at the microtubule plus-end was determined by dilution of centrosomal microtubules (●). The total assembly rate of plus- and minus-ends was determined by dilution of sheared microtubules (■). The minus-end disassembly rate was determined by subtraction of the centrosomal (plus-end) disassembly rate from the sheared (total) disassembly rate (see text).

described above, and then were sheared from the centrosomes by three passes through a 27-gauge needle. Sheared microtubules were incubated an additional 3 min to allow further elongation. These microtubules were then diluted and fixed at intervals after dilution, and free microtubules were photographed and measured. Because these microtubules have both ends free, the observed total disassembly rate of 11.4 ± 0.1 (SEM, $n = 2$) $\mu m \cdot min^{-1}$ (323 s^{-1}) represents the sum of plus-end disassembly and minus-end disassembly. The rate of plus-end disassembly obtained in a parallel experiment (4.5 μm/min) was thus subtracted from the total rate of disassembly to obtain the minus-end rate of 6.9 $\mu m \cdot min^{-1}$ (196 s^{-1}). These rates, as well as the assembly rates of egg microtubules and the corresponding assembly and disassembly rates of purified bovine brain tubulin (Mitchison and Kirschner, 1984*b*) are summarized in Table I.

Table I. Assembly and Disassembly Rates of Microtubules Assembled from Extracts of Activated Xenopus Eggs

	Plus-end	Minus-end
Assembly rate		
Extract microtubules at 23°C	2 $\mu m \cdot min^{-1} \cdot \mu M^{-1}$	0.02‡
	57 $s^{-1} \cdot \mu M^{-1}$	0.5
Phosphocellulose-purified brain tubulin at 37°C*	0.14 $\mu m \cdot min^{-1} \cdot \mu M^{-1}$	0.042
	3.8 $s^{-1} \cdot \mu M^{-1}$	1.2
Disassembly rate (at 37°C)		
Extract microtubules	−4.5 $\mu m \cdot min^{-1}$	−6.9§
	−128 s^{-1}	−196§
Phosphocellulose-purified brain tubulin*	−12.0 $\mu m \cdot min^{-1}$	−7.5
	−340 s^{-1}	−213

* From Mitchison and Kirschner (1984*b*).
‡ Not detectable. The figure given represents an upper limit.
§ Determined by subtraction, see text.

Xenopus Oocytes Contain a Plus-End–Specific Inhibitor of Microtubule Assembly

The inability of oocyte extracts to assemble microtubules from centrosomes in vitro could be due to any of several reasons. First, the oocyte extract could inactivate centrosomes. To test this possibility, centrosomes adsorbed to coverslips were incubated 15 min with oocyte extract (diluted to 15 mg/ml with BRB), rinsed briefly with BRB, and incubated with egg extract. Preincubation with oocyte extract had no effect on subsequent microtubule assembly during incubation with egg extract (not shown). Thus the oocyte extract does not irreversibly inhibit centrosome function, corroborating the results of Karsenti et al. (1984) that centrosomes microinjected into oocytes are capable of aster assembly after subsequent maturation and activation of the oocyte.

In that oocytes and eggs contain comparable pools of cytoplasmic tubulin (Heidemann and Kirschner, 1975; see above), which are equally competent to assemble in vitro (Heidemann et al., 1985), the observed difference in microtubule assembly in vitro (and the differences in microtubules in vivo) cannot be due to lack of, or irreversible inactivation of, tubulin.

The lack of assembly by oocyte extracts could result from either the presence of an inhibitory factor, or from the lack of an assembly promoting factor found in eggs. Mixing experiments were performed to distinguish these possiblities (Fig. 1, *C–E*). Increasing volumes of oocyte extract were added to a constant volume of egg extract. The total assembly volume was maintained constant by addition of BRB. Addition of increasing amounts of oocyte extract (from 2.5 to 7.5 mg/ml final concentration) resulted in a marked inhibition of microtubule assembly from the egg extracts. Because the oocyte extract contains tubulin, the total tubulin concentration actually increased with increasing amounts of oocyte extract. Thus, the oocyte extract actively inhibits assembly of microtubules; inhibition is not due to dilution of competent tubulin or lack of promoting factors found in eggs.

