

# Feedback Control of the Metaphase–Anaphase Transition in Sea Urchin Zygotes: Role of Maloriented Chromosomes

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**Abstract.** To help ensure the fidelity of chromosome transmission during mitosis, sea urchin zygotes have feedback control mechanisms for the metaphase–anaphase transition that monitor the assembly of spindle microtubules and the complete absence of proper chromosome attachment to the spindle. The way in which these feedback controls work has not been known. In this study we directly test the proposal that these controls operate by maloriented chromosomes producing a diffusible inhibitor of the metaphase–anaphase transition. We show that zygotes having 50% of their chromosomes ( $\sim 20$ ) unattached or mono-oriented initiate anaphase at the same time as the controls, a time that is well within the maximum period these zygotes will spend in mitosis. In vivo observations of the unattached maternal chromosomes indicate that they are functionally within the sphere of influence of the molecular events that cause chromo-

some disjunction in the spindle. Although the unattached chromosomes disjoin (anaphase onset without chromosome movement) several minutes after spindle anaphase onset, their disjunction is correlated with the time of spindle anaphase onset, not the time their nucleus breaks down. This suggests that the molecular events that trigger chromosome disjunction originate in the central spindle and propagate outward. Our results show that the mechanisms for the feedback control of the metaphase–anaphase transition in sea urchin zygotes do not involve a diffusible inhibitor produced by maloriented chromosomes. Even though the feedback controls for the metaphase–anaphase transition may detect the complete absence of properly attached chromosomes, they are insensitive to unattached or mono-oriented chromosomes as long as some chromosomes are properly attached to the spindle.

THE metaphase–anaphase transition is a critically important point during cell division. Not only do chromosomes synchronously disjoin and start to move towards opposite spindle poles, but also the cell becomes committed to finish mitosis and enter  $G_1$  (reviewed in Sluder and Rieder, 1993). The fidelity of chromosome segregation requires the complete and proper attachment of all chromosomes to the spindle before the cell executes the metaphase–anaphase transition. Improper chromatid distribution due to unattached or mono-oriented chromosomes ultimately has serious, often lethal, consequences for the cell lineage. This essential coordination between chromosome attachment to the spindle and anaphase onset is not left to chance; in almost all higher eukaryotic cells the metaphase–anaphase transition point appears to be subject to “checkpoint” controls (concepts reviewed in Hartwell and Wienert, 1989; Murray, 1992) that delay the metaphase–anaphase transition in response to perturbations in spindle microtubule assembly and spatial arrangement (Sluder, 1979; Sluder and Begg, 1983; reviewed in Rieder and Palazzo, 1992). That this checkpoint control is a signal transduction

pathway has been recently demonstrated by genetic studies of budding and fission yeast (Hoyt et al., 1991; Li and Murray, 1991).

Zirkle (1970; and Zirkle, R. E. 1970. *J. Cell Biol.* 47:235a) proposed that this checkpoint also monitors proper chromosome attachment through a control function that resides in unattached kinetochores. This hypothesis is consistent with the recent observation that the kinetochores of bipolar-oriented chromosomes lose a phosphorylated epitope by the time of metaphase, while unattached chromosomes retain this epitope (Gorbsky and Ricketts, 1993). McIntosh (1991) extended the Zirkle hypothesis by proposing that each centromere contains stretch sensitive enzyme(s) that produce an inhibitor of the metaphase–anaphase transition when its daughter kinetochores are not under bipolar tension. Concurrently, the inhibitor is destroyed by a hypothetical cytoplasmic pathway. When even a single chromosome is not attached to the spindle in a bipolar fashion, its centromere produces enough inhibitor to block the metaphase–anaphase transition. Once that chromosome becomes attached to both spindle poles, the centromeric enzymes are stretched and inactivated, thereby allowing the concentration of inhibitor to fall below the threshold that permits the metaphase–anaphase transition to occur.

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Whether or not maloriented chromosomes actually produce a diffusible inhibitor of the metaphase–anaphase transition, a number of observations indicate the importance of proper chromosome attachment in the controls for anaphase onset. First, Spencer and Hieter (1992) found for yeast that mutational or deletional manipulation of the centromeric DNA of a supernumerary chromosome led to a prolongation of mitosis that was independent of the rate at which this chromosome was lost. Second, injection of affinity-purified autoimmune sera containing antibodies against the CENP family of centromeric proteins into G<sub>2</sub> HeLa cells allows seemingly normal spindle assembly and chromosome attachment but blocks the cell cycle in mitosis (Bernat et al., 1990; Earnshaw et al., 1991). Similarly, injection of cultured cells with monoclonal antibodies to CENP-E, a kinesin-like centromeric protein, or the CHO1 antibody against a kinesin-like protein blocks or substantially delays entry into anaphase (Yen et al., 1991, 1992; Nislow et al., 1990, 1992). Third, substoichiometric doses of vinblastine or low doses of taxol applied to HeLa cells alter microtubule dynamics at the kinetochores and arrests the cells in mitosis despite seemingly normal spindle organization (Wendell et al., 1993; Jordan et al., 1993). Fourth, treatment of PtK<sub>1</sub> cells with the kinase inhibitor 6-dimethylaminopurine stabilizes improper chromosome orientations and more than doubles the duration of metaphase (Nicklas et al., 1993). Fifth, for spermatocytes of some mantid species anaphase does not occur and the cells eventually degenerate when a normally paired X chromosome is separate and consequently fails to congress to the spindle equator (reviewed in Nicklas and Arana, 1992). However, such a meiotic arrest with an unpaired X chromosome is not observed in all mantid species (Hughes-Schrader, 1948) and persistent, multiple autosomal malorientations in grasshopper spermatocytes treated with kinase inhibitors do not prevent the cells from entering anaphase (Nicklas et al., 1993). Furthermore, mutations that impair chiasma formation bypass the natural meiosis I arrest in *Drosophila* eggs (McKim et al., 1993).

