Control Mechanisms of the Cell Cycle: Role of the Spatial Arrangement of Spindle Components in the Timing of Mitotic Events

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ABSTRACT To characterize the control mechanisms for mitosis, we studied the relationship between the spatial organization of microtubules in the mitotic spindle and the timing of mitotic events. Spindles of altered geometry were produced in sea urchin eggs by two methods: (*a*) early prometaphase spindles were cut into half spindles by micromanipulation or (*b*) mercaptoethanol was used to indirectly induce the formation of spindles with only one pole. Cells with monopolar spindles produced by either method required an average of 3× longer than control cells to traverse mitosis. By the time the control cells started their next mitosis, the experimental cells were usually just finishing the original mitosis. In all cases, only the time from nuclear envelope breakdown to the start of telophase was prolonged. Once the cells entered telophase, events leading to the next mitosis proceeded with normal timing. Once prolonged, the cell cycle never resynchronized with the controls. Several types of control experiments showed that were not an artifact of the experimental techniques. These results show that the spatial arrangement of spindle components plays an important role in the mechanisms that control the timing of mitotic events and the timing of the cell cycle as a whole.

Spindle microtubules play a key role in coordinating the nuclear and cytoplasmic events that comprise mitosis. As a structural component of the cell they are not only essential for the proper execution of most mitotic events (3, 25), but they also function in the mechanisms that control the timing of these events (23). If microtubule assembly is specifically prevented with Colcemid, cells such as sea urchin eggs will continue through the cell cycle but spend about twice the normal amount of time in mitosis. The interphase portion of the cell cycle is of normal duration. Similar results are obtained when microtubule assembly is only partially inhibited; cells with spindles that are shorter and less birefringent than normal take significantly longer to reach anaphase onset than their normal counterparts, and the start of the next cell cycle is correspondingly delayed. Furthermore, experimental delays in the start of microtubule assembly directly determine when the cell will initiate anaphase, finish mitosis, and start the next cell cycle (23).

Although spindle microtubules are clearly involved in regulating the timing of mitotic events, the molecular basis of this regulation is unknown. Microtubules might modulate cell cycle timing by a direct feedback mechanism whereby the cell senses the fraction of the tubulin pool that is used in spindle assembly (24). Alternatively, the effect of microtubules on cell cycle timing may be indirect, for example through the activity of some microtubule-associated enzymatic process that is active only on the assembled microtubule. A third possibility is that the organization of microtubules in the mitotic apparatus determines the distribution of other organelles or cytoplasmic factors, which in turn influence timing.

To further investigate the role of microtubules in cell cycle timing, we have studied the relationship between the spatial organization of microtubules in the spindle and the timing of mitotic events. We used two independent methods to rearrange spindle microtubules without depolymerizing them. Both methods yielded identical results: cells with spindles that have altered microtubule arrangement spend substantially more time in mitosis than normal cells.

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MATERIALS AND METHODS

Lytechinus pictus (Pacific Bio-marine Laboratories Inc., Venice, CA) were maintained in modified Instant Ocean aquaria (Aquarium Systems, Mentor, OH). Gametes were obtained and handled as previously described (21). Eggs were fertilized and allowed to develop in artificial sea water or millipore-filtered natural sea water. All experiments were performed at 20–23°C.

Micromanipulation Experiments: Eggs were stripped of their fertilization envelopes at 1–2 min after fertilization by two passages through a "Nitex" screen with openings of ~80 μm (Tetko Inc., Elmsford, NY). The eggs were allowed to divide once and then were mounted in a manipulation chamber (2). To anchor the eggs, one surface of the upper coverslip was washed just before use with 0.25% (wt/vol) protamine sulfate (Sigma Grade 1; Sigma Chemical Co., St. Louis, MO) in distilled water, and then rinsed with sea water. This is the minimal treatment that gives adequate anchorage of the eggs. The eggs were spread on the treated surface of the coverslip before it was mounted on the chamber. The chamber was filled with FC-47 fluorocarbon oil (3M Co., St. Paul, MN) and the exposed face of oil capped with mineral oil.

Micromanipulation was performed with either an Ellis (5) or a modified Licht (E. Licht Co., Denver, CO) piezoelectric micromanipulator. The Licht manipulator head was controlled by a military surplus joystick assembly (No. JS7600, C and H Sales, Pasadena, CA) with a 10-turn potentiometer mounted on the handle. Observations and photographs were made with Nikon Model S or Zeiss WL microscope bodies adapted for polarization microscopy (21). Microneedles were made as previously described (2). Photographs were recorded on Kodak Plus-X film and developed in Kodak Microdol-X.

Mercaptoethanol Experiments: Fertilized eggs were immersed in 0.1 M mercaptoethanol (Sigma Chemical Co.) in sea water at metaphase of the first division and remained in the mercaptoethanol until a separate control culture reached second metaphase. They were then washed several times with sea water to completely remove the mercaptoethanol (22). About one-half hour later these eggs were mounted for observation as previously described (21). The birefringent retardation of monopolar and bipolar spindles was measured with a Zeiss Brace-Kohler compensator modified to give a digital readout in nanometers (19).

All of the experiments presented in this report were performed on the fertilized eggs of the sea urchin *L. pictus*. In addition, some experiments were repeated on the eggs of *Lytechinus variegatus*, with identical results.

Colcemid Experiments: Spindles of reduced length and birefringence were induced by "pulsing" eggs with 5×10^{-6} M colcemid for 2–3.5 min in early prophase as described previously (21). Such treatments are not sufficient to completely prevent all microtubule assembly.

RESULTS

Experimental System

Spindles of altered geometry were produced directly by micromanipulation and indirectly by mercaptoethanol treatments. To denote the method of origin and to apply terminology that is consistent with published work (13, 22) we will use the following terms in this paper: half spindles are produced by micromanipulation and monopolar spindles are induced by mercaptoethanol. As our results will show, half spindles and monopolar spindles appear to be morphologically and functionally identical. For all practical purposes, both are half of a spindle, since two of either can be brought together to give a functional bipolar spindle of normal appearance.

First and second mitoses in a *L. pictus* egg are shown in Fig. 1 to illustrate normal division and to serve as a comparison for the experimental cells shown in following plates. The progress of experimental and control cells through mitosis was determined by observing nuclear events such as nuclear envelope breakdown, the start of the normal anaphase separation of half spindles (Fig. 1 *d*) and nuclear envelope reformation. These all provide relatively precise temporal markers. For experimental cells we also used the distribution and quantity of spindle birefringence to monitor their progress into telophase. This is important because spindles of altered geometry do not show a traditional anaphase movement of chromosomes; chromosomes pop apart but the chromatids do not move to opposite poles (14). In normal cells, the birefringence of the central spindle and asters gradually increases after nuclear envelope breakdown and reaches its highest value at or shortly after anaphase onset (Fig. 1, *a–c*). In early telophase (~5 min after anaphase onset) the half-spindle birefringence fades rapidly and the asters assume a larger, more diffuse, fibrous appearance (Fig. 1, *d* and *e*).

