Respective Roles of Centrosomes and Chromatin in the Conversion of Microtubule Arrays from Interphase to Metaphase

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ABSTRACT We report the results of studies in which partially purified centrosomes, nuclei, and DNA were injected into frog's eggs, which are naturally arrested in metaphase or interphase. These results have led to an independent assessment of the contributions of the centrosome and the chromatin to the formation of the mitotic spindle and suggest a simple explanation for the transition from interphase to metaphase microtubule arrays.

Morphogenesis in cells is thought to be caused by a rearrangement of cytoskeletal elements in response to various internal or external signals. Although in some cases the signals for these morphogenetic changes are known, and in many cases specific components of the cytoskeleton have been implicated, in no case has it been possible to suggest a mechanism explaining precisely how the cytoskeletal elements are actually rearranged. During the past decade, detailed biochemical and biophysical studies of the self-assembly of actin, intermediate filaments, and microtubules have suggested various ways in which polymerization can be regulated. What is needed is some way of understanding how assembly and disassembly mechanisms of the filaments are coordinated spatially to give the observed organized changes in cell shape.

A well-studied example of a morphological change is the transition from interphase to metaphase and back to interphase. Although the details vary considerably in various plants or animals, the overall pattern is that the interphase microtubule distribution disappears to be replaced by a bipolar metaphase spindle. In some animal cells the dissolution of the interphase microtubule array is accompanied by a rounding-up of the cell. A major difference between animals and plants is that in animal cells centrioles seem to play a prominent role in mitosis, whereas plant cells do not have centrioles (except for some species of lower plants). There are several questions raised by the voluminous literature on mitosis that have not been answered by descriptive studies. These include the following: How can the interphase microtubules disappear while there is a concomitant assembly of microtubules to form the spindle in the same cell? What role does the centriole play in spindle assembly and what performs its function in plants? What produces the shape of the mitotic spindle? How many specific signals or instructions are needed to bring about the various states of spindle assembly and disassembly?

A difficulty in studying mitosis is the rapidity with which it proceeds and the difficulty in arresting the cell at a specific stage without inducing physiological imbalance. Eggs and oocytes of various invertebrate and vertebrate animals are naturally arrested at stages of meiosis or mitosis and can be made to proceed synchronously through the cell cycle in response to various signals, such as the addition of hormones or fertilization. In addition, many of these eggs can be easily microinjected, and the behavior of subcellular components can be assayed in a cytoplasmic environment that is at a specific stage of the cell cycle. Using this approach, we have looked at microtubule assembly in Xenopus eggs at metaphase and in interphase. We have examined separately the role of the chromatin and the centriole in the establishment of the metaphase spindle and the interphase microtubule arrays. Our findings suggest, first, that a difference in the threshold concentration for tubulin assembly may be the fundamental control regulating the transition between interphase and metaphase, and, second, that the DNA or chromatin specifically influences the local assembly of microtubules in metaphase cells. Though the centrioles appear to contribute to mitotic spindle assembly, the results reported here suggest that the overall pathway of spindle formation in animals may be very similar to that in plants.

Cell Cycle in Xenopus Eggs Proceeds in the Absence of the Nucleus

The unfertilized Xenopus egg is arrested in metaphase of the second meiotic division with the spindle intact. The
meiotic spindle in frogs is anastral. In mouse (14), and perhaps all vertebrates, it lacks a centriole. In some species the centriole is contributed by the sperm; in others it forms spontaneously some time after fertilization. Fertilization or activation of the Xenopus egg initiates completion of meiosis, and the egg enters interphase. At 60 min the first mitotic division begins, and cleavage ensues at 90 min (3). Thereafter, the egg divides every 30 min, alternating between M and S phases. The Xenopus egg can also be arrested at the next mitotic division by microinjection of a small quantity of cytoplasm from unfertilized eggs containing the yet uncharacterized factor called cytostatic factor (10). Cytostatic factor injection blocks cleavage and causes arrest of the chromosomes in a condensed state at metaphase on a mitotic spindle (11).

Recently, Hara et al. (6) showed that the cell cycle in early cleavage in Xenopus can proceed in the absence of the normal nuclear and cytoplasmic events. Enucleated but activated eggs fail to cleave but undergo periodic contractions of their cortex, timed with the cell cycle. They also show an oscillation of an M phase-specific cytoplasmic factor (4). The various cytoplasmic states of the activated egg dictate the behavior of injected nuclei or DNA with respect to nuclear assembly and disassembly, chromosome condensation, and DNA replication (5, 7, 9, 12, and footnote 1). Two metaphase states can be studied: the one represented by unfertilized eggs and the other produced by cytostatic factor arrest of fertilized eggs. Interphase stages can be produced by relieving these mitotic blocks (by pricking the unfertilized eggs or by injection of Ca²⁺ into cytostatic factor-arrested eggs [Fig. 1]).

