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The checkpoint control for anaphase onset does not monitor excess numbers of spindle poles or bipolar spindle symmetry

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SUMMARY

Exit from mitosis in animal cells is substantially delayed when spindle assembly is inhibited, spindle bipolarity is disrupted, or when a monopolar spindle is formed. These observations have led to the proposal that animal cells have a 'spindle assembly' checkpoint for the metaphase-anaphase transition that monitors bipolar spindle organization. However, the existence of such a checkpoint is uncertain because perturbations in spindle organization can produce unattached kinetochores, which by themselves are known to delay anaphase onset. In this study we have tested if cells monitor bipolar spindle organization, independent of kinetochore attachment, by analyzing the duration of mitosis in sea urchin zygotes and vertebrate somatic cells containing multipolar spindles in which all kinetochores are attached to spindle poles. We found that sea urchin zygotes containing tripolar or tetrapolar spindles progressed from nuclear envelope breakdown to anaphase onset with normal timing. We also found that the presence of supernumerary, unpaired spindle poles did not greatly prolong mitosis. Observation of untreated PtK_1 cells that formed tripolar or tetrapolar spindles revealed that they progressed through mitosis, on average, at the normal rate. More importantly, the interval between the bipolar attachment of the last monooriented chromosome and anaphase onset was normal. Thus, neither of these cell types can detect the presence of gross aberrations in spindle architecture that inevitably lead to aneuploidy. We conclude that animal cells do not have a checkpoint for the metaphase-anaphase transition that monitors defects in spindle architecture independent of the checkpoint that monitors kinetochore attachment to the spindle. For dividing cells in which spindle microtubule assembly is not experimentally compromised, we propose that the completion of kinetochore attachment is the event which limits the time of the metaphase-anaphase transition.

Key words: Anaphase, Cell Cycle, Checkpoint, Kinetochore, Microtubule, Mitosis, Spindle

INTRODUCTION

The equal segregation of chromosomes during mitosis requires the establishment of a bipolar spindle axis and the attachment of all sister chromatids to opposite spindle poles. Since spindle assembly involves ever changing associations of dynamically unstable microtubules, the occurrence of errors is a normal part of the mitotic process (concepts discussed by Nicklas, 1989; Rieder et al., 1994; Nicklas and Ward, 1994). Given that the amount of time required for the complete resolution of mistakes in spindle assembly can be quite variable, the cell would risk unequal chromosome distribution if the time of the metaphase-anaphase transition were determined by an invariant timing mechanism (concepts reviewed by Hartwell and Weinert, 1989).

A number of observations have led to the proposal that eukaryotic cells possess a 'spindle assembly' checkpoint that delays anaphase onset when spindle assembly or bipolarity are perturbed (Sluder, 1979; Sluder and Begg, 1983; Minshull et al., 1989; Hoyt et al., 1991; Li and Murray, 1991; Hunt et al., 1992; Kubiak et al., 1993; Andreassen and Margolis, 1994; reviewed by Murray, 1992, 1994; Earnshaw and MacKay, 1994; Gorbsky, 1995; Wells, 1996). Complete inhibition of spindle microtubule assembly significantly delays the metaphase-anaphase transition or arrests some cell types in mitosis (reviewed by Rieder and Palazzo, 1992). For somatic cells even subtle changes in spindle assembly caused by extremely low doses of microtubule poisons (Colcemid, nocodazole, or vinblastine) or the microtubule stabilizing agent taxol lead to significant delays in the metaphase-anaphase transition even though all kinetochores appear to establish functional attachments to the bipolar spindle and move to the metaphase plate (Zieve et al., 1980; Jordan et al., 1991, 1992, 1993; Rieder et al., 1994).

Importantly, the duration of mitosis is also greatly increased by defects in the spatial arrangement, or architecture, of spindle microtubules. In vertebrate somatic cells mitosis is prolonged approximately two to threefold if the cell

assembles a monopolar spindle (Bajer, 1982; Wang et al., 1983; Jensen et al., 1987). Similarly, in sea urchin zygotes mitosis is prolonged threefold when the zygotes are induced to form monopolar spindles by either of two independent methods (Sluder and Begg, 1983; also see Hunt et al., 1992). Furthermore, when the spindle in a sea urchin zygote is microsurgically cut into two half spindles, the duration of mitosis is tripled even though the cell contains the normal number of spindle poles and the normal complement of astral microtubules (Sluder and Begg, 1983). These results suggest that the controls for the metaphase-anaphase transition can detect defects in the bipolar organization of the spindle or the presence of unpaired spindle poles that are not interacting with each other.

However, the existence of a checkpoint that specifically monitors spindle architecture is open to question, because disruption of spindle organization can also produce unattached kinetochores and perturbations of microtubule-kinetochore interactions can lead to attached kinetochores that experience less than normal tension (see McIntosh, 1991). In vertebrate somatic cells even a single unattached kinetochore substantially delays the metaphase-anaphase transition (Rieder et al., 1994, 1995). In insect spermatocytes the checkpoint for the metaphase-anaphase transition appears to monitor tension at the kinetochores (Li and Nicklas, 1995; Nicklas et al., 1995).

The goal of our current study was to determine whether alterations in spindle organization delay the metaphase-anaphase transition through the action of the checkpoint that monitors kinetochore attachment or the action of a separate checkpoint that can detect an improperly organized spindle. We reasoned that we could distinguish between these two possibilities by determining the duration of mitosis in cells containing multipolar spindles with all chromosomes bioriented between pairs of spindle poles. In these cells such gross defects in spindle architecture, which directly lead to aneuploidy, should delay anaphase onset if cells do in fact have a checkpoint that monitors spindle organization. Although there have been numerous reports of multipolar spindles in cultured cells (Heneen, 1970, 1975; Pera and Rainer, 1973; Keryer et al., 1984; Gosh and Paweletz, 1987; Selitto and Kuriyama, 1988) and sea urchin embryos (Mazia et al., 1960; Sluder and Begg, 1985), the duration of mitosis in such cells relative to bipolar controls, and how it relates to kinetochore attachment, has never been explored. For our present study we worked with sea urchin zygotes and vertebrate somatic cells because the checkpoint that monitors chromosome attachment has been partially characterized in these model systems (Sluder et al., 1994; Rieder et al., 1994, 1995).