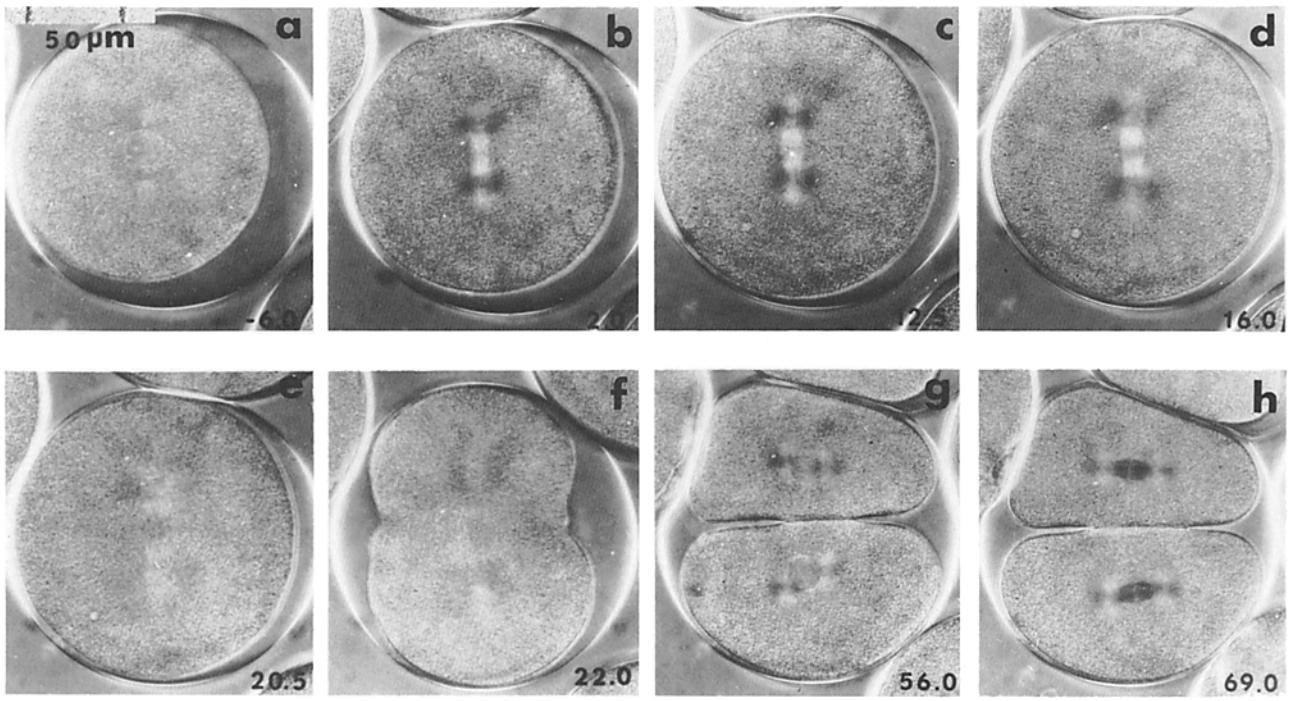

FIGURE 1 First and second mitosis in the fertilized egg of *L. pictus*. (*a*) Before first nuclear envelope breakdown; (*b*) prometaphase; (*c*) metaphase; (*d*) early anaphase; (e) telophase fading of spindle birefringenece; (*f*) early cleavage; (g) early prophase of second mitosis; (*h*) prometaphase of second mitosis. Minutes before and after first nuclear envelope breakdown are shown in the lower corner of each frame. Polarization micrographs; additive and subtractive compensation. Bar, 50 μm. × 260.

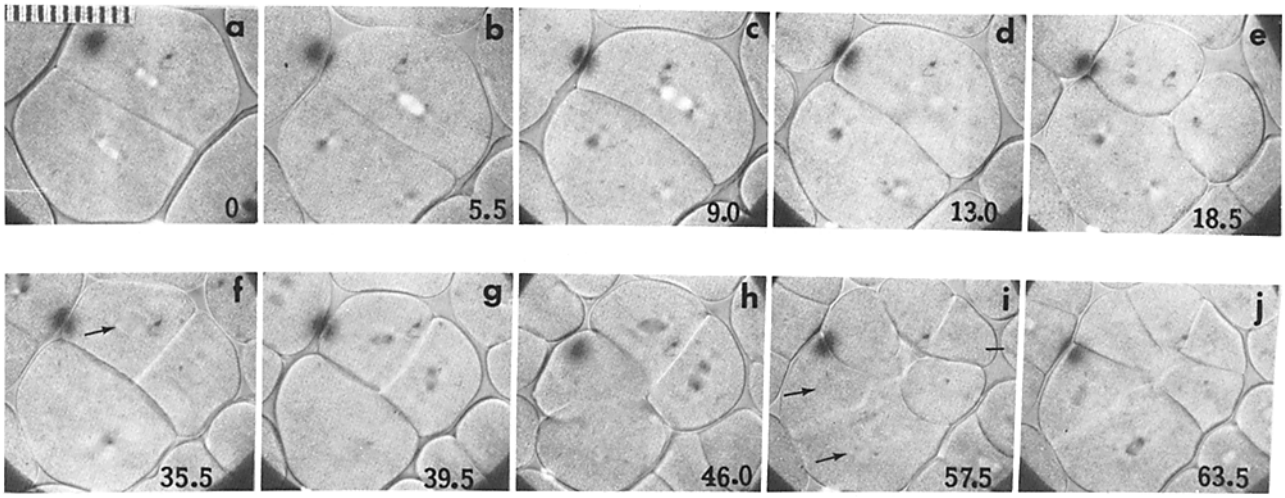

FIGURE 2 Development and timing of a cell with two half spindles; (*a*) before manipulation, both daughter cells are in prometaphase of second mitosis; (*b*) spindle in lower blastomere cut with microneedle; (*c–e*) manipulated cell stays in mitosis while control completes mitosis; (*f–h*) manipulated cell enters telophase and cleaves while control enters third mitosis; (*i*) manipulated cell reforms nuclei while control cleaves; (*j*) manipulated cell enters third mitosis while control cells are preparing for fourth division. Arrows indicate position of nuclei. Minutes after second nuclear envelope breakdown are given in the lower corner of each frame. Polarization micrographs; additive and subtractive compensation. Bar, 50 μm. × 164.

Astral microtubules are no longer focused on a small central spot; instead the aster takes on a rather squared appearance. Shortly thereafter the cell begins to cleave, nuclear envelopes start to reform around individual chromosomes or groups of chromosomes (Fig. 1*f*), and DNA synthesis begins (6, 7).

