Experimental Separation of Pronuclei in Fertilized Sea Urchin Eggs: Chromosomes Do Not Organize a Spindle in the Absence of Centrosomes

GREENFIELD SLUDER* and CONLY L. RIEDER‡ *with the technical assistance of* FREDERICK MILLER*

**Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545; and ‡Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201*

ABSTRACT We tested the ability of chromosomes in a mitotic cytoplasm to organize a bipolar spindle in the absence of centrosomes. Sea urchin eggs were treated with 5×10^{-6} colcemid for 7–9 min before fertilization to block future microtubule assembly. Fertilization events were normal except that a sperm aster was not formed and the pronuclei remained up to 70 μm apart. After nuclear envelope breakdown, individual eggs were irradiated with 366-nm light to inactivate photochemically the colcemid. A functional haploid bipolar spindle was immediately assembled in association with the male chromosomes. In contrast to the male pronucleus, the female pronucleus in most of these eggs remained as a small nonbirefringent hyaline area throughout mitosis. High-voltage electron microscopy of serial semithick sections from individual eggs, previously followed in vivo, revealed that the female chromosomes were randomly distributed within the remnants of the nuclear envelope. No microtubules were found in these pronuclear areas even though the chromosomes were well-condensed and had prominent kinetochores with well-developed coronas. In the remaining eggs, a weakly birefringent monaster was assembled in the female pronuclear area.

These observations demonstrate that chromosomes in a mitotic cytoplasm cannot organize a bipolar spindle in the absence of a spindle pole or even in the presence of a monaster. In fact, chromosomes do not even assemble kinetochore microtubules in the absence of a spindle pole, and kinetochore microtubules form only on kinetochores facing the pole when a monaster is present. This study also provides direct experimental proof for the longstanding paradigm that the sperm provides the centrosomes used in the development of the sea urchin zygote.

During mitosis in both plant and animal cells, spindle microtubules (MTs)[1] are assembled and spatially organized into a fusiform array which forms the cytoskeletal component of the mitotic apparatus. The poorly understood forces and interactions that encode the specific temporal and spatial organization of spindle MTs are of fundamental importance to cell division. They are required to generate the essential twoness of cell reproduction.

There are two conflicting views on the origin of spindle bipolarity. The traditional view holds that specialized structures (e.g., centrosomes) form the poles of the spindle and therefore determine the spindle axis (16, 18, 19). In animal cells, these spindle poles nucleate radial arrays of MTs that overlap to form the continuous or interpolar fibers of the mitotic apparatus. Subsequent interactions between the kinetochores and poles then lead to the assembly of kinetochore MTs (7, 24). Although the exact origin of kinetochore MTs is debated (recruitment from the poles vs. nucleation by the kinetochore; see references 21 and 24), the model requires that polar structures be present to attach the chromosomes to the spindle, establish their amphitelic orientation, and ensure a bipolar spindle axis. In effect, chromosomes are aligned by the poles and not vice versa. This hypothesis is supported by

[1] *Abbreviations used in this paper:* MTs, microtubules; NE, nuclear envelope.

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the observations that typical animal cells have easily recognizable centrosomes that seem to define the spindle polarity, and that the chromosomes are firmly anchored to these centrosomes by their kinetochore fibers (4, 5). In such cells, extra centrosomes can interact with chromosomes to give a multipolar spindle.

The view that spindle bipolarity is determined by discrete polar structures has been challenged in the past (28), and more recently by observations that suggest that chromosomes alone are sufficient to organize a bipolar spindle. Dietz (10), for example, observed that crane fly spermatocytes can form a functional bipolar spindle even when the two spindle poles (i.e., asters) fail to separate. Some researchers have interpreted this observation to indicate that lateral interactions between the condensed chromosomes and/or between MTs of their kinetochore fibers provide the forces that align the chromosomes and organize the spindle structure (e.g., references 19 and 35). Also, Karsenti et al. (13, 14) found that when karyoplasts, or even isolated DNA, are injected into *Xenopus* eggs, a fusiform array of MTs forms around groups of chromosomes. The formation of these spindlelike structures in the absence of demonstrable centrosomes suggested to them that chromosomes alone could organize a bipolar spindle. A similar conclusion was reached by Witt et al. (35, 36) who reported that chromosomes of Chinese hamster ovary cells can form kinetochore fibers and achieve a bipolar orientation without interacting with spindle poles. Recently, Euteneuer et al. (11) suggested that ancillary spindle poles form in Chinese hamster ovary cells recovering from colcemid block by the coalescence of kinetochore fibers. Finally, the observation that plant spindles have diffuse poles (reviewed in reference 2) is consistent with a chromosomal origin for spindle organization.

