Interconversion of Metaphase and Interphase Microtubule Arrays, as Studied by the Injection of Centrosomes and Nuclei into *Xenopus* Eggs

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ABSTRACT We have designed experiments that distinguish centrosomal, nuclear, and cytoplasmic contributions to the assembly of the mitotic spindle. Mammalian centrosomes acting as microtubule-organizing centers were assayed by injection into *Xenopus* eggs either in a metaphase or an interphase state. Injection of partially purified centrosomes into interphase eggs induced the formation of extensive asters. Although centrosomes injected into unactivated eggs (metaphase) did not form asters, inhibition of centrosomes is not irreversible in metaphase cytoplasm: subsequent activation caused aster formation. When cytoskeletons containing nuclei and centrosomes were injected into the metaphase cytoplasm, they produced spindle-like structures with clearly defined poles. Electron microscopy revealed centrioles with nucleated microtubules. However, injection of nuclei prepared from karyoplasts that were devoid of centrosomes produced anastral microtubule arrays around condensing chromatin. Co-injection of karyoplast nuclei with centrosomes reconstituted the formation of spindle-like structures with well-defined poles. We conclude from these experiments that in mitosis, the centrosome acts as a microtubule-organizing center only in the proximity of the nucleus or chromatin, whereas in interphase it functions independently. The general implications of these results for the interconversion of metaphase and interphase microtubule arrays in all cells are discussed.

Microtubules undergo major rearrangements during the mitotic cycle. In mammalian cultured cells, the extensive interphase aster of microtubules disappears between prophase and metaphase and is replaced by a spatially more restricted array of microtubules, the mitotic spindle (1, 2). At metaphase, most of the microtubules are part of the spindle; that is, fewer microtubules extend out into the cytoplasm. During anaphase and telophase, microtubules again grow out from the poles into the cytoplasm as the spindle disappears. Analogous successions of microtubule patterns are found in cleaving eggs (3, 4). In unfertilized amphibian eggs the cell cycle is arrested at second meiotic metaphase with condensed chromosomes and an intact spindle (5). The meiotic spindle appears barrel-shaped and differs from the mitotic spindles found in cleaving early embryos in that no astral fibers seem to radiate from behind the poles. (Compare Fig. 1 *A* with *B*). We explain these anastral poles by the lack of centrosomes compared with the better studied meiotic spindle of the mouse, which is similar in structure (6). Fertilization starts the cell cycle and leads to the formation of a very large interphase aster (several hundred microns in diameter) from the centrosome contributed by the sperm. In *Xenopus* this interphase aster persists until ~50 min post-fertilization when it disappears and a mitotic spindle is formed around the zygote nucleus (for review see 5).

This rapid conversion of microtubule arrays from interphase to metaphase is a fundamental problem of morphogenesis on a cellular level. Although microtubule assembly has been reproduced in vitro (7–10), and spindles can be isolated still in dynamic equilibrium with tubulin subunits (11, 12), the assembly of mitotic spindles has not been accomplished in vitro and the conversion between metaphase and interphase microtubule arrays has not been well studied.

The frog egg offers a good system intermediate between in vivo and in vitro experiments for such studies. The cytoplasm has long been shown to be dominant over the nucleus. For example, brain nuclei with a very low mitotic index rapidly undergo chromosome condensation and mitosis when injected into oocytes undergoing meiotic maturation (13). Mey-

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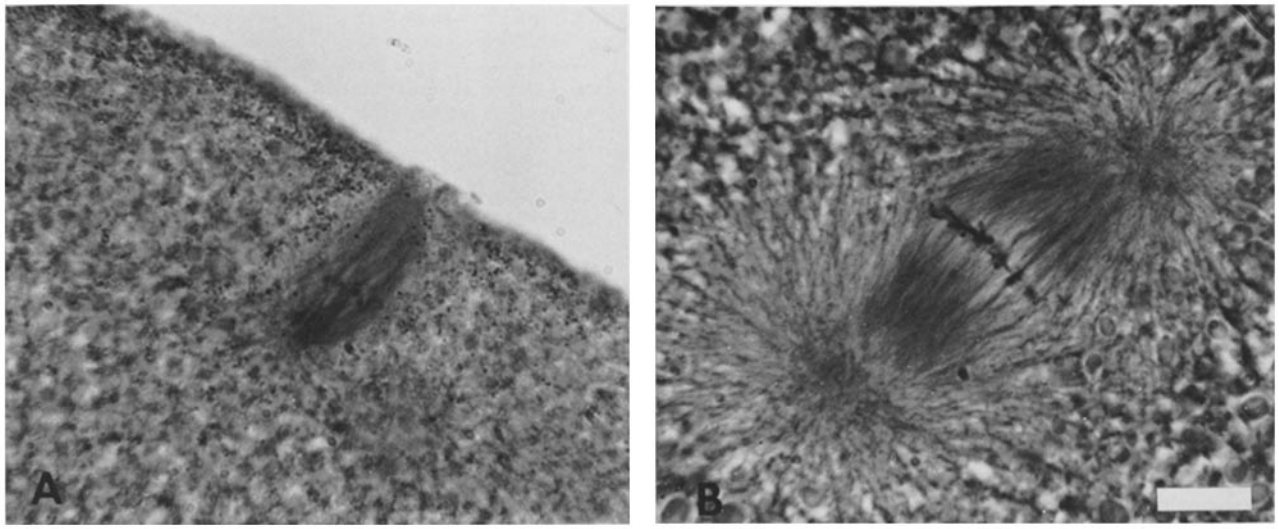


FIGURE 1 Comparison between the meiotic spindle of the second metaphase of the mature unfertilized egg (*A*), with the mitotic spindle of a cell from a late blastula *Xenopus* embryo (*B*). Note the astral microtubules coming out from the poles in the mitotic but not the meiotic spindle; paraffin section Bar, 20 μm. × 600.

erhof and Masui have shown that brain nuclei injected into unactivated *Rana* eggs at meiotic metaphase or into eggs arrested at metaphase with a cytoplasmic factor from unfertilized eggs (cytostatic factor or CSF)[1] will form spindles (14). Similarly Elinson (15) reported that sperm introduced into immature unfertilized *Rana* eggs without activation will produce spindles but no asters. Upon activation the egg enters interphase and asters form, revealing the presence of active centrosomes. Thus, at stages where the egg is in metaphase, injected nuclei form chromosomes arrayed on a microtubule spindle. At interphase stages, nuclei remain intact and asters form if centrosomes are present. In this paper, we have used the meiotic metaphase of the unactivated *Xenopus* egg as a model of the general metaphase state for mitosis as well as meiosis. The activated egg is used here as a model of interphase since injected nuclei remain intact while DNA replication occurs (16, 17). In the interphase state nuclei will also form upon injection of procaryotic DNA (18). Using the arrested cytoplasmic states as test tubes presumably containing all the components required to regulate microtubule assembly, we have tried to dissect the relative role of the nucleus (or chromatin) and centrosome in the initiation of microtubule assembly in both metaphase and interphase.

We have compared the behavior of isolated centrosomes, nuclei devoid of centrosomes, and whole cytoskeletons injected in those two cytoplasmic states. The results clearly showed that isolated centrosomes are inactive in the metaphase cytoplasm but form large asters in the interphase cytoplasm. Moreover, nuclei devoid of centrosomes condensed their chromatin and promoted microtubule polymerization in the metaphase cytoplasm but remained intact and have no discernible effect on microtubule assembly in interphase cytoplasm. When both nuclei and centrosomes are co-injected, asters or half spindles formed only in the vicinity of the condensed chromatin. We discuss the general implications of these results for understanding the interconversion of metaphase and interphase microtubule arrays in all cells.

[1] *Abbreviations used in this paper:* CSF, cytostatic factor; MMR, modified modified Ringer's.

MATERIALS AND METHODS

Preparation of Eggs: Unfertilized eggs were obtained from *Xenopus* laevis females as previously described (19). The jelly coat was removed with 2% cystein-HCl (Sigma Chemical Co., St. Louis, MO) neutralized to pH 7.8 with NaOH (19). After jelly coat removal, the eggs were washed in modified modified Ringer's (0.1 M NaCl, 2 mM KCl, 1 mM $MgSO_4$, 2.0 mM $CaCl_2$, 5.0 mM HEPES, 0.1 mM EDTA [pH 7.8]) and transferred to MMR containing 5% Ficoll (type 400, Sigma Chemical Co.). Eggs were fertilized before jelly coat removal with *Xenopus* sperm obtained from a testis surgically removed and stored as previously described (19).

Assay for CSF Activity by Cytoplasmic Transfer: 50–60 nl of donor egg cytoplasm was injected into recipient eggs that were fertilized 30–60 min before. The donor egg is considered to have CSF activity if the recipient eggs fail to cleave, up to 3 h after injection. Assays were carried out in duplicate or triplicate.

Preparation of Oocytes: A piece of ovary was surgically removed from a *Xenopus* female injected with 100 IU pregnant mare serum gonadotropin (Gestyl Organon Inc., West Orange, NJ), 3–7 d before. The oocytes were dissected out of the ovary with fine forceps and maintained in MMR. Injections were carried out in MMR. When desired, maturation was induced in vitro by incubating the oocytes overnight in modified Barth saline HEPES (MBSH) (20) (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 0.82 mM $MgSO_4$, 0.41 mM $CaCl_2$, 10 mM HEPES [pH 7.4]) containing 10 μg/ml progesterone. Mature oocytes are recognized by the appearance of a white spot at the top of the animal pole.

Preparation of Centrosomes: Centrosomes were prepared from cultured neuroblastoma cells (N115 Cells, a gift of Marshall Nirenberg, National Institutes of Health) according to two different procedures. I The first procedure involved little purification from a crude cell extract: cells were collected in Dulbecco's modified Eagle medium (DME) containing 10% fetal calf serum and centrifuged 1 min at 2,400 rpm in 10 ml sterile plastic tubes in a clinical centrifuge (Damon IEC, Needham Heights, MA). Cells are resuspended in precooled (4°C) buffer A (80 mM KCl, 20 mM NaCl, 15 mM PIPES, 0.5 mM Spermidine, 0.2 mM spermine, 2 mM EDTA, 0.5 mM EGTA, pH 6.9) containing 1% Triton × 100. Lysed cells were pelleted (1 min at 2,400 rpm in the clinical centrifuge), washed once in 10 ml of buffer A* (buffer containing 1% β mercaptoethanol, 0.25 M sucrose, and 10^{-4} M GTP), and resuspended in 1 ml of buffer A*. Microtubule organizing centers (MTOC) were removed by 10 strokes with a glass dounce. Nuclei and large cell debris were pelleted by one spin in the clinical centrifuge (2,400 rpm, 1 min) and the supernatant solution was centrifuged 15 min at 10,000 rpm in a Beckman J2-21 centrifuge (Beckman Instruments, Palo Alto, CA) using a JS13 rotor at 4°C. The pellet was resuspended into 500 μl of 10 mM K_2HPO_4/NaH_2PO_4, 0.25 M sucrose, 0.1 mM GTP, pH 7.2. This suspension was centrifuged 1 min at 2,400 rpm in the clinical centrifuge to remove contaminating nuclei and kept at 4°C. The second procedure was a purification that involved cell dissociated by low ionic

strength followed by concentration of the microtubule organizing centers on a 20% Ficoll cushion and a final purification on a 20–65% sucrose gradient. Microtubule organizing centers sediment in 55–60% sucrose after a 30-min centrifugation at 35,000 rpm in a SW40 rotor. The detailed procedure will be published elsewhere (T. Mitchison and M. Kirschner, manuscript in preparation). Aliquots from the peak gradient fractions were directly frozen in liquid nitrogen and kept at −70°C. Centrosome concentration as determined by double staining of the fractions with a human anticentrosome antibody and an antitubulin antibody is usually 5×10^7 centrosomes/ml in the peak fractions.

