

Organization and regulation of cortical microtubules during the first cell cycle of *Xenopus* eggs

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Summary

Anti-tubulin antibodies and confocal immunofluorescence microscopy were used to examine the organization and regulation of cytoplasmic and cortical microtubules during the first cell cycle of fertilized *Xenopus* eggs. Appearance of microtubules in the egg cortex temporally coincided with the outgrowth of the sperm aster. Microtubules of the sperm aster first reached the animal cortex at 0.25, (times normalized to first cleavage), forming a radially organized array of cortical microtubules. A disordered network of microtubules was apparent in the vegetal cortex as early as 0.35. Cortical microtubule networks of both animal and vegetal hemispheres were reorganized at times corresponding to the cortical rotation responsible for specification of the dorsal-ventral (D-V) axis. Optical sections suggest that the cortical microtubules are continuous with the microtubules of the sperm aster in fertilized eggs, or an extensive activation aster in activated eggs.

Neither assembly and organization, nor disassembly of the cortical microtubules coincided with MPF activation during mitosis. However, cycloheximide or 6-dimethylaminopurine, which arrest fertilized eggs at interphase, blocked cortical microtubule disassembly. Injection of p13, a protein that specifically inhibits MPF activation, delayed or inhibited cortical microtubule breakdown. In contrast, eggs injected with cyc Δ 90, a truncated cyclin that arrests eggs in M-phase, showed normal microtubule disassembly. Finally, injection of partially purified MPF into cycloheximide-arrested eggs induced cortical microtubule breakdown. These results suggest that, despite a lack of temporal coincidence, breakdown of the cortical microtubules is dependent on the activation of MPF.

Key words: MPF, confocal microscopy, sperm aster.

Introduction

Specification of the dorsal-ventral (D-V) axis of frog embryos occurs early in development, during a critical period between fertilization and first cleavage (Scharf and Gerhart, 1983). An early indicator of the D-V axis in eggs of many amphibians is the formation of a region of distinct pigmentation opposite the sperm entry point (for a recent review, see Gerhart et al., 1981). This 'grey crescent' forms midway through the first cell cycle and marks the future dorsal side of the developing embryo (Gerhart et al., 1981, 1983). Formation of the grey crescent coincides with a rotation of the egg cortex relative to the deeper yolk-filled cytoplasm (Vincent et al., 1986). Agents that arrest or block this rotation disrupt axis specification, and result in embryos with deficiencies in dorsal structures (Vincent et al., 1987; Scharf and Gerhart, 1983).

Elinson and Rowning (1988) have described an extensive array of microtubules in the vegetal cortex of fertilized eggs from both *Rana pipiens* and *Xenopus laevis* that appears halfway through the cell cycle and

persist until shortly before cytokinesis. Assembly and organization of the cortical microtubules into parallel bundles coincides with the cytoplasmic rotation required for grey crescent formation and specification of the D-V axis. Moreover, this array of microtubules is aligned in the direction of the cortical rotation. Recently, Houliston and Elinson (1991a) have suggested that some of the microtubules in the vegetal cortex are continuous with microtubules of the sperm aster, which is assembled shortly after fertilization (Stewart-Savage and Grey, 1982). The observed correspondence between microtubule organization and axis specification and the sensitivity of axis formation to physical and chemical treatments that alter or inhibit microtubule assembly (Manes et al., 1978; Vincent et al., 1987; Scharf and Gerhart, 1983) suggest that the cortical microtubule network plays an integral role in specification of the D-V axis in frog embryos (Elinson and Rowning, 1988).

Many events of the embryonic cell cycle, including chromosome condensation, nuclear envelope breakdown and spindle assembly, are regulated by the cyclic

activation of maturation promoting factor (MPF) (Lohka and Maller, 1985; Dunphy and Newport, 1988). However, neither the assembly nor disassembly of the cortical microtubule network corresponds temporally to MPF activation during mitosis (Gerhart et al., 1984; Gautier et al., 1989; Murray and Kirschner, 1989). We therefore examined the dependence of cortical microtubule assembly and disassembly on MPF activation during the first cell cycle. We report that disassembly of the cortical microtubules was inhibited by cycloheximide, 6-dimethylaminopurine, and p13, all of which inhibit activation of MPF (Murray et al., 1989; Minshull et al., 1989; Murray and Kirschner, 1989; Neant et al., 1989; Rime et al., 1989; Brizuela et al., 1987; Dunphy et al., 1988; Dunphy and Newport, 1989). Furthermore, injection of MPF into cycloheximide-arrested eggs induced breakdown of the cortical microtubules. These results suggest that, despite the difference in timing, breakdown of the cortical microtubules network is dependent upon MPF activation during mitosis of the first cell cycle. In addition, we describe the assembly and reorganization of a dense microtubule network in the animal cortex of fertilized and activated *Xenopus* eggs.

Materials and methods

Unless otherwise indicated, all chemicals were obtained from Sigma Chemical Company (St Louis, MO).

Treatment of eggs

Mature *Xenopus laevis* were obtained from *Xenopus* 1 (Ann Arbor, MI). Ovulation, fertilization, and dejelling of eggs were as described by Newport and Kirschner (1982). Alternatively, dejelled eggs were electrically activated as described by Murray and Kirschner (1989).

Cycloheximide (500 µg/ml) and 6-dimethylaminopurine (15 or 500 µg/ml) were diluted to their final concentrations in 10% modified Ringer's buffer (10 mM NaCl, 0.2 mM KCl, 0.1 mM MgSO₄, 0.2 mM CaCl₂, 0.5 mM Hepes, 0.01 mM EDTA, pH 7.8), and were added during the dejelling process. Eggs remained in the inhibitors throughout the duration of the experiment.

Fixation and immunofluorescence

Eggs were fixed in microtubule assembly buffer (80 mM potassium Pipes pH 6.8, 5 mM EGTA, 1 mM MgCl₂) containing 3.7% formaldehyde (Mallinckrodt, Paris KY), 0.25% glutaraldehyde (Ted Pella, Redding CA), and 0.2% Triton X-100 for four to five hours at room temperature, followed by incubation in 100% methanol overnight at 4°C. For comparison purposes, eggs were also fixed in the above buffer plus 0.5 µM taxol. No difference was seen between eggs fixed in the presence or absence of taxol.

Eggs were bisected, either laterally, along the animal-vegetal axis, or equatorially before processing for immunofluorescence (Gard, 1991), and were bleached overnight with H₂O₂ in methanol (Dent and Klymkowsky, 1987). Eggs were then washed in phosphate-buffered saline (128 mM NaCl, 2 mM KCl, 8 mM NaH₂PO₄, 2 mM KH₂PO₄, pH 7.2), and incubated with 100 mM NaBH₄ in PBS for 5-16 hours at room temperature to minimize autofluorescence.