The controversy concerning the origin of spindle bipolarity, and the related controversy concerning the mechanism of kinetochore MT formation and kinetochore orientation, arise from an inability in the past to differentiate experimentally between respective contributions that kinetochores and centrosomes make in forming the spindle. Although a few recent studies have attempted to determine the contributions that each of these organelles makes in forming the mitotic apparatus (6, 9, 35), these approaches are subject to criticism (reviewed in references 21, 22, and 24).

In this paper, we describe an experimental system that has allowed us to evaluate the role that chromosomes play in spindle assembly. Our goal was to determine whether chromosomes, in the absence of centrosomes, are sufficient to organize a functional bipolar spindle during mitosis in animal cells. We have characterized this system both in vivo with the light microscope and ultrastructurally using serial semithick sections. We find that under normal conditions, chromosomes cannot form a bipolar spindle in the absence of centrosomes or even in the presence of a monaster. Indeed, when centrosomes and a monaster are absent, the kinetochores do not even acquire MTs.

MATERIALS AND METHODS

Living Material and Light Microscopy: Eggs from the sea urchins *Lytechinus pictus* and *Lytechinus variegatus* were obtained by intracoelomic injection of 0.5 M KCl as described elsewhere (12). They were then treated, before fertilization, for 8–9 min with 5×10^{-6} M colcemid (Sigma Chemical Co., St. Louis, MO) in sea water. This pulse of colcemid prevents MT assembly in these eggs for at least 2 h. A few minutes after the colcemid treatment, the eggs were fertilized, and if they were to be later processed for electron microscopy, the fertilization envelopes were mechanically removed as described elsewhere (32). The colcemid-treated fertilized eggs were spread before the first nuclear envelope (NE) breakdown on a protamine sulfate–coated coverslip, and mounted in fluorocarbon oil preparation as described elsewhere (32, 33). Shortly after NE breakdown, they were irradiated for 15 s with 366-nm light to photochemically inactivate the colcemid (31) and allow MT assembly. Selected cells were irradiated, observed, and photographed with a Zeiss ACM microscope (Carl Zeiss, Inc., Thornwood, NY) modified for polarization microscopy. Additional cells were observed and photographed, with differential interference contrast optics, on an Olympus BHS microscope (Olympus Corp. of America, New Hyde Park, NY).

Electron Microscopy: Cells to be examined with the electron microscope were circled on the coverslip with a diamond objective scribe. They were then followed and photographed in vivo until the desired time for fixation. Methods used in the fixation, embedding, and serial semithick sectioning of sea urchin egs, previously followed in vivo, are described in detail elsewhere (33). Semithick (0.25 μm) serial sections of each egg were mounted on Formvar-coated slot grids and stained in uranyl acetate followed by lead citrate (23, 26). The sections were then screened for content by phase-contrast light microscopy (25). Selected ribbons of sections, which contained the male and female pronuclear regions, were viewed and photographed on the New York State Department of Health high-voltage electron microscope operated at 800 kV.