In experimental cells, the monopolar or half spindles show similar changes in birefringence during mitosis. Thus, the telophase fading of spindle birefringence will be used in this study to determine, within a few minutes, when the experimental cells have entered telophase. Although sister chromatids in monopolar spindles do split as in anaphase onset (D. Mazia, unpublished observation, and reference 14), this is not detectable in vivo with the polarization microscope.

An important feature of the sea urchin system is that the start of second mitosis normally occurs synchronously in the two daughter blastomeres (Fig. 1, *g* and *h*). Thus, while one daughter is used as an experimental cell, the other serves as an ideal timing control. In this way even very small differences in timing (~30 s) can be easily detected.

TABLE I

Time from Nuclear Envelope Breakdown to Telophase

	Micromanipulation		Mercaptoethanol		Colcemid
	Experimental	Control	Experimental	Control	Experimental
$\bar{x}$	48.0	14.5	48.9	14.9	22.2
r	20.5–63.0	8.5–22.5	14–102	8.5–22.5	14–38
s	15.1	3.7	21.2	3.53	6.6
n	7	11	24	33	44

Comparison of time from nuclear envelope breakdown to fading of spindle birefringence in telophase for experimental and control cells. All values are in minutes. Note that control cells are from the same embryos as the experimental cells. $\bar{x}$ is mean; *r* is range; *s* is the standard deviation; *n* is the number of cells. Fewer experimental cells are included in this data than control cells because we show here timing data on only the experimental cells for which we have good quantitative data. In the micromanipulation experiments, we successfully cut a total of 19 spindles but include only 7 here. For the mercaptoethanol experiments, we observed 49 cells but include only 24 here. All experimental cells, however, showed timing patterns that were qualitatively the same.

Micromanipulation Experiments

Fertilized, demembranated eggs were mounted in a micromanipulation chamber just before second nuclear envelope breakdown. In early prometaphase, the spindle of one blastomere was engaged with a microneedle and broken into two half spindles. The tip of the microneedle was inserted into the region of the metaphase plate and moved rapidly back and forth perpendicular to the spindle axis. The needle tip was then removed and reinserted in each spindle pole and pulled laterally. After one or more repetitions of this operation, the two half spindles separated and could be moved apart. With only one exception, the separated half spindles did not spontaneously reassociate to give a bipolar spindle.

The results of a typical experiment are shown in Fig. 2. Throughout the operation the birefringence of the manipulated spindle remains at roughly the same level as the control spindles. The separated half spindles often show a nonuniform distribution of birefringence; one side of the aster has more microtubules, suggesting that the chromosomes are localized to one quadrant. With time the separated half spindles become progressively more robust (Fig. 2, *b–e*). After a variable period of time, the manipulated cell finishes mitosis and starts its next cell cycle. The asters become more diffuse and fibrous, the birefringence fades, the cell cleaves, and nuclei reform (Fig. 2, *f–i*). These telophase events proceed with normal appearance and coordination except that the cleavage furrow is sometimes weak and later regresses. Later mitoses of the manipulated cell appear normal; functional bipolar spindles of normal appearance are formed (Fig. 2*j*) and the cells go through these mitoses at an approximately normal rate.

From the standpoint of cell cycle timing, the manipulated cells always spend substantially more time in mitosis than their normal counterparts (Table I). In the case shown here (Fig. 2) the manipulated cell still has birefringent, well-defined, half spindles when the control has already gone through telophase and cleaved (Fig. 2*e*). By the time the experimental cell reaches telophase, the progeny of the control cell have initiated their next mitosis and are assembling spindles (Fig. 2, *f* and *g*). However, once the experimental cell starts to enter

telophase as seen by changes in half-spindle birefringence, events proceed at an approximately normal pace. This indicates that the manipulation prolonged only the prometaphase/metaphase portion of mitosis. Once the cell cycle of the manipulated cell is prolonged, it never resynchronizes with the controls (Fig. 2, *h* and *j*).

We have performed 19 such experiments on two species of *Lytechinus* and always observe the patterns of timing described above (Table I). The time from nuclear envelope breakdown to the telophase fading of half-spindle birefringence was on average 48 min. This is about three times as long as the time normal cells take to traverse the same portion of the cell cycle ($\bar{x} = 15$ min). We also note that the variability in duration of mitosis is greater for the manipulated cells than their controls. The reason for this is not understood.

In some cases, the postmanipulation development of the experimental cell is slightly different. Occasionally a cell remains in mitosis long enough for the pole of each half spindle to split, giving two pairs of asters (Fig. 3). Although, splitting and separation of the daughter poles are normally late telophase events that prepare the cell for the next division (13), they can occur before telophase if mitosis is sufficiently prolonged. As the two asters of each half spindle move apart, the chromosomes become incorporated into a metaphase plate, yielding bipolar spindles of normal appearance. These spindles are functional in that they move chromosomes in anaphase (Fig. 3*f*) and induce a cleavage furrow.

Of the 19 cells in which we cut spindles, 6 showed the formation of bipolar spindles from half spindles as described above. In two other cases, the half spindles started to develop into bipolar spindles but the cells entered telophase before the process was complete.

Micromanipulation Control Experiments

To test whether micromanipulation can nonspecifically damage the cell, we performed a series of control manipulations. In the first we inserted the microneedle into one daughter cell and vigorously stirred the cytoplasm, closely duplicating the motions used to break spindles. In 10 such experiments neither spindle birefringence, nor the timing of mitotic events, was in any way affected. Both the control and the manipulated cells proceeded synchronously through mitosis and on into the next division.

The results of another control experiment in which we actually engaged a spindle and rotated it by ~45° is shown in Fig 4. Although the spindle is slightly distorted at first (Fig. 4*c*), the cell initiates anaphase, disassembles its spindle, cleaves, and enters the next mitosis in synchrony with the control (Fig. 4, *e–h*).

In addition, we performed three experiments in which we cut a daughter spindle into two half spindles and then pushed the halves back together again. An example of such an experiment is shown in Fig. 5. Shortly after nuclear envelope breakdown the lower spindle is cut in two (Fig. 5*b*). When the halves are pushed back together again (Fig. 5*c*), they reassociate to give a functional spindle of normal appearance (Fig. 5*e*). Later this spindle initiates anaphase and the cell goes through a normal sequence of telophase events. The next mitosis is also normal (Fig. 5*h*). The third division spindles (Fig. 5*h*) appear squat because they are tilted out of the plane of the photograph. Mitosis takes somewhat longer in these manipulated cells. These experiments show that breaking a spindle into two half spindles does not prevent normal spindle function when the two halves are subsequently recombined and argues that the manipulation is not an irreversible damage to the spindle.