Antibody incubations were as previously described (Gard,

1991) using DM1A, a monoclonal antibody specific for α -tubulin (Bloese et al., 1984, available from ICN Biomedicals, Lisle, IL) and rhodamine-conjugated anti-mouse IgG as a secondary antibody (Organon Teknika-Cappel, Malvern, PA). Eggs were dehydrated in 100% methanol, then cleared and mounted in benzyl benzoate-benzyl alcohol (A. Murray, as cited by Dent and Klymkowsky, 1987). Whole eggs were mounted in chamber slides sealed with two coverslips to allow viewing of both sides of the egg. Bisected eggs were mounted in 0.5 mm depression slides.

Eggs were examined with an MRC-600 laser-scanning confocal microscope (Bio-Rad Microsciences, Cambridge, MA) using a 60 \times (NA 1.4) objective and aperture settings that provided optical sections approximately 0.7-1.0 µm thick. The use of a 10 \times objective for increased field of view is noted in the appropriate figure legend. Projection of several consecutive optical sections (by linear summation) was used to provide extended depth of focus. The number of sections projected in each figure is indicated in the corresponding legend.

Histone kinase assays

Histone kinase activity was assayed as described previously (Gard et al., 1990).

Partial purification and injection of MPF

MPF was partially purified (approximately 2.5-fold) from *Xenopus* eggs following modifications of the procedures described by Wu and Gerhart (1980) and Lohka et al. (1988). Unactivated eggs laid into 100 mM salt solution were collected and dejellied with 5 mM dithiothreitol (DTT) in 1.5 mM Tris-HCl, pH 8.5. Eggs were then washed in ice-cold extraction buffer (EB: 80 mM β -glycerol phosphate, 20 mM EGTA, 15 mM MgCl₂) and lysed in 2 volumes of cold EB containing 10 mM DTT and 1/1000 volume protease inhibitors (100 mM phenylmethylsulfonyl fluoride, 100 mM benzamidinium-HCl, 1 mg/ml pepstatin A, 1 mg/ml phenanthroline). Lysates were centrifuged at 21,000 g for 45 minutes at 4°C. The clear cytoplasmic layer was removed from between the yolk pellet and lipid cap and precipitated with one-half volume of saturated (NH₄)₂SO₄ (33%) in EB on ice 30-45 minutes. Precipitates were collected by centrifugation at 20,000 g, 4°C for 20 minutes. The resulting pellet was resuspended in EB plus 0.3 mM γ sATP (EB- γ sATP) to a final concentration of 4-8 mg/ml, and frozen at -70°C. Aliquots were thawed prior to injection, dialysed into ice-cold EB plus 1 mM DTT for 1 hour, and brought to approximately 1 mM γ sATP.

MPF activity was assayed by injection into stage VI oocytes. Germinal vesicle breakdown (GVBD) was scored by dissection of oocytes fixed in 10% TCA 2 hours after microinjection. One unit of MPF activity was defined as the amount of injected MPF required to cause 50% GVBD in the oocytes (Wu and Gerhart, 1980).

'Inactive' MPF was prepared as described for MPF (above) with the exception that eggs were electrically activated 15 minutes prior to lysis. Protein concentrations comparable to that of the active MPF were used for injection of oocytes and cycloheximide-arrested eggs. Inactive MPF did not induce GVBD when injected into stage VI oocytes.

Injection of cyc Δ 90 and p13

50 ng of cyc Δ 90 (a gift from Michael Glotzer, Mark Solomon and Marc Kirschner) was diluted in EB buffer and injected into fertilized eggs between 0.35 and 0.75. 200 ng of p13 (a gift from John Newport) was diluted in EB buffer and injected into fertilized eggs between 0.3 and 0.6. Eggs were fixed at

intervals, prepared for immunofluorescence and examined for the presence of cortical microtubules.

Results

Microtubule organization in fertilized eggs

We have used a monoclonal antibody to α -tubulin and confocal immunofluorescence microscopy to examine the assembly, organization, and disassembly of microtubules during the first cell cycle of fertilized *Xenopus* eggs (see materials and methods). By 0.25–0.35 after fertilization (times reported are normalized to the time of first cleavage), microtubules of the sperm aster (Stewart-Savage and Grey, 1982) were observed to extend to the cortex of the animal hemisphere (Fig. 1A). Upon closer inspection, microtubules extending from the sperm aster to the nearest point on the egg surface appeared to terminate there, while adjacent microtubules bent and ran parallel to the egg surface

(Fig. 1B). The latter population formed a network of microtubules in the cortex of the animal hemisphere, extending from 1–4 μm below the egg surface. In grazing sections, outgrowth of the sperm aster and cortical microtubules gave rise to a radial pattern of microtubule organization in the animal hemisphere (Fig. 1C). Initially, these microtubules were restricted to the region of the animal cortex closest to the sperm aster. However, by 0.35–0.45, the microtubules were observed throughout the animal cortex.

By 0.35–0.45, microtubules were observed to extend from the sperm aster to the cortex of the vegetal hemisphere (not shown; Stewart-Savage and Grey et al., 1991a). Grazing optical sections of eggs fixed at this time revealed an extensive but poorly ordered meshwork of microtubules in the cortex of the vegetal hemisphere (Fig. 1D). No defined focus of microtubule assembly or other evidence of a cortical microtubule organizing center (MTOC) was observed on the vegetal surface. Between 0.45 and 0.5, coincident with the