RESULTS

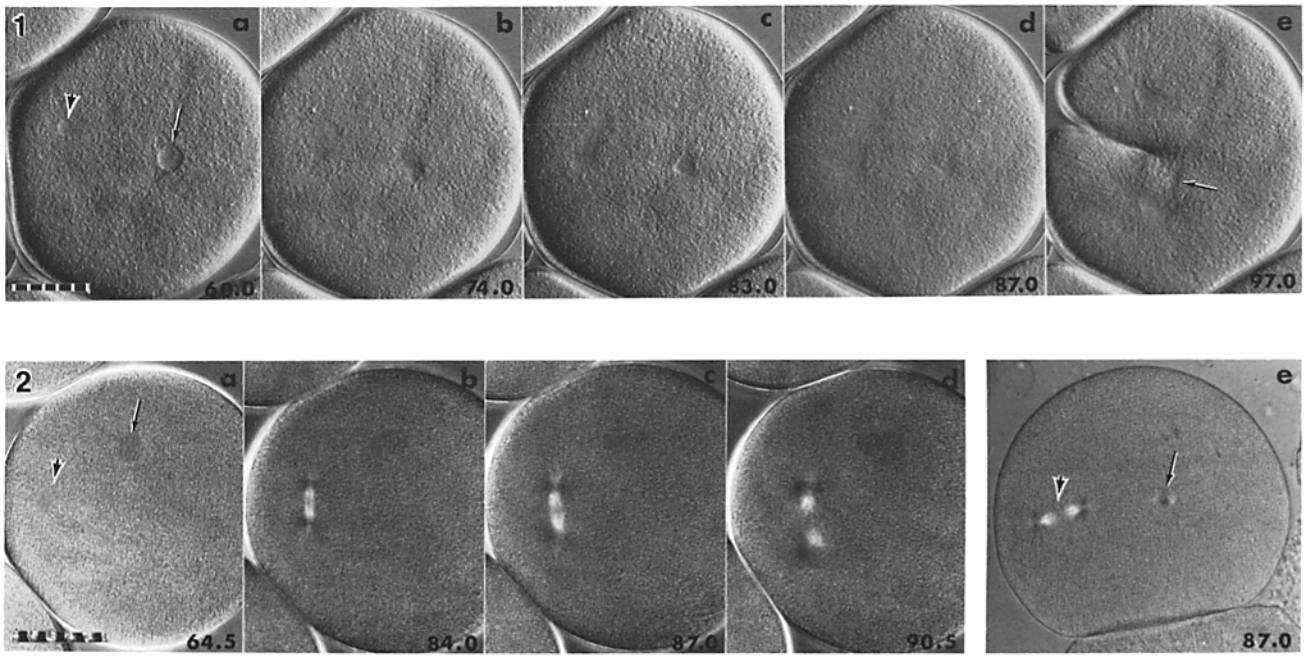
Light Microscopic Observations

Eggs were pulsed with colcemid and then fertilized. Fertilization appeared normal in that each egg elevated a fertilization envelope and only one sperm was incorporated. However, since the sperm aster did not form, pronuclear migration was inhibited and syngamy did not occur. The male pronucleus became visible, somewhere in the periphery of the egg, ~15 min after fertilization, whereas the female pronucleus remained ecentrically located in its prefertilization position (Figs. 1*a* and 2*a*). The positioning of the male pronucleus relative to the female pronucleus appeared random in a population of zygotes, presumably because the sperm can fertilize the sea urchin egg anywhere on its surface. For this study, we selected zygotes in which the female and male pronuclei were separated by almost the diameter of the cell.

At the normal time for NE breakdown, which occurs in these species ~1 h after fertilization, the female pronucleus appears at the light-microscopic level to crumple, leaving an irregular hyaline area about the same size as the original nucleus (Figs. 1*b* and 2*b*). About 10 min later, the male pronucleus appeared to crumple. This consistently observed asynchrony in NE breakdown is in accord with that found by Aronson (1) in his studies of colcemid-treated sea urchin eggs.

Shortly after both nuclei had broken down, we irradiated individual eggs on the microscope with 366-nm light for 15 s to inactivate photochemically the colcemid. Immediately after irradiation, a functional bipolar spindle of normal appearance formed around the male chromosomes (Figs. 1, *b–f* and 2*b*). With time, this spindle became more robust and its birefringence increased. Later, it initiated a normal anaphase (Figs. 1*d* and 2*d*), and the cell cleaved between the asters, starting on the side of the male spindle (Fig. 1*e*). Telophase events appeared normal, and daughter nuclei of male origin reformed on either side of the furrow. At this point, the female chromosomes were sometimes drawn into one of the male telophase asters (Fig. 1*e*, arrow).

In some cells, one of the asters moved 20 μm or more away from the male chromosomes just after irradiation. In such cases, a monopolar spindle was formed by the single remaining aster and male chromosomes; the free aster remained separate in the cytoplasm (data not shown).



FIGURES 1 and 2 (Fig. 1) Development of a zygote with separate pronuclei: differential interference contrast optics. (*a*) Before NE breakdown and irradiation. Male pronucleus is indicated by the arrowhead, female pronucleus by the arrow. (*b* and *c*) NE breakdown and spindle assembly in the male pronuclear area. Female chromosomes remain in a small hyaline area. (*d*) Early anaphase in the male spindle. (e) Telophase and cleavage. Arrow shows female chromosomes moving into male telophase aster. Minutes after fertilization are shown in lower corner of each frame. 10 μm per scale division. × 200. (Fig. 2) Development of a zygote with separate pronuclei: polarization optics. (a) After NE breakdown but before irradiation. male pronucleus is indicated by the arrowhead, female pronucleus by the arrow. (*b* and *c*) After irradiation with 366-nm light. Spindle is assembled with male chromosomes. Female chromosomes remain in a small hyaline area. (*d*) Male spindle in anaphase. Female chromosomes are still in a small hyaline area. (e) Different cell with female monaster is indicated by the arrow and a male spindle is shown by the arrowhead. Minutes after fertilization are shown in lower corner of each frame. 10 μm per scale division. × 245.