In addition, we have determined the duration of mitosis in sea urchin zygotes that contain supernumerary asters in order to examine the possibility that the spindle assembly checkpoint could monitor some aspect of centrosome-centrosome interaction (see Bailly and Bornens, 1992, for the possible involvement of centrosomes in checkpoint controls). The rationale for this portion of the study is the correlation that zygotes with a monopolar spindle stay in mitosis much longer than normal (Sluder and Begg, 1983) and have a spindle pole that is unpaired (i.e. does not have a partner with which to interact). Conceivably unpaired spindle poles have an activity that inhibits the metaphase-anaphase transition.

MATERIALS AND METHODS

Sea urchin zygotes

Lytechinus pictus and *L. variegatus* sea urchins were purchased from Marinus Inc. (Long Beach, CA) and Susan Decker (Hollywood, FL), respectively. Eggs and sperm were obtained by intracoelomic injection of 0.5 M KCl (Fuseler, 1973). Zygotes were cultured and observed in natural sea water at 18-20°C.

To produce zygotes with multipolar spindles we treated fertilized eggs for 1-6 minutes with 5×10^{-6} M Colcemid (Sigma Chemical Co., St Louis, MO) starting when syngamy was complete approximately 30 minutes after fertilization. The duration of the Colcemid treatment was adjusted for the eggs of each female to produce short barrel shaped spindles with greatly diminished asters at first mitosis (see Sluder, 1976, 1979). Although the zygotes proceeded through mitosis, the anaphase separation of chromosomes was greatly diminished and cleavage furrows either failed to form or failed to become complete. At the end of first mitosis, zygotes were mounted in fluorocarbon oil preparations as previously described (Sluder, 1979). Before second nuclear envelope breakdown (NEB) individual zygotes containing only a single nucleus or two closely spaced nuclei were irradiated for 30 seconds with 366 nm light to photochemically inactivate the Colcemid and allow the cells to assemble a full complement of spindle microtubules (reviewed by Sluder, 1991). Individual zygotes with multipolar spindles were followed and the times of nuclear envelope breakdown and anaphase onset determined by polarization microscopy with a modified Zeiss ACM microscope (Carl Zeiss Inc., Thornwood, NY) or an Olympus BH-2 microscope equipped for differential interference contrast (Olympus Corporation of America, Melville, NY). Photographs were recorded on Kodak Plus X film which was developed in Kodak Microdol-X (Eastman Kodak Inc., Rochester, NY).

For *L. pictus* control zygotes we quantified the duration of first or second mitoses in zygotes that were individually irradiated with 366 nm light for 30 seconds shortly before first or second NEB. For *L. variegatus* controls we followed untreated zygotes through first and second mitoses.

To examine chromosome distributions in multipolar sea urchin zygotes, aliquots of zygotes during second mitosis were pelleted and fixed in 3 parts 95% ethanol plus 1 part glacial acetic acid. Later drops of fixed zygotes were placed on slides and stained with 2% orcein in 75% acetic acid and covered with coverslips. The preparations were flattened by mechanical pressure and observed by phase contrast microscopy with a ×100 oil immersion objective on a Zeiss Axioscope microscope (Carl Zeiss Inc., Thornwood, NY).

To induce the formation of supernumerary asters in the presence of a bipolar (or multipolar) spindle, eggs were fertilized in natural sea water and treated 10 minutes later with hypertonic sea water (8 ml of 2.5 M NaCl added to 50 ml of sea water and adjusted to pH 8.9 with NaOH) for 15-35 minutes. The treatment duration was empirically determined for each batch of eggs in order to induce the formation of some but not too many cytasters. The zygotes were then washed twice with natural sea water and cultured in beakers prior to mounting in fluorocarbon oil preparations. The interval between NEB and anaphase onset for first mitosis was determined by polarization microscopy.

Cultured PtK1 cells

PtK_1 cells (2N = 12) were maintained and cultured for light microscope studies as previously described by Rieder et al. (1994, 1995). Since the duration of mitosis is highly temperature dependent (Rieder, 1981) the medium bathing the cells was kept at 35-37°C using a previously described custom-designed cell culture heater (Rieder et al., 1994).

Selected cells were followed by time-lapse video light microscopy using a Nikon Diaphot inverted light microscope equipped with ×100 (NA 1.25) or ×60 (NA 1.4) phase contrast objectives. Cells were illuminated with shuttered, monochromatic (546 nm) heat-filtered light obtained from a 100 W tungsten bulb. Video images, captured with a

DAGE MTI model VE1000 Newvicon tube camera (Dage MTI, Wabash, WI), were routed through an Argus 10 (Hamamatsu Photonics, Bridgewater, NJ) or an IMAGE 1 (Universal Imaging Corporation, West Chester, PA) image processor. Noise in the optical and electronic systems was eliminated by background subtraction, and recording an 8 frame jumping average. Processed images were stored on a Panasonic Model TQ 2025F optical memory disk recorder, or a Panasonic Model AG-6760P Super VHS time lapse recorder (ADCO Aerospace, Ft. Lauderdale, FL), using framing rates of 4-15 frames/minute.

We followed binucleate cells from prophase through anaphase. Additional cells with multipolar spindles were identified and filmed after nuclear envelope breakdown (NEB) through anaphase; these cells all contained one or more monooriented chromosomes at the onset of the recordings. The times of nuclear envelope breakdown (NEB), anaphase onset, and congression of the last monooriented chromosome were determined for each cell and statistical analysis was conducted using Quattro Pro 6.0 software (Novell, Oren, UT).