Cytoplasmic Extracts of Xenopus Eggs: Eggs were dejellied with 2% cysteine as described above, washed in quarter strength MMR, and activated in this medium by a 2-s electric shock (12 VAC), delivered by a Heath-Schlumberger regulated high voltage power supply. The two electrodes were separated by 2 cm. Activation of the eggs can be visualized by three criteria: (*a*) The vitelline membrane separates from the egg and the perivitelline space inflates; (*b*) 4 min after activation the pigmented animal hemisphere contracts toward the animal pole and relaxes about 10 min later; and (*c*) during this time the second polar body is extruded, the white spot at the animal pole disappears and is replaced by a very small black dot. Extracts were prepared only if >90% of the eggs were activated. 60 min after activation, eggs were collected and transferred to 1.5-ml conical Eppendorf centrifuge tubes. They were then washed three times in cold, 50 mM EGTA pH 7.0 by successively inverting the tubes and allowing the eggs to settle. After the third wash, the eggs were gently packed by a 30-s centrifugation at 1,000 rpm in the clinical centrifuge. Excess buffer was removed (only a thin meniscus was left) and the eggs were crushed by 1 to 3 pipetting through the blue tips (1-mm diam) of a 1-ml Gilson Pipetman (Gilson, France). The homogenate was then transferred to 0.8 ml Beckman ultraclear centrifuge tubes (Beckman Instruments, Inc., Palo Alto, CA) and centrifuged 1 h at 50,000 rpm in a SW 50.1 rotor. The EGTA is diluted five to tenfold by this procedure leading to an approximate final concentration of 10 mM EGTA in the cytoplasmic extract.

Microtubule Regrowth on Centrosomes in Xenopus Egg Extracts: Centrosomes were diluted 10 times in 80 mM PIPES, 1 mM EGTA, 1 mM $MgCl_2$ [pH 6.9] containing 0.1% Triton X-100, and centrifuged 10 min at 5,000 rpm onto the bottom coverslip of an observation chamber (21). The chamber was filled with 25 μl of egg extract, covered with a clean coverslip, and incubated for 10 min at room temperature. It was then quickly washed in the same buffer containing 0.1% Triton X-100 and dipped for 5 min into 0.1% glutaraldehyde diluted in the same buffer. The chambers were then rinsed in 100 ml of PBS (0.1 M NaCl, 20 mM K_2HPO_4:NaH_2PO_4 [pH 7.2]) containing 1% BSA (Sigma Chemical Co.) and 0.1% Tween 20 (Sigma Chemical Co.) for 15 min. The buffer was changed twice during this time. The same procedure was carried out without centrosomes to estimate the amount of spontaneous microtubule growth. Microtubules were stained by immunofluorescence using a monoclonal antibody (mouse anti-α-tubulin, gift of Dr. Steve Blose and James Lin, Cold Spring Harbor Laboratory) diluted 500 times and a rhodamine-labeled goat anti-mouse secondary (Capell Laboratories, Cochranville, PA) diluted 100-fold. The samples were incubated at room temperature for 15–20 min for each antibody. The washing steps were carried out over the same period of time. Observation was done using a Zeiss photomicroscope III equipped with a Leitz 63 × neofluar lens and pictures were taken on Kodak TriX film developed with Diafine.

Cells Used for Injection in Eggs: The following cell lines were used: An epithelial frog cell line (A6 cells) was maintained in culture in NCTC 102 supplemented with 10% fetal calf serum (FCS) at 26°C in a 5% CO_2 atmosphere. A mouse neuroblastoma cell line (N115 cells) was maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum at 37°C in a 10% CO_2 atmosphere. Chinese hamster ovary cells (CHO) were cultured in α-minimal essential medium without nucleosides, supplemented with 10% fetal calf serum, at 37°C in a 10% CO_2 atmosphere. Mouse L929 cells were maintained in minimal essential medium supplemented with 5% fetal calf serum, at 37°C in a 5% CO_2 atmosphere. For injection in eggs, the cells were removed from the tissue culture flasks by shaking (N115 cells) or trypsinization (all the other cell lines) and washed in the appropriate medium. They were then resuspended in buffer A and transferred to siliconized glass tubes. Cells were incubated on ice for <1 min in buffer A containing 0.1% lysolecithin (Sigma Chemical Co.). Then, an equal volume of 1 mg/ml BSA solution in buffer A was added to absorb the excess of lysolecithin. The cells were centrifuged at 1,100 rpm for 2 min in a clinical centrifuge and resuspended in buffer A for injection into eggs. When the activation of the egg induced by piercing the cortex with the needle was not desired, EGTA was added to the injection buffer at a concentration of 10 mM.

Karyoplast Preparation: Preliminary enucleation experiments have been carried out using Chinese hamster ovary cells as described by Shay et al. (22). However, the karyoplasts prepared in this way were contaminated by centrosomes (5 to 20%). We therefore tried the method described by Lucas et al. (23) using mouse L929 cells. This method which includes removal of contaminating cells and cytoplasmic fragments from the karyoplast preparation (23) gave rise on a routine basis to karyoplast preparations contaminated by <1% of centrosome-containing bodies. The purified karyoplasts were transferred to siliconized glass tubes, lysed, and injected in eggs as described for whole cells.

Immunofluorescent Staining of Karyoplasts and Cytoplasts: Karyoplasts were diluted to 10^5/ml in 80 mM PIPES. 1 mM $MgCl_2$, 1 mM EGTA, pH 6.9 regrowth buffer to which 0.1% Triton and 1% formaldehyde (Mallincrodt, Paris, Kentucky) were added. 25 ml of this suspension was centrifuged into observation chambers (21) at 5,000 rpm for 15 min (4°C) in a Beckman J2 21 centrifuge (Beckman Instruments, Inc.) with a JS13 rotor. The chambers were then washed over a 10-min period with two changes of 50 ml of PBS (20 mM K_2HPO_4:NaH_2PO_4, 100 mM NaCl [pH 7.2]) to which 0.1% Tween 20 (Sigma Chemical Co.) and 1% BSA were added.

Whole cells and cytoplasts were directly fixed on the slides or coverslips following the same method. The primary antiserum was a human serum from a patient with a linear scleroderma. This serum contains antibodies to centriole cylinders and pericentriolar material (24). Karyoplasts and cells were incubated for 20 min with a 1:100 dilution of this serum. After washing off the excess primary antiserum, rhodamine-conjugated goat anti-human immunoglobin antibodies (Cappel Laboratories) were applied at a 1:100 dilution for 20 min. Preparations were then washed with PBS containing 0.1% Tween 20 and 1% BSA. Coverslips were mounted in 90% glycerol containing 2% propylgallate (Sigma Chemical Co.). Observation was done with a Zeiss photomicroscope III using a neofluar Leitz 63 × lens. Pictures were taken on Kodak TriX film developed in Diafine.

Activation of Eggs by Pricking and Injection of Material into Eggs: 50 to 200 centrosomes, in 50 nl of 20 mM K_2HPO_4:NaH_2PO_4 were usually injected into eggs [pH 7.2]. To suppress egg activation, EGTA (10 mM) was injected with the centrosomes. Eggs were maintained into MMR + 5% Ficoll during all injections to reduce leaking. Cytoskeletons and karyoplasts were injected as described in the respective sections.

In some experiments, activation was avoided by injecting the eggs in a "nonactivating medium" (0.1 M KCl, 0.5 mM $MgSO_4$, 0.1 mM $CaCl_2$). This procedure worked perfectly with some batches of eggs and very poorly for others. Under most circumstances materials may be injected into unactivated eggs, and the eggs subsequently activated. Eggs were preincubated for 10 min in nonactivating medium, and then injected with centrosomes. 2 h later, the eggs that had not been activated (more than 70% with good batches of eggs) were transferred to MMR + 5% Ficoll and activated by pricking. (100% of the eggs activated, showing that they actually did not activate during injection in the nonactivating medium.) In many experiments where subsequent activation was not required, eggs could be prevented from activation more conveniently by injecting a 10 mM EGTA solution.

Paraffin Sections of Eggs and Histological Staining: For histology of paraffin sections, eggs and oocytes were fixed in a few milliliters of Tellysnicky's modification of Smith's fixative (0.5 g potassium dichromate, 2.5 ml glacial acetic acid, and 10 ml formaldehyde solution, diluted to 100 ml with water) for 2 to 18 h. The eggs were rinsed in several changes of tap water after which, they were dehydrated and embedded in Paraplast Plus (Lancer, St. Louis, MO). 10-μm sections were cut and floated onto slides with a dilute solution of Mayer's albumin fixative (Harleco, Gibbstown, NJ). After drying, the slides were stained with Mayer's acid hematoxylin (hematoxylin powder from Chroma-Gesellschaft, Stuttgart, Germany), which stains the chromatin dark purple, and counterstained with fast green.

Electron Microscope Study of Spindle-like Structures: Chinese hamster ovary cells were lysed in buffer A and lysolecithin containing the DNA dye Hoeschst 33258 (Bisbenzimide, Calbiochem-Behring Corp., La Jolla, CA) as described above. 50–200 cells were injected together with 10 mM EGTA to prevent activation. 90 min later, the eggs were broken open with fine forceps in a microtubule stabilizing buffer (70% RB, 30% glycerol, 1 mM GTP), under a binocular lens. Clear, ovoid bodies excluding the yolk were found. Excess adhering yolk was removed by 2–3 pipetting through a 300–600-μm Pasteur pipette tip and the bodies were transferred into 1% electron microscope grade glutaraldehyde made up in the microtubule stabilization buffer for fixation (15 min). The whole procedure lasted 2 min between egg opening and fixation. The presence of DNA in the isolated bodies was checked, before processing for electron microscopy, by observation under the fluorescent microscope. The structures containing DNA were spun down in observation chambers and postfixed in 2% glutaraldehyde in 0.1 M cacodylate buffer for 15 min. After a 15-min wash in this buffer, the structures were fixed for 30 min in 0.5% OsO_4 dehydrated in ethanol and embedded in Araldite. Sections (0.1 μm) were cut with a diamond knife, placed on oval grids (25), and counterstained with 1% aqueous uranyl acetate and 0.3% lead citrate. Sections were viewed under a Philips 400 electron microscope.

RESULTS

Centrosome Activity in Interphase and Metaphase Cytoplasm

MAMMALIAN CENTROSOMES FORM ASTERS IN ACTIVATED EGGS

Previous studies have shown that purified basal bodies from protozoa will induce asters but will not serve as parthenogenetic agents in amphibian eggs (30), whereas impure particulate fractions containing centrioles from lysed cells, or the sperm midpiece from sea urchin, will both support parthenogenesis as well as induce aster formation (26, 27).