When viewed with the polarizing microscope, the female pronucleus developed in one of two fashions. In most eggs, it remained as a small hyaline area throughout mitosis. There was no birefringence associated with the female chromosomes (Fig. 2, *b–d*), and there was no evidence of spindle formation. Rather, the chromosomes appeared, in the differential interference contrast microscope, to be randomly distributed within the area of the former nucleus.

Alternatively, a weak monaster formed in the female pronuclear area a few minutes after irradiation (Fig. 2*e*). There was no indication of bipolar spindle formation in these cells, and the monaster persisted until the cell entered telophase.

In those eggs in which the male and female pronuclei were nearby before NE breakdown, the female chromosomes became incorporated into the forming spindle after irradiation. These chromosomes then participated normally in spindle dynamics. This is in accord with the results of other studies (30, 31) in which sea urchin eggs, treated with colcemid after syngamy, were irradiated in mitosis. In such cases, all chromosomes participated normally in spindle assembly. Thus, the behavior of the female chromosomes that we observed in the present study was not an artifact of our experimental methodology.

Electron Microscopic Observations

Individual eggs with well-separated pronuclei were followed in vivo by polarized light– or differential interference contrast–microscopy. When the male spindle was in metaphase or anaphase, each egg was circled on the coverslip, fixed, and then embedded.

In the first portion of this study, we made serial semithick sections of five eggs. Sections of male and female chromosomes from a single representative egg are shown in Figs. 3–7. This egg is pictured in vivo with the polarizing microscope in Fig. 3, lower left inset. The birefringent male spindle is shown by the arrowhead in the upper left quadrant of the cell, and the female pronuclear area is shown by the arrow in the upper right quadrant. The male spindle is slightly defocused in order to include the female pronuclear area in the micrograph. Sections through the male spindle revealed a typical spindle morphology with an abundance of kinetochore and nonkinetochore MTs converging on well-defined polar areas (Fig. 3, *pa*). Each chromosome possessed conspicuous kinetochores which were attached to, and oriented toward, their respective polar area by kinetochore fiber MTs (arrow in Fig. 3, and lower right inset in Fig. 3).

In contrast to the male chromosomes, the female chromosomes in the same egg did not become associated with any spindle structure (Figs. 4–7). The chromosomes appeared to be distributed randomly within the pronuclear area (Fig. 4). These female chromosomes appeared well-condensed and had prominent kinetochore plates with well-developed coronas (Fig. 4, inset, and Figs. 5–7). However, no MTs could be found near the kinetochores or in the pronuclear area. In all five cells examined, the female chromosomes were surrounded by a loosely organized sheath of membranes consisting of two closely apposed unit membranes interspersed with stacks of membranes reminiscent of annulate lamellae (Fig. 4, asterisks). These membrane elements were extremely convoluted and always fenestrated with clear channels between