In all of our experiments, manipulated cells never appeared unhealthy relative to other cells in the preparation, and observations never had to be terminated because of later cell death.

Mercaptoethanol Experiments

As an entirely independent means of producing spindles of altered geometry, we induced the formation of monopolar spindles using the mercaptoethanol technique developed by Mazia et al. (13).

For a complete discussion of the logic behind the induction of monopolar spindles, the reader should see references 13 and 22. Briefly, monopolar spindles are indirectly induced by putting the splitting and replication of mitotic centers out of phase with each other. The effect is not due to nonspecific damage to the cell because identical results can be obtained by prolonging mitosis with Colcemid at microtubule-specific doses or by micromanipulation (Sluder and Begg, manuscript in preparation).

The experiment is performed by treating eggs with mercaptoethanol at metaphase of the first division for ~1 h. Upon removal of the drug, the eggs immediately form a tetrapolar spindle and later cleave into four cells. At the next mitosis, each daughter cell forms a monopolar spindle.

To easily compare the timing of cells containing monopolar spindles with those containing bipolar spindles, we made use of a fortuitous occurrence. Sometimes in mercaptoethanol-treated eggs, one cleavage furrow regresses and the egg cleaves

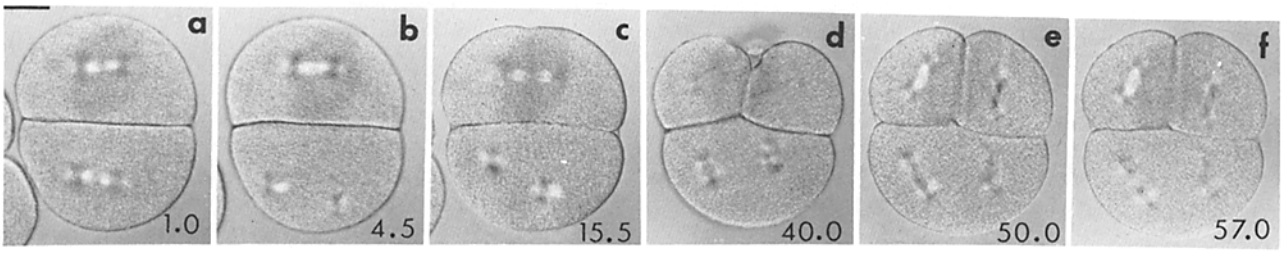

FIGURE 3 Development and timing of a cell with two half spindles. (a) Before manipulation, two daughter cells in early prometaphase of second mitosis; (*b*) spindle of lower blastomere cut in half with microneedle; (*c–f*) manipulated cell stays in second mitosis while the control cell completes mitosis and goes well into its third division; (*d–f*) in the manipulated cell the asters of the two half spindles split and move apart. The chromosomes become aligned into metaphase plates yielding two functional bipolar spindles; minutes after second nuclear envelope breakdown are given in the lower corner of each frame. Polarization micrographs; additive and subtractive compensation. Bar, 40 μm. × 175.

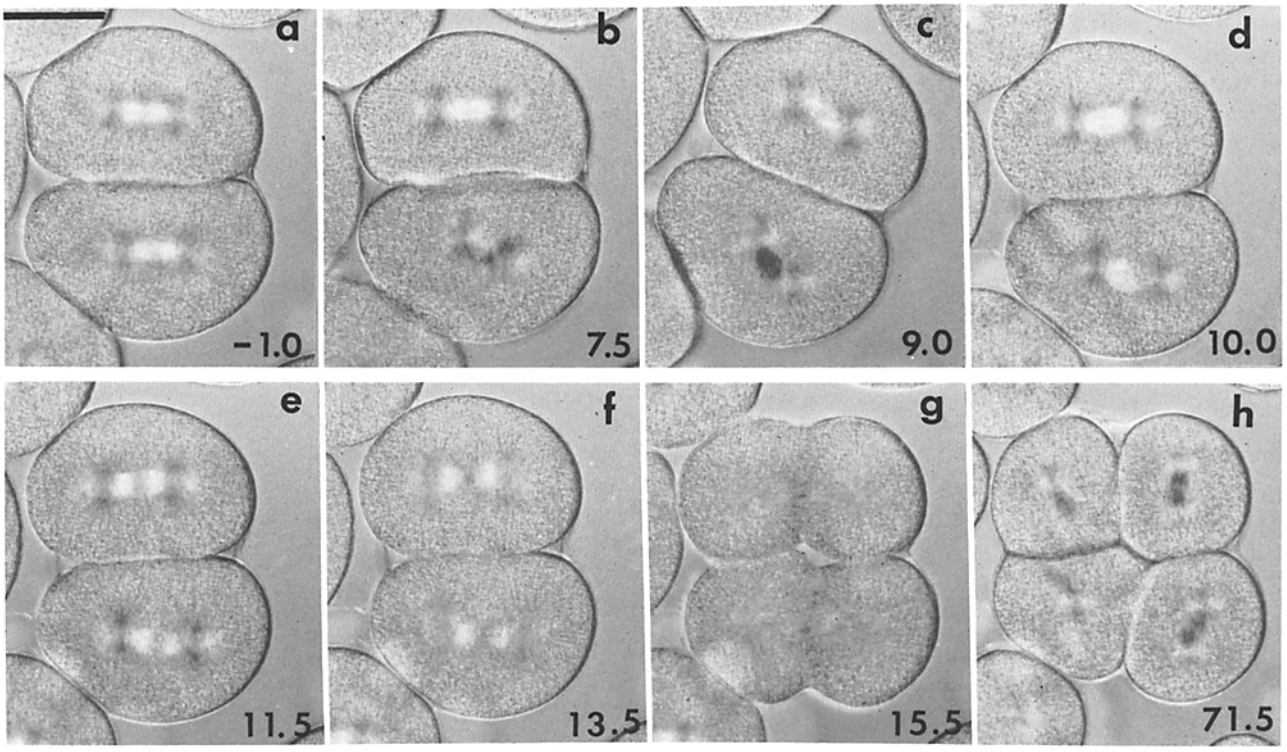

FIGURE 4 Control experiment for micromanipulation technique. (*a*) Before manipulation. Two daughter cells in early prometaphase of second division; (*b–d*) the spindle is engaged with the microneedle and rotated ~45°. With time the spindle returns to its original position. The microscope stage was rotated in frame *c* to show the manipulated spindle better; (*e–g*) the manipulated cell initiates anaphase, goes through telophase and cleaves in synchrony with the control blastomere; (*h*) progeny of both original cells enter third mitosis and assemble spindles synchronously. Minutes before and after the start of the manipulation are shown in the lower corner of each frame. Polarization micrographs; additive and subtractive compensation. Bar, 20 μm. × 625.