Oocyte extracts also inhibited the assembly of bovine brain tubulin onto either centrosomes or axonemes (Figs. 5 and 6). Centrosomes and axonemes were incubated with a constant amount of brain tubulin and increasing amounts of oocyte extract in solution. A constant reaction volume was maintained by addition of BRB. With tubulin alone substantial assembly of microtubules was observed from centrosomes (Fig. 5 *A*), and axonemes exhibit the characteristic fast- and slow-growing ends previously described (Allen and Borisy, 1974; plus- and minus-ends, respectively) with a ratio of lengths of 3 to 1. Addition of 15 μl (one-fourth of the reaction volume, containing 450 μg of soluble protein) of oocyte extract markedly inhibited assembly of centrosomal microtubules (Fig. 5 *B*). Assembly from axonemes revealed that inhibition occurred predominantly at the plus-end. Little or no inhibition is observed at the minus-end, resulting in axonemes with microtubules of equal length at both ends. Results were quantitated by measuring microtubules from the plus- and minus-end of axonemes (plus-ends were identified by their tendency to fray). Histograms showing the length distributions of plus- and minus-ends, and a plot of mean microtubule lengths versus added oocyte protein are shown in Fig. 6. At intermediate amounts (200–300 μg of protein) the oocyte extract inhibited assembly at the plus-end only, with a resulting shift in the average plus-end microtubule length from 15 to 7 μm. At higher amounts of oocyte extract (450 μg of protein) assembly at the plus-end was inhibited such that the assembly at both axoneme ends was equal (here observed to be ~5 μm). At even higher concentrations (>500 μg) assembly from both ends was completely inhibited, and no microtubule assembly was observed from axonemes or centrosomes (not shown). The dramatic difference in the effect of the oocyte extract on the microtubule ends suggests that the inhibition is a specific effect, rather than a nonspecific inhibition of all microtubule assembly. The end-specific promotion of microtubule assembly by egg extract (see below) also serves as a control for nonspecific effects of added protein on microtubule assembly.

Several preliminary experiments have been performed to address the nature of the inhibitory factor. Inhibitory activity is heat labile (5 min at 100 °C) and is retained after overnight dialysis against BRB. Activity is removed from extracts by adsorption to DEAE cellulose. Extracts containing inhibitory activity are stable after freezing in liquid nitrogen and can be stored for extended periods at −70°C.

Activated Eggs Contain an End-Specific Promoter of Microtubule Assembly

In contrast to the strong inhibition of microtubule assembly in oocyte extracts, assembly of microtubules from extracts