RESULTS

Sea urchin zygotes with multipolar spindles

We compared the duration of mitosis (here taken as the time from NEB to anaphase onset) for normal zygotes with zygotes containing multipolar spindles in which all chromosomes were attached in a bipolar fashion between pairs of spindle poles. To produce zygotes with tripolar or tetrapolar spindles, we treated fertilized eggs with Colcemid before first mitosis to diminish, but not fully prevent, spindle microtubule assembly. As a consequence, the zygotes assembled short barrel shaped spindles with very small asters (Fig. 1A) (also see Sluder, 1976). As the zygotes proceeded through first mitosis the extent of anaphase chromosome separation was greatly reduced (Fig. 1B). As a result, the zygotes reformed only a single nucleus or two closely spaced nuclei (Fig. 1C). Although a cleavage furrow may have been initiated in some cases, rarely did it become complete due to the substantial diminution of the asters (Rappaport, 1969). The zygotes were then irradiated during interphase with 366 nm light to photochemically inactivate the Colcemid and allow the assembly of a full complement of spindle microtubules at second mitosis (reviewed by Sluder, 1991). At second mitosis the zygotes assembled tetrapolar spindles (Fig. 1D-E, and see Fig. 3A) or when two of the spindle poles failed to separate, tripolar spindles. Fixed and stained preparations revealed that the chromosomes were all

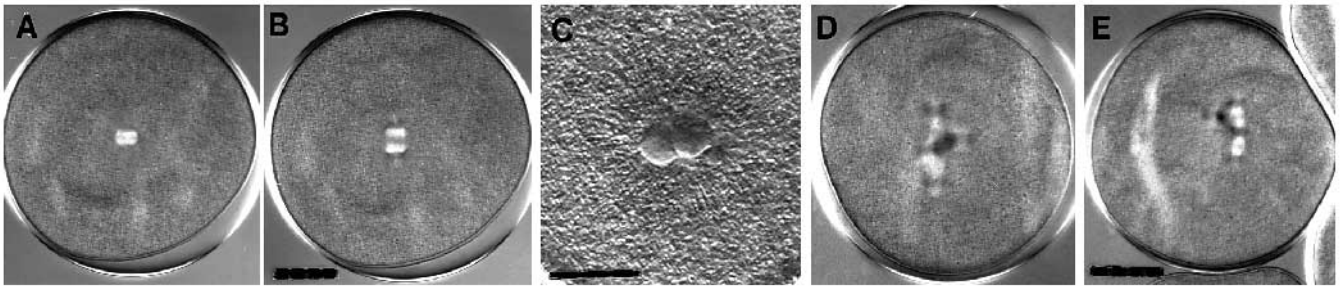

Fig. 1. Induction of multipolar spindles in sea urchin zygotes. (A) Colcemid induced assembly of a short barrel shaped spindle at first mitosis. (B) Early anaphase in the same zygote: chromosome separation is greatly reduced. (C) Either a single nucleus or two closely spaced daughter nuclei reform in telophase (shown here for a different zygote at higher magnification with differential interference contrast optics). The cleavage furrow does not form or fails to become complete. (D) Shortly before second nuclear envelope breakdown zygotes are irradiated with 366 nm light to photochemically inactivate the Colcemid. Shown here is an example of a tetrapolar spindle assembled at second mitosis. (E) Another example of a tetrapolar spindle. (A,B,D,E) Polarization optics. (C) Differential interference contrast optics. Bars: 40 μm (B,E); 30 μm (C).

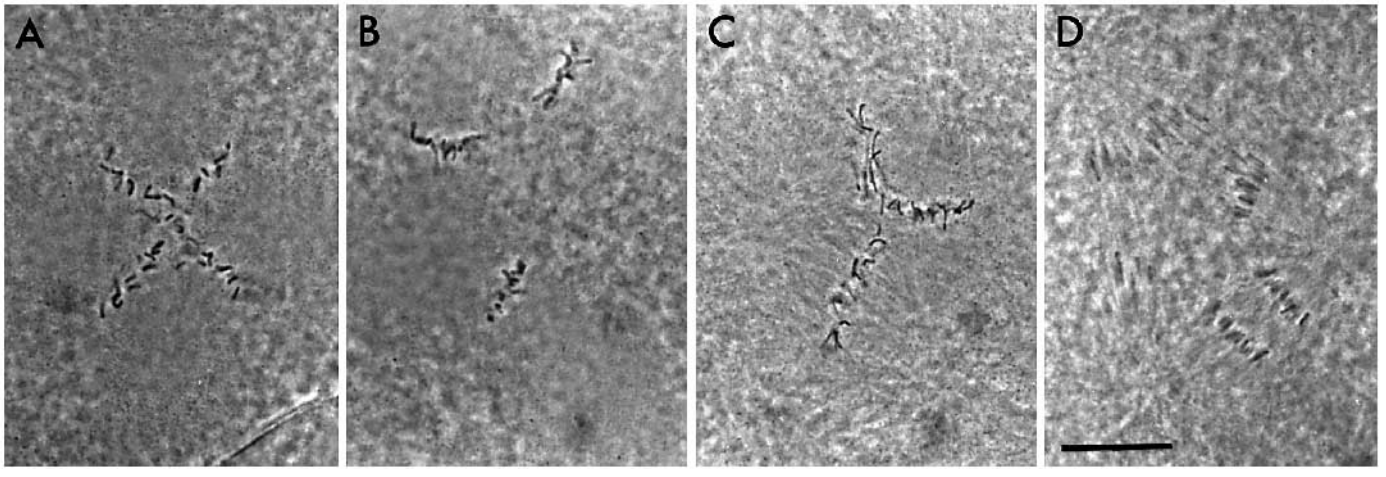

Fig. 2. Fixed and stained preparations showing examples of chromosome distribution in sea urchin zygotes with multipolar spindles. (A) Chromosomes all bioriented between pairs of spindle poles of a tetrapolar spindle. (B) Tetrapolar spindle with three metaphase plates. (C) Chromosome distribution in a tripolar spindle. (D) Early anaphase in a tripolar spindle; all chromatids are attached to spindle poles. Phase contrast optics. Bar, 10 μm.