**Centrioles Are Active in Interphase Cytoplasm but Inactive in Mitotic Cytoplasm²**

Centrioles may be isolated from mammalian cells and purified between 2,000- and 5,000-fold (Mitchison, T., and M. Kirschner, unpublished data). These centrioles, for example, purified from mouse neuroblastoma cells, contain about 5% tubulin by weight, all of which can be accounted for by the tubulin in the centriole. In vitro, these centrosomes nucleate about 50 microtubules, with the proper polarity (Mitchison, T., unpublished observation). In extracts of frog's eggs, these centrosomes will also efficiently nucleate assembly under conditions under which there is no observable spontaneous polymerization. They also support parthenogenetic development.

When injected into activated eggs (interphase state), the centrosomes induce the formation of large asters within 20

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² Most of the materials and methods used in this work have been described (2, 9). When necessary, new methods are described in the figure legends. Since the injection event resumes the metaphase arrest and activates the egg cell cycle, the metaphase state of the cytoplasm is not directly accessible. Activation was prevented, when desired, by adding 10 mM EGTA to the injection medium.

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1 Newport, J., and M. Kirschner. Regulation of the cell cycle during early Xenopus development. *Cell.* In press.
min (Fig. 2a). Approximately one aster grows per centrosome injected. However, when centrosomes are injected into un-activated eggs (metaphase state), no asters are formed (Fig. 2b). Similarly, centrosomes injected into fertilized eggs ar-rested at metaphase with cytoplastic factor fail to form asters. Moreover, asters that first formed in the interphase cytoplasm (activated eggs) and that then were returned to the metaphase state (by cytoplastic factor injection) disappear. These results show clearly that centrosomes, which are fully competent to act as microtubule-organizing sites, are active in interphase cytoplasm and inactive in metaphase cytoplasm.

It is easily shown that the centrosomes are not destroyed or irreversibly inhibited. Centrosomes can be injected into un-fertilized eggs under conditions under which the egg remains in the metaphase state. After several hours the eggs can be activated by pricking, at which time asters form. Centrosomes can even be injected into immature oocytes, which are then allowed to mature in vitro. When they are arrested naturally at meiotic metaphase, no asters form. However, after activation, asters appear and their formation depends completely on the previous injection of centrosomes. Therefore, centrosomes are active in interphase and inactive in metaphase, and this activity is reversibly expressed.

Injection of Crude Nuclei into Metaphase Cytoplasm Induces Spindle Formation

The surprising failure of centrosomes to function in meta-

FIGURE 4  Behavior of lambda DNA injected into unactivated eggs. Eggs were injected with 2–5 ng of lambda DNA in 20 mM phosphate buffer containing 10 mM EGTA and squashed after various times in the presence of the DNA dye bisbenzimide. Fluorescence microscopy. (a–c) X 900. (d) X 220. (a) At 5 min, threads of DNA were spread over a large area in the egg cytoplasm. (b) After 1 h, the DNA coalesced into thick fibers localized into several spherical structures that appeared in the cytoplasm. (c) After 3 h, the DNA condensed into small structures looking like ill-defined condensed chromosomes. (d) Low magnification of the area shown in c. The condensed DNA is localized in a large spherical structure that resisted the squashing process.
Injection of Karyoplast Nuclei into Metaphase Cytoplasm Induces Spindle Formation

To test the effect of the nucleus proper on the activity of centrosomes it was necessary to render nuclei free of contaminating centrosomes. Karyoplasts, which are nuclei surrounded by a plasma membrane but free of most cytoplasmic contaminants, can be prepared from cultured cells by treatment with cytochalasin B and centrifugation. About 99% of a given preparation of karyoplasts are free of centrioles (16). We have confirmed this by staining with an antibody specific for pericentriolar material. The karyoplasts lacking centrosomes were lysed with lysolecithin and injected into eggs. In activated eggs (interphase cytoplasm) the karyoplast nuclei swell, but there is no sign of microtubule assembly or aster formation, indicating that functionally as well as structurally no centrosomes are present (Fig. 3a). In unactivated eggs (metaphase cytoplasm), the nuclear envelope of the karyoplast nuclei apparently disappears, the chromosomes condense, and a large number of fibers can be seen to grow around the chromatin (Fig. 3b). These fibers rearrange to form spindles lacking a sharp focus. The rearrangement of the fibers and chromosomes is similar to that described for the plant spindle, as exemplified by work with Haemanthus (13). When several nuclei are in proximity to each other, a ring of fibers first surrounds the group of nuclei. In 30 min this rearranges itself into a circle, with the chromosomes on the inside and broad microtubule foci at intervals around the circle. These arrangements are similar to the corresponding structures seen with crude nuclei but have much broader foci, with no fibers radiating toward the cytoplasm. These results clearly demonstrate that in metaphase cytoplasm the spindle can assemble in the absence of centrosomes and that the resulting spindle resembles that of plants.