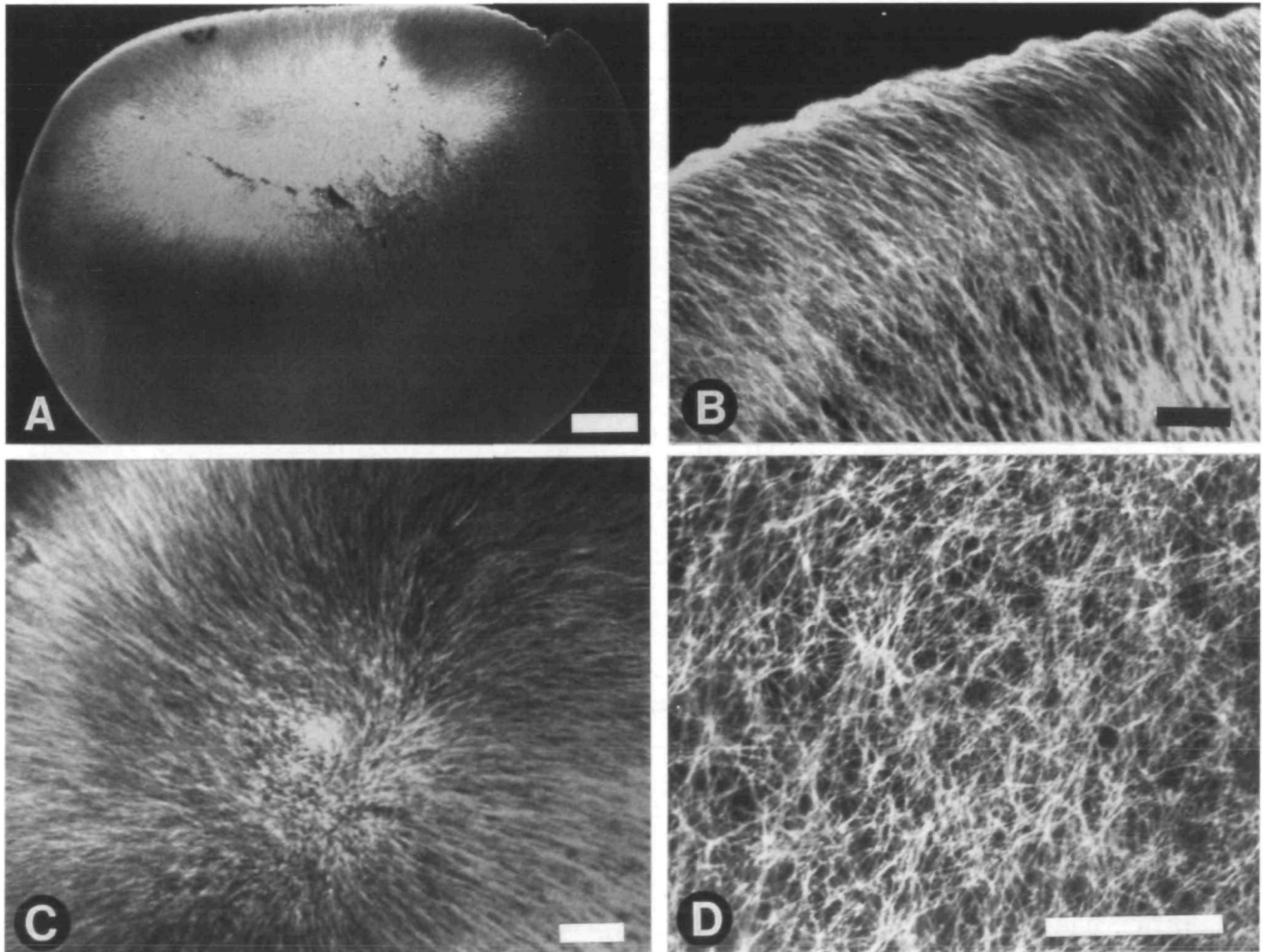


Fig. 1. Appearance of microtubules in the egg cortex coincides with outgrowth of the sperm aster. (A) A cross-section of a fertilized egg at 0.25. Assembly of the sperm aster is apparent in the animal hemisphere (10 \times objective). (B) At higher magnification, microtubules of the sperm aster have reached the animal cortex by 0.25 (a projection of 6 images at 1 μm intervals). (C) Grazing sections of the animal cortex reveal the radial organization of cortical microtubules at 0.25 (a projection of 10 images at 1 μm intervals). (D) Grazing sections of the vegetal cortex at 0.35 reveal a poorly organized microtubule network (a projection of 3 images at 1 μm intervals). Scale bar is 100 μm in A; 25 μm in B, C and D.