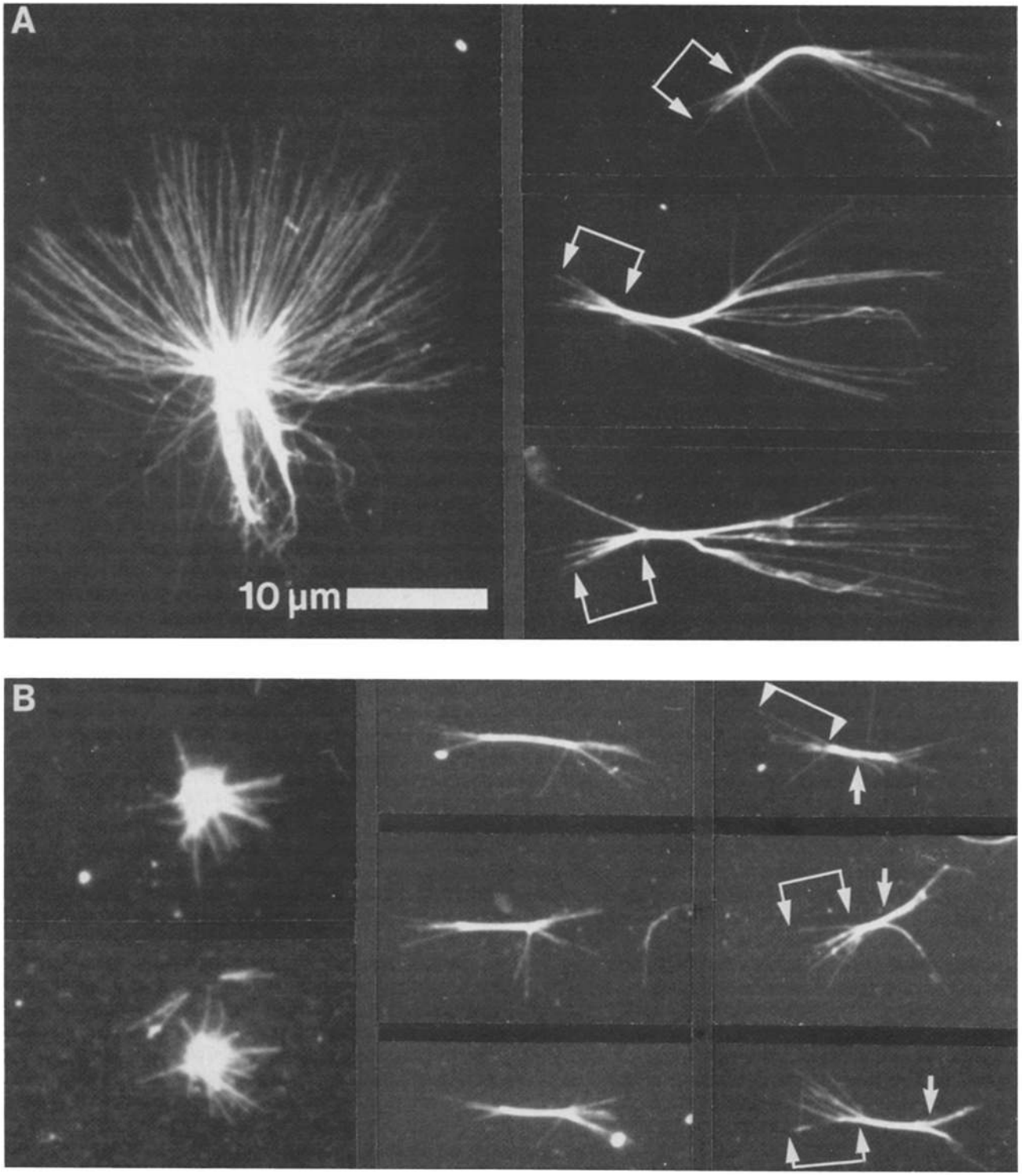

Figure 5. Oocyte extract contains a plus-end–specific inhibitor of assembly of purified bovine brain tubulin. Assembly of phosphocellulose-purified brain tubulin (2.5 mg/ml) was nucleated by centrosomes and axonemes for 15 min at 37°C. In the absence of oocyte extract (*A*) large microtubule asters (20–30 μm in diameter) are assembled from centrosomes. Axonemes exhibit characteristic growth polarity, with the slow-growing, or minus-end, microtubules (bracketed by *arrows*) approximately one-third the length of the plus-end microtubules. Addition of 450 μg of oocyte extract protein to the assembly reaction (*B*) results in a marked inhibition of assembly from centrosomes, of which two examples are shown. Six typical axoneme profiles are shown. Note that in most cases the microtubules at both axoneme ends are of comparable length, preventing assignment of polarity by difference in growth rates, but indicating that oocyte extract preferentially inhibits the plus-end. In three cases shown the axoneme has frayed (*single arrows*), a characteristic of the distal or plus-end, allowing determination of polarity with some degree of certainty. The minus-end microtubules (bracketed by *arrows*) in these cases are similar in length to those of the control, while the plus-ends show nearly complete inhibition of assembly. Bar, 10 μm.