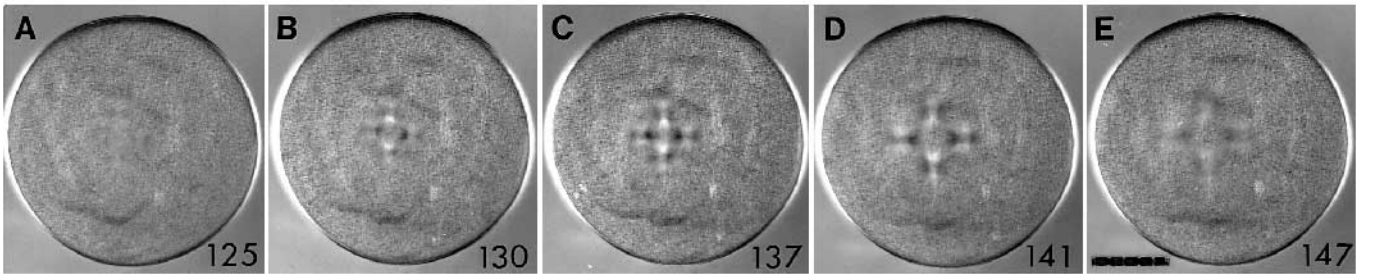

Fig. 3. Progression of second mitosis in a zygote with a tetrapolar spindle. (A) Shortly before second nuclear envelope breakdown. (B and C) Prometaphase assembly of the spindle. (D) Early anaphase. (E) Onset of spindle disassembly in early telophase. Minutes after fertilization are shown in the lower corner of each frame. Polarization optics. Bar, 50 μm.

attached in a bipolar fashion between pairs of spindle poles (Fig. 2A-C). Despite the multipolarity, spindle assembly, anaphase, and telophase events appeared to be normal (Fig. 3A-E). Anaphase onset, as seen by microtubule distribution in the polarization microscope and chromosome disjunction in fixed/stained preparations, was synchronous for all parts of the multipolar spindles. For zygotes fixed in anaphase, all chromosomes appeared to be attached to spindle poles and we observed no lagging chromosomes in the region between the poles (Fig. 2D). The chromosomes appear to be randomly distributed to pairs of spindle poles, consistent with the findings of Mazia et al. (1960) and Bibring (1962) for chromosome distribution on multipolar spindles induced by prolonging mitosis with mercaptoethanol.

For *L. pictus* controls we used zygotes that were individually irradiated with 366 nm light for 30 seconds shortly before first or second NEB. For *L. variegatus* controls we used untreated zygotes at first and second mitoses. The first control mitosis allows us to directly compare the timing of zygotes with bipolar and multipolar spindles in the original cell volume. The second control mitosis corresponds directly to the number of mitoses the experimental zygotes have undergone. For untreated and irradiated control zygotes we found that second mitosis is approximately one to two minutes shorter than first mitosis (also see Sluder, 1979).

Our data are summarized in Table 1. For *L. pictus* or *L. variegatus* zygotes there was no statistically significant difference between the duration of second mitosis for zygotes with tripolar and tetrapolar spindles. For *L. pictus* zygotes the duration of the multipolar second mitosis was not significantly different from the first mitosis in irradiated controls and on average approximately one minute longer than second mitosis in irradiated controls, a difference that is not statistically significant. For *L. variegatus* zygotes the duration of multipolar second mitosis (tripolar and tetrapolar values pooled) was not significantly different from the duration of first mitosis in untreated controls. Multipolar second mitosis was on average 1.2 minutes longer than second mitosis in untreated controls, which is a statistically significant difference (t=2.21, 0.05>P>0.02). That second mitosis is normally two minutes shorter than first mitosis, suggests that multipolar zygotes at

Table 1. Minutes from NEB to anaphase onset: multipolar and control zygotes

	Mean	*n*	s.e.m.	Range
L. pictus				
Tripolar spindles	14.4	20	0.78	9-22
Tetrapolar spindles	14.0	55	0.42	7-19
Supernumerary centrosomes	17.3	27	0.6	12-25
Control 1st mitosis	14.3	47	0.30	10-20
Control 2nd mitosis	13.4	46	0.53	8-28
L. variegatus				
Tripolar spindles	10.6	7	0.48	8-12
Tetrapolar spindles	11.4	10	0.54	9-15
Pooled data	11.1	17	0.38	8-15
Control 1st mitosis	11.7	32	0.28	9-15
Control 2nd mitosis	9.8	22	0.39	7-13

Values for mean, standard error of the mean (s.e.m.), and range are given in minutes. *n*, the number of zygotes followed in each category.

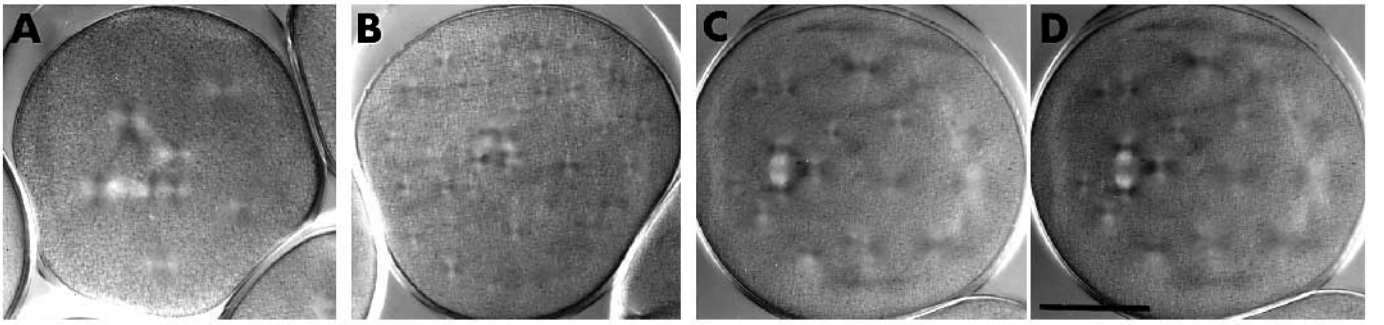

Fig. 4. First mitosis in zygotes with supernumerary asters and the formation of multipolar spindles. (A) Example of a zygote with a few cytasters. (B) Example of a zygote with many cytasters, only some of which are visible in this plane of focus. (C and D) Before and after anaphase onset for a zygote with supernumerary asters. Polarization optics. Bar, 50 μm.

