Anaphase Onset in Vertebrate Somatic Cells Is Controlled by a Checkpoint That Monitors Sister Kinetochore Attachment to the Spindle

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Abstract. To test the popular but unproven assumption that the metaphase-anaphase transition in vertebrate somatic cells is subject to a checkpoint that monitors chromosome (i.e., kinetochore) attachment to the spindle, we filmed mitosis in 126 PtK_1 cells. We found that the time from nuclear envelope breakdown to anaphase onset is linearly related ($r^2 = 0.85$) to the duration the cell has unattached kinetochores, and that even a single unattached kinetochore delays anaphase onset. We also found that anaphase is initiated at a relatively constant 23-min average interval after the last kinetochore attaches, regardless of how long the cell possessed unattached kinetochores. From these results we conclude that vertebrate somatic cells possess a metaphase-anaphase checkpoint control that monitors sister kinetochore attachment to the spindle. We also found that some cells treated with 0.3–0.75 nM Taxol, after the last kinetochore attached to the spindle, entered anaphase and completed normal poleward chromosome motion (anaphase A) up to 3 h after the treatment—well beyond the 9–48-min range exhibited by untreated cells. The fact that spindle bipolarity and the metaphase alignment of kinetochores are maintained in these cells, and that the chromosomes move poleward during anaphase, suggests that the checkpoint monitors more than just the attachment of microtubules at sister kinetochores or the metaphase alignment of chromosomes. Our data are most consistent with the hypothesis that the checkpoint monitors an increase in tension between kinetochores and their associated microtubules as biorientation occurs.

THE transition from metaphase to anaphase is a key cell cycle event that commits the cell to exit mitosis and enter a new interphase (reviewed in Murray, 1992; Sluder and Rieder, 1993). Since the equal segregation of chromosomes at mitosis is predicated on each acquiring a bipolar attachment to the spindle before anaphase onset, the metaphase-anaphase transition must be tightly coordinated with proper completion of chromosome attachment. Ensuring the essential coordination of these events would be relatively straightforward if the mitotic portion of the cell cycle was fixed in duration, but sufficiently long so that all chromosomes had time to acquire a proper attachment before disjoining. However, the stochastic nature of spindle formation makes chromosome attachment an unpredictable and error-prone process. In vertebrate cells a chromosome first attaches when one of its kinetochores, usually the one closest to and facing a spindle pole at nuclear envelope breakdown (NEB),[1] interacts with microtubules (MTs) growing from that pole (reviewed in Rieder, 1990). As a result of this interaction the chromosome "monoorients" and moves towards that pole. To establish biorientation the unattached sister kinetochore must acquire MTs from the distal pole which is often many micrometers away. Since polar MTs are dynamically unstable structures that rarely grow as long as the spindle interpolar axis, and since their density rapidly decreases with increasing distance from the pole, the distal kinetochores on monooriented chromosomes may remain unattached for highly variable periods of time (reviewed in Rieder, 1990, 1991). Additional temporal variability in completing proper chromosome attachment also arises in response to resolving errors made during the initial attachment process (e.g., correcting malorientations; see Ault and Rieder, 1992).

Checkpoint controls are signal transduction pathways that block progression of the cell cycle until the event being monitored is completed (reviewed in Hartwell and Wienert, 1989;

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1. *Abbreviations used in this paper*: NEB, nuclear envelope breakdown; MT, microtubule.

Murray, 1992). Genetic analyses of yeast mitosis (Hoyt et al., 1991; Li and Murray, 1991), as well as functional studies on higher eukaryotic cells (e.g., reviewed in Sluder and Rieder, 1993), clearly reveal that the metaphase-anaphase transition in animal cells is subject to a checkpoint control that delays anaphase onset in response to a number of experimental perturbations. These include, e.g., the inhibition of spindle MT assembly (Sluder, 1979; Kung et al., 1990), rearrangement of spindle architecture (Sluder and Begg, 1983; Hunt et al., 1992), mutational or deletional alterations of centromeric DNA (Spencer and Hieter, 1992), the presence of unpaired homologues during meiosis I (Callan and Jacobs, 1952), antibody-induced disruptions of centromeric organization (e.g., Tomkiel et al., 1994), and the modification of MT dynamic instability by low doses of vinblastine or Taxol (Jordan et al., 1991, 1992, 1993; Wendell et al., 1993). Given the diverse interventions that delay the metaphase-anaphase transition, the processes or events monitored by this checkpoint remain unknown.