Given the considerable interest in how the cell controls the metaphase–anaphase transition, we have directly tested the applicability of the diffusible inhibitor model (Zirkle, 1970; McIntosh, 1991) to the feedback controls for the metaphase–anaphase transition operating in sea urchin zygotes. These zygotes are similar to most somatic cells in that inhibition of spindle microtubule assembly substantially delays the metaphase–anaphase transition. Also, delays in spindle microtubule assembly after nuclear envelope breakdown delay anaphase onset by exactly the same amount of time (Sluder, 1979). The finding that cutting the spindle into two half spindles causes the zygote to stay longer in mitosis than normal indicates that microtubules do not influence the time of the metaphase–anaphase transition through some bulk property dependent upon total tubulin polymer (Sluder and Begg, 1983). The feedback controls in these zygotes appear to be able to detect the absence of any properly attached chromosomes. Blastomeres in which all chromosomes are mono-oriented to one or two asters spend almost threefold the normal amount in mitosis (Sluder and Begg, 1983) and zygotes containing normal asters but no chromosomes have a cell cycle that is longer and more variable than normal (Sluder et al., 1986).

To determine if the feedback controls for the metaphase–anaphase transition in these zygotes depend upon maloriented

chromosomes producing a diffusible inhibitor of this transition, we compare the time from nuclear envelope breakdown to anaphase onset for zygotes having 50% of the chromosomes completely unattached or mono-oriented to the spindle against normal controls. Should unattached kinetochores or unstretched centromeres produce a diffusible inhibitor of the metaphase–anaphase transition, we reasoned that half of the chromosomes (~20) should effectively block or delay anaphase onset. For our purposes, the great advantage of the sea urchin zygote system over cultured cells is that the rapid cell cycle and relatively consistent timing of mitotic events of these zygotes allows us to obtain quantitative data on the time from nuclear envelope breakdown to the metaphase–anaphase transition for a sufficient number of cells to make meaningful comparisons.

## Materials and Methods

### Living Material

*L. pictus* and *L. variegatus* sea urchins were purchased from Marinus Inc. (Long Beach, CA) and Susan Decker (Hollywood, FL), respectively. With the exception of the stained preparations of chromosomes all work was performed on *L. pictus* zygotes. Eggs and sperm were obtained by intracoelemic injection of 0.5 M KCl (Fuseler, 1973).

To produce zygotes with only 50% of the chromosomes attached to the spindle, unfertilized eggs were treated for 4–8 min with  $5 \times 10^{-6}$  M Colcemid (Sigma Chemical Co., St. Louis, MO) to prevent future sperm aster assembly and hence, block pronuclear fusion. After Colcemid treatment, the eggs were fertilized and cultured in natural sea water at 16–19°C. Shortly before the expected time of first nuclear envelope breakdown the zygotes were mounted in fluorocarbon oil preparations as previously described (Sluder, 1979). Just after nuclear envelope breakdown of both pronuclei, individual zygotes were irradiated for 15–30 s with 366 nm light (reviewed in Sluder, 1991) to photochemically inactivate the Colcemid and allow the cells to assemble spindle microtubules. Individual zygotes were followed at 19°C and the times of sperm nuclear envelope breakdown and anaphase onset determined by polarization microscopy with a modified Zeiss ACM microscope. Control zygotes were similarly treated with Colcemid after pronuclear fusion to prevent future microtubule assembly and irradiated just after nuclear envelope breakdown. In the preparations of experimental zygotes some fortuitously showed pronuclear fusion; these were also used as controls. Photographs were recorded on Kodak Plus X film which was developed in Kodak Microdol-X.

To determine the duration of mitosis without microtubule assembly zygotes were treated for 12 min with  $5 \times 10^{-6}$  M Colcemid after pronuclear fusion to prevent microtubule assembly. They were mounted on fluorocarbon preparations and individually followed with an Olympus BH-2 microscope equipped for differential interference contrast to determine the times of nuclear envelope breakdown and nuclear envelope reformation (determined as the first clearly visible appearance of karyomeres). To control for possible toxic side effects of the Colcemid treatment, zygotes were treated for 12 min with the same concentration of B-lumicolchicine (Sigma Chemical Co.). This isomer of colchicine does not bind to tubulin or prevent microtubule assembly but shows the same toxic side effects as the native compound (reviewed in Sluder, 1991).

Real time characterization of chromosome behavior in living zygotes was accomplished by treating zygotes with 1 µg/ml Hoechst 33342 (Sigma Chemical Co.) for 20 min in natural sea water and following them with a Zeiss Axiovert microscope equipped with differential interference contrast and fluorescence. Fluorescence illumination was provided by a 100 W high pressure mercury arc lamp. Zygotes were observed with a 40×, 1.0 NA plan apochromat objective. Images were taken with a Photometrics CC200 cooled CCD camera, with a metachrome-coated Thompson CCD chip, coupled to the trinocular of the microscope with a 3× tube factor coupling. Images were taken using 1–2-s exposures shuttered both at the light source and the camera with Uniblitz shutters (Vincent Associates, Rochester, NY). The epiillumination filtration consisted of a short pass (<365 nm) emission filter and a heat cut filter to remove infrared radiation. In some experiments the heat cut filter was removed to produce an image of the cell body. Images were transferred via the RS170 port of the camera controller to a Panasonic

TQ2028F OMDR. For image processing we used an ITEX 151 Image Processor (Imaging Technology Corp., Woburn, MA). Image greyscales were determined using a built in algorithm which samples 420 regions throughout the image. Subsequent images were redigitized and displayed over a set greyscale range for each sequence. The insert image in Fig. 3 *d* was sharpened using digital deconvolution within the arithmetic logic unit of the image processor employing a  $3 \times 3$  kernel with central value 9 and all other values  $-1$  and digitally magnifying by  $2\times$ . Images on the video screen were photographed on 35 mm Plus-X film and developed in Microdol-X.

### Stained Preparations

To better document the disjunction of unattached maternal chromosomes Colcemid-treated eggs were fertilized and the cultures were irradiated with 366 nm light after most of the male pronuclei had broken down. Approximately 10 min later aliquots of zygotes were pelleted and fixed in three parts 95% ethanol plus one part glacial acetic acid at 3-min intervals. 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  $100\times$  oil immersion objective.