In the present work, we have used well characterized centrosomes, (centrioles and adherent pericentriolar material) purified from neuroblastoma cells according to the procedure outlined in Materials and Methods (for a fuller characterization of the centrosomes, see T. Mitchison and M. Kirschner, manuscript in preparation). These centrosomes nucleated microtubule assembly when incubated with mammalian brain tubulin depleted of microtubule-associated proteins (T. Mitchison and M. Kirschner, in preparation). They will also induce microtubule assembly in concentrated extracts of activated *Xenopus* eggs, as shown in Fig. 2*a*. Spontaneous assembly under the same conditions was small (Fig. 2*b*). Injection of a large number of centrosomes into activated *Xenopus* eggs resulted in the formation of a large number of asters (Fig. 3, *a* and *b*) and consequent abortive furrowing of the cortex. At the proper dilution of 5–10 centrosomes per egg, about half of the injected eggs produced blastulae and a few went on to more advanced stages. The frequency of blastulae that formed varied from 20 to 60% according to the batch of eggs, and haploid tadpoles were occasionally obtained showing that the centrosomes used can indeed induce parthenogenesis. In summary by both in vivo and in vitro criteria, mammalian centrosomes can act as microtubule organizing centers in activated frog eggs.

In the following sections we discuss the activity of centrosomes in various cytoplasm states of the egg. Fig. 4 shows schematically the various states utilized in this paper; the condition of the endogenous nucleus is diagrammed so that the conditions for experimentally inducing the states is apparent. Fully grown oocytes are in a G2-like state with a tetraploid genome (their last round of DNA synthesis had

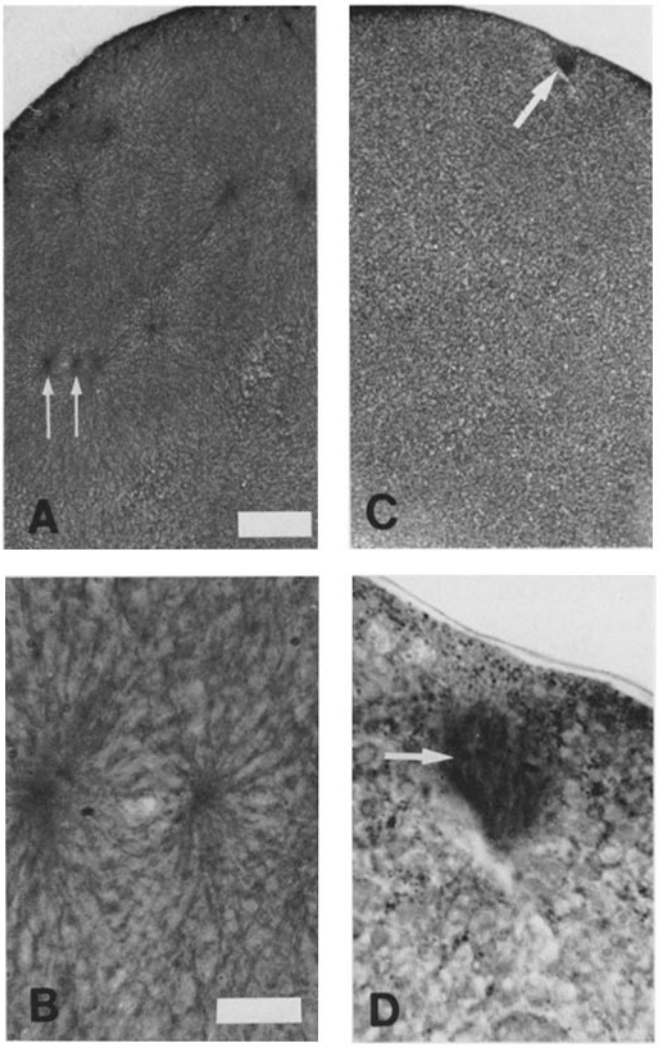


FIGURE 3 Centrosome activity in activated and unactivated eggs (paraffin sections). (*A*) Centrosomes are injected in phosphate buffer (20 mM, pH 7.2 and the eggs fixed 2 h later. Numerous asters are observed throughout the cytoplasm (arrows); (*B*) enlargement of the two asters shown by an arrow in *A*; (*C*) centrosomes are injected in phosphate buffer 20 mM, pH 7.2 containing 10 m EGTA; (*D*) enlargement of the meiotic spindle seen in *C*. The arrow shows the metaphase plate of chromosomes. Bars: (*A* and *C*) 100 μm; (*B* and *D*) 20 μm. × 100 (*A* and *C*); × 600 (*B* and *D*).

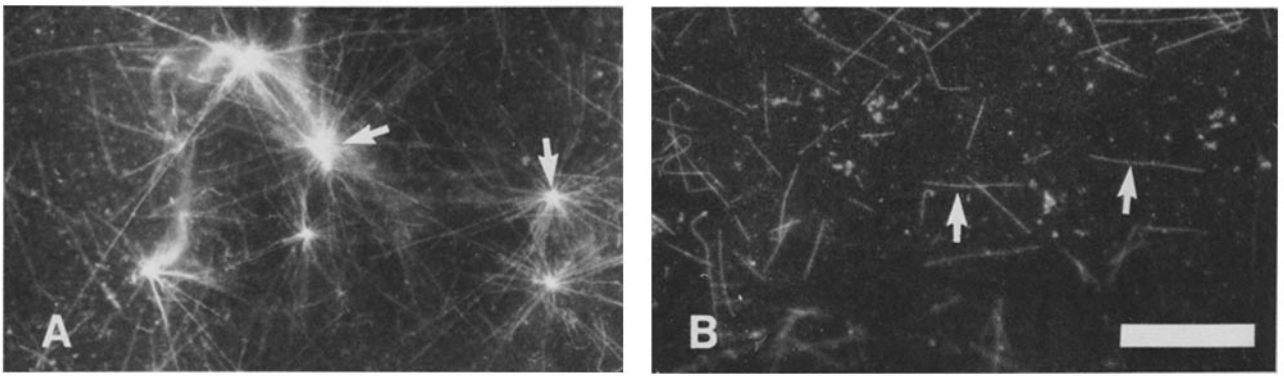


FIGURE 2 Nucleation of microtubule growth on centrosomes purified from N115 cells in a *Xenopus* egg cytoplasmic extract. (*A*) Centrosomes were sedimented on a glass coverslip and the extract added for 10 min at room temperature. After fixation, the coverslips were processed for immunofluorescence using a tubulin antibody (arrows show asters); (*B*) same as *A*, without centrosomes (arrows show a few individual microtubules). For experimental details, see Materials and Methods. Immunofluorescence microscopy Bar, 20 μm. × 900.

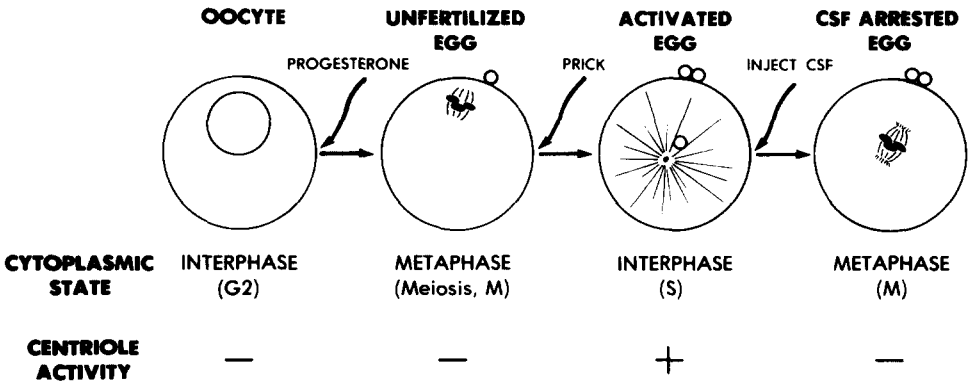


FIGURE 4 Cytoplasmic states of the oocyte and egg.

been completed months earlier). The oocyte has a large germinal vesicle that probably stores many of the nuclear components needed in the rapid cleavage period. In vitro, progesterone caused the initiation of meiosis which resulted in an unfertilized egg that was arrested in metaphase of the second meiotic division. One polar body that already had been extruded is shown. Fertilization or activation by pricking caused the unfertilized egg to complete meiosis and pass into an interphase state where DNA synthesis begins. The interphase and mitotic states alternated at 21°C every 30 min for 12 cleavages after the first 90-min cleavage period. At any point, injection of CSF will cause arrest at the next metaphase (for review see 5).

CENTROSOMES FAIL TO FORM ASTERS ON UNACTIVATED EGGS

Because the act of injecting centrosomes into unfertilized eggs causes activation, it is difficult to assess whether centrosomes are active in the cytoplasm of unactivated eggs. Two methods were used to avoid activation. In the first, EGTA was co-injected along with centrosomes. Injection of EGTA has been shown to block activation of sea urchin eggs (28). In *Xenopus*, EGTA blocked the cortical contractions and the elevation of the vitelline membrane. The surface contraction waves that indicate progress through the cell cycle (29) were not initiated. Cytostatic factor, which has been shown to be Ca^{++} sensitive in vitro and that is normally broken down within 10 min after egg activation was preserved by EGTA injection. The injection medium must contain at least 5 mM EGTA for activation to be blocked; this produced an internal EGTA concentration of ~250 μM. As shown in Fig. 3, *c* and *d*, active centrosomes injected into unactivated eggs with 10 mM EGTA, produced no asters and the eggs maintained their meiotic spindle. However, when EGTA was injected more than 5 min after activation, cytostatic factor activity was destroyed and injected centrosomes induced aster formation.

Two difficulties with the EGTA blockage of activation were apparent: Eggs blocked from activation by EGTA could not be activated subsequently by pricking. Therefore it was difficult to determine whether the centrosomes were irreversibly inhibited by EGTA, and therefore whether EGTA might have produced some nonphysiological modification of centrosome activity. Second, Ca^{++} is an important regulator of microtubule assembly as well as many other cell processes and it is not clear whether, in addition to the effect of EGTA in blocking activation, EGTA was affecting microtubule assembly.

The other useful and reversible method we used to block activation was to replace NaCl with KCl in the usual amphibian Ringer's solution. The effectiveness of this medium varied with the batch of eggs. Usually, the activated eggs were easily recognized: these were discarded, whereas the unactivated eggs were retained for examination. Eggs injected with centrosomes in nonactivating medium like those injected with EGTA failed to form asters and preserved their meiotic spindles; yet, as discussed earlier, they retained the ability to be activated subsequently by pricking when replaced in the usual diluted Ringer's solution.

Fertilized eggs injected with cytoplasm of unfertilized eggs (CSF) arrest at metaphase and these eggs cannot be reactivated by pricking (14). When 100 centrosomes were injected into CSF-arrested fertilized eggs, only 2 or 3 asters are found after 3 h. Approximately the same number of asters were found in fertilized eggs injected with buffer instead of centrosomes. In the same experiment, in fertilized eggs, about 40 asters were produced.

Injection of CSF into eggs already containing asters caused their depolymerization. Centrosomes were first injected into eggs and asters were allowed to grow for 45 min at which time the eggs received 60 nl of CSF. The presence of asters was surveyed in eggs fixed at various times after CSF injection. The number of asters in the cytoplasm of eggs that did not receive CSF was about 25 over 2.5 h. On the other hand, the number of asters found per egg arrested with CSF dropped to about 2 after CSF injection.