An important but unresolved question is whether the metaphase-anaphase transition is inhibited or delayed by the presence of one or more chromosomes that have not established a proper bipolar attachment, against the background of an otherwise fully formed spindle. Based on cine records Zirkle (1970) concluded that "newt cells, like most others, never start anaphase" in the presence of monooriented chromosomes. As a result he, and others (Hartwell and Weinert, 1989; Earnshaw et al., 1991; McIntosh, 1991; Spencer and Hieter, 1992; Gorbsky and Ricketts, 1993; Sluder and Rieder, 1993), envision that the attachment of kinetochores to spindle MTs plays an important role in the metaphase-anaphase checkpoint control pathway. However, the notion that anaphase is delayed in vertebrate cells until all kinetochores are properly attached to the spindle is a subjective impression that has yet to be proven. Indeed, Zirkle (1970) found no correlation between when the last pair of congressing kinetochores reached the spindle equator and the duration of metaphase—a phase of mitosis that cannot be estimated quantitatively. Furthermore, it is well established that animal somatic cells (e.g., newts, Rieder and Alexander, 1989; PtK, Roos, 1976) can enter anaphase in the presence of one or more monooriented chromosomes. Such an event would be expected to occur periodically if, as suggested from the conclusions of Snyder et al. (1982) and Vandre and Borisy (1989), anaphase is initiated a consistent interval after NEB. Finally, most recently, Sluder et al. (1994) found that anaphase onset is not delayed in experimentally manipulated sea urchin zygotes when 50% of the chromosomes are monooriented to one pole or remain unattached to the spindle. The uncertainty in how unattached kinetochores influence the timing of anaphase onset in vertebrates is due, in part, to a lack of reliable quantitative information on the duration of mitosis in a system where kinetochore attachment and behavior can be clearly and continuously monitored.

To determine if monooriented chromosomes delay anaphase onset in vertebrate somatic cells we have systematically followed individual untreated PtK_1 cells and correlated the duration of mitosis, defined as the interval between NEB and anaphase onset, with the amount of time each cell contained one or more monooriented chromosomes. Our results clearly demonstrate that anaphase in PtK_1 is delayed even by a single unattached kinetochore, and that the duration of mitosis is related to the time it takes for the last monooriented chromosome to become attached to the spindle in a bipolar fashion.

Materials and Methods

Cell Culture

Stock cultures of the female rat kangaroo kidney epithelial cell line PtK_1 ($2n = 12$), initially purchased from American Type Culture Collection (batch F-10679; batch date 9/1/92; passage no. 69; Rockville, Maryland), were grown within 75-cm^2 T-flasks at 37°C in Hepes-buffered L-15 medium containing 12% fetal calf serum (see Rieder, 1981). For study the cells were enzymatically removed from the flasks and pipetted into Petri dishes containing medium and 25-mm^2 glass coverslips. After a 1–2-d incubation at 37°C the coverslips were mounted in Rose (1954) chambers. These closed chambers allow individual cells to be viewed at high resolution yet contain enough media to support continued growth of the culture for 4–5 d.

Some mitotically active Rose chamber cultures were treated with 1.0 μM Colcemid (Sigma Chemical Co., St. Louis, MO) in conditioned medium. By contrast, for experiments with Taxol individual cells were followed by video microscopy within the Rose chamber from prophase until the last monooriented chromosome bioriented and congressed. At this time the culture was perfused with 0.3–3 nM Taxol (Calbiochem Corp., San Diego, CA) in serum-free L-15 medium containing 1.0% DMSO. 14 PtK_1 cells were also perfused, before NEB, with serum-free L-15 medium containing 1.0% DMSO but no Taxol.

Time-Lapse Video-enhanced Light Microscopy

Selected cells were followed from prophase through cleavage by time-lapse video-enhanced light microscopy using framing rates of 4–15 frames/min. The equipment employed included Nikon Diaphot and Nikon Diaphot 200 inverted light microscopes equipped, respectively, with phase contrast or differential interference contrast optics. Both of these microscopes were housed in rooms maintained at 20–22°C. Cells were illuminated with shuttered, monochromatic (546 nm) heat-filtered light obtained from 100W tungsten or mercury bulbs. They were viewed and followed with either 100× phase-contrast (NA = 1.25) or 60× differential interference contrast (NA = 1.40) objectives and a 0.85 NA condenser. Video images, obtained with a DAGE-MTI model VE1000 Newvicon tube camera (Dage-MTI Inc., Wabash, MI), were routed through an Argus 10 (Hamamatsu Photonics, Bridgewater, NJ) or IMAGE 1 (Universal Imaging Corp., West Chester, PA) image processors. Optical and electronic noise was eliminated by background subtraction, and recording an eight frame jumping average. Processed images were stored on Panasonic Model TQ 2025F optical memory disk recorders (ADCO Aerospace, Ft. Lauderdale, FL).

Since the duration of mitosis is highly temperature dependent (e.g., see Rieder, 1981) the medium bathing the cells was kept at 35–37°C using a custom-designed and constructed Rose chamber heater. In brief this heating system consisted of a rectangular (115 × 65 × 9 mm) aluminum block with a rectangular hole in the center just large enough to accommodate a Rose chamber. The temperature of the Rose chamber was monitored by thermocouples within the block and within the chamber. These two temperature sensors were coupled into an electronic control circuit that maintained the chamber at a steady-state temperature. Since the objective lens is an effective heat sink, the cells were slightly cooler than the surrounding medium. However, since this factor was consistent throughout all experiments, and since the microscopes were kept at 20–22°C, no attempt was made to warm the objective.