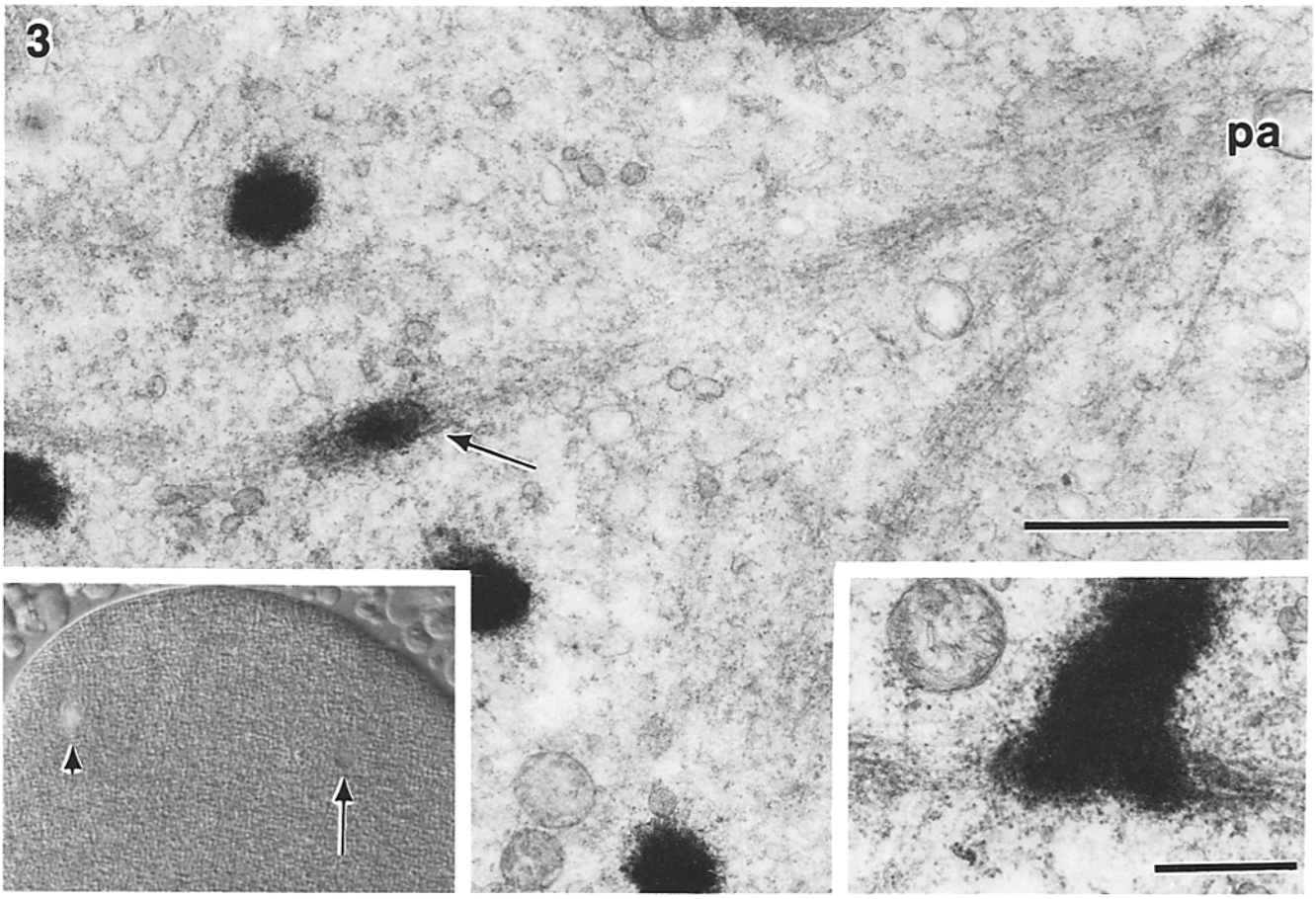


FIGURE 3 Electron micrograph from a portion of a male spindle. One kinetochore is indicated by the arrow, *pa* is polar area. × 17,500. Bar, 2.0 μm. (*Left inset*) Same cell before fixation as seen in the polarizing microscope. Arrowhead denotes male spindle, arrow denotes female chromosomes. (*Right inset*) Chromosome from a different section of the same male spindle. Note the well-defined kinetochores and well-developed bundle of kinetochore MTs. (*Right inset*) × 19,500. Bar, 1.0 μm.

the cytoplasm and pronuclear area. Often, yolk granules and mitochondria were found directly adjacent to the chromosomes (Figs. 4 and 7).