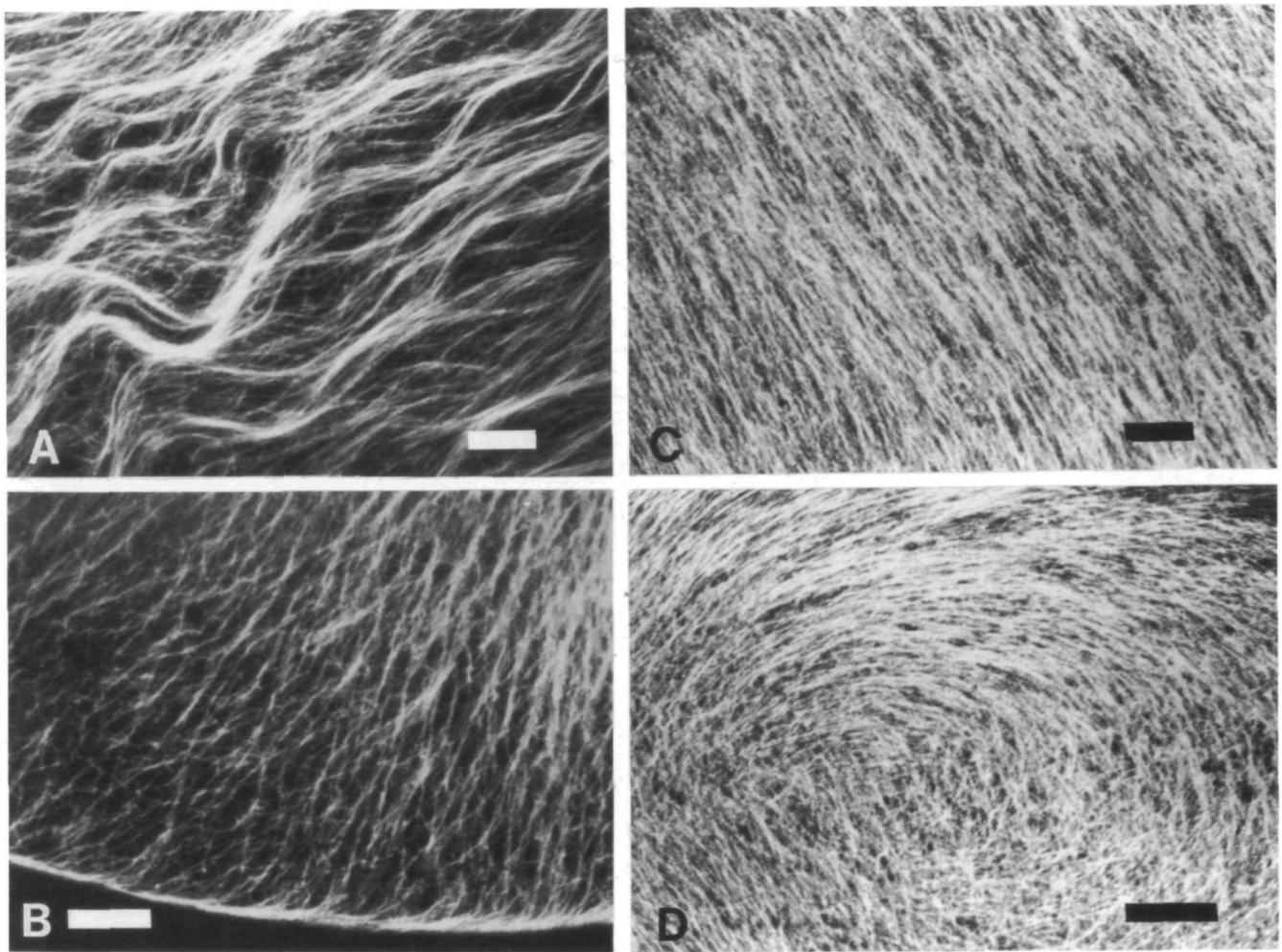


Fig. 2. Reorganization of the cortical microtubule network during the cortical rotation (0.5 to 0.75). (A) Microtubules in the vegetal cortex of an egg fixed at 0.65 are bundled into a parallel array (a projection of 13 images at $0.5\ \mu\text{m}$ intervals). (B) Cross-sections of the vegetal hemisphere at 0.5 reveal a dense network of radially organized cytoplasmic microtubules, which merge with the band of microtubules in the vegetal cortex (a projection of 6 images at $1\ \mu\text{m}$ intervals). (C) Microtubules of the animal cortex of an egg fixed at 0.75 are organized into a parallel array (a projection of 8 images at $0.5\ \mu\text{m}$ intervals). (D) A microtubule spiral in the animal cortex of an egg fixed at 0.5, (a projection of 9 images at $0.5\ \mu\text{m}$ intervals). All scale bars are $25\ \mu\text{m}$.

onset of cortical rotation, the initially disordered microtubules of the vegetal cortex became organized into numerous wavy parallel bundles, corresponding to the cortical microtubule array described by Elinson and Rowning (1988) and Houliston and Elinson (1991a). Examples of these bundles in eggs fixed at 0.65 are shown in Fig. 2A. The tight packing of these bundles made resolution of individual microtubules difficult or impossible. Bundles were observed to splay apart into smaller groups of microtubules, which then merged into larger adjacent bundles, giving rise to a highly interconnected network. Serial optical sections of the cortex revealed that the cortical microtubule bundles were restricted to a distinct yolk-free region extending from approximately 1 to $5\ \mu\text{m}$ below the egg surface, consistent with observations of Elinson and Rowning (1988).

Cross-sections of the vegetal hemisphere revealed a dense network of cytoplasmic microtubules extending from the sperm aster to the vegetal cortex. Individual cytoplasmic microtubules were traced over distances as long as $100\ \mu\text{m}$, whereas microtubule bundles could be traced distances of $300\text{--}500\ \mu\text{m}$. In contrast to the findings of Houliston and Elinson (1991a), we did not observe a significant population of short microtubule fragments in the egg cytoplasm. In favorable sections, individual microtubules of the cytoplasmic array were observed to merge into the cortical microtubule network, which appeared as a bright, fluorescent band in cross-section (Fig. 2B).

Between 0.45 and 0.5 the initial radial organization of cortical microtubules in the animal hemisphere became disrupted. At this time, microtubules in the animal cortex became bundled, though these bundles often

appeared straighter, shorter, and less pronounced than those in the vegetal cortex of the same egg (compare Fig. 2C, showing cortical microtubules in the animal hemisphere at 0.75, with vegetal microtubules at 0.65 in Fig. 2A). In many eggs, microtubule bundles in the animal hemisphere appeared to spiral outward from a central focus that was distinct from the center of the original radial organization (Fig. 2D shows such a microtubule spiral at 0.5), similar to the microtubule spirals observed by Elinson and Rowning (1988). However, these microtubule spirals were most commonly found in the lateral regions of the animal hemisphere (in 23 of 32 eggs examined), and were only rarely found in the more equatorial regions of the vegetal hemisphere (less than 1% of the eggs examined throughout our experiments). At later times (0.55–0.65), the focus of spiral organization appeared more prominent, though the extent of spiral organization varied considerably. Microtubules and microtubule bundles in the animal cortex appeared to spiral outward from this focus toward the lateral regions, to become oriented into a roughly parallel array aligned with the vegetal microtubule array. We have never observed more than one region of spirally organized microtubules per individual egg.

Microtubule organization in activated eggs

We also examined the organization of cortical and cytoplasmic microtubules in unfertilized eggs after electrical activation. Optical cross-sections of activated eggs fixed at approximately 0.3 (normalized to a first cleavage in fertilized eggs) revealed a dense array of radially organized microtubules in the animal hemisphere (not shown), similar to those observed by Houlston and Elinson (1991a). These microtubules radiated from a central region deep within the egg cytoplasm and extended to the animal cortex (Fig. 3A). Upon closer examination, microtubules appeared to radiate from one or two distinct foci located deep within the animal hemisphere (Fig. 3B). Microtubules from this cytoplasmic array extended to the animal cortex, forming a radial array of cortical microtubules similar to that observed in fertilized eggs (not shown).

By 0.4, a poorly organized network of microtubules appeared at the vegetal cortex of activated eggs. Between 0.45 and 0.55, these microtubules became organized into parallel bundles similar to those seen in fertilized eggs (Fig. 3C). Cross-sections of activated eggs fixed after 0.4 revealed a dense network of cytoplasmic microtubules radiating from the central cytoplasm that extended to and merged with microtubules in the vegetal cortex (not shown).

Protein synthesis is required for cortical microtubule disassembly

The network of cortical microtubules in fertilized eggs persists through M-phase (0.7) and is disassembled during, or just prior to, cytokinesis (0.95–1.0; Elinson and Rowning, 1988). To address the dependence of cortical microtubule assembly and disassembly on the