Data Analysis

For this study we filmed over 126 PtK_1 cells from before NEB through telophase. We found that both NEB and anaphase onset occurred over approximately 1 min, and that it took a similar amount of time to establish with certainty when a monooriented chromosome biorients and initiates congression. Because of this we rounded all of our data to the nearest minute.

The following parameters were established from the video records for every cell: (*a*) the time of NEB; (*b*) the time of anaphase onset; (*c*) the number of monooriented chromosomes at 20, 25, 30, 35, 40, and greater than 40 min after NEB; and (*d*) the time when the last monooriented chromo-

some initiated congression relative to NEB. These data were entered manually into Quattro Pro 5.0 (Borland International, Inc., Scotts Valley, CA) and STATISTIX II (NH Analytical Software, Roseville, MN) for plotting and one-way analyses of the variance (AOV).

Results

Previous attempts to determine the influence of monooriented chromosomes on the timing of anaphase onset (i.e., Zirkle, 1970) relied on estimating the duration of "metaphase," which is traditionally defined to start when all of the kinetochores are positioned on the spindle equator. Since all mono- and bioriented chromosomes in vertebrate somatic cells undergo constant and often substantial oscillatory motions across the metaphase plate (Fig. 1, *E–G*), determining when metaphase begins is subjective and thus somewhat arbitrary (see McIntosh, 1991). To eliminate this ambiguity we determined the full duration of mitosis, as defined by the period from NEB to anaphase onset (see also Vandre and Borisy, 1989). Both of these events can be determined with a temporal accuracy of ≤1 min. This approach provides the only truly accurate way of addressing the issues considered by our work.

Video records of 126 cells revealed that the duration between NEB and anaphase onset is highly variable in PtK_1 ranging from 23 to 198 min with an average of 50 ± 2 min (Table I *A*; see Figs. 1, 2, and 3 *A*). None of the cells filmed entered anaphase in the presence of a monooriented chromosome.

This analysis was restricted to those cells in which the replicated centrosomes had separated, or were separating, at the time of NEB. In ~10% of the cells (not included in the 126-cell sample), spindle pole separation was delayed for a variable time after NEB, producing a transient monopolar spindle that persisted for up to 15–20 min (see Rattner and Berns, 1976). We eliminated these cells from our analysis because variability in the timing of centrosome separation increases the variability in the duration of prometaphase.

Table I. Minutes from Nuclear Envelope Breakdown to Anaphase Onset

	Number of cells	Average and SEM	Range
A. All Cells	126	50 ± 2	23–198
B. When:			
No monooriented chromosomes 20 min after NEB	57	38 ± 1	23–61
Monooriented chromosomes 20–29 min after NEB	30	44 ± 2	32–72
Monooriented chromosomes 30–39 min after NEB	11	59 ± 3	48–83
Monooriented chromosomes 40 or more min after NEB	28	77 ± 5	53–198
C. When:			
One monooriented chromosome 20–29 min after NEB	18	42 ± 1	32–54
One monooriented chromosome 30–39 min after NEB	11	59 ± 3	48–83
One monooriented chromosome 40 or more min after NEB	12	70 ± 4	55–89

The Effect of Monooriented Chromosomes on the Duration of Mitosis

In PtK_1 and other vertebrate somatic cells, kinetochore fibers form asynchronously on sister kinetochores after NEB. As a result, shortly after NEB each cell contains a variable number of monooriented chromosomes, the majority of which acquire a bipolar orientation and congress within 25 min after NEB (Figs. 1 and 2). As noted by Roos (1976) for PtK_1, "a slight rotation around the long axis, at