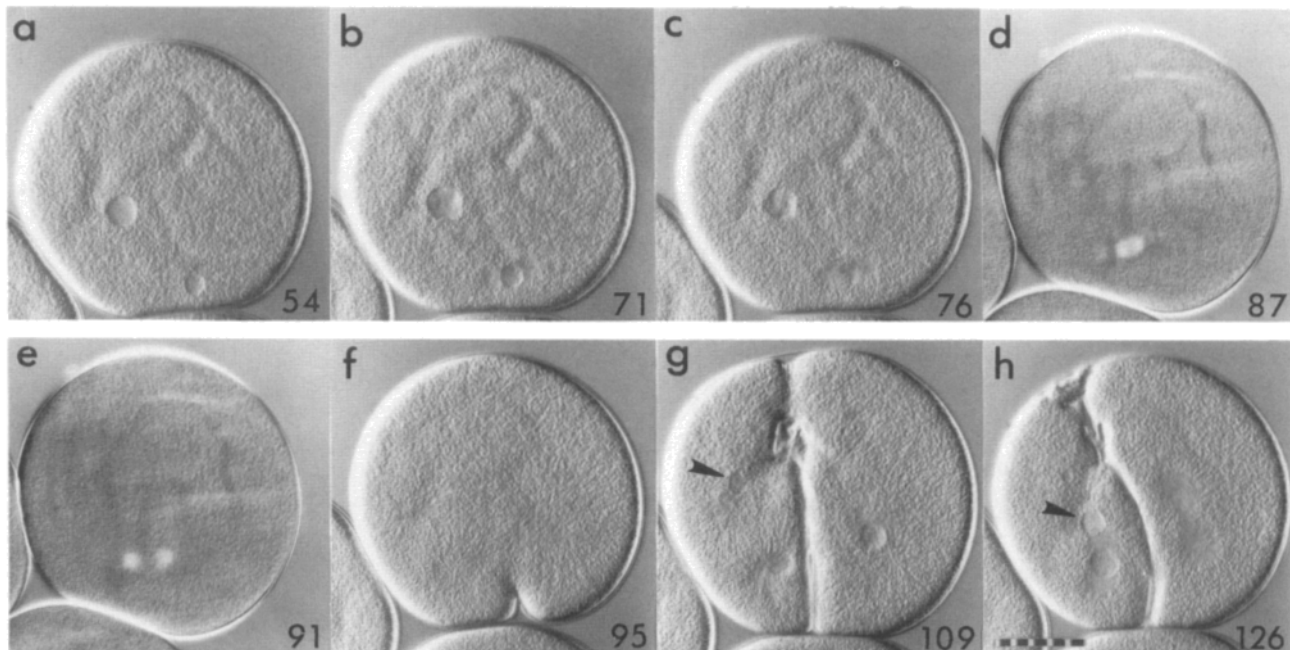
## Results

### Experimental System

We produced zygotes in which 50% of the chromosomes are unattached to the spindle by treating unfertilized eggs with Colcemid to prevent future microtubule assembly. Fertilization events in such treated eggs are normal with the exception

that a sperm aster is not assembled and pronuclear fusion consequently does not occur. The male pronucleus becomes visible at a random position at the surface of the zygote  $\sim 15$  min after fertilization and is always smaller than the female nucleus (Fig. 1 *a*). At the onset of mitosis both pronuclei break down with the female pronucleus leading in 74 of 78 cases (Fig. 1 *b*). The female pronucleus breaks down before the male pronucleus by an average of 7.5 min ( $n = 74$ , range = 0–33 min.). It is not readily apparent why nuclear envelope breakdown should be asynchronous, and this is an issue we are currently exploring.

Shortly after breakdown of the male pronucleus (Fig. 1 *c*) we irradiate individual zygotes on the microscope with 366 nm light to photochemically inactivate the Colcemid. After irradiation a functional bipolar spindle is assembled in association with the male chromosomes (Fig. 1 *d*) because the sperm contributes the centrosome used in development (reviewed in Sluder, 1992). The male spindle assembles with normal kinetics and anaphase appears normal (Fig. 1 *e*). Throughout mitosis the female pronuclear area typically remains as a small irregular hyaline area of approximately the same size as the original pronucleus. In a minority of cases a small monaster is assembled at the site of the female pronucleus with the maternal chromosomes all mono-oriented around its periphery (see Sluder and Rieder, 1985). This monaster represents the expression of the residual microtubule organizing center of the maternal centrosome (reviewed in Sluder, 1992). In telophase the reforming female nucleus



**Figure 1.** Development of a *L. pictus* zygote with 50% of its chromosomes unattached to the spindle. (*a*) Larger female and smaller male pronuclei prior to first mitosis. (*b*) Start of nuclear envelope breakdown for the female pronucleus; the male nucleus remains intact. (*c*) After the breakdown of the male pronucleus the zygote is irradiated to inactivate the Colcemid and allow the assembly of a functional bipolar spindle. (*d*) The spindle in the same cell shown with polarization optics. The maternal chromosomes remain in a small hyaline area. The birefringent streaks seen predominantly in the upper quadrant of the zygote are out of focus images of folds in the fertilization envelope. (*e*) Early anaphase for the spindle. (*f*) Telophase and the onset of cleavage. (*g*) Daughter nuclei organized around the paternal chromosomes are seen on either side of the cleavage furrow in the lower portion of the zygote. The reforming nucleus containing the maternal chromosomes is indicated by the arrowhead. (*h*) This “maternal” nucleus moves towards the “paternal” nucleus in that blastomere. Later both maternal and paternal chromosomes participate in spindle assembly. (*a–c* and *f–h*) Differential interference optics; (*d* and *e*) polarization optics. Minutes after fertilization are shown in the lower corner of each frame.  $10\ \mu\text{m}$  per scale division.

is typically drawn into one of the telophase asters and fuses with one of the male nuclei (Fig. 1, *g* and *h*, arrowheads).

Our previously published correlative light and serial section ultrastructural analysis of this experimental system has shown that the maternal chromosomes are distributed randomly within the highly convoluted and fenestrated remnants of the pronuclear envelope (Sluder and Rieder, 1985). The chromosomes are well condensed and have prominent kinetochores with well-developed coronas but are not associated with any microtubules. The maternal chromosomes are clearly in contact with the cell cytoplasm because yolk granules and mitochondria are often found in close proximity to the chromosomes. For zygotes that assemble a monaster at the female pronuclear area, the chromosomes are mono-oriented with just the kinetochores facing the monaster focus attached to microtubules. Within the same zygotes, the paternal chromosomes have prominent kinetochores which are attached to and oriented toward opposite spindle poles by kinetochore microtubules.

To generate controls which had undergone the same experimental manipulations, fertilized eggs were treated with Colcemid after pronuclear fusion and individually irradiated with 366 nm light just after first nuclear envelope breakdown. We also followed zygotes from the preparations of experiments in which pronuclear fusion fortuitously occurred. First and subsequent mitoses are normal for control zygotes from both sources.