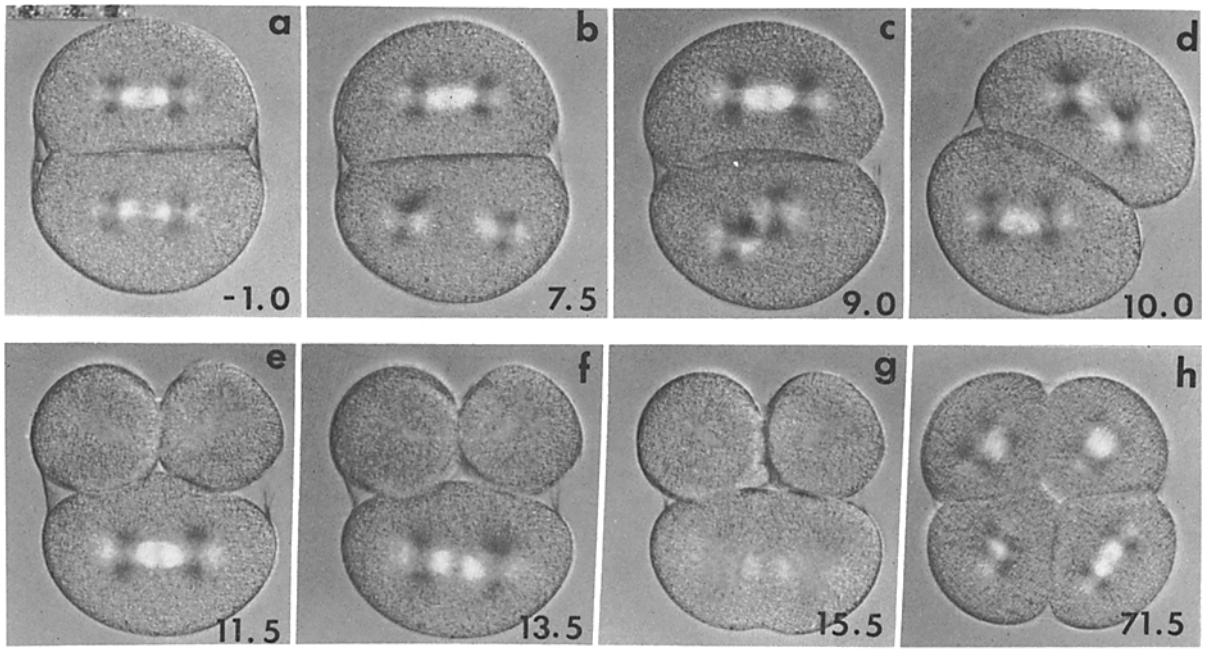

FIGURE 5 Spindle cut in half and put back together. (*a*) Before manipulation, two daughter cells in early prometaphase of second mitosis; (*b*) spindle in lower cell is cut with a microneedle; (*c*) several minutes later the half spindles are pushed back together; (*d*) these reassociate to give a normal, functional spindle. In this frame the microscope stage is rotated to show the manipulated spindle; (*e*) the manipulated spindle is in late metaphase, the control cell has completed mitosis; (*f–g*) anaphase and telophase in the manipulated cell appear normal; (*h*) progeny of both original cells enter third mitosis and assemble normal bipolar spindles. Some spindles are tilted and thus appear short. Minutes before and after the manipulation are shown in the lower corner of each frame. Polarization micrographs, additive and subtractive compensation. 10 μm per scale division. × 625.

into three cells rather than four. This gives one daughter cell with a bipolar spindle (two monopolar spindles that have come together), and two daughters each with a monopolar spindle. The morphology, development, and timing of such bipolar spindles are normal. Thus, we have an ideal internal control; all cells being compared have been through the same experimental regime, only the number and spatial arrangement of the poles being different.

The timing of cells with monopolar spindles is similar to that of cells with half spindles produced by micromanipulation. Cells with monopolar spindles spend substantially more time in mitosis than do their counterparts with bipolar spindles. As shown in Fig. 6, *a–e* the birefringence of the monopolar spindle starts to fade in telophase (Fig. 6 *d*) only when the control cell has completed mitosis and is well into interphase.

As in the micromanipulated cells, only the prometaphase/metaphase portion of mitosis is prolonged; telophase events proceed with normal timing. Also, the cell cycle never resynchronizes with the control, but remains permanently out of phase.

The amount of time cells with monopolar spindles spend in mitosis is highly variable, even among equivalent cells of the same embryo. In extreme cases, cells with monopoles can spend as much as seven times longer in mitosis than normal cells. These points are illustrated in Fig. 7. The upper left cell stays in mitosis longer than the control, but does go into telophase well ahead of its sister (Fig. 7, *d* vs. *j*). Such asynchrony between two daughters with monopoles is not uncommon. This case also illustrates the tremendous amount of time a cell with a monopole can spend in mitosis (upper right cell). This cell stays in mitosis for >102 min while the control goes through three full divisions. By comparison, control cells spend, on average, 15 min between nuclear envelope breakdown and the start of telophase.

Fig. 7 also shows an example of a case in which a monopolar

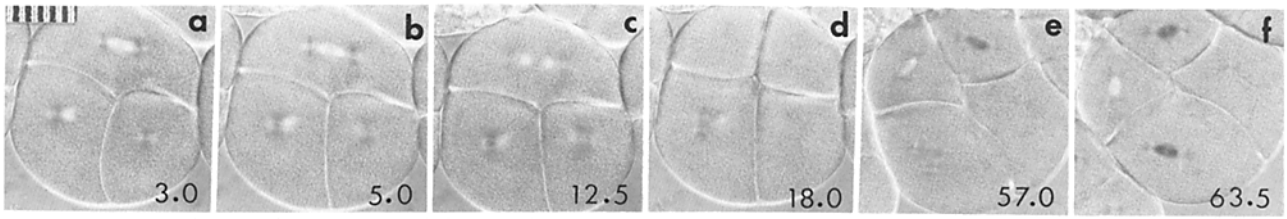

FIGURE 6 Mercaptoethanol experiment. Cell has cleaved into three after removal of the mercaptoethanol. Upper cell has two poles and forms a bipolar spindle that is used as a control. (*a*) Prometaphase, lower left cell has a monopolar spindle, lower right cell has only an aster; (*b–d*) control cell initiates anaphase and finishes mitosis. The cell with a monopole remains in mitosis and is just entering telophase when the control has already started its next cell cycle. (*e*) The progeny of the control cell have entered the next mitosis. The cell that had a monopole does not cleave and reforms a single nucleus; (*f*) the cell that once had a monopole enters the next mitosis and forms a functional bipolar spindle of normal appearance. Polarization micrographs, additive and subtractive compensation. Minutes before and after second nuclear envelope breakdown are given in the lower corner of each frame. 10 μm per scale division. × 175.