CENTROSOMES ARE NOT DESTROYED IN THE UNACTIVATED EGG CYTOPLASM

We have shown in the previous sections that centrosome activity is suppressed in unactivated as well as CSF-arrested eggs. In both cases the cell cycle was arrested at metaphase. However, the unactivated egg was arrested at the second meiotic metaphase and the centrosomes could be destroyed in this cytoplasm. This is a serious possibility since centrosomes were eliminated at some point during oogenesis, and the unactivated frog egg apparently does not contain a centrosome. To test their stability in unactivated eggs (Table I), centrosomes were injected into eggs maintained in the nonactivating medium described in Materials and Methods and incubated for 2 h in Ringer's solution. At this point none of the injected eggs contained asters. Six of these eggs were then activated and examined 2 h later. All of them contained ~20 asters as compared with 16 for eggs directly activated by injection of centrosomes. Eggs, injected with buffer in nonactivating medium, transferred to Ringer's solution, and then activated, did not contain asters. Thus centrosomes were

TABLE I

Centrosome Stability in Nonactivated Eggs

Centrosomes injected in	No. of asters found per egg	No. of eggs containing a meiotic spindle	No. of injected eggs
Unactivated* eggs	0	4	7
Unactivated‡ eggs subsequently activated	23 ± 6	0	8
Activated§ eggs	16 ± 3	0	4

* Eggs maintained in nonactivating medium for injection and then transferred to MMR. Eggs are fixed 2 h after centrosome injection.

‡ Eggs maintained in nonactivating medium during injection, then transferred to MMR. 2 h later, pricked for activation, and fixed after a further 2 h.

§ Eggs maintained in nonactivating medium for 2 h, then transferred to MMR, injected with centrosomes, and fixed 2 h later.

present for at least 2 h in unactivated eggs and became fully functional after activation.

We have also injected centrosomes into oocytes, matured the oocytes in vitro with progesterone, and activated the mature oocytes. Immature oocytes produced no asters after centrosome injection in agreement with previous studies (30, 31). After maturation into the unactivated egg, no asters were formed. However, when the mature oocytes (unactivated eggs) were activated, the number of asters formed was similar to when centrosomes were directly injected into oocytes matured in vitro into unactivated eggs. Therefore, incubation for ~18 h in the oocyte cytoplasm before and during meiosis did not diminish the subsequent ability of centrosomes to form asters after activation. In addition, these results show that oocyte maturation and germinal vesicle breakdown are necessary (31, 32) but not sufficient steps for the expression of centrosome activity.

Behavior of Nuclei in Interphase and Metaphase Cytoplasm

NUCLEI CONTAINING CENTROSOMES REMAIN INTACT AND FORM LARGE ASTERS WHEN INJECTED INTO ACTIVATED EGGS

Whole cytoskeletons containing nuclei and centrosomes have been injected into activated eggs. No major morphological modification of the nuclei was apparent during 40–50 min following injection. The nuclear envelope seemed to remain intact and a large aster grew from a point close to each nucleus as shown in Fig. 5. Nuclear envelope breakdown, chromatin condensation, and spindle-like figures occurred, however, at later time points when the egg cytoplasm entered a mitotic state (5) and the cell cycle started a 30-min oscillation period.

NUCLEI CONTAINING CENTROSOMES FROM STABLE METAPHASE-LIKE SPINDLES WHEN INJECTED INTO UNACTIVATED EGGS

Although purified centrosomes demonstrated no capacity for microtubule nucleation in unactivated *Xenopus* eggs, crude preparations of nuclei induced spindle formation. Fig. 6 shows a time course of spindle assembly after injection of permeabilized *Xenopus* A6 epithelial cells into unactivated eggs.

15 min after injection, the chromatin had already started to condense (Fig. 6*A*). At this stage no asters could be seen around the nuclei or anywhere else in the cytoplasm. After 30 min, chromosomes condensed on what may be a prometaphase-like spindle (Fig. 6*B*). Often several asters surrounded the mass of chromosomes. These asters probably originated from centrosomes contributed by several nuclei. Asters were always in areas adjacent to chromatin. By 90 min after injection the astral arrays reached a stable, metaphase-like configuration. In some cases where individual nuclei were present, normal bipolar spindles were formed (Fig. 6*C*). However, where several microtubule nucleating centers were found in the same region, star-like configurations were produced (Fig. 6*D*). The condensed chromosomes found in the center of these structures (small arrows, Fig. 6*D*), were surrounded by several foci (large arrows) arrayed in an almost perfect ring. One characteristic of both bipolar and star-shaped configurations was the absence of many fibers pointing away from the centers. During this time, the endogenous nucleus remained at metaphase.

The induction of spindle formation was not restricted to cells of frog origin. Chromatin condensation and spindle formation occurred with CHO and L929 fibroblasts, and N115 neuroblastoma cells of mammalian origin as well. For some reason the L929 mouse fibroblasts always tended to form tangled masses of chromosomes whereas other cells yielded individual chromosomes. However, the bipolar spindles and star configuration formed from L929 cells were similar to those produced by other cells. These results clearly demonstrate that metaphase spindles and related structures will assemble from crude cell fractions exposed to the cytoplasm of unactivated eggs. This contrasts to the failure of purified centrosomes to assemble microtubule arrays in the same cytoplasmic environment.

NUCLEI, FREE OF CENTROSOMES, ORGANIZE MICROTUBULE ARRAYS IN UNACTIVATED EGGS

It has been reported by Zorn et al. that karyoplasts produced from mouse L929 cells are free of centrosomes (33). The assay

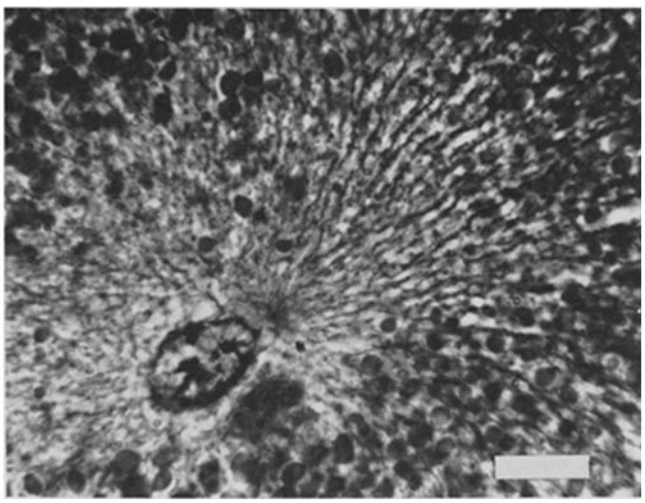


FIGURE 5 Behavior of permeabilized frog A6 cells injected into activated eggs. Cells were permeabilized and injected in eggs with buffer A without EGTA. Eggs were fixed 40 min after injection; paraffin section. The nucleus remains intact and a large aster has formed. Bar, 20 μm. × 600.

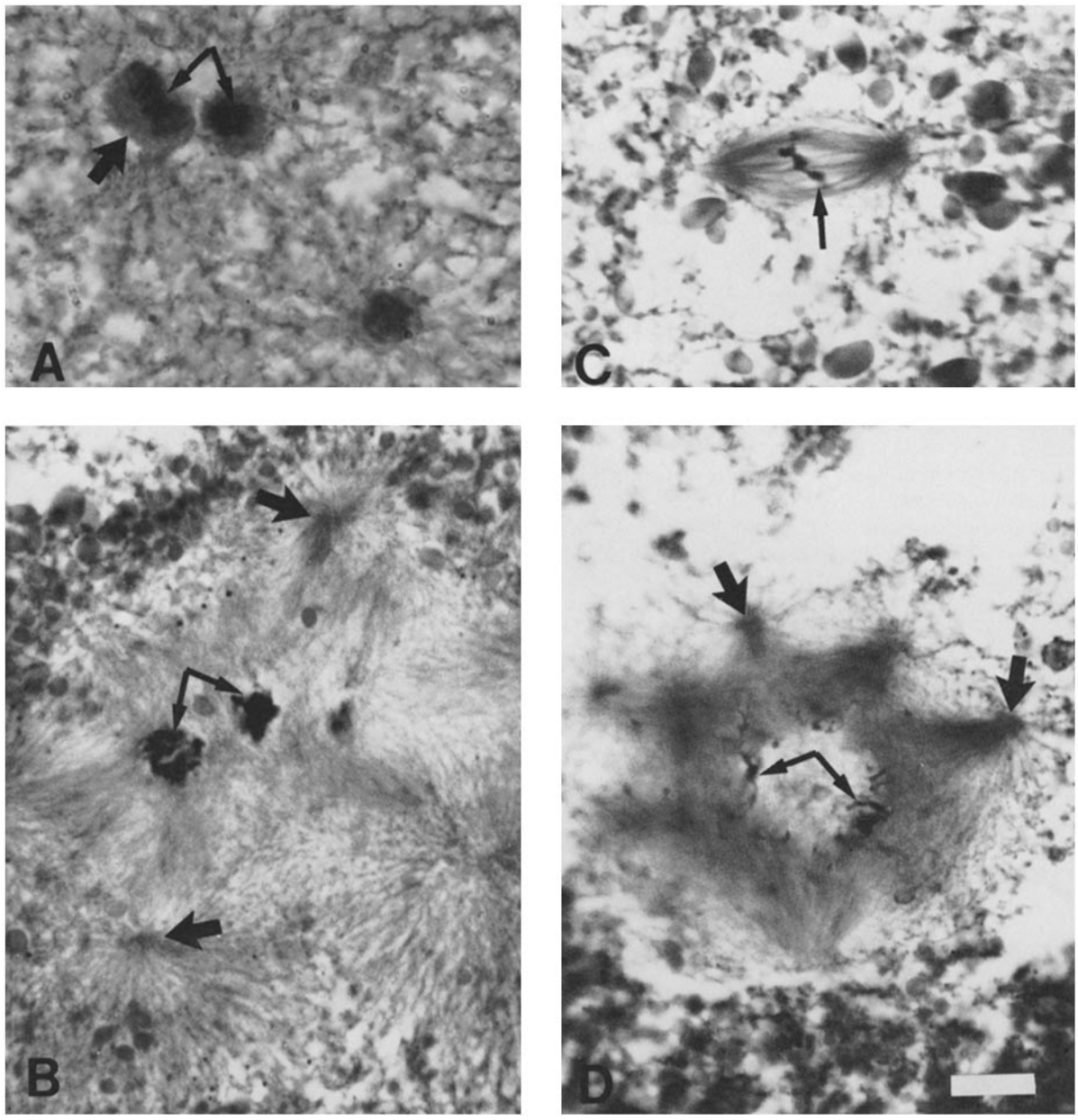


FIGURE 6 Behavior of permeabilized frog A6 cells injected into nonactivated eggs. Cells were permeabilized and injected in eggs with buffer A containing 10 mM EGTA as described in Material and Methods; paraffin sections. (*A*) 15 min after injection, chromatin starts to condense (small arrows) and remnants of the original cell cytoskeleton are still around the nuclei (fat arrow). No asters are visible at this time. (*B*) 30 min after injection, nuclear envelope is clearly broken down, chromosomes are condensed in a "prophase-like" way (small arrows), the cell cytoplasm has been dispersed, and the egg yolk is excluded by asters (fat arrows). (*C*) 90 min after injection, individual nuclei form almost normal "meiotic-like" spindles with condensed chromosomes on a metaphase plate (arrow). (*D*) After the same incubation time, aggregated nuclei form star-like structures made of a central circle of condensed chromosomes (small arrows) surrounded by half spindles, the poles of which contain very small asters (fat arrows). Bar, 20 μm. × 600.