#### ***Time of Anaphase Onset in the Presence of Maloriented Chromosomes***

Zygotes of *L. pictus* with 50% of the chromosomes unattached to the spindle initiate anaphase at the same time as the controls, ~19 min after nuclear envelope breakdown at 18–19°C (Table I). The extent to which female pronuclear envelope breakdown precedes that of the male pronucleus has little influence on the nuclear envelope breakdown to anaphase onset duration for the male spindle. When female nuclear envelope breakdown precedes that of the male by <8 min, anaphase is initiated on average 19.7 min ( $n = 45$ ,  $s = 6.4$  min) after male pronuclear envelope breakdown. When the female nucleus breaks down 8 or more min before the male pronucleus, anaphase is initiated on average 18.8 min ( $n = 29$ ,  $s = 5.7$  min) after male pronuclear envelope breakdown.

To address the concern that these zygotes will spend a maximum of only 19 min in mitosis before spontaneously

returning to interphase regardless of chromosome attachment, we completely block spindle microtubule assembly with a pulse of Colcemid administered after pronuclear fusion. We then record the time from nuclear envelope breakdown to nuclear envelope reformation for individual zygotes. We use nuclear envelope reformation (seen as the first appearance of karyomeres) as a measure of the end of mitosis in this experiment because the time of chromosome disjunction cannot be reliably determined in vivo by differential interference contrast optics. Chromosome disjunction in Colcemid-treated zygotes precedes karyomere formation by several minutes, the same interval found in control zygotes (Sluder, 1979). The results (Table I) show that zygotes of *L. pictus* spend almost 60 min between nuclear envelope breakdown and reformation when spindle microtubule assembly does not occur. Qualitatively, the same is true if the Colcemid is administered before pronuclear fusion. The lumicolchicine controls (Table I) show that this prolongation of mitosis is not due to toxic side effects of the Colcemid (rationale reviewed in Sluder, 1991).

Whether the maternal chromosomes are completely unattached or are mono-oriented to either a monaster or to one of the asters of the male spindle does not influence the time of anaphase onset for the male spindle. This is most easily seen in cases in which the reformed female nucleus at the end of first mitosis remains slightly separated from one of the male nuclei after cleavage (Fig. 2 *a*, arrowheads). For the two cases shown here, the maternal chromosomes mono-orient to one aster of the spindle and organize a birefringent “fan” to one side of the aster during second mitosis (Fig. 2, *b–e*, arrows). The maternal chromosomes are located near the periphery of the “fan” (see Leslie, 1992). Despite mono-oriented chromosomes on one of the daughter spindles, anaphase onset is synchronous in both blastomeres (Fig. 2, *c* and *e*).

#### ***Time of Unattached Chromosome Disjunction***

We were concerned that the 10–63- $\mu$ m separation between the unattached or mono-oriented chromosomes and the spindle metaphase plate might mean that they are too far away to inhibit the metaphase–anaphase transition, assuming that they would be capable of doing so when closer to the spindle. To determine if the unattached chromosomes behave as if they are within the sphere of influence of the spindle we labeled the chromosomes and determined the times of chromosome disjunction in the spindle and the unattached chromosomes in vivo. Times of nuclear envelope breakdown were determined for individual zygotes with differential interference contrast optics using 15–30-s sampling intervals. This frequent sampling interval and the rapid collapse of the round nuclear profile allows us to determine within <1 min the times of pronuclear breakdown.

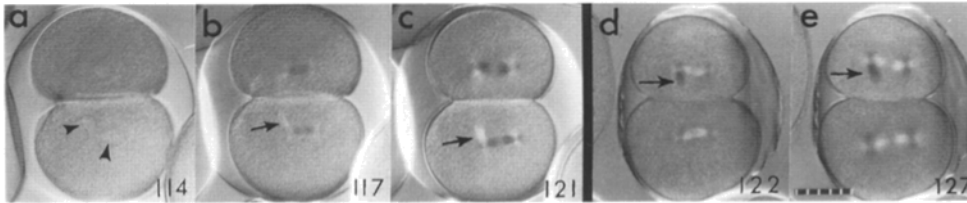
Shortly after male pronuclear breakdown, the zygote was irradiated 4–8 s with 366 nm light through the epi-fluorescence optics to inactivate the Colcemid and allow spindle assembly. Thereafter we followed the zygote by fluorescence microscopy. Shortly before the expected time of spindle anaphase onset individual zygotes were followed at 30–60-s intervals with 1–2-s exposures of 366 nm light. As soon as the spindle initiated anaphase we followed the zygote at 30-s intervals with 1–2-s exposures of 366 nm light. We used this

**Table I.**

Minutes from Nuclear Envelope Breakdown to:

	Average	<i>n</i>	SEM
Anaphase Onset			
100% of chromosomes attached	19.33	78	0.69
50% of chromosomes attached	19.31	49	0.67
Nuclear Envelope Reformation			
Colcemid	59.8	29	2.75
lumicolchicine controls	23.0	28	0.91

The number of cells examined and the standard error of the mean in minutes are shown by *n* and SEM, respectively. All data are taken from *L. pictus* zygotes followed at 18–19°C.



**Figure 2.** Time of anaphase onset is normal in the presence of mono-oriented chromosomes; shown for two *L. pictus* zygotes during second mitosis. (a) In the lower blastomere the nucleus containing the maternal chromosomes remains slightly

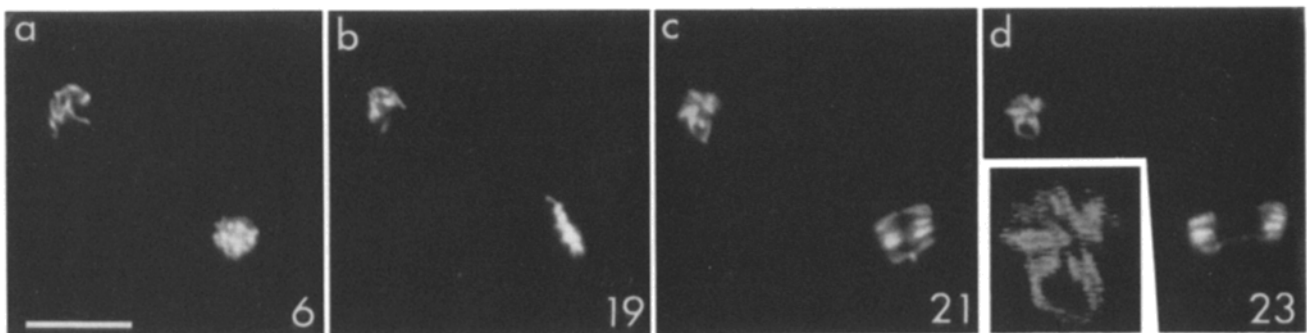
separated from the nucleus with paternal chromosomes (arrowheads). (b) After synchronous nuclear envelope breakdown, the maternal chromosomes monoorient to one aster and organize a birefringent "fan" of microtubules to that side of the aster (arrow). (c) Anaphase onset is synchronous in both blastomeres. (d) Another zygote showing maternal chromosomes monooriented to one side of an aster in the upper blastomere (arrow). (e) Anaphase onset is synchronous in both blastomeres. Minutes after fertilization are shown in the lower corner of each frame. Polarization optics. 10  $\mu$ m per scale division.