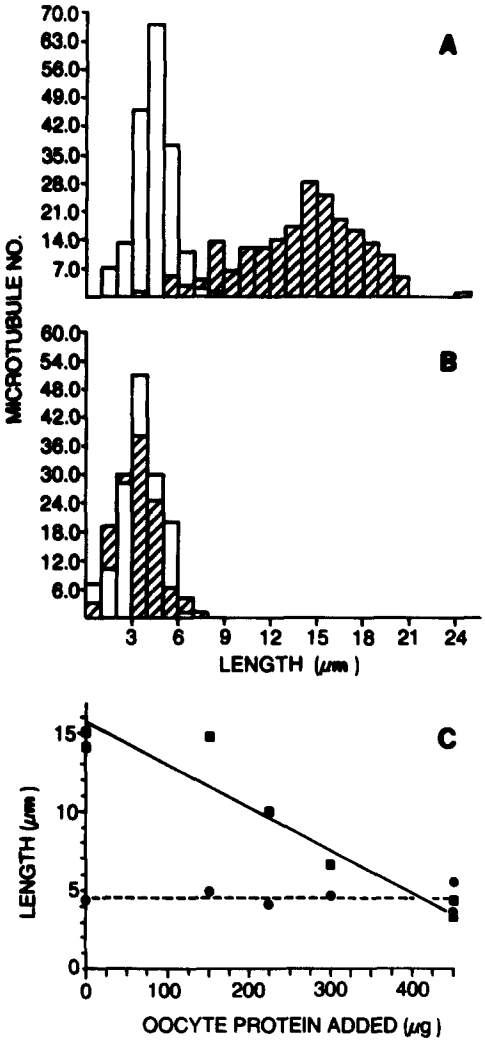

Figure 6. Quantitative analysis of the inhibition of microtubule assembly by oocyte extract. Microtubules from the experiment shown here were photographed and measured. Histograms of the microtubule length distributions for the plus- (*hatched bars*) and minus-eyed (*open bars*) in the (*A*) absence and (*B*) presence of 450 μg of oocyte extract protein are shown. In *B* only those axonemes where fraying of the plus-end allowed determination of polarity were measured. In *C* the mean lengths of plus- (■) and minus-end (●) are plotted vs. increasing amounts of oocyte extract proteins added. Inhibition of assembly of phosphocellulose-purified brain tubulin exhibits a unique specificity for the microtubule plus-end.

of activated eggs is very rapid. These results suggest that the egg contains a factor that accelerates microtubule assembly. This conclusion was confirmed by the ability of the egg extract to promote the assembly of brain tubulin in a dose-dependent manner. Extracts from activated eggs were frozen in liquid nitrogen and thawed to destroy the self-assembly activity described above, presumably by inactivating the endogenous tubulin. Extracts that had been frozen and thawed, and were incapable of microtubule assembly by themselves, were then mixed with a constant amount of bovine brain tubulin, and incubated with centrosomes or axonemes as nucleating sites. Egg extracts dramatically accelerated the assembly of microtubules from centrosomes as shown in Fig. 7, *A* and *B*, and quantitatively in Fig. 8, *C–D*. The promotion of centrosomal microtubule assembly was linearly dependent upon the amount of added egg protein, reaching a two-fold promotion in the experiment shown. Much greater stimulation of assembly is observed (see accompanying article) using purified fractions containing the promoting factor from egg extracts. Assembly from axonemes in the presence of egg extract revealed that assembly promotion was specific for the microtubule plus-end (Figs. 7 and 8, *A* and *B*). The greater heterogeneity of plus-end microtubule length in the presence of the egg extract (Fig. 8 *B*) could be due to elongation of axoneme B-subfiber microtubules (note the presence of more than nine microtubules at the axonemes plus-end in Fig. 7 *B*), which is not as efficient as A-subfiber elongation. Microtubule assembly at the axoneme minus-end was observed in the presence of egg extract, but was not accelerated over the minus-end assembly observed with tubulin alone (see *arrows* in Fig. 7, *A* and *B*, and also Fig. 8, *A* and *B*).

Discussion

We have studied the assembly of microtubules in cytoplasmic extracts from two distinct stages in *Xenopus* oogenesis: the oocyte (arrested at meiotic prophase) and the artificially activated egg (entering interphase after completion of meiosis). These two stages of oogenesis were chosen for their dramatic difference in microtubule assembly properties in vivo (Heidemann and Gallas, 1980; Heidemann et al., 1985; Heidemann and Kirschner, 1975; Karsenti et al., 1984).

Microtubule assembly in an in vitro assay using centrosomes or axonemes as nucleation centers was found to accurately reflect the assembly properties observed in the source material. Extracts prepared from oocytes (with tubulin concentrations as high as 20 μM) were unable to support microtubule assembly, consistent with the small number of microtubules in intact oocytes, and the instability of microtubules microinjected into oocytes (Heidemann and Kirschner, 1975). Extracts of activated eggs, on the other hand, supported extensive microtubule assembly, consistent with the dramatic enlargement of the sperm aster after fertilization (Gerhart, 1980; Stewart-Savage and Grey, 1982).

Several possible explanations exist for the differences in microtubule assembly observed in oocyte and egg extracts in vitro. Immunoblot analysis revealed that oocyte and egg extracts contained comparable pools of tubulin, as had previously been found by Heidemann and Kirschner (1975) using a colchicine-binding assay. Intrinsic differences in tubulin subunits from these different stages of oogenesis could also result in different assembly properties. However, purified spindle and cytoplasmic tubulins from sea urchin or surf clams exhibit no differences in their biochemical or assembly properties (Keller and Rebhun, 1982; Suprenant and Rebhun, 1984, *a*, *b*). Heidemann et al. (1985) have also found no difference in the assembly competence of *Xenopus* tubulin isolated from either oocytes or eggs. Therefore, it is unlikely that differences in tubulin pool size or subunit composition are responsible for the observed lack of microtubule assembly in oocyte extracts in vitro, or in intact oocytes.