in this case involved the examination of 15,000 thin sections in the electron microscope. Using the same preparative procedure, we have produced karyoplasts substantially free of centrosomes. These are shown by phase contrast microscopy in Fig. 7*A*. The percentage of karyoplasts containing a centrosome in these preparations, has been determined using an antibody to pericentriolar material obtained from a human autoimmune serum (24, 34). Whole L929 cells have a centrosome associated with each nucleus (shown by immunofluorescence in Fig. 7*D*). The cytoplasts remaining after cytochalasin enucleation also each have a centrosome associated with them (shown by immunofluorescence in Fig. 7*C*). However, the karyoplasts (shown in Fig. 7*A* by phase contrast) have no detectable centrosomes associated with them (Fig. 7*B*). In six preparations, the contamination of karyoplasts by centrosomes varied from 0.5 to 2%. As a functional criterion for the absence of a centrosome, cytoskeletons made from karyoplasts did not induce microtubule nucleation in vitro as do those made from whole cells (T. Mitchison, unpublished results). In addition, the absence of contaminating centrosomes, could also be verified by a bioassay. Injection of centrosomes under activating conditions into *Xenopus* eggs

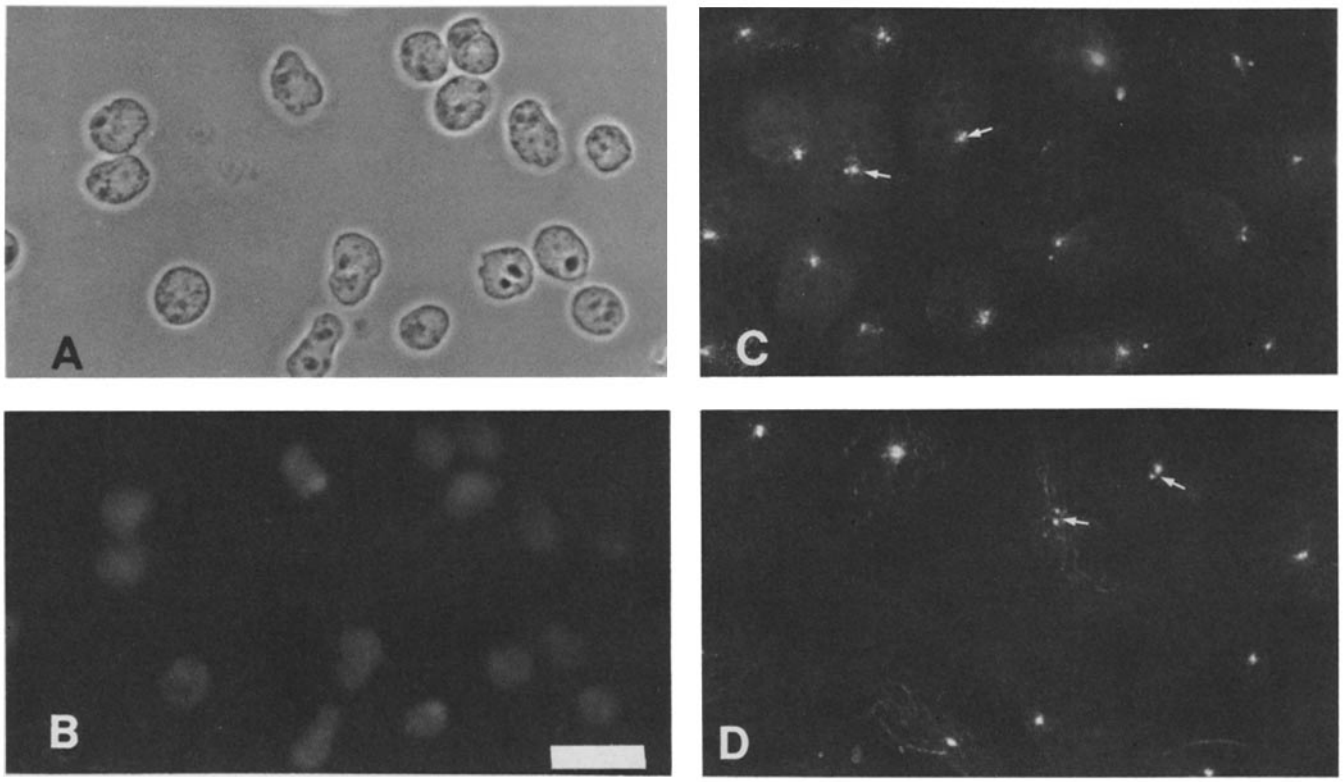
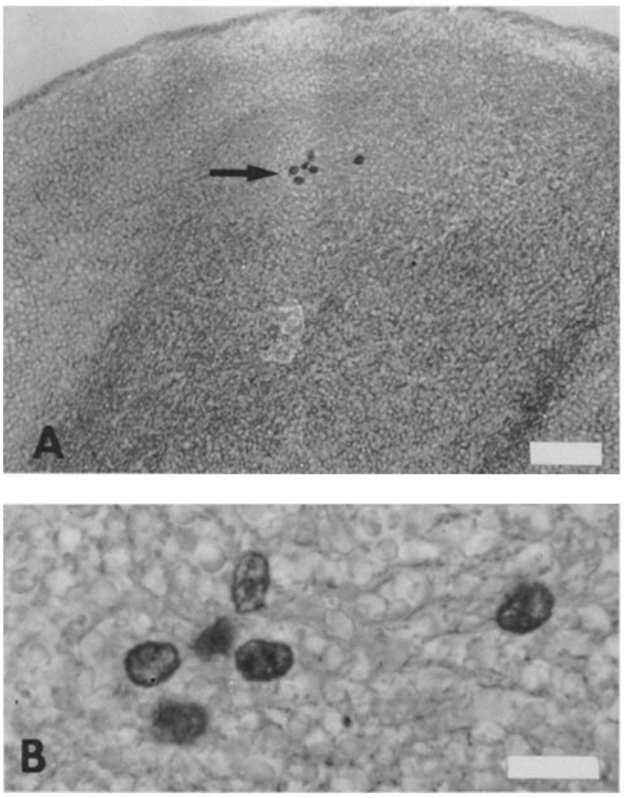


FIGURE 7 Karyoplasts do not contain centrosomes. Karyoplasts and cytoplasts were obtained from L929 cells. They have been stained by indirect immunofluorescence, using a human anticentrosome antibody as described in Materials and Methods. Immunofluorescence microscopy. (*A*) Karyoplasts visualized by phase contrast microscopy. (*B*) Centrosomes are not detected on those karyoplasts by immunofluorescence. The nuclei are visible by the presence of a light background staining. (*C*) The centrosome is well stained in L929 cells by this antibody (arrows). (*D*) Centrosomes are also stained in cytoplasts (bright white dots, sometimes in pairs, arrows). Bar, 20 μm. × 600.



caused aster formation and led to parthenogenesis. The injection of lysed karyoplasts into activated eggs did not lead to aster formation (Fig. 8*A*). A higher magnification (Fig. 8*B*) shows that these eggs contain nuclei with probably intact nuclear envelopes but no detectable aster fibers. This contrasts with the results obtained when whole cells (Fig. 5) or centrosomes were injected into activated eggs (Fig. 3).

When karyoplast nuclei free of centrosomes were injected into unactivated eggs, spindle-like structures formed (Fig. 9). Fibrous, yolk excluding area appeared around the condensed chromatin but no star-shaped structures like those formed around permeabilized whole cells could be found. Instead, when several nuclei were present together, a large oval array of fibers enveloped the mass of condensed chromatin (Fig. 11*A*).

The pathway of spindle formation on injected karyoplasts was somewhat different from what was observed when whole cells were injected. Asters (or fibrous arrays emanating from

FIGURE 8 Behavior of karyoplasts from L929 cells injected into activated eggs. Karyoplasts were permeabilized and injected in eggs with buffer A as described in Materials and Methods; paraffin sections. (*A*) 30 min after injection, nuclei are still intact (arrow). No asters are found in the cytoplasm. (*B*) Enlargement of the nuclei shown in *A*: The nuclear envelope seems still intact, chromatin is not condensed, and nuclei are surrounded by yolk platelets. Bars: (*A*) 100 μm; (*B*) 20 μm. × 100 (*A*); × 600 (*B*).

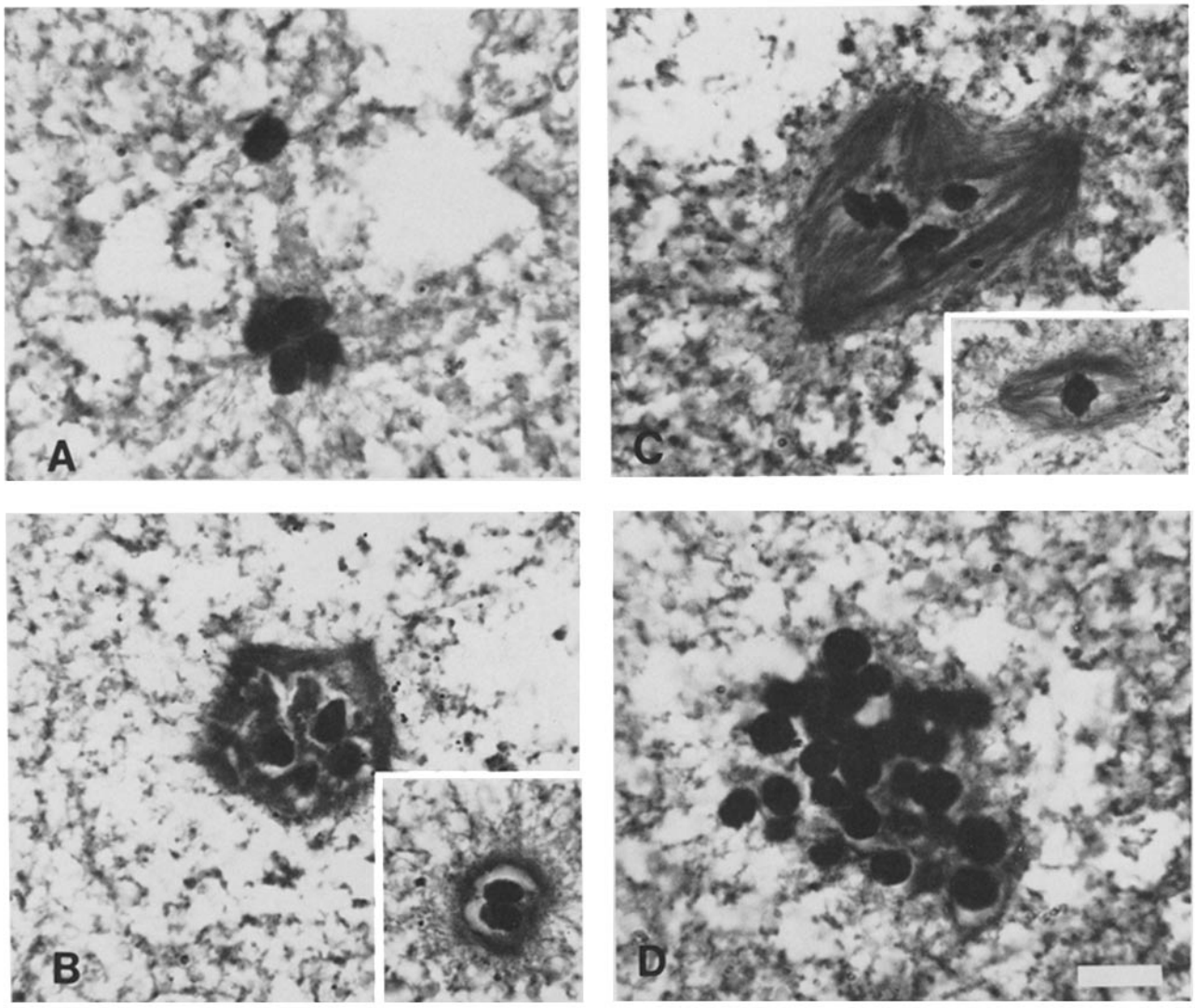


FIGURE 9 Behavior of karyoplasts from L929 cells injected in nonactivated eggs. Karyoplasts were permeabilized and injected in eggs with buffer A containing 10 mM EGTA, as described in Materials and Methods; paraffin sections. (*A*) 15 min after injection, the chromatin starts to condense but no microtubules are visible. (*B*) 30 min after injection, a fibrous material appears at the periphery of the condensed chromatin. No astral figures are visible. (*C*) 60–90 min after injection, the fibrous material is reorganized into spindle-like structures around ill-defined masses of condensed chromatin. (*D*) Eggs preincubated for 15 min into 10 μg/ml nocodazole have been injected with karyoplasts and further incubated for 60 min in nocodazole. Chromatin is condensed, but no fibrous material or spindle-like structure could be found around. Bar, 20 μm. × 600.