intermittent observation protocol because it does not produce any visible abnormalities in anaphase chromosome disjunction. We estimate that we can determine the time of spindle anaphase onset within 1–2 min for chromatids lying in the plane of focus. The disjunction of unattached maternal chromosomes (anaphase onset without chromosome movement) is seen as the doubling of at least one individual chromosome or an obvious one step increase in chromosome number within the plane of focus. We rarely directly observed the doubling of more than a few chromosomes in each zygote because the bulk of the chromosomes were typically clumped together, only a few chromosomes were in the shallow depth of focus of the objective lens, and the two chromatids of any given chromosome had to separate in this plane of focus to be seen. Since all chromosomes of Colcemid-treated sea urchin zygotes and other types of cells synchronously disjoin in the absence of spindle microtubules (Sluder, 1979; Rieder and Palazzo, 1992), we have no reason to suspect that only some of the unattached chromosomes disjoin during the *in vivo* observations. Furthermore, Brownian motion of the chromosomes into and out of the exact plane of focus meant that in some cases we did not observe the precise time of chromosome disjunction. Therefore, our assignment of the time of unattached chromosome disjunction was probably late in some instances; this may have contributed to some of the scatter in our timing data.

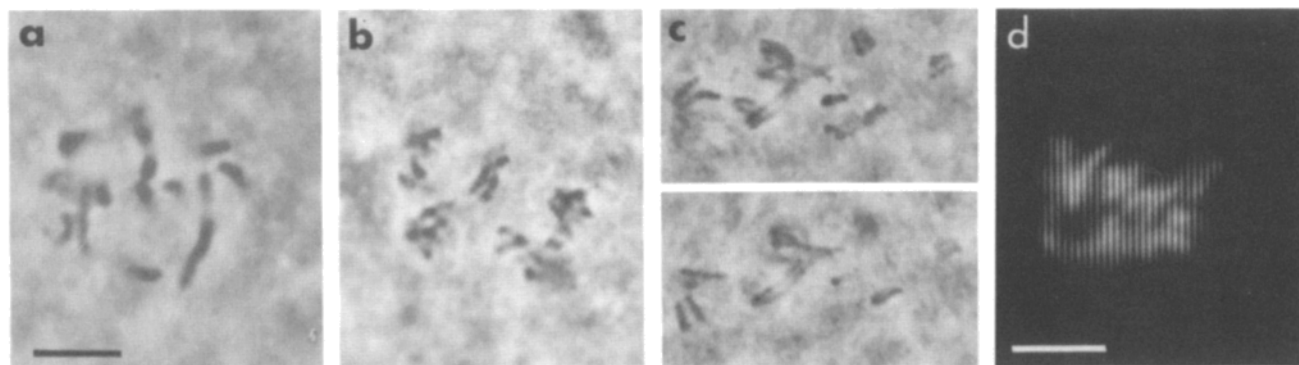
In many cases we observed cytoplasmic movements at the time of spindle anaphase onset that cause both the spindle and the unattached maternal chromosomes to translate within the cytoplasm.

An example of such an experiment shown in Fig. 3. After nuclear envelope breakdown of the male pronucleus, the maternal and paternal chromosomes are randomly distributed in the nuclear areas (Fig. 3 a). Irradiation with 366 nm light inactivates the Colcemid and the paternal chromosomes become aligned on the metaphase plate of the spindle (Fig. 3 b). At anaphase onset the disjunction of the paternal chromosomes is synchronous and easily seen (Fig. 3 c). For this particular example the female pronucleus broke down 5 min before the male pronucleus, yet the maternal chromosomes disjoined 5 min after spindle anaphase onset (Fig. 3 d). As is often the case, it is difficult to photograph the disjunction of the maternal chromosomes. An enlarged and digitally sharpened image of the maternal chromosomes is shown in Fig. 3 d (inset). The three chromosomes that have disjoined with both chromatids in this plane of focus are seen at 12, 5, and 9 o'clock positions.

Other examples of the disjunction of unattached maternal chromosomes are shown in Fig. 4. The first three panels show chromosomes in zygotes that were fixed, stained, and observed by phase contrast microscopy; the last panel shows chromosomes photographed *in vivo* by fluorescence micros-



**Figure 3.** Behavior of unattached chromosomes during mitosis. In this case the female pronucleus breaks down 5 min before the male pronucleus. (a) Maternal (upper left) and paternal chromosomes shortly after male pronuclear breakdown. (b) Spindle assembly and alignment of the paternal chromosomes on a metaphase plate (lower right). The organization of the spindle is normal but not visible in this micrograph. (c) Early anaphase for the spindle; the maternal chromosomes have not disjoined. (d) Maternal chromosomes disjoin 5 min after spindle anaphase onset. Inset shows an enlarged and digitally sharpened image of the maternal chromosomes; daughter chromatids of three chromosomes can be seen in this plane of focus. Fluorescence optics. Minutes after male pronuclear envelope breakdown are shown in the lower corner of each frame. Bar, 20  $\mu$ m.