Microtubule assembly is sensitive to ionic conditions such as Ca^{2+} concentration and pH, and it is difficult to assess with certainty the role these factors play in assembly in extracts (Weisenberg and Rosenfeld, 1975). Note, however, that the observed differences in microtubule assembly between

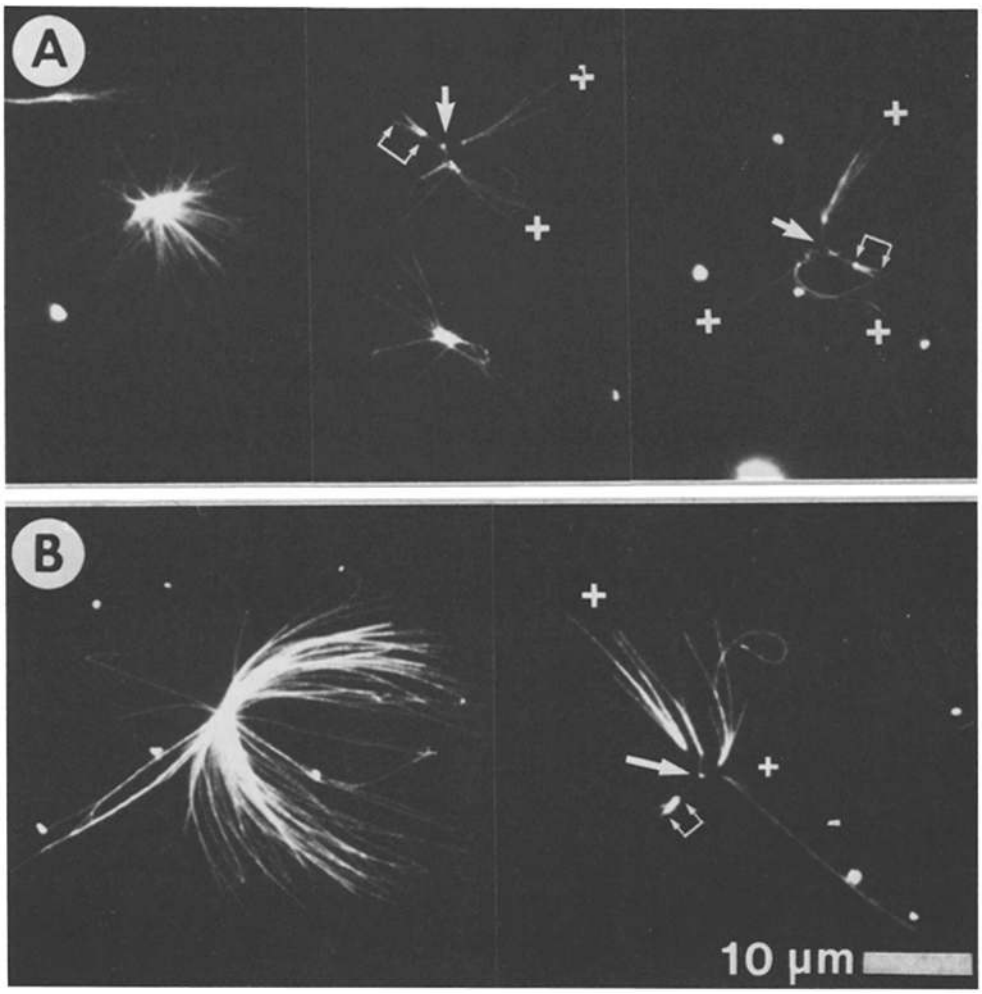

Figure 7. Extracts of activated *Xenopus* eggs promote plus-end assembly of purified bovine brain tubulin. Centrosomes and axonemes were used to nucleate assembly of phosphocellulose-purified brain tubulin (2.5 mg/ml for 5 min at 37°C) in the (*A*) absence or (*B*) presence of 450 μg of egg extract protein. The egg extract had been previously frozen and thawed to inactivate endogenous tubulin (see text). Small microtubule asters were observed in the sample without added egg protein. Axonemes in these samples exhibited the polar assembly characteristic of microtubules. The slow-growing or minus-end is denoted by *brackets*, plus-end microtubules by +. Addition of 450 μg of activated egg extract (*B*) results in a promotion of assembly from centrosomes. Assembly of microtubules at the axoneme plus-end was also promoted. Note that more than nine microtubules have elongated from the axoneme plus-end (+), suggesting elongation of the axoneme B-subfibers (see text). However, microtubules at the axoneme minus-end (*brackets*) were not substantially longer than those in the control (compare *brackets* in *A* and *B*). Bar, 10 μm.

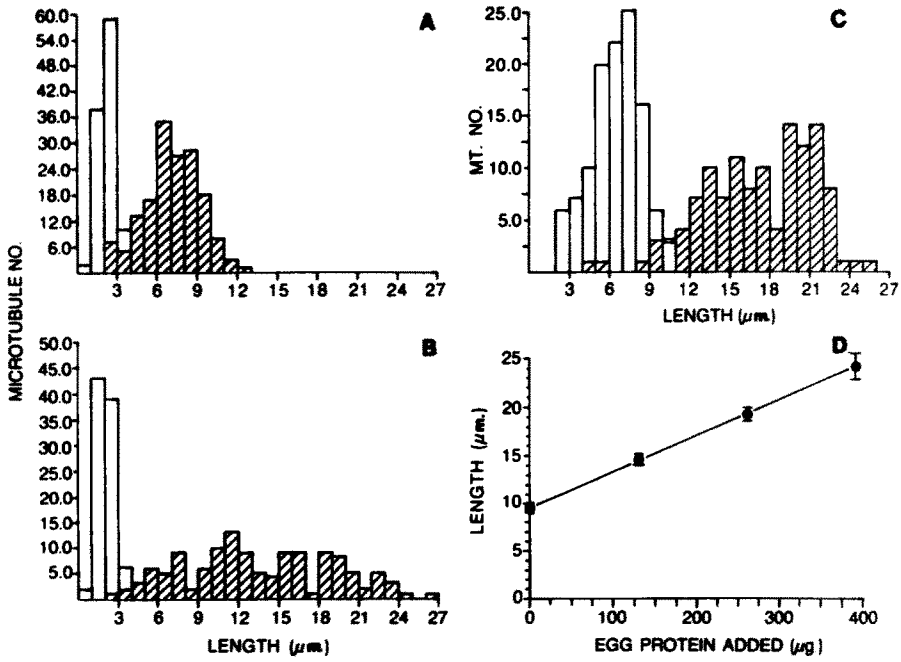