second mitosis progress through mitosis at a similar rate to those in first mitosis. Perhaps this is related to the fact that second division multipolar zygotes have the same cytoplasmic volume as control zygotes at first mitosis.

Sea urchin zygotes with unpaired spindle poles

Two findings suggest the possibility that an unpaired spindle pole, that cannot interact with a partner pole, has an activity that delays the metaphase-anaphase transition in sea urchins. Zygotes with one or two monopolar spindles spend threefold the normal time in mitosis when the spindle poles do not interact with each other and when 50% of the chromosomes have unattached kinetochores (all chromosomes monooriented) (Sluder and Begg, 1983). However, when 50% of the chromosomes are completely unattached in the presence of a bipolar spindle (half the total number of kinetochores are unattached), progression through mitosis is perfectly normal (Sluder et al., 1994). In the first case the poles are unpaired and in the other they are paired; the percentage of unattached kinetochores is the same in both cases.

To test if unpaired spindle poles delay anaphase onset we determined the time from NEB to anaphase onset in zygotes that contained supernumerary asters that were not associated with the spindle (Fig. 4). Should unpaired spindle poles activate the checkpoint for the metaphase-anaphase transition, anaphase onset should be delayed. We produced extra poles (classically called cytasters) by fertilizing eggs and shortly thereafter treating them with hypertonic sea water. This is essentially the same regime used for parthenogenetic activation of unfertilized eggs with the exception that we use fertilization, not an artificial agent, to activate the eggs (see Loeb, 1913). Cytasters contain centrioles, sometimes in greater than normal numbers (Kato and Sugiyama, 1971; Miki-Nomura, 1977; Kuriyama and Borisy, 1983; Kallenbach, 1985), and can act as functional spindle poles as determined by their capacity to interact with the spindle to form a multipolar spindle (Fig. 4) and to double between mitoses (not shown). We could vary the number of extra poles by changing the duration of exposure to hypertonic sea water; longer treatments produced more cytasters (compare Fig. 4A to B). The morphology and sequence of spindle events during mitosis was normal in zygotes containing extra asters with the exceptions that zygotes containing cytasters close to the spindle formed multipolar spindles (Fig. 4C and D) and more than one cleavage furrow formed in telophase (not shown).

The interval from NEB to anaphase onset in first division zygotes containing up to approx. 30 supernumerary centrosomes was on average 17.3 minutes (Table 1) with no systematic difference in timing between zygotes that contained a few supernumerary asters and those that contained many. This is on average 3 minutes longer than the corresponding interval for first division controls and second division zygotes with multipolar spindles.

PtK_1 cells with multipolar spindles

We followed 62 binucleate cells from nuclear envelope breakdown through anaphase onset (approximately 3% of the cells in a growing PtK_1 culture are binucleate, see Heneen, 1970). We also filmed an additional 33 cells with monooriented chromosomes starting after NEB when it was evident that they were forming a multipolar spindle. None of the cells we followed entered anaphase in the presence of a monooriented chromosome. Chromosome behavior could be unambiguously followed in 44 of the 62 binucleate cells filmed from NEB to anaphase onset and in 25 of the 33 cells filmed after NEB. In the remaining 26 cells chromosome behavior could not be clearly determined prior to anaphase onset and were thus excluded from our analysis.

Of the 44 binucleate cells we filmed from NEB to anaphase onset, 14 formed tripolar spindles (Figs 5-6), and 30 formed

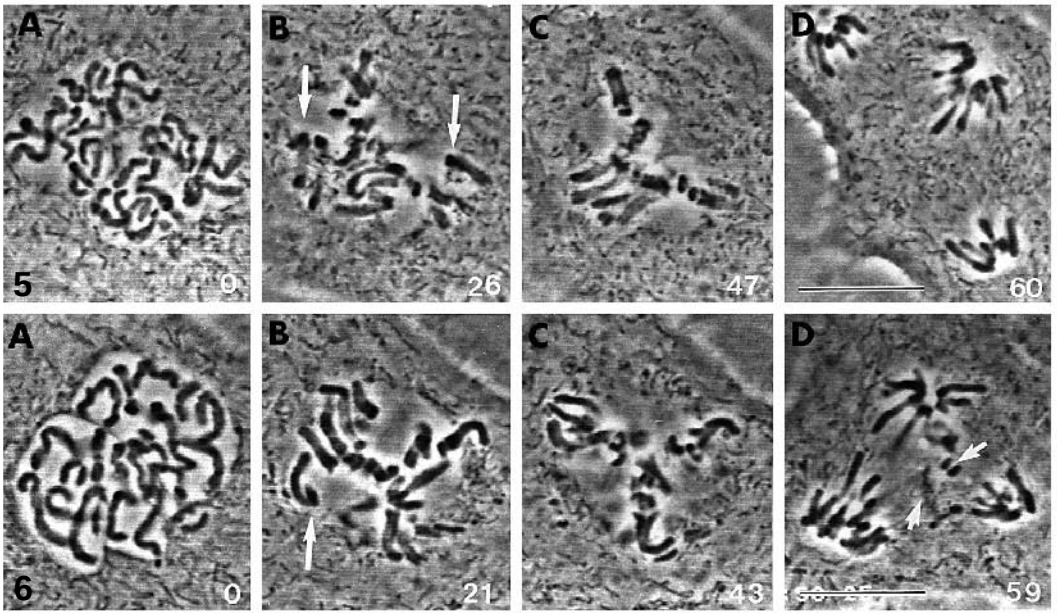

Fig. 5. Selected video-frames of a binucleated PtK1 cell progressing through a tripolar mitosis. (A) NEB. (B) The last two monooriented chromosomes (arrows) initiate congression at about the same time. (C) Anaphase onset 47 minutes after NEB. (D) Telophase. This cell entered anaphase 21 minutes after the last monooriented chromosomes acquired a bipolar attachment and no chromatids remained stranded between the poles during anaphase. Minutes after first frame shown at lower right corner of each frame. Phase contrast optics. Bar, 20 μm.