**Figure 4.** Examples of unattached chromosome disjunction during mitosis. (a–c) Fixed and stained preparations of unattached maternal chromosomes. (a) Maternal chromosomes before disjunction. (b) Example of maternal chromosomes after disjunction; only some of the chromosomes are seen in this focal plane. (c) Two optical sections through the same group of disjoined maternal chromosomes. (d) Disjoined maternal chromosomes seen in vivo by fluorescence microscopy. In this plane of focus four disjoined chromosomes (eight chromatids) are present in the upper right hand quadrant of the grouping. Bars, 5  $\mu$ m.

copy. Fig. 4 a shows an optical section through maternal chromosomes that have not yet disjoined. Fig. 4 (b–d) show maternal chromosomes that have disjoined; note that the chromosomes appear thinner and are obviously paired when both chromatids are in the focal plane. Fig. 4 c shows two optical sections through the same group of unattached maternal chromosomes. In Fig. 4 d four disjoined chromosomes (eight chromatids) are present in the upper right hand quadrant of the grouping of chromosomes.

Even though the female pronucleus breaks down first, we find that the unattached maternal chromosomes always disjoin after spindle anaphase onset typically by a few minutes (summarized in Table II). To determine whether or not the disjunction of the maternal chromosomes is correlated with the time of spindle anaphase onset, we establish the time of female nuclear envelope breakdown as zero time and plot the times of maternal chromosome disjunction against the times of paternal chromosome disjunction (Fig. 5). In 18 cases, for which we have times of nuclear envelope breakdown, the correlation coefficient for these data sets is 0.90 indicating that the disjunction of the maternal chromosomes is temporally linked to the anaphase onset for the spindle. For these cases there is no significant correlation between the

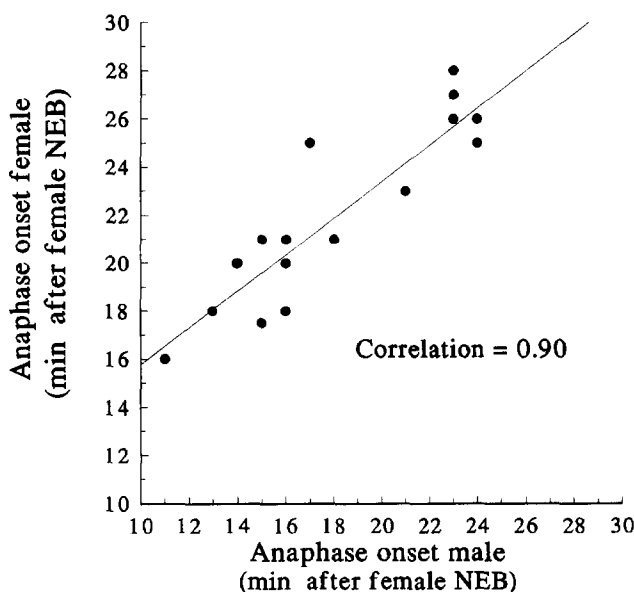
time of maternal chromosome disjunction and the amount of time female nuclear envelope breakdown precedes male nuclear envelope breakdown (correlation coefficient 0.49). For an additional seven cases in which we did not observe the times of nuclear envelope breakdown the maternal chromosomes disjoined on average 4 min (range 1.5–7 min) after spindle anaphase onset.

In addition, we followed three cases in which the pronuclei were sufficiently close to each other that the maternal chromosomes became mono-oriented around one of the asters of the spindle. In the case shown in Fig. 6 the mono-oriented chromosomes disjoin 2 min after spindle anaphase onset

**Table II.**

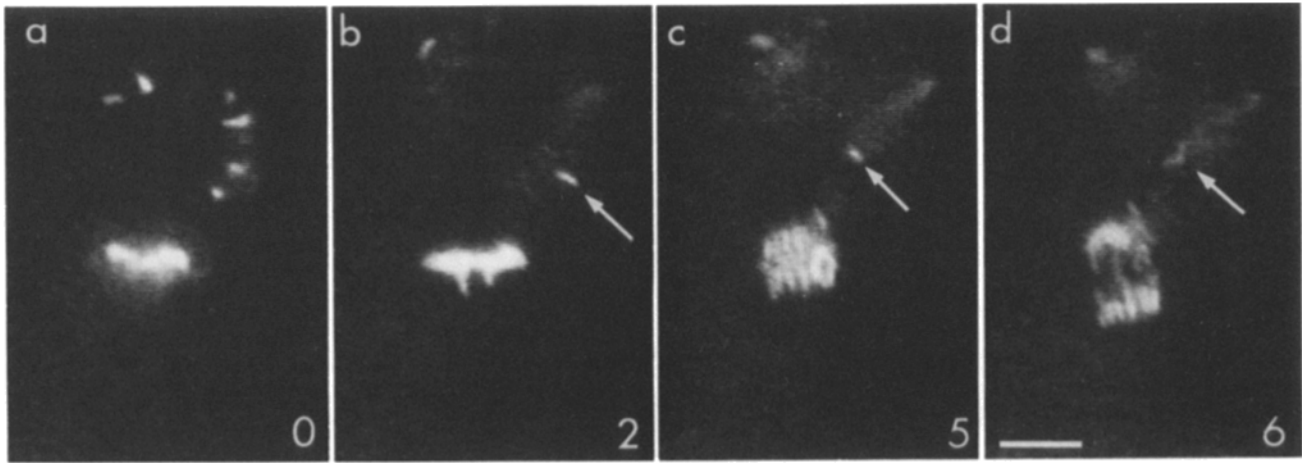
	Average	n	SEM
Female NEB leads by:	5.4	19	0.60
Paternal chromosome disjunction leads by:	3.9	26	0.40
Duration of mitosis:			
Paternal chromosomes	13.6	18	0.86
Maternal chromosomes:	21.2	18	1.37

The female pronucleus always breaks down first. The paternal chromosomes on the spindle always disjoin at anaphase onset before the unattached maternal chromosomes disjoin. NEB stands for nuclear envelope breakdown. Duration of mitosis is the time from nuclear envelope breakdown to the disjunction of the chromosomes. All values are minutes except n, the number of cells examined. The number of cells examined and the standard error of the mean in minutes are shown by n and SEM, respectively. All data are taken from *L. pictus* zygotes followed at 20–22°C; as a consequence, the duration of paternal chromosome mitosis is shorter than that shown in Table I.



**Figure 5.** Test of the correlation between the times of spindle anaphase onset and the disjunction of the unattached or mono-oriented maternal chromosomes. The time of female pronuclear breakdown is chosen as the onset of mitosis or time zero. The interval from this zero time to the disjunction of the maternal chromosomes (ordinate) is plotted against the interval from zero time to anaphase onset for the paternal chromosomes (abscissa). The linear regression line is drawn through the points.