Figure 8. Data from the experiment shown here were quantitated by photographing, measuring, and plotting the length distributions of plus- (*hatched bars*) and minus-end (*open bars*) microtubules assembled from axonemes in the (*A*) absence and (*B*) presence of 450 μg of egg protein. Egg extract promotes the assembly of plus-end microtubules, but does not promote assembly at the minus-end. The promotion of plus-end assembly is also apparent in the length distribution of centrosomal microtubules (*C*) in the absence (*open bars*) or presence (*hatched bars*) of 450 μg of egg protein. Addition of increasing amounts of egg protein results in a linear increase in the mean length of centrosomal microtubules (*D*, at least 100 microtubules measured per point, ± SEM).

extracts made from *Xenopus* oocytes and eggs were apparent over a wide range of cytoplasmic protein concentrations, representing dilutions of cytoplasm into assembly buffer of 5–50-fold (see Fig. 2). The retention of distinct assembly properties under such conditions in which Ca^{2+} and pH can be more carefully controlled suggests that the observed differences in assembly properties, in particular the lack of assembly in oocyte extracts, are not due to ionic conditions in the in vitro reactions.

Alternatively, oocytes might lack some necessary cofactor that promotes microtubule assembly in eggs. To test this hypothesis we assayed the effect of oocyte extracts on microtubule assembly by mixing them with either egg extracts or purified brain tubulin. The oocyte extracts inhibited microtubule assembly in egg extracts in a dose-dependent manner. This progressive inhibition suggests that oocytes possess an activity that actively suppresses microtubule assembly rather than lacking an essential promoting activity. Oocyte extracts also inhibit assembly of purified tubulin that itself contains no assembly factors.