Fig. 6. Binucleated PtK1 cell progressing through a tripolar mitosis. (A) NEB. (B) The last monooriented chromosome (arrow) has become bioriented. (C) Anaphase onset 43 minutes after NEB. (D) Telophase. This tripolar cell entered anaphase 22 minutes after the last monooriented chromosome initiated congression, and several chromatids were stranded between the spindle poles during anaphase (arrows in d). Minutes after first frame shown at lower right hand corner of each frame. Phase contrast optics. Bar, 20 μm.

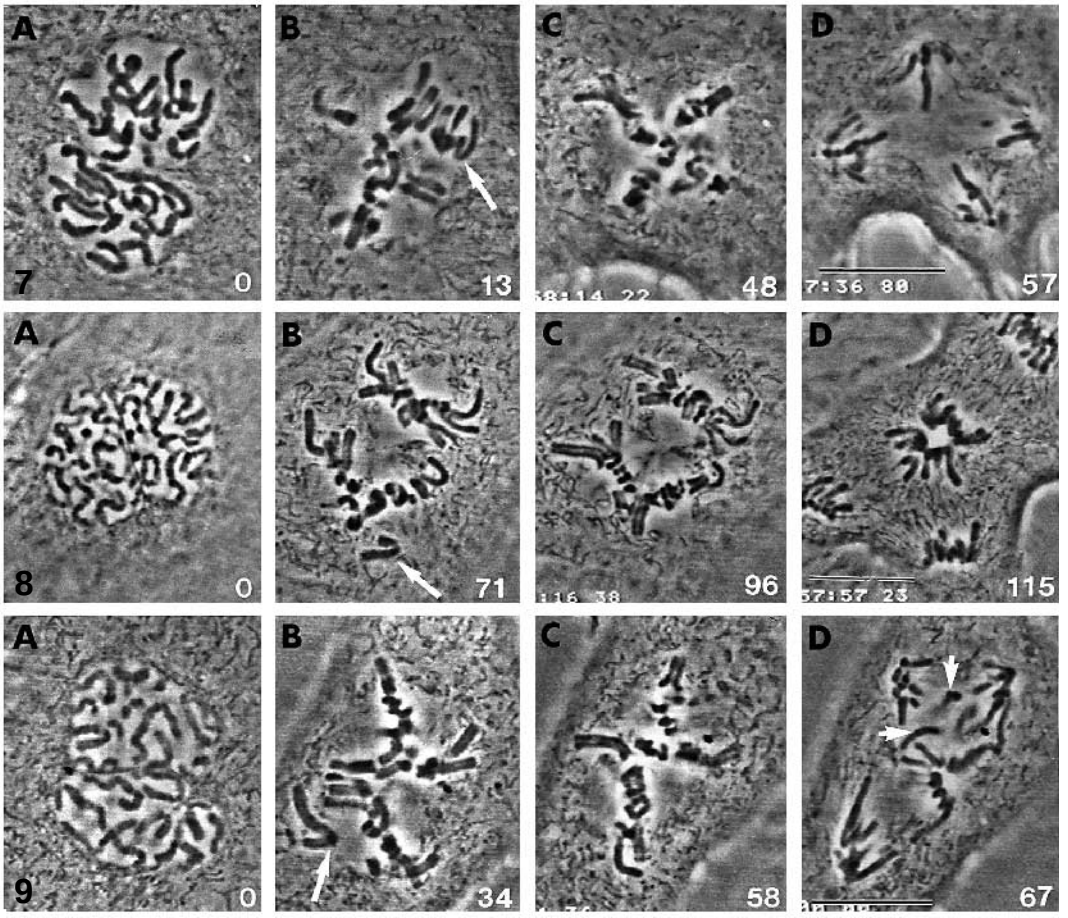

Fig. 7. Binucleated PtK cells progressing through a tetrapolar mitosis. (A) NEB. (B) Prometaphase. In this cell the last monooriented chromosome, marked by the arrow, initiated congression 16 minutes after this picture (29 minutes after frame A). (C) Anaphase onset 48 minutes after NEB and 19 minutes after the last monooriented chromosome became bioriented. (D) Telophase. No chromatids were stranded between the poles during anaphase. Minutes after first frame shown at lower right corner of each frame. Phase contrast optics. Bar, 20 μm.

Fig. 8. Binucleated PtK cells progressing through a tetrapolar mitosis. (A) Just prior to NEB. (B) The last monooriented chromosome (arrow) has become bioriented. (C) Anaphase onset 96 minutes after NEB. (D) Telophase. This tetrapolar cell entered anaphase 25 minutes after the last monooriented chromosome initiated congression. No chromatids are stranded between the poles during anaphase. Minutes after first frame shown at lower right corner of each frame. Phase contrast optics. Bar, 20 μm.