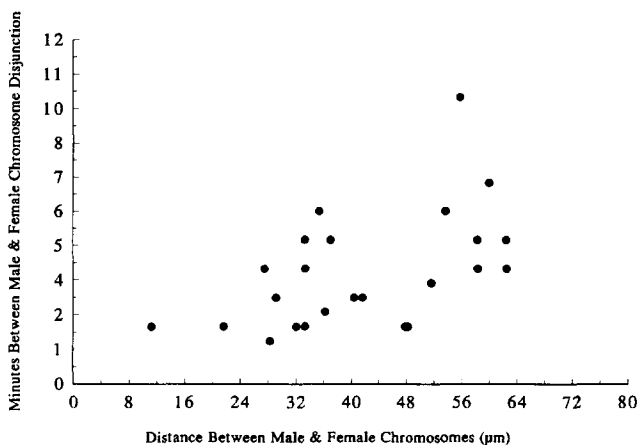
**Figure 6.** Behavior of mono-oriented chromosomes during mitosis. Male and female pronuclei were close together and after assembly of the spindle, all the maternal chromosomes became mono-oriented around the upper aster. (a) Six mono-oriented maternal chromosomes are imaged in this plane of focus. (b) Another plane of focus showing a maternal chromosome of interest (*arrow*) close to the metaphase plate. (c) Shortly after spindle anaphase onset; this maternal chromosome (*arrow*) has not disjoined. (d) This maternal chromosome disjoins 2 min after spindle anaphase onset. Fluorescence optics. Minutes after the first frame of this series are shown in the lower corner of each frame. Bar, 10  $\mu$ m.

(Fig. 6 d, *arrow*). For the other two cases the mono-oriented chromosomes disjoined 1 and 6 min after spindle anaphase onset. In one other case we observed a spindle in which some of the paternal chromosomes were mono-oriented; the mono-oriented chromosomes disjoined 1 min after spindle anaphase onset.

That unattached or mono-oriented chromosomes always disjoin after spindle anaphase onset raises the possibility that the molecular events of the metaphase anaphase transition originate in the central spindle and propagate outward. For the 25 cases in which we could make unambiguous distance measurements, we have plotted the difference in maternal and paternal chromosome disjunction times against the closest distance between the metaphase plate just prior to anaphase onset and the unattached or mono-oriented chromosomes (Fig. 7). The sampling interval after spindle anaphase onset and the technical difficulties of visualizing

the exact moment for the disjunction of maternal chromosomes contribute to the scatter in our data. We find that the time between paternal and maternal chromosome disjunction is positively correlated with distance using the non-parametric Spearman's ranked coefficient correlation test ( $P < .02$ ). However, we cannot tell if the relationship between time and distance is linear or more complex since there is substantial scatter in the data and a linear correlation test gives a correlation coefficient of only 0.52.

In the course of following *in vivo* chromosome behavior we did not detect any functional differences between maternal and paternal chromosomes. When the initially separate maternal chromosomes are close enough to the central spindle, they become drawn into the spindle. There they appear to attach properly, join the metaphase plate, and start anaphase chromosome movement at the same time as the paternal chromosome (data not shown).



**Figure 7.** For 25 zygotes with separate pronuclei we plot the difference in times of maternal and paternal chromosome disjunction against the distance between the two chromosome groups.

## Discussion

The sea urchin zygote clearly has feedback control mechanisms that delay the metaphase-anaphase transition when spindle microtubules are not assembled (Sluder, 1979) and when no chromosomes are properly attached to the spindle (Sluder and Begg, 1983; Sluder et al., 1986). The negative feedback model specifying that improperly attached chromosomes produce a diffusible inhibitor of the metaphase-anaphase transition could provide a rational explanation for these observations (Zirkle, 1970; McIntosh, 1991).

By blocking pronuclear fusion we have devised an experimental system that allows us to test whether or not unattached or mono-oriented chromosomes produce such an inhibitor of the metaphase-anaphase transition. Our results show that zygotes with 50% of their chromosomes unattached to the spindle initiate anaphase at the same time as the controls, 19 min after nuclear envelope breakdown. This does not simply reflect the maximum amount of time these

zygotes will spend in mitosis before spontaneously returning to interphase; Colcemid-treated zygotes that do not assemble any spindle microtubules stay almost threefold longer in mitosis than normal. Lumicolchicine controls show that this prolongation of mitosis is not a toxic side effect of the Colcemid as we used it (see Sluder, 1991).

Whether the maternal chromosomes are completely unattached or mono-oriented has no functional consequence for the time of anaphase onset. In the cases in which most, if not all, of the maternal chromosomes are mono-oriented around one of the spindle poles at first mitosis, anaphase onset for the spindle occurs at the normal time. For those cases in which the duplicated maternal chromosomes mono-orient to one of the second division spindles (Fig. 2), probably more chromosomes are maloriented than properly attached; nevertheless, anaphase onset in that blastomere occurs synchronously with the same embryo control blastomere. Being directly engaged with an aster, these maloriented chromosomes presumably would be close enough to the spindle to exert an inhibitory influence if they were capable of doing so.

We have no reason to suspect that the maternal chromosomes are functionally different than the paternal chromosomes from the standpoint of their ability to trigger the checkpoint control for the metaphase-anaphase transition. At the onset of first mitosis the maternal and paternal chromosomes have duplicated once; by the time of second mitosis they have replicated twice. Given that the assembly of all new chromosomes takes place from a common pool of subunits, it is unlikely that maternal and paternal chromosomes could have significantly different compositions.

We have addressed the possible concern that the completely unattached chromosomes may be sufficiently distant from the spindle so as to be incapable of influencing the timing of anaphase onset even if they possibly could when closer to the spindle. In vivo observations show that disjunction of the unattached maternal chromosomes always follows spindle anaphase onset even though the female pronucleus breaks down first. Since the time of maternal chromosome disjunction is correlated with the time of spindle anaphase onset, the unattached chromosomes behave as if they are functionally within the sphere of influence of the molecular events of the metaphase-anaphase transition in the spindle.

These results show that even if the unattached or mono-oriented chromosomes produce a diffusible inhibitor that delays anaphase onset, it must be very weak, given the number of inappropriately attached chromosomes involved (~20), or it is not diffusible. In either case, this putative inhibitory factor would be functionally ineffective as part of a surveillance checkpoint mechanism for the metaphase-anaphase transition point.