The identity of the inhibitory factor in the oocyte remains to be established. The retention of inhibitory activity after extended dialysis and its heat lability suggest that it is a macromolecule. Bryan et al. (1975) found that RNA and other polyanions in cytosol could inhibit the assembly of cycled brain microtubule proteins by binding and sequestering microtubule-associated proteins (MAPs). *Xenopus* oocyte extract, however, inhibits the assembly of MAP-free tubulin, suggesting that it acts directly on tubulin. The dramatic promotion of tubulin assembly by egg extracts also serves as a control for nonspecific effects of protein or nucleic acids on assembly.

A surprising feature of the inhibitory activity in the oocyte extract was its specificity for the microtubule plus-end. Adding increasing amounts of oocyte extract to an assembly reaction containing axonemes as nucleating centers resulted in progressive inhibition of the fast-growing, plus-end. Only at higher concentrations is microtubule assembly at both ends completely inhibited. The mechanism by which this plus-end–specific inhibition is achieved has not yet been determined. Several possibilities for its mode of action exist, such as binding directly to the microtubule plus-end, thereby capping that end. Alternatively, the inhibitor might function by binding to tubulin monomer in a manner which inhibits addition to the plus-end but not the minus-end, similar to the mechanism of action proposed for profilin (Tilney et al., 1983), an inhibitor of actin assembly.

The biological role played by this inhibitor is not understood. Preliminary evidence suggests that inhibitory activity is lost during the first meiotic division in vitro (data not shown) and does not reappear during the ensuing meiotic cycle or during the subsequent cleavage cycle. Assembly of a large cytoplasmic microtubule aster after fertilization (the sperm aster) is implicated in cytoplasmic rearrangements that determine the embryonic axis during development (Gerhart, 1980; Gerhart et al., 1983; Manes and Barbieri, 1977). It is possible that the inhibitory activity serves to prevent premature microtubule assembly in oocytes that might interfere with subsequent later development of the egg.

In contrast to the lack of assembly from oocyte extracts, extracts from activated eggs assembled extensive microtubule arrays from centrosomes in vitro. The assembly rate was linearly dependent on extract tubulin concentration with a slope of 2 $\mu m \cdot min^{-1} \cdot \mu M^{-1}$ (57 $s^{-1} \cdot \mu M^{-1}$), and an *x*-intercept, or critical concentration of 0.4 μM at 22–23°C (determined graphically from Fig. 3 and calculated from the measured on- and off-rate constants). This rate dependence on concentration is 15-fold greater than that observed for purified bovine brain tubulin at 37°C. (Mitchison and Kirschner, 1984*b*), suggesting that the egg contains a factor capable of significantly altering the rate of microtubule assembly.

Microtubule assembly in egg extracts is restricted to the microtubule plus-end. No microtubules were observed to elongate from the minus-end of *Tetrahymena* axonemes after 5 min of incubation in extracts with tubulin concentrations as high as 6 μM (17 mg/ml total protein). We estimate that we can detect microtubules as short as 0.5 μm by immunofluorescence (though we cannot accurately measure microtubules this size). This places an upper limit on the assembly rate at the minus-end of 0.02 $\mu m \cdot min^{-1} \cdot \mu M^{-1}$ (0.5 $s^{-1} \cdot \mu M^{-1}$). This rate is 100-fold less than that observed at the microtubule plus-end, while the difference in assembly rates of the plus- and minus-end of microtubules assembled from brain tubulin is typically about threefold. Promotion of bovine brain tubulin by egg extract also exhibits specificity for the plus-end, as does a protein factor we have isolated from *Xenopus* eggs which promotes microtubule assembly in vitro (see accompanying article).

Measurement of the dilution-induced disassembly rates of extract microtubules (-4.5 $\mu m \cdot min^{-1}$ and -6.9 $\mu m \cdot min^{-1}$ for the plus- and minus-ends respectively) revealed that the egg microtubules are also significantly more stable than those assembled from purified brain tubulin, again with the greatest effect seen at the plus-end. Extract microtubules are also more resistant to disassembly induced by cold (not shown). Thus the dramatic plus-end microtubule assembly observed in egg extracts is brought about by both a 15-fold increase in the tubulin on-rate and a two- to threefold decrease in the tubulin off-rate.