Fig. 9. Binucleated PtK cells progressing through a tetrapolar mitosis. (A) NEB. (B) The last monooriented chromosome has become bioriented (arrow). (C) Anaphase onset 58 minutes after NEB. (D) Telophase. Several chromatids are stranded between the poles during anaphase (white arrows). This cell entered anaphase 24 minutes after the last monooriented chromosome initiated congression. Minutes after first frame shown at lower right corner of each frame. Phase contrast optics. Bar, 20 μm.

tetrapolar spindles (Figs 7-9). We found that the great majority of tripolar spindles formed in our study contained metaphase plates that were shaped like a 'Y' or a 'T' (Figs 5-6). Three cases, however, formed tandem spindle arrays in which the poles were positioned in a roughly linear fashion with a metaphase plate between all three spindle poles. The position of the poles in tetrapolar spindles varied considerably (Figs 7-9) but most were positioned at the corners of a square to produce a 'X' shaped metaphase plate (Figs 7 and 9) or in a zig-zag pattern to produce a 'Z' shaped alignment of chromosomes (not shown). In 65% of the cells with tetrapolar spindles and 44% of cells with tripolar spindles, one or more chromatids failed to move toward a pole during anaphase, remaining positioned in the region between two or more poles (Figs 6 and 9). These stationary chromatids were not derived from monooriented chromosomes, because all chromosomes had fully congressed before anaphase onset. Instead we suggest, based on ultrastructural data on kinetochore orientation in multipolar PtK_1 spindles (Heenen, 1975; Gosh and Paweletz, 1987), that the kinetochore on such chromatids was not free to move after anaphase onset because it was attached by microtubules to two poles. This interpretation is consistent with our observation that the greater the number of spindle poles, the higher the incidence of stranded chromosomes during anaphase.

The average time from NEB to anaphase onset was 57

Table 2. Minutes from NEB to anaphase onset: multipolar and control PtK cells

	Mean	*n*	s.e.m.	Range
Tripolar spindles	57	14	5	31-90
Tetrapolar spindles	57	30	3	29-96
Bipolar spindles	50	126	2	23-198

Minutes from congression of last monooriented chromosome to anaphase onset: multipolar and control PtK cells

	Mean	*n*	s.e.m.	Range
Tripolar spindles	27	19	3	11-49
Tetrapolar spindles	24	50	1	10-44
Bipolar spindles	23	126	1	9-48

Values for mean, standard error of the mean (s.e.m.), and range are given in minutes. *n*, the number of cells followed in each category. The values for cells with bipolar spindles come from Rieder et al. (1994).

minutes for both cells with tripolar or tetrapolar spindles (data summarized in Table 2). These averages are not significantly different from the average 50 minute duration of mitosis in mononucleated cells forming normal bipolar spindles (Rieder et al., 1994). Thus, the average duration of mitosis in multipolar PtK_1 cells is the same as that in normal cells.

We have previously shown that the time from when the last free kinetochore attaches to the spindle and anaphase onset averages a constant 23 minutes regardless of how long the cells contained monooriented chromosomes (Rieder et al., 1994). Here we found for cells containing tripolar spindles that anaphase was initiated on average 27 minutes after the last monooriented chromosome bioriented. For cells containing tetrapolar spindles this interval averaged 24 minutes (see Table 2). There is no statistically significant difference between the average values for cells containing tripolar and tetrapolar spindles. Also, there is no significant difference between the averages for either class of multipolar spindles and the average for control cells.

DISCUSSION

The hypothesis that cells have a checkpoint that monitors proper bipolar spindle symmetry is appealing because defects in this parameter inevitably produce aneuploidy to the same extent as persistently monooriented or unattached chromosomes. However, the fact that experimental perturbations of spindle assembly, geometry, or polarity can also produce unattached kinetochores, which by themselves will delay anaphase onset, prompted us to empirically test if cells have a 'spindle assembly' checkpoint that is truly independent of the checkpoint that monitors kinetochore attachment.

Sea urchin zygotes

For zygotes of both species we found that the duration of second mitosis was the same whether they contained tripolar or tetrapolar spindles. More importantly, the duration of mitosis for *L. pictus* zygotes with multipolar spindles was the same as that for irradiated first division controls and not significantly longer than that for second mitosis controls. For *L. variegatus* zygotes with multipolar spindles the time from NEB to anaphase onset was not significantly different from that for first division controls and on average 1.2 minutes longer than that in second mitosis controls. Since a 1-2 minute variation from the mean duration of mitosis is well within the range of values found in a normal second division population, we do not think that this difference is the consequence of a checkpoint mechanism for the metaphase-anaphase transition. Second division zygotes with multipolar spindles appear to show the same timing as first mitosis controls. The fact that both populations have the same cytoplasmic volume raises the possibility that cell size has some bearing on the pathways that lead to the metaphase-anaphase transition.

These results reveal that the mechanisms that control the metaphase-anaphase transition in sea urchin zygotes do not detect gross defects in spindle architecture, even those that will directly lead to aneuploidy. This leaves us with the question of why zygotes with one or two monopolar spindles spend threefold the normal time in mitosis (Sluder and Begg, 1983) if they cannot detect the disruption of bipolar spindle symmetry. Previous work with sea urchin zygotes has provided evidence that these zygotes initiate anaphase onset at the normal time with half of the chromosomes completely unattached or monooriented to one of the spindle poles and the remainder attached properly to a bipolar spindle (Sluder et al., 1994). Perplexing, however, is the fact that the metaphase-anaphase transition is markedly delayed when the same total number of kinetochores are unattached under conditions in which the chromosomes are all monooriented on two monopolar spindles in the same cell (Sluder and Begg, 1983). This striking difference in the times of the metaphase-anaphase transition does not appear, at face value, to be a function of how many kinetochores are unattached. Unless the inhibitory activity produced by unattached kinetochores is extremely local or in low concentration due to its diffusion into a large cytoplasmic volume, possibilities that we are now testing, some other spindle parameter is being monitored in zygotes with monopolar spindles.