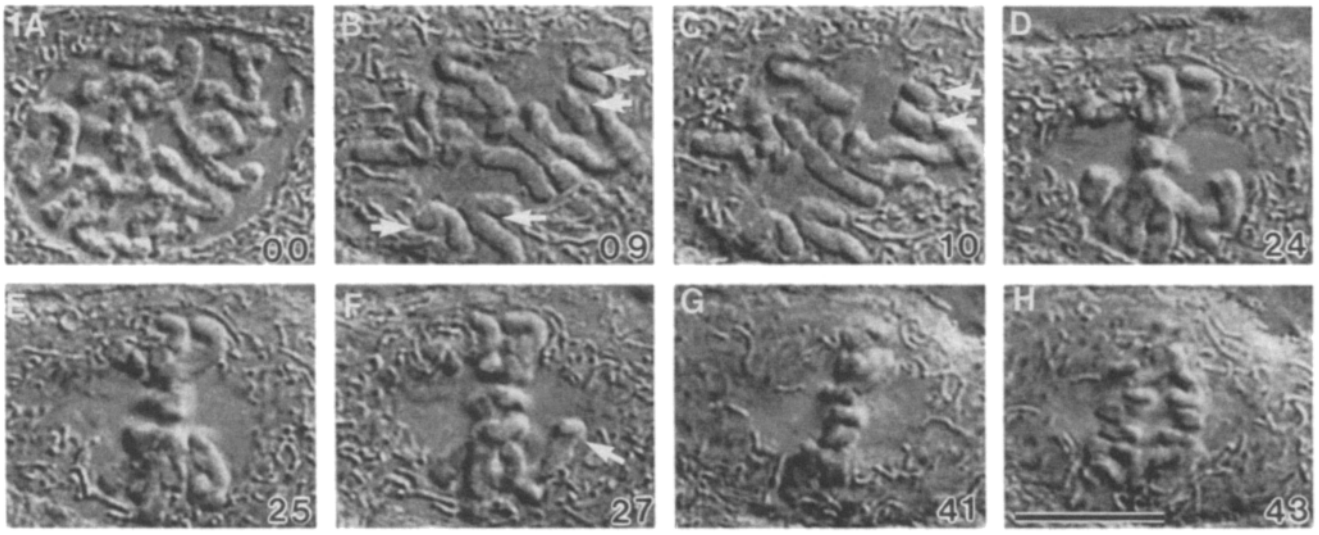


Figure 1. Selected video frames from a DIC recording of a PtK_1 cell in which the last monooriented chromosome bioriented within 24 min of NEB. After NEB (*A*) several chromosomes form a monopolar attachment and monoorient to each pole (*B, arrows*). Examination of the video record reveals that two of these chromosomes (*C, arrows*) acquired a biorientation between *B* and *C*. By *D* all of the monooriented chromosomes are bioriented and congressing to the spindle equator (*E*). One bioriented chromosome subsequently undergoes a substantial poleward displacement during an oscillation (*F, arrow*). Anaphase onset is initiated 41 min after NEB (*G* and *H*), or 17 min after the last monooriented chromosome bioriented. Time in min at lower right corner of each frame. Bar, 10.0 μm.

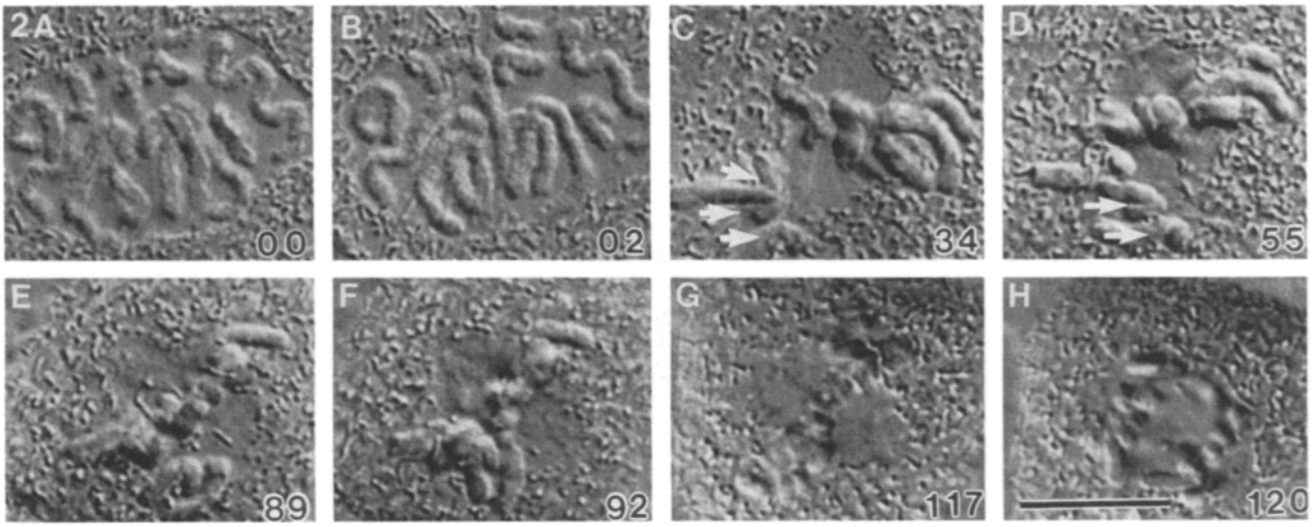


Figure 2. Same conditions as in Fig. 1 except this cell had monooriented chromosomes (*C* and *D*, *arrows*) until the last two bioriented 89 min after NEB (*E*). Anaphase is initiated 117 min after NEB (*G*), or 28 min after the last monooriented chromosome bioriented. Time in min at lower right hand corner of each frame. Bar, 10.0 μm.

least of the kinetochore region and the arm segments proximal to it, precedes congression of most" monooriented chromosomes. We used this visual criterion, as well as the ensuing sustained motion of the chromosome towards the forming metaphase plate, to establish from our video record when each monooriented chromosome acquired a bipolar attachment to the spindle.

To determine the extent to which monooriented chromosomes prolong the duration of mitosis, we plotted for each cell the period between NEB and anaphase onset against the time it took for the last monooriented chromosome to initiate congression after NEB. This scatter plot (Fig. 3 *B*) reveals a significant correlation ($r^2 = 0.85$) between these two variables. The longer it takes for the last monooriented chromosome to initiate congression, the greater the time between NEB and anaphase onset. Indeed, in our most extreme example anaphase onset did not occur until the last monooriented chromosome congressed almost 3 h after NEB.