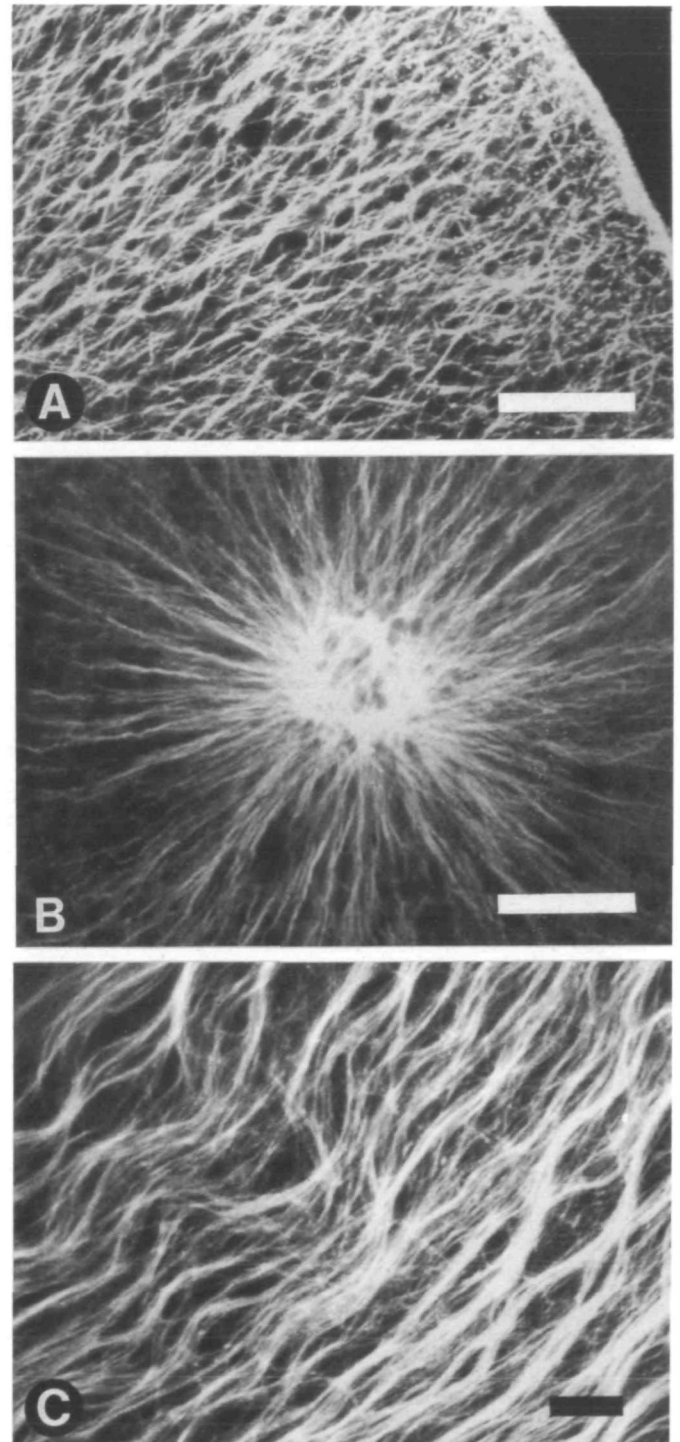


Fig. 3. Organization of cytoplasmic and cortical microtubules in parthenogenically activated eggs. (A) A cross-section of an activated egg at 0.65 reveals a dense array of radially organized cytoplasmic microtubules extending to the animal cortex. (B) A cross-section of a foci of microtubules deep in the animal hemisphere of an activated egg fixed at 0.75. (C) Grazing sections of the vegetal cortex of an activated egg at 0.55 reveal an extensive network of microtubule bundles (a projection of 6 images at 2 μm intervals). All scale bars are 20 μm .

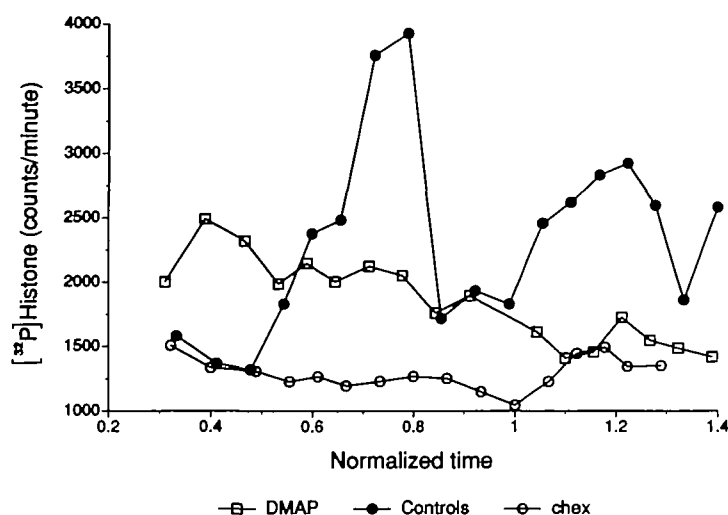


Fig. 4. Cycloheximide and DMAP block activation of MPF/histone kinase. Extracts prepared from untreated, cycloheximide-treated, and DMAP-treated eggs at the indicated times after fertilization (normalized to first cleavage) were assayed for MPF/histone kinase activity (Gard et al., 1990). Peaks in MPF/histone kinase activity in untreated eggs at 0.75 and 1.2 correspond to M-phase of the first two cell cycles.

MPF cycle, we examined the effects of several agents known to inhibit the activation of MPF.

Cycloheximide arrests the cell cycle of early *Xenopus* embryos by blocking synthesis of cyclins, which are required for activation of MPF (Minshull et al., 1989; Murray and Kirschner, 1989). Addition of cycloheximide (500 μ g/ml) shortly after fertilization (before 0.1) arrested eggs in interphase preceding the first mitotic division, as indicated by low levels of histone kinase activity (Fig. 4) and inhibition of cytokinesis (not shown). Examination of the arrested eggs by confocal immunofluorescence microscopy revealed that the assembly and alignment of the microtubules in cycloheximide-arrested eggs were indistinguishable from untreated controls (Fig. 5B,C). However, the cortical microtubules failed to disassemble in cycloheximide-arrested eggs, persisting long after CMBD occurred in untreated eggs fertilized and fixed in parallel (compare Figs 5A and D; Fig. 6). Cortical microtubules were routinely observed in cycloheximide-arrested eggs 180 minutes after fertilization (approximately 2.0–2.4), by which time normal embryos have completed three or more division cycles. In extreme cases, cortical microtubules were seen in cycloheximide-arrested eggs up to 300 minutes (3.2–4.0) after fertilization. However, the parallel array of the microtubule bundles became progressively less well ordered with extended arrest in cycloheximide (Fig. 5D). This loss of organization may result from the continued dynamics (growth and shrinking) of the cortical microtubules, after the completion of the cortical rotation in cycloheximide-arrested eggs (Vincent et al., 1987). The eventual loss of cortical microtubules in cycloheximide-arrested eggs was usually accompanied by other changes in the

appearance of the egg cortex and cytoplasm, which we interpreted as death and degeneration of the egg.

Disassembly of cortical microtubules requires protein phosphorylation

6-dimethylaminopurine (DMAP), a purine analogue that inhibits protein phosphorylation without blocking protein synthesis, has been shown to arrest the cell cycle of starfish and mouse oocytes by blocking MPF activation (Neant et al., 1989; Rime et al., 1989). Fertilized *Xenopus* eggs transferred to 500 μ g/ml DMAP early in the first cell cycle (before 0.1) failed to cleave and arrested in interphase as judged by low levels of histone kinase activity (Fig. 4). The time course of appearance of microtubules in the vegetal cortex of DMAP-arrested eggs was indistinguishable from that in untreated eggs. However, DMAP disrupted the subsequent organization of cortical microtubules into parallel bundles (compare Fig. 5E and F with Figs 1D and 2A) and blocked CMBD. 95% of the DMAP-arrested eggs examined between 1.0 and 2.0 (up to 180 minutes after fertilization) retained a substantial network of cortical microtubules ($n=65$, Fig. 6).