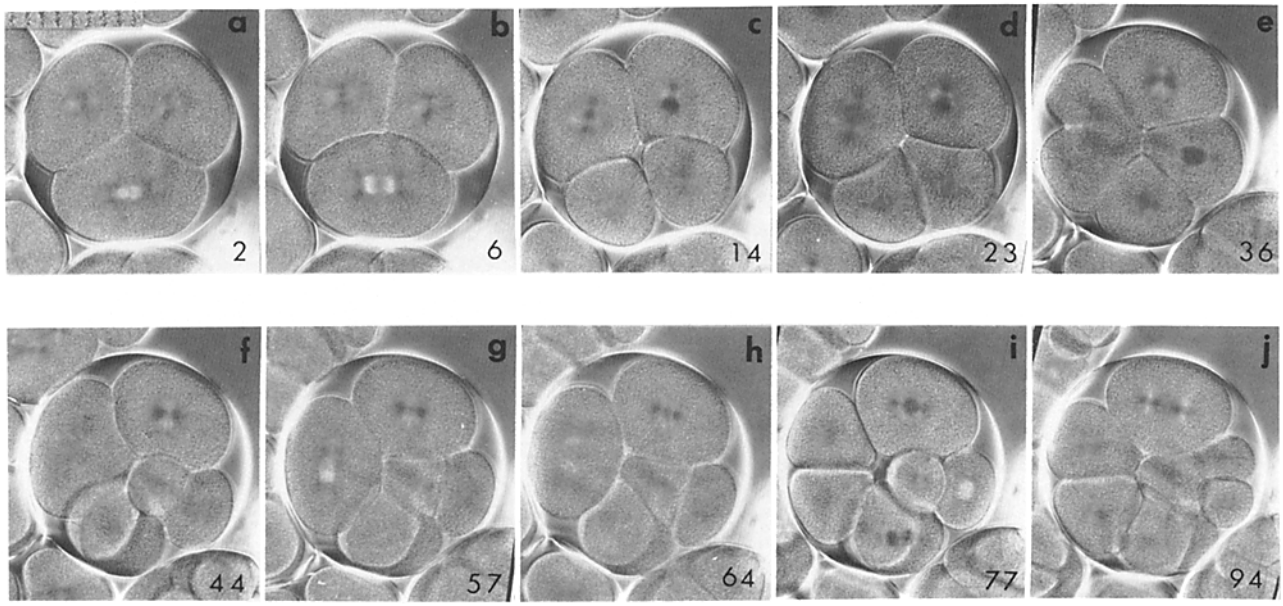

FIGURE 7 Mercaptoethanol experiment. This egg has cleaved into three cells after removal of mercaptoethanol; lower cell has two poles and thus forms a bipolar spindle. (*a–e*) The upper left cell stays in mitosis longer than the control. The aster of the monopole splits yielding a bipolar spindle and the cell initiates an abortive cleavage furrow; (*e*) the microscope stage was slightly rotated counterclockwise to better show the monopole in the upper right cell; (*a–j*) the upper right cell remains in mitosis while the control cell goes completely through three mitoses. The monopolar spindle eventually does split and the two asters move apart giving a bipolar spindle; minutes after second nuclear envelope breakdown are given in the lower corner of each frame. Polarization micrographs, additive and subtractive compensation. 10 μm per scale division. × 210.

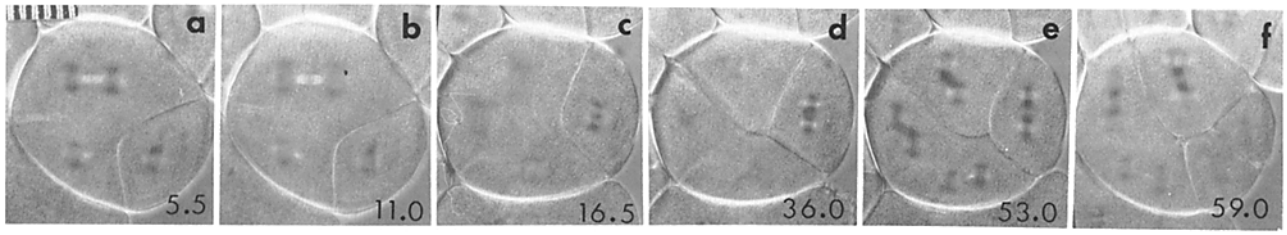

FIGURE 8 Mercaptoethanol experiment; monopolar spindle in the same cytoplasm as a bipolar spindle. Here two cleavage furrows have regressed giving a cell with only a monopolar spindle (lower right) and a larger cell having both a monopole and a bipole in the same cytoplasm. (*a*) Mid prometaphase; (*b*) bipolar spindle initiates anaphase; (*c–d*) its birefringence fades in telophase, and a furrow cleaves the cell. (*b–d*) The monopolar spindle in the common cytoplasm shows synchronous development. (*e*) Daughter nuclei from the bipole and the single nucleus from the monopole all enter the next mitosis synchronously. (a–e) The monopole alone in a cell shows substantially different timing. This cell stays in mitosis throughout the time the larger cell takes to finish the original mitosis and enter the next. (*c* and *e*) The cell with only a monopolar spindle stays in mitosis long enough that the monopole splits and the asters move apart giving a functional bipolar spindle. (*e*) this spindle is shown in anaphase. The microscope stage was rotated slightly counterclockwise at the time of *c*. Minutes after nuclear envelope breakdown are shown in the corner of each frame. Polarization micrographs, additive and subtractive compensation. 10 μm per scale division. × 170.

spindle splits during mitosis, producing two asters. As in the case of half spindles produced by micromanipulation, these asters separate. The chromosomes become incorporated into a metaphase plate (Fig. 7, *g–j*) giving a bipolar (albeit aneuploid) spindle of normal appearance that later initiates a normal anaphase. Although this cell spends much more time in mitosis than a cell with a normal bipolar spindle, the sequence, morphology, and timing of the telophase events are normal.

We performed this type of experiment on 41 eggs of *L. pitcus* and 8 eggs of *L. variegatus*; in all cases, the cells with monopolar spindles took significantly more time to traverse in mitosis than cells of the same embryo that had bipolar spindles (Table I).

Monopolar and Bipolar Spindles in a Common Cytoplasm

To determine whether monopolar spindles have unusual stability or whether they impart unusual properties to cells, for example by releasing a hypothetical cell cycle inhibitor, we studied the timing of cells that had a monopole in the same cytoplasm as a bipolar spindle. A typical example of such an experiment is shown in Fig. 8. Here a furrow regresses, leaving two cells, one with only a monopole and the other with both a monopole and a bipole in a common cytoplasm. Nuclear envelope breakdown and the start of spindle assembly occurred synchronously for all three nuclei. The development of the bipole shows normal timing and morphology; anaphase onset occurs at approximately the normal time, 10–15 min after nuclear envelope breakdown, and the birefringence quickly fades a few minutes later (Fig. 8 *b*). The development of the monopole in the same cytoplasm shows similar timing; its birefringence fades at the same time that the bipole enters anaphase (Fig. 8, *a–d*). Later, the cell cleaves across the axis of the bipole (Fig. 8 *d*) and three nuclei reform synchronously (two from the bipole and one from the monopole). In comparison, the timing of the cell with only a monopolar spindle is dramatically different. It remains unchanged (Fig. 8 *e*) while the other cell completes mitosis, goes through interphase, and starts its next mitosis. This cell finishes mitosis about one full cell cycle behind the cell with the bipole/monopole combination (Fig. 8 *f*).