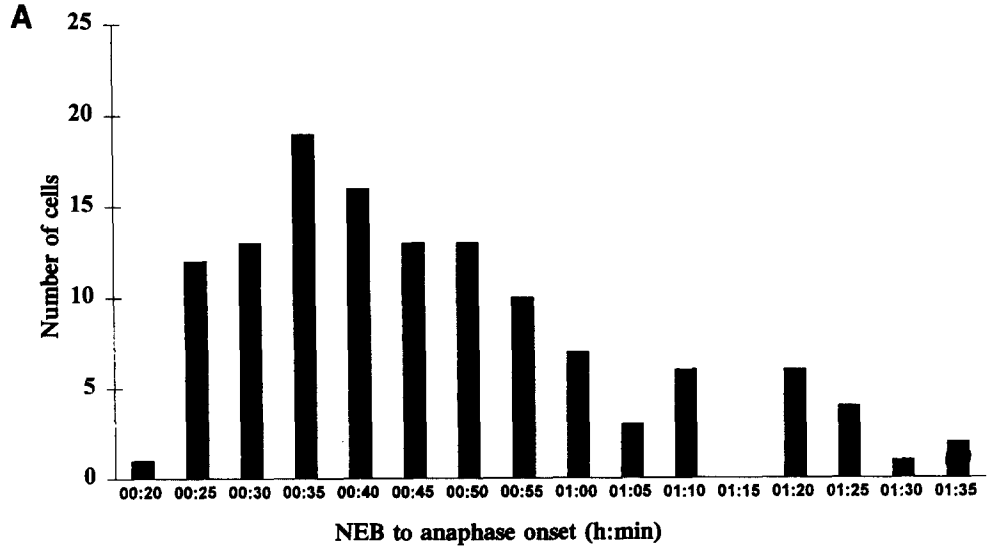
To better display the data we divided the cells into four categories: those in which all of the chromosomes were bioriented 20 min after NEB, and those that contained one or more monooriented chromosomes 20–29 min (e.g., Fig. 1), 30–39 min, and ≥40 min (e.g., Fig. 2) after NEB. The data are summarized in Table I *B* and displayed graphically in Fig. 3 *C*. Cells lacking monooriented chromosomes 20 min after NEB entered anaphase on average 38 ± 1 min after NEB ($n = 57$; range = 23–61 min). By contrast cells that contained monooriented chromosomes for longer periods entered anaphase correspondingly later (Table I *B*). Relative to cells in which all chromosomes were bioriented 20 min after NEB, anaphase onset occurred over twofold later in cells that contained monooriented chromosomes 40 or more min after NEB (Table I *B*; Figs. 2 and 3 *C*).

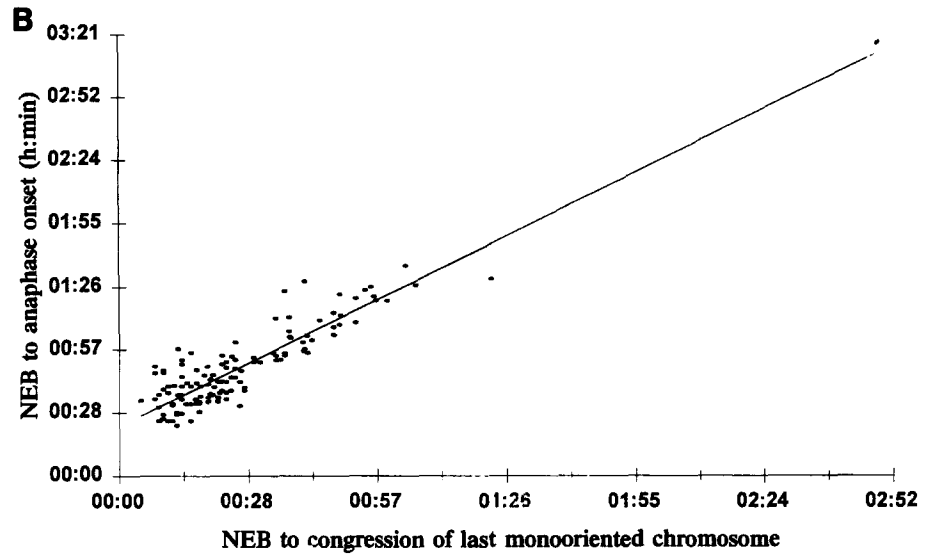
We next wanted to determine whether a single monooriented chromosome prolongs mitosis in PtK_1 cells. Our results (Table I *C*) clearly reveal that the longer a cell contains just a single monooriented chromosome the later it enters anaphase. The duration of mitosis for cells containing one monooriented chromosome ≥30 min past NEB is significantly different from that of cells lacking monooriented chromosomes 20 min after NEB ($P = 0.0001$). Thus, the onset of anaphase in PtK_1 cells is delayed by a single monooriented chromosome.