CMBD occurs normally in M-phase-arrested eggs

Cyc Δ 90, a truncated cyclin protein that is resistant to proteolysis, has been shown to arrest the cell cycle of *Xenopus* egg extracts in M-phase (Murray et al., 1989). Microinjection of cyc Δ 90 before the completion of mitosis arrested fertilized eggs in M-phase as judged by high levels of histone H1 kinase activity, chromosome condensation and inhibition of cytokinesis (data not shown). Assembly and organization of the cortical microtubules into parallel arrays occurred normally in eggs injected with cyc Δ 90. However, in contrast to eggs arrested in interphase (with cycloheximide or DMAP), 95% of the cyc Δ 90-injected eggs showed complete disassembly of the cortical microtubules despite complete inhibition of cytokinesis (Table 1).

Injection of p13 inhibits cortical microtubule breakdown

To test directly the requirement of MPF activation for CMBD, we examined the effect of p13, a specific inhibitor of MPF, on the cortical microtubule network.

Table 1. Injection of MPF induces disassembly of cortical microtubules in fertilized, cycloheximide-arrested eggs

Experimental treatment	% CMBD†
Fertilized eggs	
Controls	94% (76/81)
Injected with cyc Δ 90	95% (59/62)
Cycloheximide-arrested eggs	
Uninjected	11% (6/59)
Injected with EB Buffer	14% (6/42)
Injected with active MPF	92% (20/22)
Injected with inactive MPF	14% (5/36)

†Eggs tallied from 1.0 to approximately 2.4 normalized time. CMBD was assayed by confocal immunofluorescence.

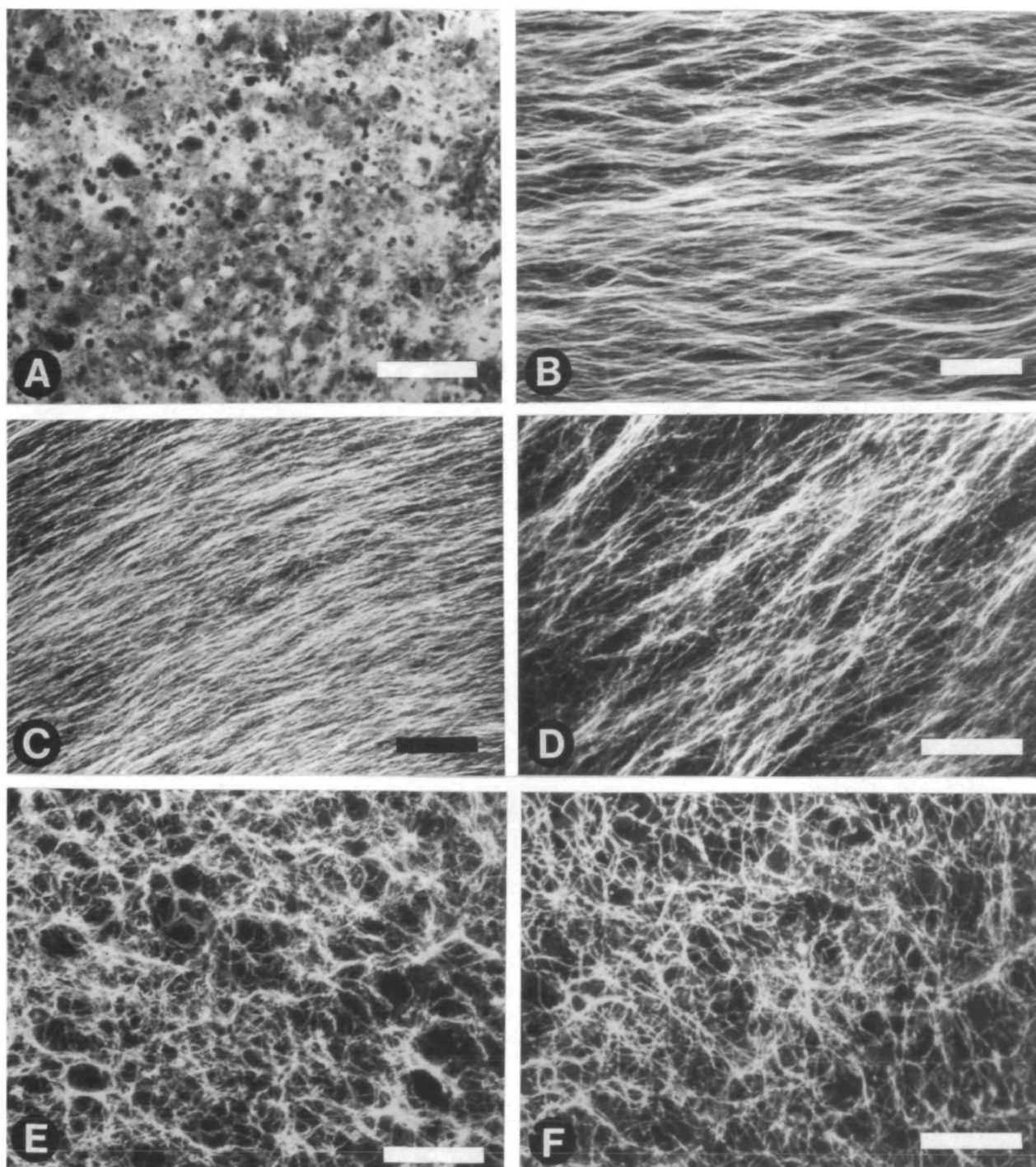


Fig. 5. Inhibition of cortical microtubule disassembly by cycloheximide and DMAP. (A) A grazing section of a fertilized egg at 1.0 reveals complete disassembly of the cortical microtubule networks. (B) Microtubules in the vegetal cortex of a cycloheximide-arrested egg at 0.75 are nearly indistinguishable from those in untreated eggs (compare with Fig. 2A; a projection of 5 images at 0.5 μm intervals). (C) Microtubules of the animal cortex in a cycloheximide-treated egg at 0.75 are indistinguishable from those in untreated eggs (compare with Fig. 2C; a projection of 5 images at 0.5 μm intervals). (D) Cortical microtubules are still apparent in the vegetal cortex of a cycloheximide-treated egg at 1.85, (a projection of 12 images at 0.5 μm intervals). (E and F) Poorly organized microtubule networks are apparent in the vegetal cortex of a DMAP-treated eggs fixed at 0.65 (E) and 1.45 (F; compare with Figs 1D and 2A). Scale bars are 20 μm .