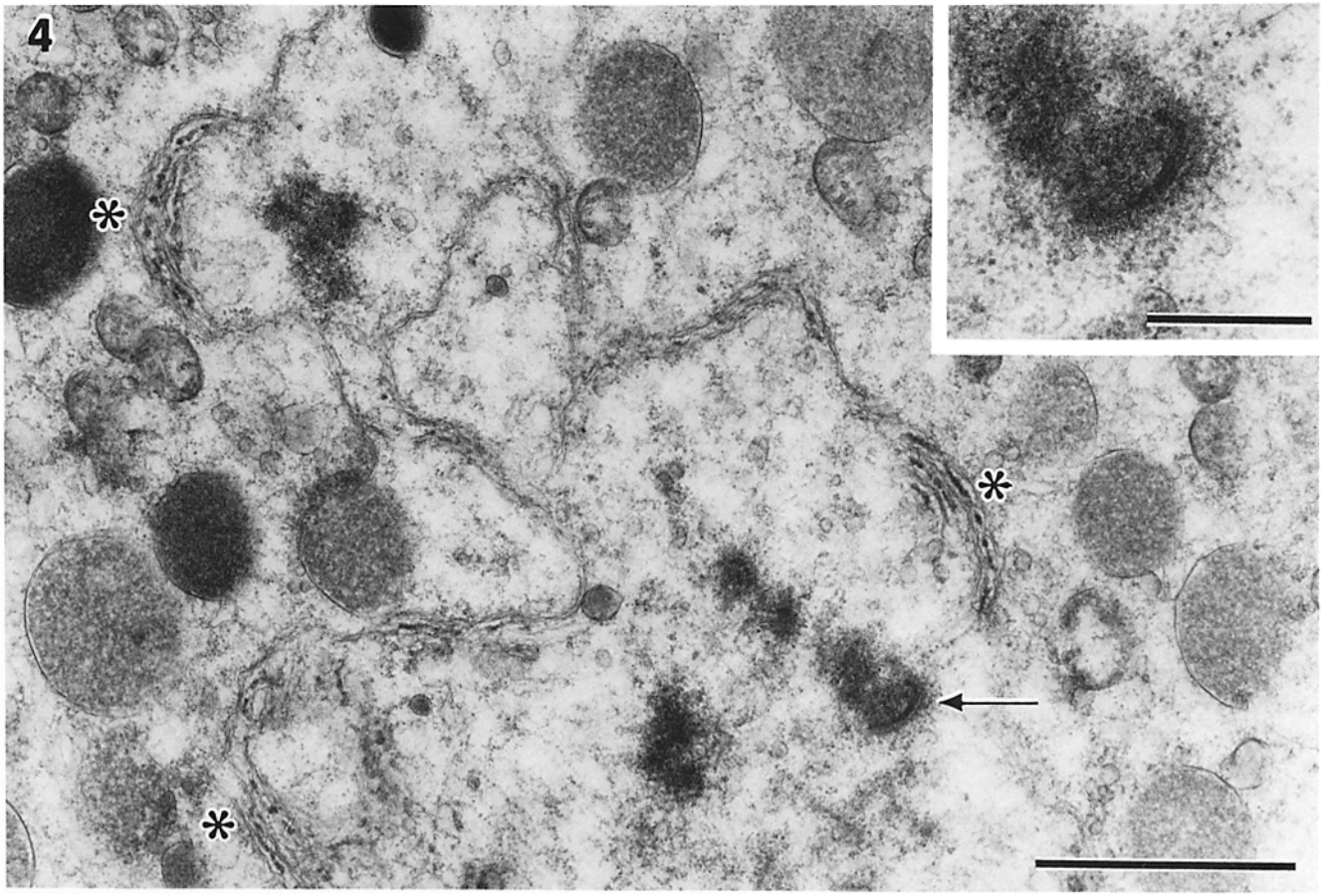
In the two additional eggs serially sectioned during this part of the study, the female pronuclear area contained karyomeres. Each of the female chromosomes was individually and intimately enveloped by NE material. The chromosomes in these telophase pronuclei lacked kinetochores and no MTs could be found in the vicinity of the reforming nucleus (data not shown).

An additional five eggs, each of which contained a weak monaster in the vicinity of the female pronucleus (see Fig. 2*e*, arrow), were also serially sectioned and examined with the high-voltage electron microscope. The results of this part of the study will be presented in detail elsewhere and will therefore only be summarized here. In brief, each of these female pronuclear areas contained a single monaster around which the chromosomes were grouped. This monaster lacked centrioles, contained an abundance of radially arrayed membranes, and had small patches of pericentriolarlike material into which numerous radially oriented MTs terminated. The chromosomes were truly mono-oriented around the monaster: Those kinetochores facing the monaster center were attached to MTs that formed a prominent fiber. The sister kinetochores, facing away from the monaster, lacked MTs. Similar mono-orientation of chromosomes has been previously described in sea urchin eggs containing monopolar spindles (17) or monasters (20).

DISCUSSION

In this study, we have tested the explicit form of the hypothesis that the chromosomes and their kinetochore fibers in dividing animal cells can organize a bipolar spindle without specialized polar structures. We developed an experimental system that allowed us to follow the behavior of chromosomes in a mitotic