If the metaphase-anaphase checkpoint is relieved when the last monooriented chromosome biorients (as proposed e.g., by Zirkle 1970, McIntosh 1991), then the cell should enter anaphase after a relatively constant interval from when the last monooriented chromosome attaches in a bipolar fashion to the spindle. On average anaphase begins 23 ± 1 min ($n = 126$; range = 9–48 min) after the last monooriented chromosome initiated congression (summarized in Table II). Interestingly, the average time between these two events did not differ significantly ($P = 0.75$) between cells in which congression of the last monooriented chromosome was initiated early (≤20 min) or late (≥40 min) relative to NEB.

The Inhibition of Kinetochore Attachment by Colcemid Significantly Delays Anaphase Onset

Up to this point we have shown that unattached kinetochores delay anaphase onset in normal, untreated PtK_1 cells. However, it is known that PtK_1 cells enter anaphase in the complete absence of kinetochore attachment, as e.g., when spindle MT assembly is inhibited by Colcemid (reviewed in Rieder and Palazzo, 1992). To determine the duration of mitosis in Colcemid, relative to untreated cells, we filmed 11 PtK_1 cells treated continuously with 1.0 μM Colcemid 1–7 h before NEB. In all cases we found that the complete inhibition of kinetochore attachment to spindle MTs by Colcemid prolongs mitosis well beyond the normal range. 1–3 h after NEB each chromosome within the cell can be resolved as two chromatids connected along their length (Fig. 4 *A*). Although slow rounding of the cells made it progressively more difficult to follow the fate of all chromosomes, none of the cells systematically examined by through-focus phase-contrast or differential interference-contrast microscopy had disjoined chromatids 4–5 h after NEB (Fig. 4, *B* and *C*). The onset of anaphase is signaled by chromatid disjunction (Fig.





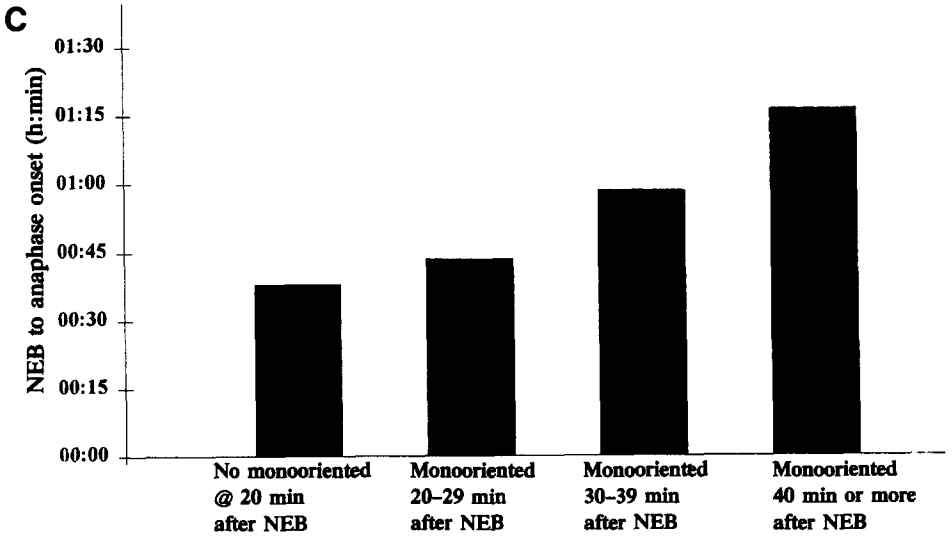


Figure 3. (*A*) Histogram plotting the number of cells versus the duration of spindle formation (NEB to anaphase onset) rounded to the nearest 5 min. This plot does not include a single cell that took over 3 h to complete spindle formation. Note that the duration from NEB to anaphase onset in PtK is highly variable. (*B*) Scatter plot in which the duration of spindle formation is plotted against the time it takes for the last monooriented chromosome in the cell to biorient relative to NEB. Note the strong positive linear relationship (r^2 = 0.85) between these two variables. (*C*) The average duration between NEB and anaphase onset versus the time the last monooriented chromosome initiates congression relative to NEB. Note that the longer the cell has monooriented chromosomes the longer anaphase onset is delayed relative to NEB.

Table II. Minutes between Biorientation of the Last Monooriented Chromosome and Anaphase Onset

	Number of cells	Average and SEM	Range
A. All Cells	126	23 ± 1	9–48
B. When last chromosome biorients:			
a. <20 min after NEB	57	24 ± 1	9–45
b. 20–29 min after NEB	30	20 ± 1	11–35
c. 30–39 min after NEB	11	25 ± 2	18–37
d. 40 or more min after NEB	28	24 ± 2	14–48

4, *C* and *D*) and is followed ~20 min later by chromosome clumping and the formation of a restitution nucleus. During this time, the cells exhibited vigorous surface blebbing and distortions (Fig. 4 *E*) before reflattening over the next hour (Fig. 4 *F*). In four cases a restitution nucleus formed on average 6 h and 26 min (range = 6 h 2 min to 6 h 48 min) after NEB. The remaining seven cells were still in mitosis when filming was terminated 4–9 h after NEB.