Injection of Both Karyoplast Nuclei and Centrosomes into Metaphase Cytoplasm Induces Polar Spindles

The spindles formed with crude nuclei differ from those

![Figure 5](attachment:figure5.png)
formed with karyoplast nuclei in the lack of astral fibers in the former and the focused nature of the spindle fibers in the latter. To see if the crude nuclear distribution involved structural elements other than nuclei and centrosomes, we attempted to reconstitute this distribution by simultaneously injecting karyoplast nuclei and centrosomes. When both nuclei and centrosomes were injected into metaphase cytoplasm, some multipolar spindles were found to have well-focused poles with small radiating asters. Asters were seen only in very close proximity to the condensed chromatin. As expected, no asters were present in regions of the cytoplasm free of chromatin. These results suggest that in metaphase centrosomes are active in the region near the nucleus or chromatin. These experiments leave unclear whether it is the chromatin, other nuclear components, or cytoplasmic material adhering to the karyoplast nucleus that is required for spindle assembly.

High Molecular Weight DNA Injected into Metaphase Cytoplasm Promotes Microtubule Assembly

Recently, Forbes et al. (2) have shown that DNA from various sources, including bacteriophage lambda, will spontaneously assemble into nucleuslike structures when injected into interphase eggs. The same DNA introduced into the metaphase cytoplasm of unactivated eggs does not promote the formation of nuclei. Five minutes after injection, long threads of DNA can be visualized in squashes of eggs stained with the DNA dye bisbenzimide (Hoechst) (Fig. 4a). The DNA is then packaged into small structures looking like irregularly condensed chromatin. This packaging takes over 3 h (Fig. 4b and c). The condensed DNA is always found in large (50–200 μm) spherical structures excluding the yolk (Fig. 4d). These structures contain a large amount of fibrous material organized around the condensed DNA, as is shown in the paraffin sections of Fig. 5c and d. One hour after injection into activated eggs (interphase cytoplasm), most of the DNA has formed chromatin, but only a part of it has assembled into nuclei (Fig. 5b). No fibres are apparent around the free chromatin or the nuclei (Fig. 5b). In contrast, after the same incubation time, a large amount of fibres have already assembled around the DNA injected into unactivated eggs (metaphase cytoplasm) (Fig. 5a). This occurs although the DNA has not reached its final level of condensation (Fig. 4). No fibrous material has been observed around the condensed DNA in unactivated eggs incubated for 2 h in 10 μg/ml nocodazole. This strongly suggests that the fibers observed in the absence of nocodazole are primarily microtubules. Moreover, thin sections made through the fibrous areas and observed by electron microscopy contain a large amount of microtubules (Fig. 6). DNA of bacteriophage T4 and E. coli origin promotes a local polymerization of microtubules when injected into unactivated eggs. However, the plasmid PBR322 and the single-stranded or replicative form of the phage M 13 fail to induce any visible local assembly of microtubules in

**Figure 6** The fibrous areas surrounding the condensed DNA contain numerous microtubules. Transmission electron microscopy. (a) × 25,000. (b) × 45,000. (See reference 2 for technical details.) (a) The star indicates the cytoplasmic area where the DNA is localized. Microtubules grow along this cytoplasmic domain. Note that the area containing the microtubules is very rich in ribosomelike particles (bottom part of the picture). (b) Microtubules at higher magnification.

**Figure 7** Fragments of bacteriophage T4 DNA prepared by sonication. T4 DNA (0.5 mg/ml) was sonicated at low intensity with a Bransonic 350 (Branson Sonic Power Co., Danbury, CT) for various lengths of time. The resulting DNA fragments were analyzed on an 0.8% agarose gel in Tris-borate, EDTA buffer at 100 V for 3.5 h. Ethidium bromide staining. (a) T4 DNA. (b) Sonicated for 5 s. Higher mol wt ≈ 23 kb. (c) Sonicated for 10 s. Higher mol wt ≈ 10 kb. (d) Sonicated 20 s. Higher mol wt ≈ 6 kb. (e) Sonicated 30 s. Higher mol wt ≈ 3 kb. (f) Molecular weight markers (Hind III cut of lambda DNA).
the metaphasic cytoplasm. This is related to their small size, as we shall see.