a local or point source) were never present during the process of spindle assembly with karyoplasts. 15 min after injection almost no fibers could be discerned (Fig. 9*A*), whereas by 30 min, a local array of fibers enveloped one or several nuclei, in which the chromatin had condensed. By 60 to 90 min the arrays of fibers developed a bipolarity, seen most clearly around single nuclei (Fig. 9*C*). The arrays were completely sensitive to the antimicrotubule drug, nocodazole (Fig. 9*D*). These experiments demonstrate that nuclei free of centrosomes can generate nonastral spindle arrays in unactivated *Xenopus* eggs.

CENTROSOME ACTIVITY IS PROMOTED BY CHROMATIN PROXIMITY IN UNACTIVATED EGGS

The results reported above suggest that in nonactivated eggs, centrosomes nucleate microtubules exclusively in the proximity of condensed chromatin. To test this notion, we co-injected purified centrosomes and karyoplast nuclei into unactivated eggs to see whether astral arrays could be produced. In these experiments 1,000 centrosomes and between 50 to 200 karyoplast nuclei were injected per egg. A low magnification view (Fig. 10*A*) shows that asters were not produced generally in the cytoplasm as was the case when purified centrosomes were injected alone into the egg. However, in the vicinity of condensed chromatin, asters were produced (Fig. 10*B*, large arrows). When several nuclei are clustered, the effect of adding centrosomes to karyoplasts was even more striking (Fig. 11). Although the added centrosomes did not recreate the exact symmetry of the star-like forms produced with aggregated extracted whole cells, the general arrangement of organizing sites as half spindles around a core of condensed chromatin was reconstituted and was distinctly different from those obtained with karyoplasts alone.

Evidence that centrosomes are active in association with the condensed chromatin has been confirmed by electron

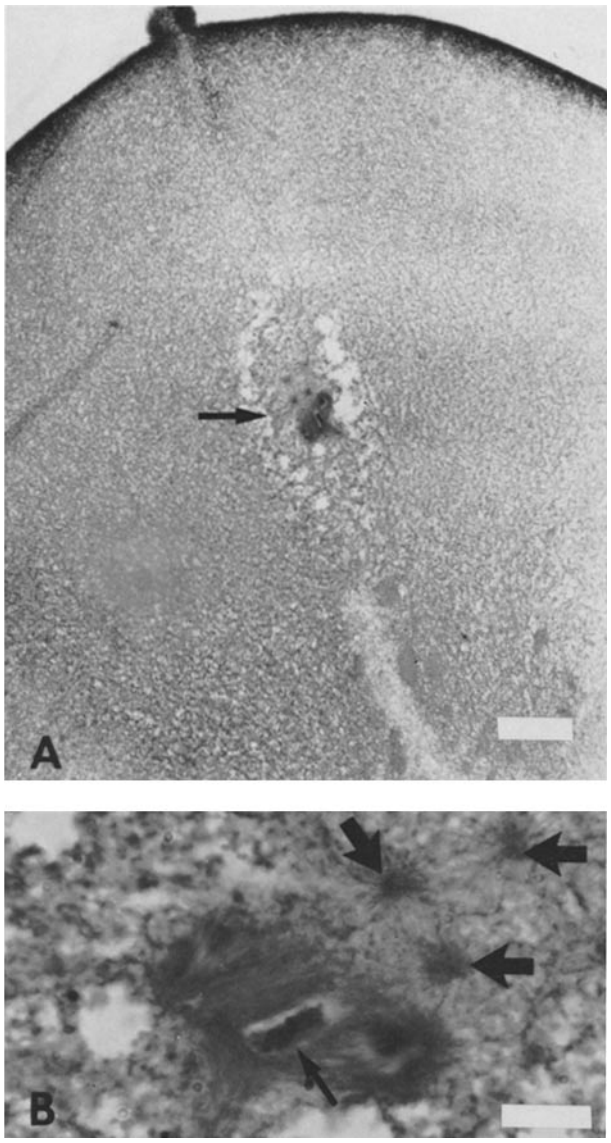


FIGURE 10 Behavior of karyoplasts co-injected with purified centrosomes in unactivated eggs. Centrosomes were mixed with karyoplasts in buffer A containing 10 mM EGTA and injected into eggs. 30 min after injection, eggs were fixed and processed for paraffin sections. (*A*) At low magnification, no asters are visible in the egg cytoplasm. Condensed chromatin with fibrous material around is shown by the arrow. (*B*) At higher magnification, condensed chromatin (small arrow) is surrounded by a spindle-like structure and three small asters (fat arrows). Bars: 100 μm (*A*); 600 μm (*B*). × 100 (*A*); × 600 (*B*).

microscopy. The large size of the *Xenopus* egg, however, makes normal serial sectioning time consuming. We have found that it is possible to manually isolate the spindle arrays in a microtubule-stabilizing buffer, and examine them in the electron microscope after standard fixation. Upon opening of eggs injected with nuclei, translucid, ovoid bodies, excluding the yolk were seen under a low power dissecting microscope. Such structures were pipetted, fixed, and observed under the fluorescent microscope. After staining with bisbenzimide they contained a ring of fluorescent chromosomes approximately half way between the center and the periphery of the structure. The size of such bodies varied between 50 and 200 μm, depending probably upon the number of nuclei that contributed to the structure. Observation of serial sections in the electron microscope, made through such structures, showed that they are built of several bundles of microtubules pointing outward from the mass of chromatin.

An overall view of microtubule bundles is shown in Fig. 12*A* at low magnification and at higher magnification in Fig. 12*B*. The aligned and converging microtubules are surrounded by various organelles such as yolk platelets, lipid globules, mitochondria, and a complex membranous system. The microtubule bundles are interspersed with condensed chromatin (Fig. 12*C*) and a ground substance that is partly made up of ribosome-sized particles (Fig. 12*B*). No obvious microtubule cross-links are visible. The regions at the ends of the bundles are coated by a dense network of membranes, shown in Fig. 12*F* at higher magnification. Similar membranes are occasionally present along microtubules inside the bundles. In various sections through this particular structure, seven centrioles surrounded by radial microtubules were found at the periphery of the microtubule bundles, most often in the membranous zone. One centriole is visible close to the bundle shown in the upper left of Fig. 12*A*. All of the centrioles found have microtubules emanating from them at various angles as shown in Fig. 12, *D* and *E*. Condensed chromatin surrounded by microtubules has been found, but although some kind of interactions are suggested by the pictures (Fig. 12*C*), true kinetochores have not been observed, although they may simply have been missed.

In summary the electron microscope analysis showed that the multipolar spindle structures that form in unactivated eggs upon injection of permeabilized cells contain centrioles that are active in nucleating microtubules. They also contain a large number of other microtubules and condensed chromatin. As expected from the light microscopy, the centrioles are located at the periphery of the complex, although a precise location at the focus of microtubule bundles has not been possible to establish in all cases. The centrioles do not seem to be the source of most of the microtubules found in the bundles. Neither does it seem reasonable to attribute the existence of all these microtubules to the kinetochores. The function of the membranous material found conspicuously around the tips of the bundles is unclear.

The Critical Concentration for Spontaneous Microtubule Polymerization Appears Higher in Unactivated Than in Activated Xenopus Eggs

The failure of individual centrosomes to nucleate microtubule assembly in unactivated eggs, and yet to serve as efficient sites of assembly in activated eggs suggest that the mitotic and interphase state may differ in their capacity for microtubule polymerization. As an indication of the threshold or critical concentrations for microtubule assembly, we have incubated eggs in various concentrations of D_2O and examined the concentration required for spontaneous microtubule assembly (30, 35). It has been previously shown that D_2O will induce microtubule formation in eggs but not in oocytes (35).

As shown in Table II and Fig. 13, spontaneous tubulin polymerization occurs in the cytoplasm of unactivated eggs incubated in Ringer's solution containing 40% D_2O. In activated eggs, the minimum D_2O concentration found to promote tubulin polymerization in the cytoplasm was only 30%. Thus, activation leads to a small but consistent decrease of the minimum concentration of D_2O required to induce microtubule polymerization. This suggests that, whatever the

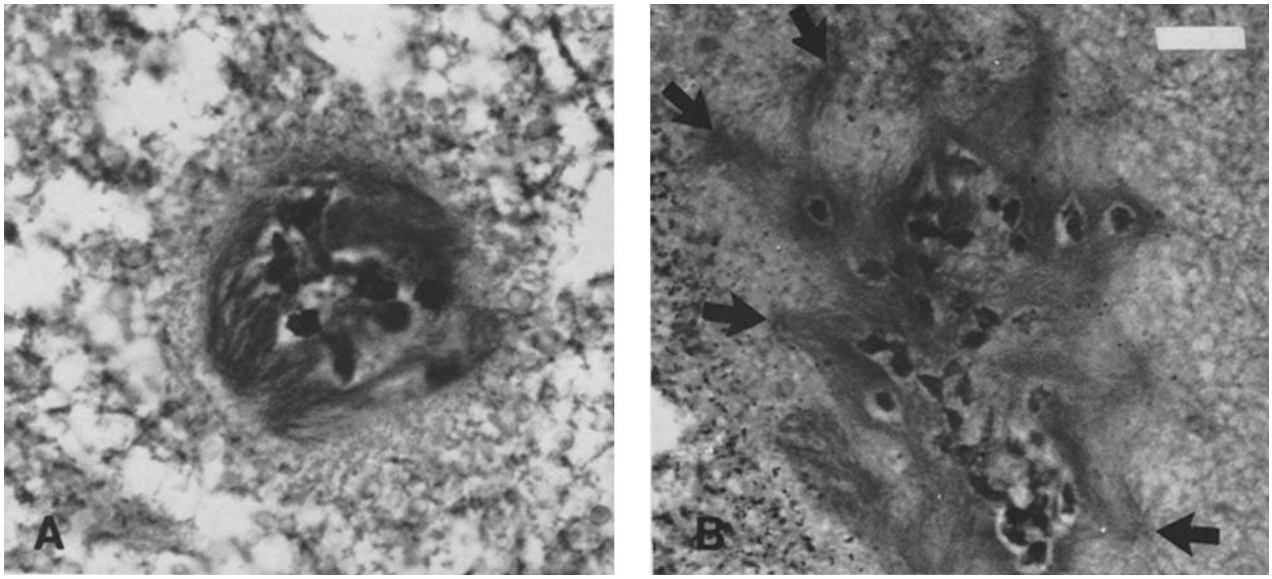


FIGURE 11 Comparison between the behavior of karyoplasts injected alone or with purified centrosomes in unactivated eggs. 90 min after injection, the eggs were fixed and processed for paraffin sections. (*A*) Karyoplasts injected without centrosomes. An ovoid array of microtubules forms around the condensed chromatin. (*B*) Karyoplasts co-injected with centrosomes as in Fig. 10. Several focused microtubule bundles (fat arrows) form around the mass of condensed chromatin. Bar, 20 μm. × 600.

mechanism, tubulin polymerization is favored in the cytoplasm of activated as compared with unactivated eggs.