Eggs injected with p13 prior to mitosis exhibited normal assembly and organization of cortical microtubules into parallel arrays. However, CMBD and cytokinesis in

p13-injected eggs were significantly delayed or blocked when compared to untreated eggs (Fig. 7). Cortical microtubules were observed up to 125 minutes after

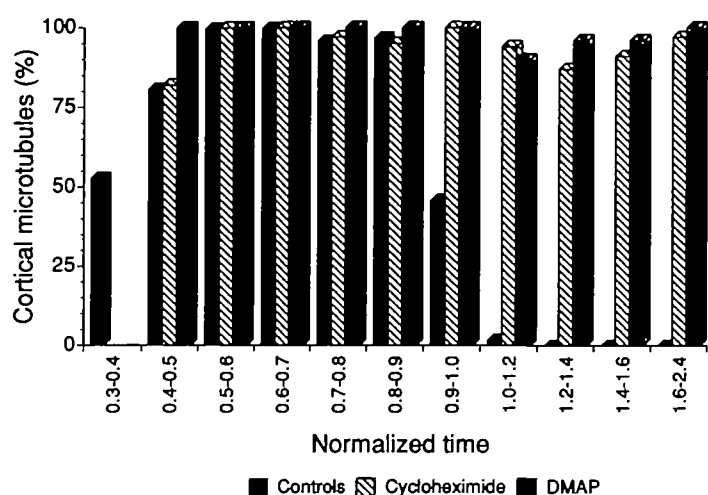


Fig. 6. Inhibition of cortical microtubule disassembly by cycloheximide and DMAP. The presence or absence of microtubules in the vegetal cortex of untreated, cycloheximide-treated, and DMAP-treated eggs was assayed at the indicated times after fertilization (normalized to first cleavage) by confocal immunofluorescence microscopy (see text for discussion of cortical microtubule organization). An average of 28 eggs (15-140) were examined at each time point, with the exception of 0.4-0.5, for which only three DMAP-treated eggs were scored. Microtubules were apparent in the vegetal cortex of both cycloheximide- and DMAP-treated eggs long after disassembly of the cortical microtubule network in untreated eggs. The first three cleavages in untreated eggs occurred at ≈ 1.0 , 1.3, and 1.7.

fertilization (approximately 1.4), the latest time point taken.

MPF induces disassembly of cortical microtubules in cycloheximide-treated eggs

To test further the dependence of CMBD on MPF activation, we examined the effect of injected MPF on cortical microtubules in cycloheximide-arrested eggs. CMBD occurred in 92% of the cycloheximide-arrested eggs injected with 5-10 units of partially purified MPF, typically 25-35 minutes after injection (see Table 1). In contrast, CMBD was observed in only 14% of cycloheximide-arrested eggs injected with 'inactive' MPF (prepared from activated eggs, Materials and methods). Uninjected cycloheximide-arrested eggs, or eggs injected with EB- γ ATP buffer alone, showed 11% and 14% CMBD, respectively (Table 1).

Discussion

As a preface to our investigation of cortical microtubule disassembly in fertilized *Xenopus* eggs, we reexamined the normal progression of microtubule assembly and organization during the first cell cycle after fertilization. Though our observations are in general agreement with previous descriptions of cortical microtubule organization in *Xenopus* eggs (Elinson and Rowning, 1988; Houliston and Elinson, 1991a,b), several differences are worth noting.

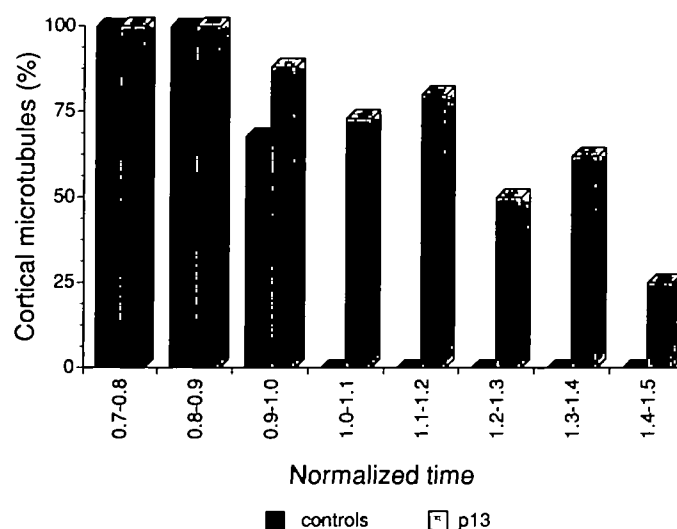


Fig. 7. Injection of p13 inhibits cortical microtubule disassembly. The presence or absence of microtubules in the vegetal cortex of untreated eggs and eggs injected with 200 ng p13 was determined at the indicated times after fertilization (normalized to first cleavage) by confocal immunofluorescence microscopy. A total of 175 p13-treated eggs were scored (6-29 per time point).

In contrast to previous reports (Elinson and Rowning, 1988; Houliston and Elinson, 1991a,b), we observed dense networks of microtubules in the animal cortex of fertilized eggs. Optical sections revealed that the cortical microtubules of the animal hemisphere are continuous with microtubules of the sperm aster, which first reach the animal cortex at approximately 0.25-0.35 of the first cell cycle (normalized time; Stewart-Savage and Grey, 1982; Houliston and Elinson, 1991a; this report). Upon reaching the animal cortex, microtubules of the sperm aster were observed to bend and run parallel to the egg surface, giving rise to a radially organized network of cortical microtubules. These observations suggest that, contrary to earlier reports (Elinson and Rowning, 1988), the cortex of *Xenopus* eggs does not exhibit dramatic regional differences in microtubule assembly.

Poorly organized microtubule networks were initially observed in the vegetal cortex of fertilized eggs at 0.35-0.45, earlier than previously reported (Elinson and Rowning, 1988; Houliston and Elinson, 1991a). The initial disorder of the cortical microtubules in the vegetal hemisphere strongly contrasted with the radial organization of the cortical microtubules apparent in the animal hemisphere. This difference could result from the proximity of the sperm aster to the animal cortex, as well as the differences in the size and density of yolk platelets encountered by the microtubules growing through the animal and vegetal hemispheres.

The cortical microtubule networks of both hemispheres become substantially reorganized beginning at 0.45 to 0.5 of the first cell cycle, coincident with the cortical rotation responsible for specification of the D-V axis (Elinson and Rowning, 1988; Houliston and Elinson, 1990a; this report). Microtubules in the vegetal

cortex become organized into parallel bundles aligned with the direction of cortical rotation (Elinson and Rowning, 1988). The initial radial pattern of microtubules in the animal cortex was also disrupted. The majority of microtubules in the animal cortex were swept into a parallel array that was continuous with the microtubules of the vegetal cortex.