Our finding that the disjunction of unattached or mono-oriented chromosomes always follows anaphase onset in the spindle, typically by a few minutes, suggests that the molecular changes that allow chromatid separation may not be initiated globally but rather start in the central spindle and diffuse or actively propagate outward. For example, there may be a propagating activation of the proteolytic activity that both degrades cyclin B and releases the chromatids from each other (Holloway et al., 1993). This is consistent with our observations that chromosomes mono-oriented to one aster of the spindle disjoin 1–2 min after spindle anaphase onset. Also, our data, despite scatter, suggest that the delay

in unattached chromosome disjunction may be related to their distance from the spindle metaphase plate.

However, we cannot rule out the possibility that the delay in unattached chromosome disjunction simply reflects the extra time needed for these chromosomes to manifest their separation without spindle microtubules to allow their active movement apart.

### **Role of Chromosome Attachment in Zygotes and Somatic Cells**

Although unattached or mono-oriented chromosomes in the presence of a normal spindle do not delay the metaphase-anaphase transition in sea urchin zygotes, a number of studies on somatic cells link defects in chromosome attachment to the spindle with delays in anaphase onset. This raises the possibility that somatic cells and sea urchin zygotes regulate the metaphase-anaphase transition differently. That this difference is due to the rigid programming of the embryonic cell cycle, such as early *Xenopus* and *Drosophila* embryos (discussed in Murray, 1992), does not apply to sea urchin zygotes. The timing of the metaphase-anaphase transition in sea urchin zygotes, like somatic cells, is clearly sensitive to alterations in spindle microtubule assembly and spindle organization (Sluder, 1979; Sluder and Begg, 1983; Sluder et al., 1986). In addition, early sea urchin zygotes are similar to somatic cells in that injection of the CHO1 antibody to a kinesin-like protein can block the cells in mitosis (Nislow et al., 1990, 1992; Wright et al., 1993).

A better explanation for this difference is to postulate that the strength or properties of the feedback controls for the metaphase-anaphase transition are variable from one type of cell to another (Murray, 1992). In sea urchin zygotes perhaps the feedback controls efficiently monitor spindle microtubule assembly but are not sensitive to proper chromosome attachment. However, we are concerned that this explanation may be too simple, because the feedback controls operating in sea urchin zygotes are not dependent solely on microtubule assembly. Microsurgical cutting of the spindle into two half spindles shows that the metaphase-anaphase transition is substantially delayed in the absence of any properly attached chromosomes even though astral number, aster size, and the microtubule content of the cell are essentially normal (Sluder and Begg, 1983; Sluder et al., 1986). In fact, zygotes with all chromosomes mono-oriented spend approximately as much time in mitosis as those in which microtubule assembly has been completely inhibited.

In the context of these observations it is important to note that zygotes containing a bipolar spindle whose microtubule content and astral size have been greatly diminished with Colcemid enter telophase slightly later than normal but in about half the time as those with all chromosomes mono-oriented to one or two asters (Sluder and Begg, 1983). This comparison indicates that the checkpoint mechanisms are less sensitive to total microtubule quantity and astral size than chromosome attachment and/or the overlap of spindle microtubules of opposite polarities. The possibility that the checkpoint for anaphase onset monitors the overlap of spindle microtubules of opposite polarities is consistent with the finding that microinjection of antibodies to kinesin-like proteins arrests sea urchin zygotes in mitosis (Wright et al., 1993).



Together with our present results these observations indicate that the checkpoint for the metaphase-anaphase transition in sea urchin zygotes can efficiently detect the complete absence of any properly attached chromosomes. However, as long as the zygote has a haploid number of chromosomes properly attached to the spindle, the checkpoint pathway does not detect multiple unattached or mono-oriented chromosomes. This, of course, begs the question of how the zygote can detect the absence of any chromosome attachment but yet be insensitive to 50% of the chromosomes being completely unattached to microtubules or mono-oriented to one spindle pole.

For somatic cells the checkpoint controls for the metaphase-anaphase transition appear to be able to detect weak or abnormal chromosome attachment to the spindle. However, the way in which improper chromosome attachment triggers the checkpoint mechanisms remains uncertain. For example, a high resolution temporal and spatial analysis of chromosome movements in newt lung cells has shown that ~75% of the time during the prometaphase/metaphase portion of mitosis sister kinetochores "exhibited coordinated and opposite phases of motion with respect to their poles" (Skibbens et al., 1993). Tension across the centromere seems to cause one kinetochore to reverse direction and actively move away from its pole, perhaps through the action of plus end-directed microtubule based motor molecules (Hyman and Mitchison, 1991). If maloriented chromosomes in this cell type produce an inhibitor of the metaphase-anaphase transition, intermittent stretching of centromeres must be sufficient to prevent the production of this inhibitor.

An alternative possibility that sister kinetochore attachment to microtubules, without bipolar tension, causes inhibitor production to cease is thus far not strongly supported. Perturbation of kinetochore assembly by injection of antibodies against centromeric proteins (Bernat et al., 1990; Yen et al., 1991), inhibition of microtubule plus end dynamics by substoichiometric doses of microtubule inhibitors (Wendell et al., 1993; Zieve et al., 1980), and microtubule stabilization by low doses of taxol (Jordan et al., 1993) leads to a metaphase arrest in HeLa cells. Some of these arrested cells have bipolar spindles of normal appearance with all daughter kinetochores attached to microtubules and aligned on the metaphase plate.

## Conclusions

Our results show that the checkpoint mechanism for the metaphase-anaphase transition in sea urchin zygotes cannot detect maloriented chromosomes as long as there are some chromosomes attached to the spindle in a normal fashion. Therefore, improperly attached chromosomes do not produce a diffusible inhibitor of this transition point. That somatic cells may be able to detect the presence of a single maloriented chromosome indicates that this checkpoint may have different functional properties in sea urchin zygotes and somatic cells. This may reflect the different problems these two types of cells normally face. In early sea urchin zygotes, the asters are large, microtubule density high, and the chromosomes small. Thus, establishment of bipolar chromosome attachment may normally have a very low and thus acceptable error rate. Gross defects in spindle assembly may

be a more common problem and thus, is a defect that efficiently triggers the checkpoint pathway. Somatic cells, on the other hand, have small asters and chromosome malorientation is common during the early stages of spindle assembly; therefore, it may be of real importance for the cell to have a means of detecting even a single maloriented chromosome. Whether or not maloriented chromosomes actually produce a diffusible inhibitor of the metaphase-anaphase transition in somatic cells remains to be determined.

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