Additional experiments of this type show that there does not seem to be any "dosage" effect. A cell that has two monopolar spindles in the same cytoplasm as a bipolar spindle shows the same timing as a cell with only a bipolar spindle. These observations rule out the possibility that a monopole can retard the development of a bipolar spindle in a common cytoplasm. Occasionally, there is a slight lag (1–3 min) in the telophase disassembly of the monopolar spindle. This may reflect the time necessary for the cellular changes induced by the bipolar spindle to reach the monopole.

Diminished Spindles

Previous work showed that the quantity of spindle microtubules influences the duration of mitosis (23). Eggs treated with low doses of Colcemid assemble short, barrel-shaped spindles of reduced length and birefringence. Such cells spend up to twice the normal amount of time between nuclear envelope breakdown and anaphase onset. To determine whether the increase in mitotic duration experienced by cells with monopolar spindles was due to the altered microtubule arrangement or to a lower amount of tubulin polymer in the cell, we compared the timing of cells with monopolar spindles to that of cells with diminished bipolar spindles, produced by treating cells with Colcemid. Colcemid at the dosages used here produces no detectable side effects (23).

A comparison of typical monopolar, bipolar, and diminished bipolar spindles is shown in Fig. 9, all printed to the same scale. The spindle length between the chromosomes and pole is approximately the same in the monopolar and normal bipolar spindles. Also, the extent of astral development is the same. The diminished spindle, however, is less than half as long (21) and has tiny asters. In monopoles the birefringence of the region between the chromosomes and the pole is the same or slightly higher than that of the corresponding region of the bipole, ~2.5 nm at metaphase. However, the birefringence of the central portion of diminished spindles, such as the one shown here, is 1–1.3 nm at metaphase, or half that of the normal bipole or monopole (see reference 21 for additional data).

Both the microtubule distribution and birefringence indicate that monopolar spindles have at least as much or more total polymerized tubulin than do diminished bipolar spindles. Any differences in cell cycle timing between the two should be due to microtubule arrangement, not number.

Table I shows a comparison of the duration of mitosis (nuclear envelope breakdown to the telophase fading of spin-

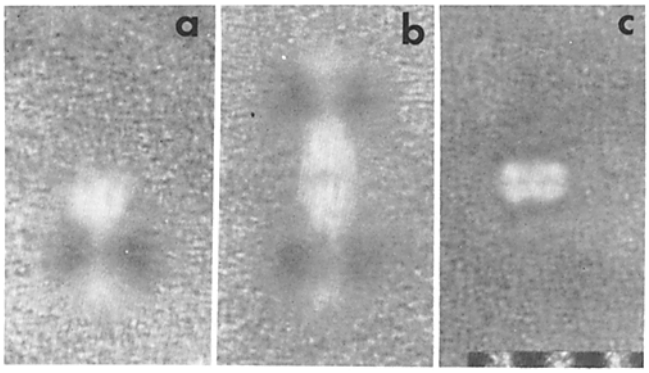

FIGURE 9 Size comparison for (a) monopolar spindle, (b) normal bipolar spindle, (c) Colcemid-diminished spindle. All photographs are printed at the same magnification. The birefringence of the region between the chromosomes and the pole is the same in the monopolar and bipolar spindles. The measured birefringence of the diminished spindle is about half that of the other two. Polarization micrographs, additive compensation. Bar, 10 μm. × 700.

dle birefringence) for cells containing monopolar spindles with cells that have diminished bipolar spindles. The data show that cells with *monopolar* spindles spend substantially *more time* in mitosis than cells with diminished spindles. Thus, two poles, even though much smaller than normal, enable a cell to go through mitosis faster than cells with one large pole.

DISCUSSION

The work presented here further characterizes the functional nature of the mechanisms that control the rate at which a cell traverses the mitotic portion of the cell cycle. Previous work has shown that both the time and quantity of microtubule assembly determine when the cell will initiate anaphase, finish mitosis, and start the next cell cycle (23). Here we show that the spatial arrangement of spindle microtubules is also an important parameter of these control mechanisms. Regardless of how we change the organization of the spindle, mitosis is substantially prolonged in every case. Generally cells with half spindles or monopolar spindles remain in mitosis until the controls have entered their next division. In one case (Fig. 7), a cell with a monopolar spindle remains in prometaphase long enough for its control cell to go completely through three divisions.

Only the prometaphase/metaphase portion of mitosis is prolonged. Telophase events and the initiation of the subsequent mitotic cycle proceed with normal timing. The system behaves as if timing is no longer sensitive to altered spindle geometry once the cell enters telophase and is committed to finishing mitosis. This pattern of temporal relationships, a variable prometaphase/metaphase and an invariant telophase/interphase, is identical to that observed when Colcemid is used to specifically modulate the time and extent of microtubule assembly (23). This strengthens our conclusion that we are studying the same phenomenon and are characterizing another facet of the way microtubules operate in the mechanisms that control the timing of mitotic events.

These results also demonstrate that in altering the spatial organization of microtubules of the spindle we are not simply inhibiting the expression of any individual mitotic event, but are profoundly affecting the progression of the cell through the cell cycle. Furthermore, the observation that the experimental cells never resynchronize with the controls rules out the possibility that the cell cycle in sea urchin eggs is governed solely by a continuous biochemical oscillator similar to the one proposed to govern the cell cycle of *Physarum* (10, 26). The explicit form of this model predicts that prolonging any one mitotic division should not prevent the subsequent mitosis from occurring on schedule.

The phenomena observed in the present study are not peculiar to the two species of sea urchins we used. Workers using other species of sea urchin (4) and, more recently, primary explants of amphibian lung cells (1) report, in passing, that cells with monopolar spindles spend more time in mitosis than equivalent cells with bipolar spindles. It is important to note that in the latter study, the monopolar figures often resulted from the failure of the two spindle poles to separate at the onset of mitosis. These cells have a normal number of centrioles, chromosomes, and other cellular components, yet their timing is altered. Thus, the rearrangement of spindle elements alone is sufficient to change the timing of mitotic events.