One aspect of a cell with one or two monopolar spindles is that the centrosome(s) are unpaired, that is they do not interact with another centrosome. Perhaps a centrosome, when unpaired, has an activity that delays anaphase onset (see Bailly and Bornens, 1992, for a discussion of the possible involvement of centrosomes in checkpoint controls). To test this possibility we determined the times from NEB to anaphase onset for *L. pictus* zygotes containing supernumerary asters (cytasters) that were not associated with the spindle. The supernumerary asters we induced in these zygotes behave as functional spindle poles in that they can interact with the spindle to form multipolar spindles and can reproduce between mitoses. Also, previous studies have shown that such cytasters are organized by centrosomes that have essentially normal ultrastructure, albeit sometimes with more than the normal number of centrioles (Kato and Sugiyama, 1971; Miki-Nomura, 1977; Kuriyama and Borisy, 1983; Kallenbach, 1985).

We found that zygotes containing a bipolar or a multipolar spindle in the presence of supernumerary asters take on average 17 minutes to reach anaphase onset, 3 minutes longer than the first division controls. Although this is a statistically significant difference, we do not think that this represents a checkpoint inhibition of anaphase onset for two reasons. First, recognized checkpoints in sea urchin zygotes cause delays in cell cycle progression that are well beyond the range of times found in a normal population (see Sluder, 1979; Sluder and Begg, 1983; Sluder et al., 1995). For sea urchin zygotes, durations of mitosis that are plus or minus 3 minutes from the mean are within the normal range. Second, zygotes with one or two monopolar spindles take on average 48-49 minutes to reach the metaphase-anaphase transition (Sluder and Begg, 1983), a prolongation of mitosis that is at least tenfold greater than the 3 minutes observed in zygotes with supernumerary asters.

Our finding that the number of cytasters in a zygote does not influence the duration of mitosis suggests that the distance between supernumerary asters or the distance of these asters from the spindle did not influence our results. These data indicate that the checkpoint control for the metaphase-anaphase transition does not operate at the centrosome level by monitoring greater than normal centrosome number or the lack of centrosome-centrosome interaction. Furthermore, the observation that zygotes with one or two monopolar spindles in the

same cytoplasm show the same prolongation of mitosis (Sluder and Begg, 1983) indicates that zygotes do not simply differentiate between one and two spindle poles.

Somatic cells

We analyzed 69 PtK_1 cells that had grossly defective spindle organization and found that the average time from NEB to anaphase onset was not significantly different from that for normal cells. However, for the purpose of this study the interval of interest was the length of time from when the last mono-oriented chromosome established a bipolar connection to the spindle until anaphase onset. Since PtK_1 cells have a checkpoint that delays anaphase until the last kinetochore is attached to the spindle (Rieder et al., 1994, 1995), a checkpoint that monitors spindle organization, should it exist, would not become apparent until the checkpoint for kinetochore attachment is relieved.

For PtK_1 cells with tripolar or tetrapolar spindles we found that the time between attachment of the last unattached kinetochore and anaphase onset did not significantly differ in mean or range from the corresponding interval in cells with normal bipolar spindles. Thus, we conclude that once the checkpoint for kinetochore attachment is relieved, PtK_1 cells are not subject to an additional checkpoint control that delays anaphase in response to defects in spindle bipolarity. This raises the question of why low doses of drugs affecting microtubule assembly (e.g. Colcemid, vinblastine, taxol) or the intranuclear injection of antibodies against centromere proteins greatly delay anaphase in vertebrate somatic cells when all sister kinetochores appear to be properly attached to the bipolar spindle (Bernat et al., 1990; Earnshaw et al., 1991; Jordan et al., 1991, 1992, 1993; Rieder et al., 1994; Tomkiel et al., 1994). Our results suggest that these agents delay the metaphase-anaphase transition by their effect on kinetochore-microtubule interactions, not by subtly altering the 3-dimensional organization of spindle microtubules. Perturbations which stabilize microtubule plus ends at kinetochores or disorganize the kinetochore/centromeric region of the chromosomes could lead to changes in tension at the kinetochore, a parameter which has recently shown to be monitored in insect spermatocytes (Li and Nicklas, 1995; Nicklas et al., 1995).

Why do cells lack a checkpoint to monitor proper spindle bipolarity?

Given that spindle multipolarity inevitably leads to aneuploidy, our results raise the question of why do neither sea urchin zygotes or somatic cells have a checkpoint that monitors this important parameter? A possible answer is that checkpoint mechanisms offer a direct selective advantage only for defects that the cell can ultimately resolve. For example, naturally occurring chromosome monoorientation or the late separation of spindle poles are errors that the cell typically can and does eventually correct. By contrast, somatic cells and zygotes do not appear to have a mechanism to correct for the presence of too many spindle poles, with the exception of the few systems that normally enter mitosis with multiple asters such as early mouse zygotes (Schatten et al., 1986) and N115 cells (Ring et al., 1982). Indeed, somatic cells with experimentally fragmented spindle poles (Keryer et al., 1984; Sellito and Kuriyama, 1988) or with spontaneously occurring ectopic spindle poles (Rieder et al., 1986) do not eliminate supernumerary poles and consequently multipolar divisions always ensue. Thus, a checkpoint for the metaphase-anaphase transition that monitors bipolar spindle symmetry would serve no functional purpose for either somatic cells or developing zygotes.

In conclusion, we propose that as long as a cell assembles spindle microtubules when it comes into mitosis the completion of kinetochore attachment limits when it will initiate the metaphase-anaphase transition. In saying this we do not imply that spindle microtubules per se cannot have a role in the pathways that lead to the metaphase-anaphase transition. Evidence for their importance in sea urchin zygotes comes from the finding that Colcemid treated zygotes with greatly diminished spindles stay longer in mitosis than normal (Sluder, 1979). Also, Andreassen and Margolis (1994) provide evidence that nocodazole treated somatic cells with many unattached kinetochores spontaneously finish prolonged mitosis sooner when they have more residual astral microtubules. Our point, however, is that under normal circumstances spindle microtubule assembly is not experimentally compromised; thus, the 'spindle assembly' checkpoint is in practice the 'kinetochore attachment' checkpoint.

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