FIGURES 4–7 (Fig. 4) Survey electron micrograph of the female pronuclear area from the same cell shown in Fig. 3 (see arrow in left inset). Prominent kinetochore is indicated by the arrow. Stacks of membranous elements are indicated by the asterisks. Note the numerous yolk granules in and around the nuclear area. × 17,000. Bar, 2.0 μm. (*Inset*) Higher magnification of the kinetochore indicated by the arrow. × 43,000. Bar, 0.5 μm. (Figs. 5–7) Electron micrographs of other sections from the same female pronuclear area as shown in Figs. 3 and 4. Note that the well-developed kinetochores (arrows) are devoid of MTs. A mitochondrion is denoted by *m* in Fig. 7. (Fig. 5) × 30,000; bar, 1.0 μm. (Fig. 6) × 42,000; bar, 0.5 μm. Fig. 7 × 34,000; bar, 0.5 μm.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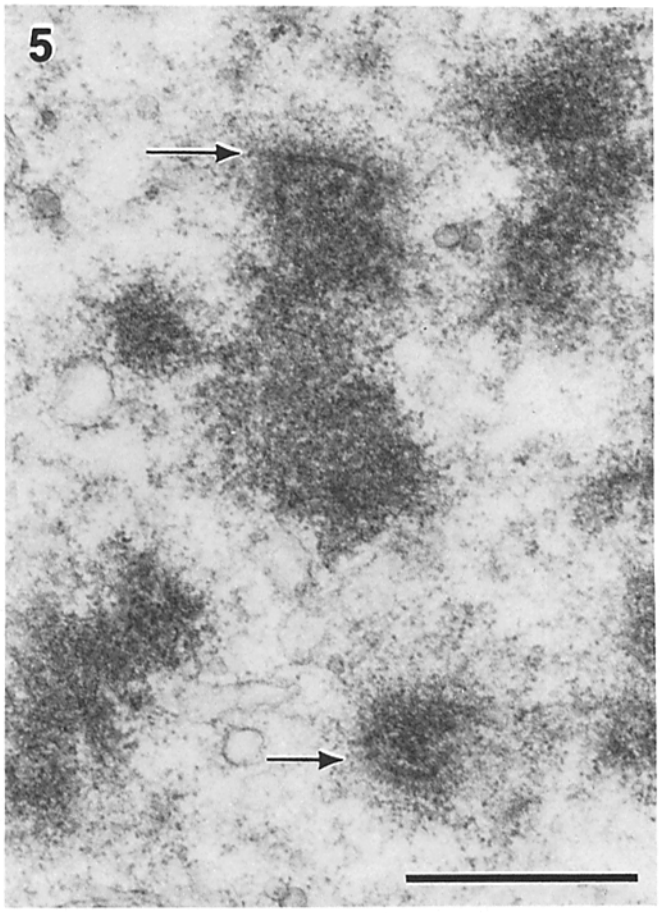
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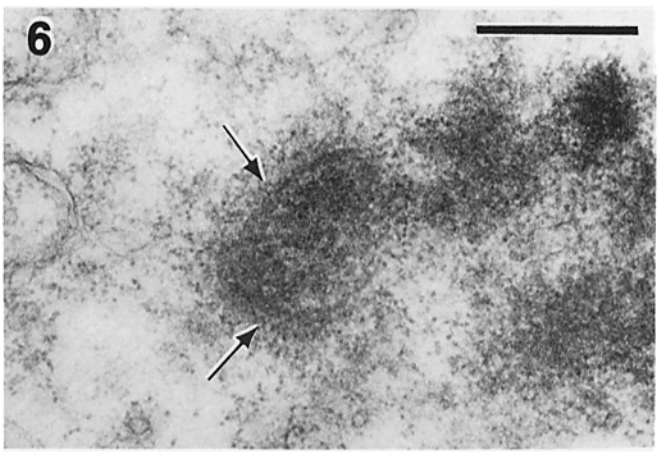
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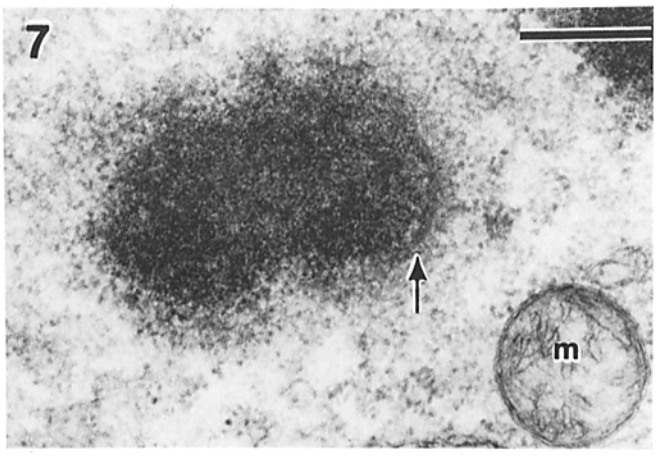
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cytoplasm when they were unequivocally free from centrosomal influence. In all cases examined, we found that chromosomes alone were not sufficient to organize a bipolar spindle. The chromosomes either remained randomly distributed in the nuclear area or became arrayed around the focus of a monaster. In the former cases, the well-defined kinetochores did not acquire MTs. Only in the latter cases, when a monaster formed in the female nuclear area, were MTs associated with kinetochores, and then only with kinetochores facing the astral focus.