Although centrosomes did not nucleate the growth of astral microtubules in unactivated eggs under normal conditions, asters appeared if the eggs were incubated in 20% D_2O (Table II). Several large asters were clearly formed in unactivated eggs injected with centrosomes, which were incubated in 30% D_2O (Table II, Fig. 13). When unactivated eggs were incubated in 40% D_2O, spontaneous microtubule assembly in the form of small astral foci occurred both in the presence and absence of injected centrosomes (Fig. 13). It has been noted previously (35, 31) that spontaneous microtubule assembly induced by D_2O does not occur randomly but in the form of small foci, suggesting that some microtubule organization can take place in the cytoplasm in the absence of a structurally defined organizing center. Thus D_2O induces microtubule assembly at lower concentrations in activated eggs than in unactivated eggs. Although asters form spontaneously around injected centrosomes in activated eggs, they will form around injected centrosomes in unactivated eggs incubated in low concentrations of D_2O.

TABLE II

Differential Effect of D_2O on Aster Formation in the Cytoplasm of Activated and Nonactivated Eggs

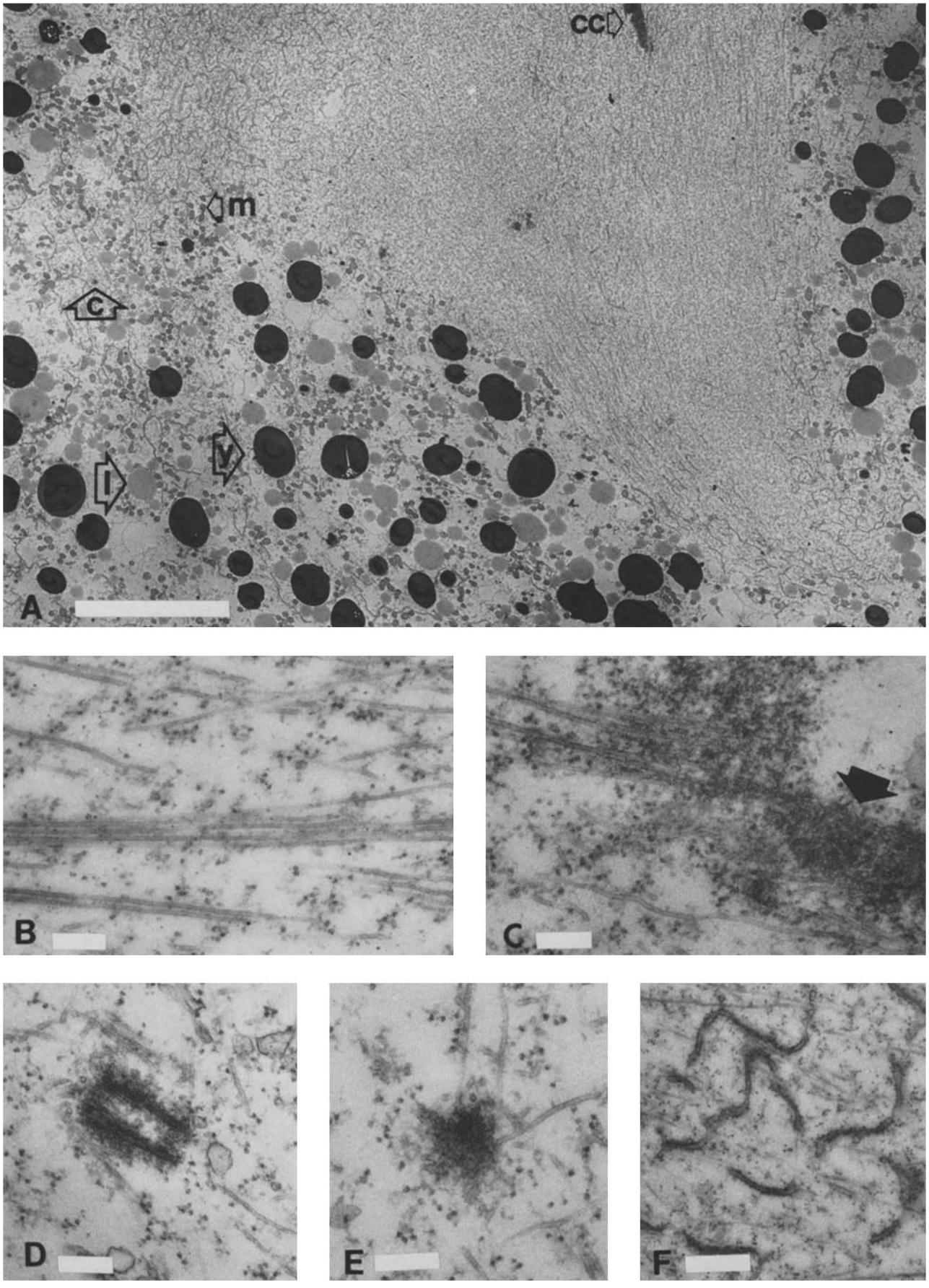
	Nonactivated eggs injected with		Activated eggs injected with	
D_2O	Buffer	Centrosomes	Buffer	Centrosomes
%, vol/vol				
0	0	0	0	69 ± 10
10	0	0	0	ND
20	0	5.5 ± 2	0	ND
30	0.8 ± 2	18 ± 5	*	ND
40	*	*	*	ND
50	*	*	*	ND

n = 6; *ND*, not determined; values equal the number of asters.
* Numerous asters.

DISCUSSION

In this paper we have tried to dissect the relative contribution of the centrosome and nucleus to the process of spindle formation. We have studied the capacity of the centrosome to nucleate microtubule assembly under conditions of metaphase arrest and under conditions in which the egg normally sets up an interphase aster. In the metaphase state no functional centrosome exists in the egg so we had to inject active centrosomes to probe whether the cytoplasm can use them to nucleate aster assembly. The partially purified centrosomes from neuroblastoma cells are good probes for this. They are active in their ability to (*a*) nucleate microtubule assembly in vitro from purified tubulin; (*b*) nucleate assembly in extracts

FIGURE 12 Electron micrographs of isolated spindle structures. (*A*) General view of two microtubule bundles. A centriole is present at the tip of the upper left bundle (*c*). *cc*, condensed chromatin; *y*, yolk; *l*, lipid; *m*, mitochondria. (*B*) Detail of the microtubule containing area. Many microtubules remain equidistant from each other over long distances. No obvious lateral connections could be distinguished. (*C*) Chromatin, with associated microtubules (arrow, chromatin). (*D* and *E*) Serial sections through a centriole. The typical centriole structure is shown in *D*, while two sections later, the pericentriolar material associated with microtubules was found, *E*. (*F*) An example at higher magnification of the aspect of the branched membranes network present at the tip of the microtubule bundles. Bars: (*A*) 10 μm; (*B–E*) 0.2 μm; (*F*) 0.5 μm. × 28,000 (*A*); × 52,500 (*B*); × 52,000 (*C*); × 51,000 (*D*); × 60,000 (*E*); × 23,800 (*F*).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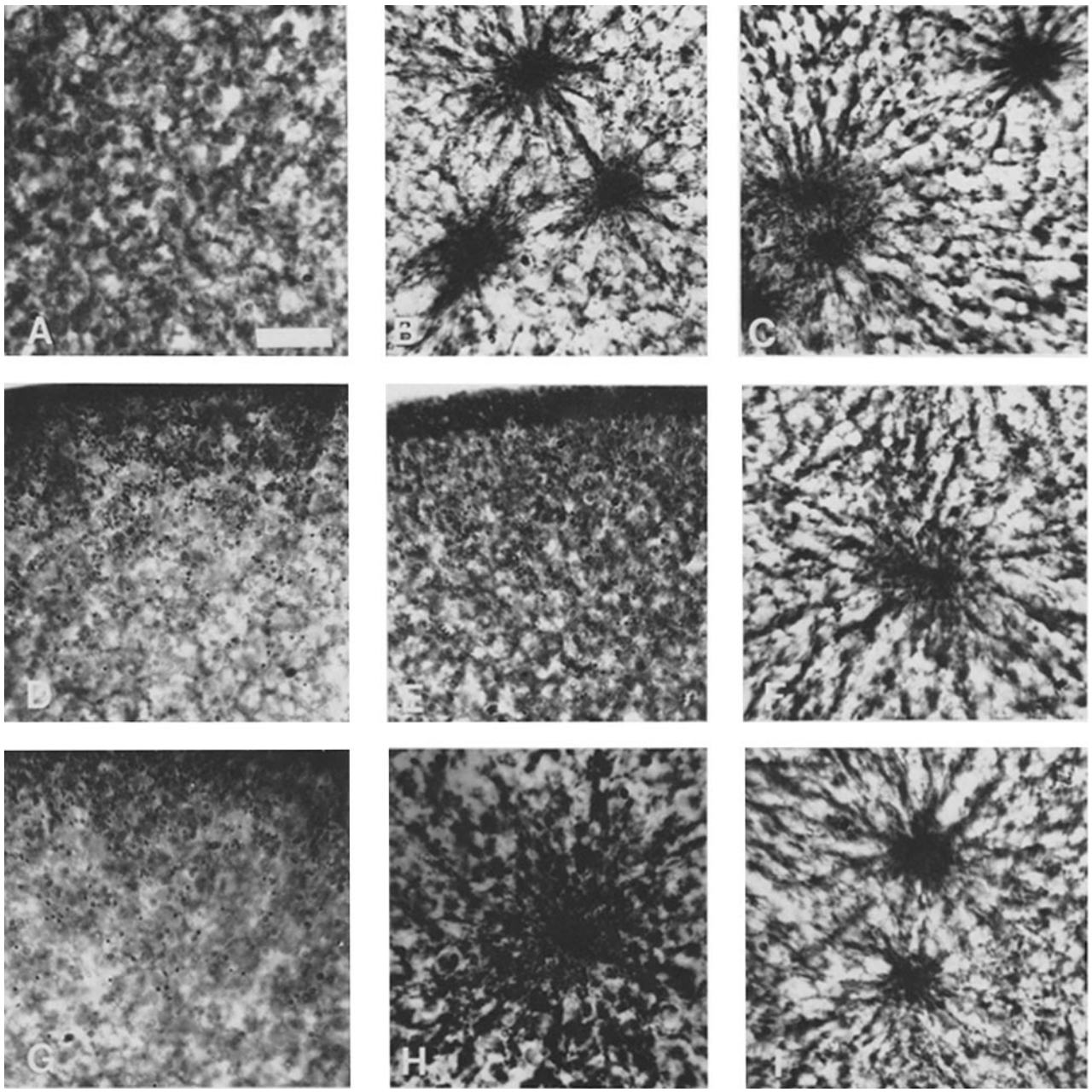

FIGURE 13 Comparison of the effect of D_2O concentration on aster formation in activated and unactivated eggs. Activated eggs: unfertilized eggs were activated by injecting 50 nl of a 20 mM phosphate buffer and incubated for 90 min in MMR + 5% Ficoll, containing (*A*) 20% D_2O, (*B*) 30% D_2O, and (C) 40% D_2O. Spontaneous aster formation occurs in the presence of 30% D_2O. Unactivated eggs: unfertilized eggs were injected with 10 mM EGTA in 20 mM phosphate buffer and incubated for 90 min in MMR + 5% Ficoll containing (*D*) 20% D_2O, (*E*) 30% D_2O, and (*F*) 40% D_2O. Spontaneous aster formation occurs in the presence of 40% D_2O. Unactivated eggs injected with centrosomes: unfertilized eggs were injected with centrosomes in the presence of 10 mM EGTA, 20 mM phosphate buffer and incubated for 90 min in MMR + 5% Ficoll containing (G) 20% D_2O, (*H*) 30% D_2O, and (*I*) 40% D_2O. Aster formation occurs in 30% D_2O; paraffin sections. Bar, 20 μm. × 600.

from activated eggs; (*c*) nucleate aster formation on injection into activated eggs; (*d*) replace the sperm centrosome in supporting cleavage in parthenogenesis.