It is possible that some of the observed differences in assembly between egg microtubules and brain tubulin reflect differences between the tubulin subunits themselves. Tubulins purified from eggs of sea urchins (Detrich and Wilson, 1983; Keller and Rebhun, 1982; Suprenant and Rebhun, 1984*a*) and surf clams (Suprenant and Rebhun, 1984*b*), for example, have been found to exhibit lower critical concentrations for assembly (4–8 μm) at physiological temperatures (17°C) than bovine brain tubulin (20 μm at 37°C). In our experiments microtubule assembly from *Xenopus* egg cytoplasm exhibits a critical concentration of 0.4 μm at 21°C. This extremely low value, the dramatic increase in tubulin on-rate compared to brain tubulin, and the promotion of brain tubulin assembly in vitro by egg extract, all suggest that the dramatic assembly observed in egg extract (and intact eggs) is not an intrinsic property of the tubulin subunits. Rather, the rapid assembly observed reflects the presence of accessory factors which modulate assembly, analogous to the MAPs identified in vertebrate brain and cultured cells (Olmsted, 1986).

It is remarkable that both factors described, the oocyte inhibitor and the promoting activity in eggs, exhibit preferential activity at the plus end. Allen and Borisy (1974) observed preferred plus-end elongation of axoneme microtubules from crude brain extracts, suggesting that end-specific factors may

exist in brain extracts. However, purified brain microtubule protein (containing tubulin, MAPs 1 and 2, and τ proteins) was not found to exhibit such end specificity. We also found that purified τ protein promotes assembly of brain tubulin equally at both plus- and minus-ends (see accompanying article). The finding of end-specific microtubule assembly factors in *Xenopus* eggs and oocytes is of extreme interest. Because most, if not all, microtubules are anchored at their minus-ends and grow at their plus-ends in vivo, it may only be necessary to regulate activity at the plus-end to control overall microtubule assembly. The existence of a plus-end–specific inhibitor of assembly in oocytes and a plus-end–specific promoter of assembly in eggs could be sufficient to regulate microtubule assembly during oogenesis.

After fertilization the sperm aster enlarges to fill the animal hemisphere of the egg (Stewart-Savage and Grey, 1982), requiring an incredible rate of microtubule assembly of roughly 30–50 $\mu m \cdot min^{-1}$. The highest assembly rates we could conveniently measure in egg extracts were 12 $\mu m \cdot min^{-1}$, at 6 μM tubulin. Since the tubulin concentration in the egg is 20–25 μM, the assembly rates in vitro can be extrapolated to 40–50 $\mu m \cdot min^{-1}$. This rate is sufficient to account for the rapid enlargement of the sperm aster and is 10-fold higher than the rate of assembly observed in interphase cultured cells (3–4 $\mu m \cdot min^{-1}$; Schulze and Kirschner, 1986).

To date, our experiments to analyze microtubule assembly in extracts from unfertilized (unactivated) eggs have yielded inconsistent results. A major difficulty encountered appears to be the lability of the metaphase state of unfertilized egg cytoplasm. Unfortunately, several agents that are commonly used to stabilize the metaphase state of cytoplasm, such as high concentrations of beta-glycerophosphate and NaF (Gerhart and Kirschner, 1984), have proven to inhibit microtubule assembly in vitro. We are continuing our efforts to develop conditions in which we can compare the microtubule assembly characteristics of metaphase (unfertilized egg) and interphase (activated egg) cytoplasm.

The results in oocytes and activated eggs, however, suggest the action of two novel microtubule assembly factors. The lack of microtubule assembly in the oocyte can be satisfactorily explained by the presence of a plus-end–specific inhibitory activity. The rapid rate of microtubule assembly in the interphase egg (fertilized or activated) can be reproduced in vitro, and is explained by the existence of a plus-end promoter of microtubule assembly. Isolation and characterization of this plus-end–specific promoter of microtubule assembly is reported in the following article.

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Note Added in Proof: We have recently measured the assembly rates of tubulin purified from *Xenopus* eggs. At 20°C, egg tubulin assembles at rates of 0.08 $\mu m \cdot min^{-1} \cdot \mu M^{-1}$ (2.3 $s^{-1} \cdot \mu M^{-1}$) at the plus-end and 0.02 $\mu m \cdot min^{-1} \cdot \mu M^{-1}$ (0.6 $s^{-1} \cdot \mu M^{-1}$) at the minus-end. These rates should be compared with those obtained for assembly of microtubules from egg cytoplasm shown in Table I.

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