Several observations show that this inability of the chromosomes to organize a bipolar spindle by themselves was due to the natural properties of the cell, not some peculiarity of the experimental system. First, the male chromosomes in the same cytoplasm formed a functional bipolar spindle in association with centrosomes. Second, we observed cases in which the male and female pronuclei were fortuitously close together before NE breakdown. After irradiation, a bipolar male spindle was assembled and all the female chromosomes became incorporated into this spindle. Third, other studies showed that the colcemid doses and the 366-nm-light irradiation used here have no adverse effects on the ability of chromosomes and centrosomes to establish a normal spindle (30, 31).

Our present observation that chromosomes in a mitotic cytoplasm cannot organize a bipolar spindle is consistent with demonstrations that animal cells can form monopolar spindles. Experimental manipulation of the reproduction of spindle poles can produce monopolar spindles in sea urchin eggs (17). From a functional standpoint, these monopolar spindles are truly half of a spindle in that two of them can come together to form a normal bipolar spindle. The kinetochores facing away from the pole are devoid of MTs, and there is no trace of a second pole (17). In addition, Bajer (3) has reported cases of monopolar spindles in newt lung cells. In all of these cases, if chromosomes without centrosomal influence were sufficient to organize a bipolar spindle, then one could not expect to find monopolar (or even monastral) spindles. All spindles would be bipolar with one astral and one anastral spindle pole.

In considering our present results, we are left with a basic question. If chromosomes alone are not sufficient to organize a bipolar spindle, then how can one explain the formation of anastral bipolar spindles? For the cells of higher plants, the amorphous material sometimes seen at the poles of the spindle (e.g., reference 15) may well anchor the chromosomes and define the bipolarity of the spindle. In fact, the bipolarity of the spindle in *Haemanthus* endosperm cells appears to be determined even before NE breakdown (29). Similarly, the anastral poles of mouse eggs contain amorphous material that can be stained with antibodies to centrosomes (8). Presumably this material organizes the bipolarity of the spindle in these eggs. Presently, there is no obvious explanation for Dietz's clear demonstration that crane fly spermatocytes can form bipolar spindles without asters (see reference 10). Either a portion of the pericentriolar material must have split off from the aster to organize the anastral pole, or spindle assembly in crane flies depends on unusual mechanisms.

In light of our present results, the observations of Ring et al. (27), that a cell line with multiple microtubule organizing centers divides in a bipolar fashion, remain an open question. Possibly they have demonstrated the existence of a specialized nonchromosomal mechanism that serves to bring multiple microtubule organizing centers together. The existence of such a mechanism is certainly necessary for the viability of the cell line.

Also, Karsenti et al. (13, 14) found that arrays of fibers (probably MTs) are assembled around centrosome-free karyoplasts or bacteriophage lambda-DNA that have been injected into *Xenopus* eggs. They claim that these MTs form anastral spindles similar to those found in plant cells. However, these arrays are unlike spindles in that the chromosomes lack kinetochore fibers and similar aggregates form around injected DNA that lacks centromeric sequences. Furthermore, anaphase chromosome movement has never been demonstrated for the injected chromosomes. All available evidence indicates that these workers are observing the formation of MT tactoids around groups of chromosomes.

One of the interesting findings of our study is that the female chromosomes cannot form kinetochore fibers when centrosomes or monasters are not present. The kinetochores are well-defined but totally devoid of MTs. Since the membranous lamellae surrounding the chromosomes are sufficiently fenestrated to allow for the entry of mitochondria into the nuclear area, these kinetochores are accessible to the cellular pool of tubulin. At face value, these results are inconsistent with those of others (9, 11, 35) working on tissue culture cells who report MT nucleation by kinetochores in the absence of centrosomal influence. The essential difference between our approach and theirs might be that they are looking at a system rapidly recovering from a colcemid or nocodazole block. At the start of recovery, these cells may have a relatively high concentration of free tubulin to drive MT assembly. We, on the the other hand, are looking at an equilibrium in which the female kinetochores must compete for tubulin with the male centrosomes. Alternatively, our results can be interpreted to indicate that kinetochore fibers are formed from centrosome-nucleated MTs.

Finally, our present work provides direct experimental proof for the long-standing paradigm that the centrosomes used in the development of the sea urchin egg comes from the sperm only. The original claim for the paternal origin of centrosomes came from examination of fixed preparations of normally fertilized sea urchin eggs. The drawings shown were consistent with the paradigm but did not prove it (34).

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Note Added in Proof: Recently, H. Schatten et al. (1982, *Eur. J. Cell Biol.* 27:74–87) showed that griseofulvin can block pronuclear movements in sea urchin eggs. At mitosis, a bipolar spindle is formed in association with the male chromosomes.

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