Many of the eggs examined during the period from 0.5–0.8 exhibited a region in which microtubules were swept into a spiral organization. Similar microtubule spirals were observed by Elinson and Rowning (1988) in the vegetal cortex of a small number of eggs. These authors concluded that the microtubule spirals represented the lateral hubs or axes of cortical rotation. In contrast, we found microtubule spirals to be much more frequent in the animal hemisphere. In addition, we never observed more than one microtubule spiral per egg, making it difficult to reconcile this spiral organization with a bipolar axis of rotation. Microtubule spirals have also been observed in the cortex of fertilized sea urchin eggs (Harris et al., 1980), which are not known to undergo the cortical rotation observed in frog eggs. Thus, the functional relationship between the microtubule spirals, observed in *Xenopus* and sea urchin eggs, and cortical rotation remains unknown.

Optical cross-sections of fertilized eggs fixed after 0.5 revealed a dense network of radially organized cytoplasmic microtubules. In contrast to recent observations of sectioned eggs (Houliston and Elinson, 1991a), we did not observe a significant population of short microtubules in the cytoplasm of fertilized eggs. This discrepancy may result from differences in the fixation and processing of eggs for immunofluorescence microscopy. In our studies, individual microtubules of the cytoplasmic array could be followed through adjacent sections for as much as 100 μm , and were probably much longer. Microtubule bundles could be followed for distances up to 500 μm , extending from the organizing center in the egg interior to the cortex. In favorable sections, radial microtubules were observed to bend and merge with the band of cortical microtubules. Our observations extend those of Houliston and Elinson (1991a), and suggest that the majority of cortical microtubules in fertilized *Xenopus* eggs are continuous with those of the sperm aster.

Cross-sections of activated eggs revealed a dense array of radially oriented cytoplasmic microtubules (Houliston and Elinson, 1990a; this report), which appeared to be nucleated from a region deep in the animal hemisphere. The MTOC responsible for nucleation of this 'activation aster' has not been extensively studied. Unfertilized *Xenopus* eggs lack functional centrosomes (Gerhart, 1980). However, a recent report suggests that taxol-induced asters in extracts of *Xenopus* eggs contain centrosome components, but no identifiable centrioles (Verde et al., 1991). We have recently found that *Xenopus* eggs contain a store of protein components sufficient to assemble several thousand centrosomes in the absence of protein synthesis (Gard et al., 1991). Upon parthenogenic activation, a maternal store of centrosome components

might function as an MTOC, nucleating assembly of the dense network of cytoplasmic microtubules observed in activated eggs. The majority of the cortical microtubules in activated eggs appeared to be continuous with the microtubules of this activation aster.

The conclusion that cortical microtubules of fertilized and activated eggs are continuous with radially organized cytoplasmic microtubules places several constraints on previously proposed models for the cortical rotation required for the specification of the D-V axis (Elinson and Rowning, 1988). The uninterrupted network of radial microtubules from deep in the egg interior to the cortex rules out a shear zone between the cortical microtubule band and the interior cytoplasmic mass. In addition, the intricate interwoven appearance of the cortical microtubule band makes it unlikely that the rotational shear zone lies within this microtubule-filled region of cytoplasm. We therefore conclude, in concurrence with the recent reports of Houliston and Elinson (1991a,b), that the microtubules and underlying yolk-filled cytoplasm move as a unit relative to the overlying layer of cortical cytoplasm.

Disassembly of the cortical microtubules is dependent on MPF activation

Cortical microtubules persist through mitosis and are disassembled just prior to, or during, cytokinesis (Elinson and Rowning, 1988). Thus, neither the assembly and organization, nor the disassembly of the cortical microtubule network coincides with the mitotic activation or inactivation of MPF, a major regulator of the cell cycle in *Xenopus* embryos (Wu and Gerhart, 1980; Dunphy and Newport, 1988).

Despite this lack of temporal correspondence, our results suggest that MPF activation during the first mitotic cycle is required for subsequent disassembly of the cortical microtubule network. Neither cycloheximide nor DMAP, both of which block MPF activation and mitosis, noticeably affected the initial assembly of cortical microtubules early in the first cell cycle. However, both agents arrested eggs in interphase, blocking the subsequent breakdown of the cortical microtubule array. These results suggest that, although protein synthesis, (namely cyclins, Minshull et al., 1989; Murray and Kirschner, 1989) is necessary, it is not sufficient to induce cortical microtubule breakdown (CMBD), and that other steps involving protein phosphorylation are also necessary. p13, a specific inhibitor of MPF (Brizuela et al., 1987; Dunphy et al., 1988), also delayed or blocked CMBD, suggesting that MPF activation was a necessary step for microtubule disassembly. In contrast, arrest in M-phase by injection of cyc Δ 90, a truncated cyclin resistant to degradation (Murray et al., 1989), did not block disassembly of the cortical microtubule network, indicating that the transition from active to inactive MPF is not a requirement for CMBD. Finally, partially purified MPF efficiently induced microtubule disassembly when microinjected into cycloheximide-arrested fertilized eggs. Together, these results suggest that activation of MPF is necessary

for inducing breakdown of the cortical microtubule network at the end of the first cell cycle.

A substantial delay of 20-25 minutes normally separates MPF activation at M-phase from disassembly of the cortical microtubule network at the end of the cell cycle. Similar delays were observed between microinjection of MPF and CMBD in cycloheximide-arrested eggs. Several mechanisms might account for these delays. First, the signaling pathway between MPF activation and breakdown might be indirect. Recently, it has been shown that the addition of MPF or MAP kinase modulates microtubule assembly in *Xenopus* egg extracts (Gotoh et al., 1991; Verde et al., 1990) suggesting microtubule assembly in eggs might be regulated by a phosphorylation cascade. Alternatively, the endwise depolymerization of the long cortical microtubules, at published rates of 12 to 17 $\mu\text{m min}^{-1}$ (Belmont and Mitchison, 1990), could account for the observed delay between MPF activation at M-phase and loss of the cortical microtubule network. However, the presence of a mitotically activated microtubule severing factor (Vale, 1991) could substantially reduce the time needed for disassembly of the cortical microtubule network. Further investigation will be required to establish conclusively the cause of the observed lag between MPF activation and CMBD.

Though having no observed effect on the initial assembly of the cortical microtubule array, DMAP was found to block the reorganization of cortical microtubules into parallel bundles, which normally coincides with cortical rotation. The reason for this block has not yet been determined. We are currently investigating the effect of DMAP on cortical rotation in fertilized and activated *Xenopus* eggs.

In summary, our observations suggest that microtubules in the cortex of both animal and vegetal hemispheres of fertilized *Xenopus* eggs are continuous with microtubules of the sperm aster. Furthermore, disassembly of the cortical microtubule networks is dependent on the activation of MPF during the cell cycle. The cortical microtubules of *Xenopus* eggs thus provide an interesting model for studying the regulation of microtubule organization and disassembly by the cell cycle in vivo, and its role in the developmentally important cytoplasmic rearrangements.

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