Anaphase Onset Can Be Delayed in Cells Containing Fully Congressed Chromosomes by Modifying the Dynamic Behavior of Spindle Microtubule Ends with Taxol

To examine the influence of MT dynamics on the metaphase-anaphase checkpoint, independent of kinetochore attachment, individual PtK_1 cells were followed from NEB until the last monooriented chromosome bioriented and completed congression. At this point the culture was perfused with 0.3–3 nM Taxol, in serum-free L-15 media containing 1% DMSO. Within 5 min after perfusing metaphase cells with this range of Taxol concentrations, the amplitude and frequency of chromosome oscillatory motions were greatly diminished. For most cells, anaphase onset was delayed relative to controls even though spindle bipolarity and the equatorial alignment of chromosomes was maintained over the next 1–2.5 h (Figs. 5 and 6). After this progressive cell rounding made it difficult to clearly follow chromosome distribution and behavior. As previously reported (Jordan et al., 1993) significant shortening of the spindle did not occur at these low Taxol doses.

As a control we perfused prophase cells with serum-free L-15 media containing 1% DMSO. Under this control situation PtK_1 cells entered anaphase 60 ± 6 min (n = 14; range = 30–119 min) after NEB, and 21 ± 2 min (n = 14; range = 12–39 min) after the last monooriented chromosome initiated congression. These numbers are essentially the same as those summarized in Tables I and II for untreated cells.

1–3 nM Taxol. Although a minority of cells (5/19) perfused with this range of Taxol concentrations entered anaphase within the normal 9–48-min time range, anaphase onset in the majority of the cells (14/19) was delayed well beyond the range of controls (summarized in Fig. 7). Anaphase onset in four of these cells, as signaled by chromatid disjunction, occurred on average 7 h, 54 min (range = 5 h, 19 min to 11 h, 45 min) after congression of the last chromosome. In these cells the chromatids exhibited no chromosome motion after disjoining. Approximately 20 min later they began to decondense and the cell initiated vigorous blebbing activity but did not complete cleavage. Over the next 30–60 min these cells reflattened onto the substrate and could be seen to contain a restitution nucleus or numerous micronuclei (see Jensen et al., 1987). In the remaining 11 cells chromosome disjunction had not occurred by the time filming was terminated 2 or more h after the drug was added.

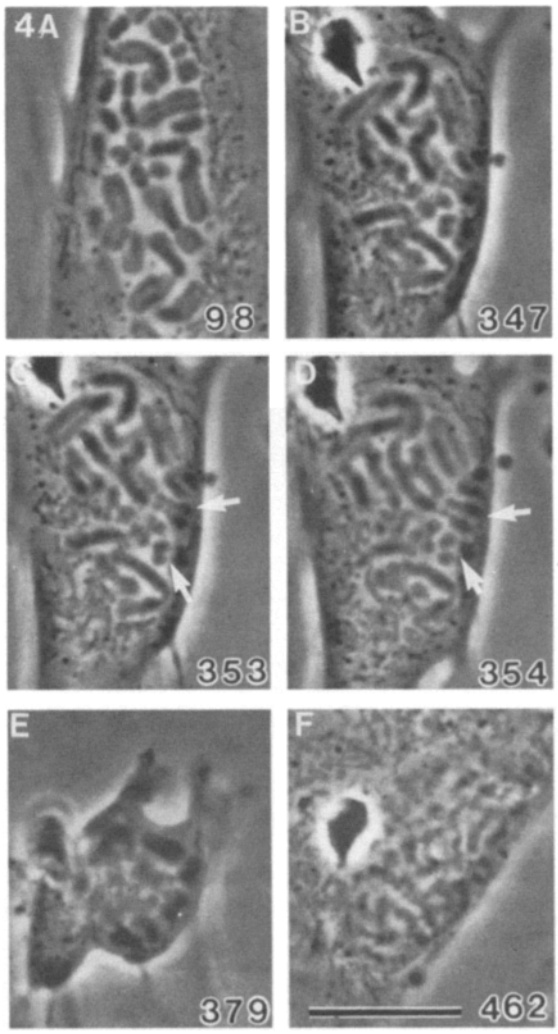


Figure 4. Selected video frames from a phase-contrast recording of a cell proceeding through mitosis in the presence of 1.0 μM Colcemid. Time in min from NEB is noted at the bottom right of each frame. Each chromosome is clearly resolvable as two chromatids, joined along their length, 1–2 h after NEB (*A*). Anaphase onset and the disjunction of chromatids occurs between *C* and *D* (arrows; note clear examples of disjunction). Approximately 25 min later the cell rounds and begins to violently bleb as it exits mitosis (*E*) and reflattens on the coverslip (*F*). Bar, 10.0 μm.