Ta DNA was sonicated for various periods of time to produce fragments of DNA of various sizes (Fig. 7). It was found that the extent of local microtubule assembly decreases with the average size of the DNA. DNA smaller than 10 kb fails to promote any detectable local microtubule assembly (Fig. 8a–c). The reverse experiment has been done with PBR322. The circular double-stranded DNA (4.3 kb) has been cut by restriction enzymes (Eco RI and Hind III) and religated by the Ta ligase to produce polymers larger than 10 kb (Fig. 9). Although PBR322 is totally unable to promote microtubule assembly in unactivated eggs, the ligated form is a strong promoter of local polymerization (Fig. 10). Even high concentration of low molecular weight DNA (100 ng/egg) fails to induce assembly. Various anionic polymers such as frog's liver RNA, dextran sulfate (500 kd), or heparin have no activity in inducing any kind of microtubule polymerization in the eggs.

The results reported here suggest that it is not the anionic properties of DNA that promote microtubule assembly. Rather it is the assembly of DNA into chromatin, which in the metaphase state accumulates and/or activates components, that promotes local polymerization. Interestingly, this does not have to come about by the prior assembly of an interphase nucleus but can occur directly by formation of metaphase chromatin. It is not clear why small DNA does not promote microtubule assembly. In any case, the present results suggest that in a given cytoplasmic environment there is a relationship between the local mass of chromatin and the number of microtubules assembled around it. If verified, this may prove to be important in explaining the size of the spindle in relation to the number of chromosomes.

Metaphase and Interphase Cytoplasm Differ in Their Ability to Induce Microtubule Assembly

As an indirect measure of the polymerizability of tubulin in eggs, we examined their response to different concentrations of D₂O. As reported (8), D₂O induces microtubule assembly in unfertilized eggs but not in oocytes (8). The assembly takes the form of small foci of polymerization, which displace yolk and other vesicles and which are readily observed with the aid of the light microscope. When this assay was applied to eggs in metaphase or interphase, a consistent and significant difference was observed in the D₂O concentration necessary to induce spontaneous polymerization. Thirty percent D₂O is required to induce assembly in activated eggs in interphase. Unactivated eggs, at metaphase, require 40%. When we injected centrosomes into metaphase cytoplasm (unactivated eggs) and incubated the eggs in various concentrations of D₂O, we found that asters formed in 20% D₂O. These data suggest diminished polymerizability of tubulin in the metaphase state, because higher D₂O concentrations were required to induce spontaneous assembly. In addition they demonstrate that D₂O can overcome the inhibition of centrosome-dependent aster assembly in eggs arrested in a metaphase state.

Mechanism of the Mitotic-Interphase Transition of Microtubule Arrays

The experiments reported here suggest some new approaches to the questions raised earlier on how the interphase microtubule array is converted to the spindle, how the spindle gets its shape, what role the centriole has in spindle assembly, and how many signals are required to assemble the spindles.
Here we report four key observations: (a) isolated centrosomes are active in interphase and inactive in metaphase cytoplasm; (b) isolated karyoplast nuclei as well as purified DNA have no effect on microtubule assembly in interphase cytoplasm but induce microtubule assembly in metaphase cytoplasm; (c) centrosomes plus nuclei reconstitute essentially normal astral spindles in metaphase cytoplasm; and (d) it is easier to induce spontaneous polymerization with D₂O in interphase cytoplasm, suggesting that tubulin polymerizability is greater in interphase than in metaphase.

These observations suggest that an overall deficiency in microtubule assembly in metaphase is overcome locally in the egg, directly or indirectly, by condensed chromatin. This global reduction of microtubule polymerizability during metaphase would result in the depolymerization of the interphase aster. The local promotion of microtubule assembly in the vicinity of the chromatin would allow for the assembly of the spindle at the same time. It is not clear how chromatin induces localized microtubule polymerization, but it is clear that the DNA does not require specific sequences found only in eukaryotic organisms. The centrosome is clearly not essential to the assembly of the spindle. However, centrosomes in the vicinity of the chromatin are activated and influence the overall microtubule distribution. The determination of the shape of the spindle is probably complex, but it certainly must involve, as a large component, the asymmetric distribution of a microtubule-promoting activity activated by the metaphase chromatin.

The microinjection experiments were designed primarily to assay components required for spindle formation. Yet these experiments may also contribute to our understanding of the steps of spindle formation. The metaphase state, whether in the unfertilized egg or in cleaving eggs arrested by cytostatic factor, is stabilized at a unique point in the cell cycle. Nevertheless, nuclei, centrosomes, or DNA injected into a cell in this arrested cytoplasmic state will initiate specific discrete events leading to the formation of a metaphase spindle, condensed chromatin, and a dispersed nuclear envelope. The rearrangements of the microtubules leading to a metaphase state seem very similar to those of early prophase and prometaphase, suggesting that in the metaphase-arrested egg, all the information necessary for the early mitotic events is present. If this is true, it may suggest that the mitotic-interphase conversion is a two-step process, with all the details of spindle assembly “hard wired” into the individual components, which merely respond to the new environment. Recent experiments conducted in our laboratory suggest that DNA synthesis may be regulated by a similar two-state mechanism.

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REFERENCES