Control Experiments

When viewed in the simplest fashion, the techniques of micromanipulation and mercaptoethanol serve as controls for each other. Micromanipulation directly gives half spindles and avoids possible chemical injury to the cell, whereas mercaptoethanol indirectly induces half spindles while avoiding mechanical damage. In both cases the response of the cell is identical from the standpoint of timing.

Direct tests for possible mechanical injury show that neither stirring of the cytoplasm nor rotating the spindle (without breaking it) alter timing; the operated cell divides synchronously with its control. Furthermore, when we push separated half spindles back together again, the halves always reassociate to give a spindle of normal appearance and function.

Mercaptoethanol also does not appear to produce any nonspecific damage to the cells. It indirectly induces the formation of monopolar spindles by prolonging mitosis, thereby altering the reproduction of mitotic centers (13, 22). Furthermore, when a furrow fails to separate two monopoles, they can associate to form a bipolar spindle of normal appearance and function. Such cells have undergone exactly the same treatment as the cells with monopoles; the only difference is in the number of spindle poles and possibly the number of chromosomes. Also, later mitoses in cells that once had monopoles are normal.

Rearranging Other Spindle Components

In rearranging spindle microtubules, we have also altered the distribution of other spindle components such as vesicles, centrosomal structures, and chromosomes. This is especially true in the micromanipulation experiments in which we cut spindles in half after they had started to assemble. In principle we could possibly influence the timing of the cell cycle by altering either the distribution of microtubules within the spindle or the arrangement of other spindle components. However, for the purposes of this paper it is not necessary to resolve this issue. We know from earlier work with Colcemid that experimental manipulations of the extent and time of microtubule assembly alone are sufficient to influence cell cycle timing (23). Thus, the basic principle we wish to demonstrate does hold: spatial relationships between elements

within the spindle are an important aspect of the pathways that control the timing of mitotic events. It is not sufficient to think of mitosis (and the cell cycle) as being controlled simply by the sequential appearance of freely diffusable substances (9, 10, 12, 15). We must also look to the interactions between properly positioned cytoplasmic components of the spindle for the mechanisms that control the mitosis portion of the cell cycle.

Transition Points

The temporal relationships observed in both our present study and in the earlier Colcemid study point to the existence of an event or physiological change that is an important transition point in the cell cycle. This is the event that commits the cell to initiate telophase, finish mitosis, and start the next cell cycle. In a normal cell, the splitting of the kinetochore regions at anaphase onset is the first visible manifestation that this event has occurred. However, from the standpoint of cell cycle timing, it is a matter of indifference whether the chromosomes actually move apart or not. Once this transition point has been reached, the chain of telophase events is set in motion; cleavage, reformation of nuclei, and the start of the next cell cycle follow in rapid succession. Such a transition point was first suggested by Mazia and termed a "point of no return" (12). Although we cannot specify in molecular or ionic terms the identity of this event, we can detect it by a change in the functional properties of the cell cycle. Before this point the progress of the cell through mitosis is flexible, while after its occurrence the cell cycle proceeds through mitosis normally, irrespective of the state of its microtubules. This is not surprising since anaphase and telophase are the time when the cell normally disassembles its spindle microtubules anyway.

While we still do not know how spindle microtubules influence the timing of the mitotic portion of the cell cycle, our present work elucidates some of the basic properties of these controls. We know that the spindle must exert a positive effect on the cell to trigger the transition point. Since a monopolar spindle does not slow the timing of the normal spindle, it cannot be acting to repress the system.

Our results are not consistent with the hypothesis that the cell is monitoring the utilization of the tubulin pool. Cells with half spindles have about the same amount of tubulin polymer as those with normal spindles, yet the timing of the cell cycle is profoundly different. Furthermore, cells with diminished bipolar spindles always go through mitosis faster than cells with an equivalent or greater amount of microtubule polymer in a monopolar or half-spindle configuration.

Similarly, our data are inconsistent with models that predict that microtubules influence the cell cycle through some associated enzyme that works in concert with the quantity of assembled tubules to regulate levels of control substances. Clearly the total amount of microtubule polymer is not as important as the arrangement of existing microtubules. If such an enzyme did exist, for example a dyneinlike ATPase that moves organelles at mitosis, its importance for the cell cycle must depend upon a properly organized microtubular scaffolding, rather than on the total amount of microtubule polymer.

At present, the most attractive possibility for the role of microtubules in the timing mechanism is that they provide the cell with a structural scaffolding that has the proper geometry to allow some rate-limiting process to take place, such as the specific spatial localization of substances or organelles within the cell. For example, vesicles that are thought to be physiologically active in Ca^{++} sequestration are collected into the spindle (11, 16, 17, 20), and their accumulation is Colcemid sensitive (16, 17, 23). Recent work suggests that the level of free intracellular Ca^{++} during mitosis can influence when the cell will initiate anaphase (8, 27). However, our data indicate that any simple scheme for redistribution, such as movement of substances toward or away from the cell cortex, is probably naive. When we compare the timing of monopolar spindles or half spindles to the timing of spindles greatly diminished with Colcemid, we find that the small bipolar spindles always allow the cell to traverse prometaphase faster. Neither considerations of the total amount of microtubule polymer nor microtubule length (astral "reach") can explain this observation. Conservative estimates of total polymer using the magnitude and distribution of birefringence show that monopolar spindles have at least as much total polymer as do diminished spindles. Cells with half spindles clearly have more polymer. In addition, Colcemid preferentially depolymerizes astral microtubules (18, 21); diminished spindles have such small asters that they could not reasonably be expected to localize as much in the way of substances or organelles as monopolar or half spindles. Thus, an important conclusion that can be drawn from our work is that the cell cycle behaves as if the control mechanisms are more sensitive to microtubule arrangement than to microtubule number or length.

Conclusions

The work presented here, taken together with the results of the previous Colcemid study (23), shows that the assembly of spindle microtubules in a spatially correct fashion is a vitally important facet of the mechanisms that control the timing of mitosis. Perhaps the reason for this is that the critical aspect of mitosis is the establishment and maintenance of a number of specific spatial relationships within the dividing cell. Before the cell commits itself to anaphase, the poles must separate to establish a division axis; the chromosomes must be aligned on the metaphase plate; and daughter chromatids must be oriented to opposite poles. Failure to maintain these spatial relationships results in aneuploidy and the eventual loss of viability. To minimize the chance of mistakes, the control mechanism has two important features. First, it allows for wide temporal tolerances for the whole process. As we have seen, the duration of prometaphase/metaphase is flexible, and the cell cycle allows more than enough time for the assembly of the labile spindle structure (23). Second, the control mechanism is set up such that both the quantity and the spatial arrangement of spindle microtubules determine when the cell will go through the critical transition point that commits it to finish mitosis and start the next cell cycle. Thus, the cell has a way to ensure that it is ready to divide correctly before it actually commits itself to completing the process.

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