We have found that centrosomes cannot form asters in unactivated eggs that contain a meiotic spindle at metaphase. The centrosomes are not destroyed by egg or oocyte cytoplasm, since activation of the egg restores centrosome activity.

Apparent conflicting results have been published as to whether asters can form before activation. Elinson (15) argued that the sperm centrosome will induce a large aster only after activation when whole sperm was introduced into immature *Rana* eggs. On the other hand, Heidemann and Kirschner (31) found that asters will form on injected basal bodies immediately after germinal vesicle breakdown during oocyte maturation in *Xenopus*. To clarify this point for *Xenopus*, experiments were done with centrosomes free of an association with the sperm pronucleus. It is important that the centrosome be free of the associated nucleus since we have found here that nuclear material can affect centrosome activity. The present results clearly demonstrate that centrosome

activity develops after egg activation. The observation of Heidemann and Kirschner can be most easily explained by the existence of a transient cytoplasmic state during maturation, which would allow aster formation. Recently, Gerhart, Wu, and Kirschner (48) found that maturation promoting factor goes through two cycles during meiosis, dropping to undetectable levels about an hour after the breakdown of the germinal vesicle and increasing to a stable high value thereafter. It is therefore possible that aster assembly occurs soon after germinal vesicle breakdown, as reported by Heidemann and Kirschner (31) during the brief time when maturation promoting factor is low.

Although centrosomes are inactive in the unactivated cytoplasm the formation of spindles from whole permeabilized cells seems to involve the centrosome. This paradox finds its resolution in the experiments in which karyoplast nuclei and a mixture of centrosomes and karyoplast nuclei have been injected into the metaphase cytoplasm of unactivated eggs.

Permeabilized karyoplasts are devoid of centrosomes (33) as well as pericentriolar material as judged by the absence of staining produced by the human anticentrosome antibody. Moreover, karyoplasts are devoid of any microtubule-nucleating material that can function in interphase since no asters grow from them after injection in activated eggs. Yet karyoplast nuclei will assemble a microtubule array around them in unactivated eggs. This array undergoes a number of structural modifications resulting in an arrested metaphase spindle having an indistinct focus at the poles, and no astral fibers emanating away from the spindle. The pathway of assembly suggests localized polymerization followed by a gradual reorganization. It is interesting that a metaphase state of the cytoplasm induces an organized sequence of events perhaps analogous to prophase and prometaphase. This may suggest that the sequential pathway for achieving the typical metaphase plate on a spindle does not depend on a sequence of regulatory signals but on the response of the nucleus to a given cytoplasmic state.

The karyoplast spindle, like the meiotic spindle and unlike the mitotic spindle is anastral. Spindles formed by injection of permeablized cells are different: they have highly focused poles and display some astral fibers. Similar astral fibers form in multipolar spindles produced from several whole cells. These astral arrays can be also reproduced by co-injection of centrosomes and karyoplasts. Only centrosomes close to the dissolving nucleus form foci for microtubule growth. Electron microscopy revealed that these centrosomes are active as foci for microtubules. An unmistakable conclusion from these experiments is that association with the nucleus renders the centrosome active in an overall cytoplasmic state environment where it is inactive by itself.

Why are isolated centrosomes active in the activated egg, the interphase state, and inactive in the unactivated egg (and cytostatic factor arrested egg), the metaphase state? There are two general possibilities. The first possibility is that the centrosome is reversibly modified is such a way that it functions in one state and does not in the other. The second is that the centrosome activity does not change but the conditions for tubulin assembly change so that microtubule growth off individual centrosomes can occur during interphase but not in metaphase. The finding that centrosomes become active in unactivated eggs incubated in D_2O concentrations subthreshold for spontaneous assembly suggests that there is no overall biochemical mechanism that inactivates centrosomes in unactivated eggs. Similarly, the fact that centrosomes associated with the nucleus retain their activity while centrosomes in the same cytoplasm at some distance from the nucleus are inactive, again suggests that centrosomes are not modified in the unactivated egg. Although it is possible that D_2O or nuclear proximity independently reverse the centrosome modification, it seems more likely that they both act directly on microtubule assembly.

We should note here that the unactivated Xenopus egg is arrested at the second metaphase of meiosis and there may be some differences between the meiotic and mitotic spindle. In particular the absence of a morphologically defined centrosome in the mouse meiotic spindle and its apparent functional absence in the frog may indicate that this is a general property of vertebrates (5, 6). However, we have shown here that somatic nuclei injected into this meiotic cytoplasm form mitotic spindles with defined asters and the overall pattern of spindle assembly, nuclear membrane breakdown, and chromosome condensation are very similar if not identical in meiosis and mitosis. Therefore we feel the use of meiotic cytoplasm (and CSF-arrested cytoplasm) is a good model for spindle assembly in the mitotic state.

The frog egg faithfully represents the transitions between metaphase and interphase. Our results of microinjection of nuclei, karyoplasts, and centrosomes as well as sensitivity of polymerization to D_2O suggest the following overall mechanism for the interphase mitotic conversion, which may be applicable to many cells. (*a*) Interphase and mitotic cells differ in their overall ability to assemble microtubules. In particular the critical concentrations for polymerization in interphase cells is lower than in mitotic cells. (*b*) The critical concentration for microtubule assembly in interphase cells is low enough to permit nucleated polymerization on centrosomes but high enough to prevent spontaneous microtubule assembly. This condition is most readily explained in terms of the polarity of the microtubule and the difference in the critical concentrations for polymerization of the two ends of the microtubule (36). (*c*) The critical concentration for microtubule assembly globally in the mitotic cytoplasm is so high that microtubule growth on centrosomes is prevented; hence centrosomes are inactive. (*d*) During prophase the nucleus locally reduces the critical concentration for tubulin polymerization. The nucleus may release factors that affect the local pH or Ca^{++} concentrations, it may release microtubule-associated proteins or capping proteins, or it may release regulatory molecules like kinases or phosphatases. (*e*) For this reason, in the area around the nucleus even in the absence of the centrosome, both spontaneous and nucleated assembly of microtubules can occur. (*f*) When centrosomes are present near the chromatin, nucleated assembly from centrosomes competes effectively with other forms of nucleated or spontaneous assembly and greatly influences the form of the mitotic spindle. (*g*) In the case of karyoplasts injected into eggs, spindles are definitely not formed by two independent asters. The ultimate bipolar shape of the spindle may be determined by the local microtubule promoting activity of the nucleus. Microtubule cross-linking and aggregation may also contribute to the karyoplast spindle morphology. It is also likely that kinetochores can affect spindle morphology. However, most of the microtubules in the spindle do not seem to interact directly with chromatin. (*h*) During anaphase, there is a reversal of the mitotic state. Globally the threshold concentration for microtubule growth drops, and cytoplasmic

asters become more prominent. As the microtubule promoting factors get reincorporated into the interphase nucleus, or are suppressed, the spindle dissolves away in favor of the interphase aster.

This model, if correct, may be an oversimplification. It ignores some of the cell cycle changes in the centrosome that have been reported (37, 38). The model, however, does suggest in a simple way how the centrosome can rapidly change the microtubule arrays it nucleates. It predicts an important role for the nucleus in microtubule assembly during mitosis, which must be considered more seriously to explain the existence of cells that divide both mitotically and meiotically without centrioles (6, 39). It explains why during prometaphase there is a period of reduced microtubule polymerization, (4, 47) and how depolymerization of the interphase aster can occur and still allow polymerization of mitotic microtubules (1, 2).

The patterns of microtubule growth in the karyoplast nuclei is similar to that which has been seen in *Haemanthus* (40). The syncytial nuclei in this plant undergo mitosis without a centriole. When mitosis starts, the interphase array depolymerizes and the microtubules form a ring surrounding the nucleus in a pattern very similar to what is observed here with the karyoplasts in *Xenopus* eggs. The reorganization of the circumferential microtubules into a spindle is also very similar to the karyoplast spindles and may involve lateral aggregation between microtubules. Many aspects of spindle formation must, therefore, be independent of the centrioles.

Harris has discussed extensively evidence for differences between the interphase aster and the mitotic spindles (4). She contrasts the higher number of microtubules in the spindle region relative to the aster and concludes that "there might be something in the zygote nucleus which catalyzes microtubule polymerization."

The exact nature of the factors that oscillate in the cytoplasm to regulate the overall threshold concentration for polymerization are of course unknown. Harris (4) proposes Ca^{++} as a major factor both for local and global control. Clearly Ca^{++} is the prime candidate for releasing the egg from the unactivated state or from the arrested state caused by cytostatic factor (J. Newport and M. Kirschner, unpublished results). Yet high levels of EGTA are only effective in suppressing the activation of the cell cycle for the first 5 min after pricking and then have no further effect on subsequent cleavages. In addition, Rink et al. (41) have been unable to detect transitions in Ca^{++} levels during the cell cycle in amphibians using a Ca^{++} electrode. Neither of these experiments is definitive because the Ca^{++} levels and changes may be below the binding constant for EGTA under in vivo conditions and below the sensitivity of the Ca^{++} electrode. It may also be that the changes are small or local and that the cell can ultimately accomodate itself to the injected EGTA. However, another candidate for global regulation of the cytoplasmic state along the cell cycle is maturation promoting factor, which is high during mitosis and low during interphase (42, 48). Maturation-promoting factor is detectable in mitotic somatic cells and eggs of starfish and amphibians (41, 47). Recently maturation promoting factor has been shown induce the nuclear membrane breakdown and chromosome condensation in embryos arrested at the end of S-phase in *Xenopus* (46). Perhaps this factor also indirectly regulates the global threshold concentration for microtubule assembly. As for the local factors released by the nucleus, we have very little information and there are many ways by which microtubule assembly can be regulated. The frog egg offers a possible approach to assay and identify them.

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