0.3–0.75 nM Taxol. We next perfused 28 cells after the last monooriented chromosome bioriented with 0.3–0.75 nM Taxol—doses that were close to the threshold for an effect. As summarized in Fig. 7 anaphase onset was not delayed, relative to controls, in 17 of these cells. However, in 6 cells anaphase onset occurred 2–3 h (Fig. 5) after congression of the last chromosome—well beyond the range of controls. Importantly, these cells exhibited seemingly normal chromosome motion to the poles (anaphase A) in the continued presence of Taxol (e.g., Fig. 5), even though this event was initiated well after untreated control cells would have entered

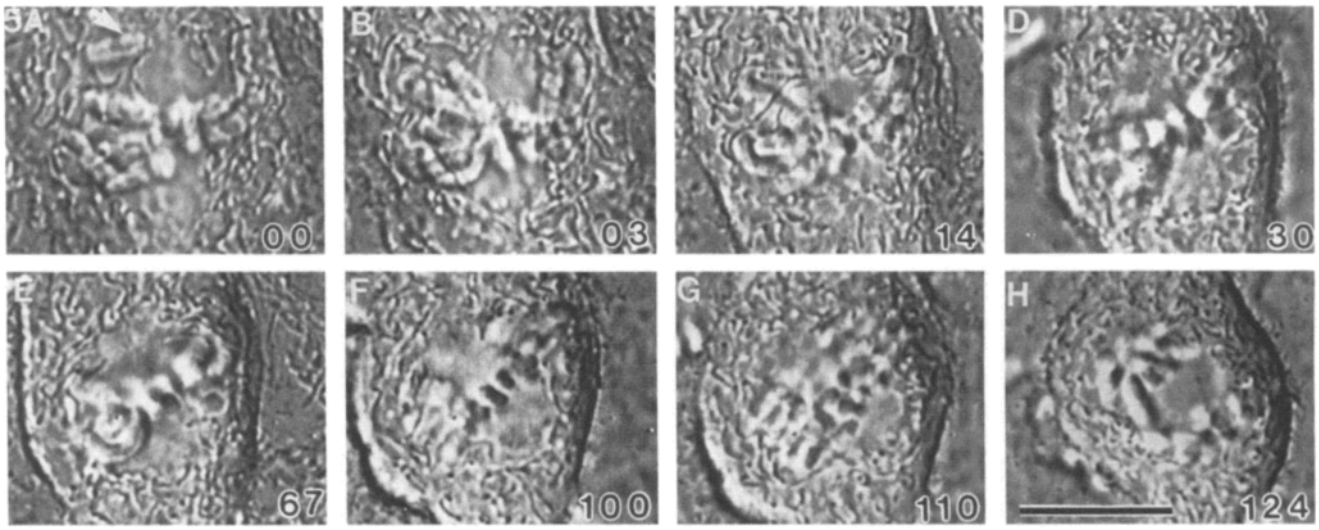


Figure 5. Selected video frames from a DIC recording of a metaphase cell treated with 0.6 nM Taxol immediately after the last monooriented chromosome (*A*, *arrow*) bioriented and congressed (*B*). The cell was treated immediately after *B*. Time in min from *A* is at the bottom right corner of each frame. Note that the spindle remains bipolar, and the chromosomes remain bioriented, from the time the drug is added (*B*) until anaphase onset (3 min before *G*). In this example Taxol delays anaphase onset 104 min compared with an average of 23 min for controls. Bar, 10.0 μm.

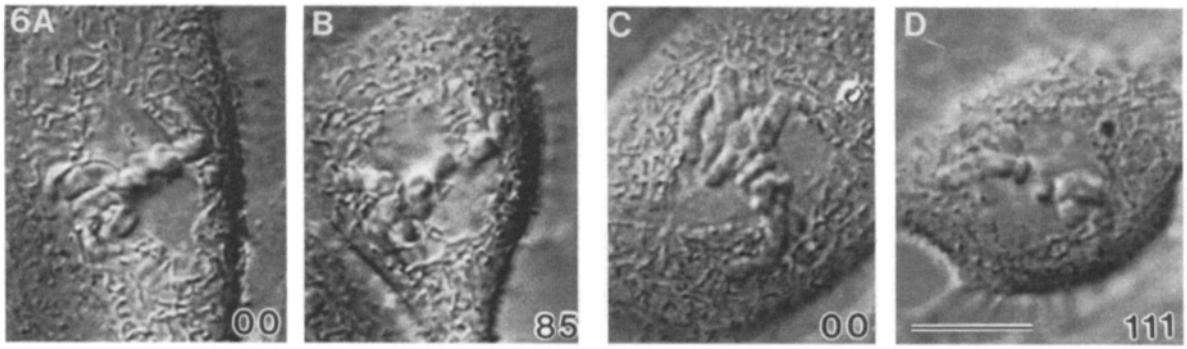


Figure 6. Selected DIC video frames from two PtK cells (*A–D*) treated with 0.5 nM Taxol after (*A*) and during (*C*) congression of the last monooriented chromosome. Taxol was added immediately after the *A* and *C* frames were recorded. In both cases anaphase onset was considerably delayed beyond the normal range even though the chromosomes remain fully congressed on bipolar spindles. Bar, 10.0 μm.

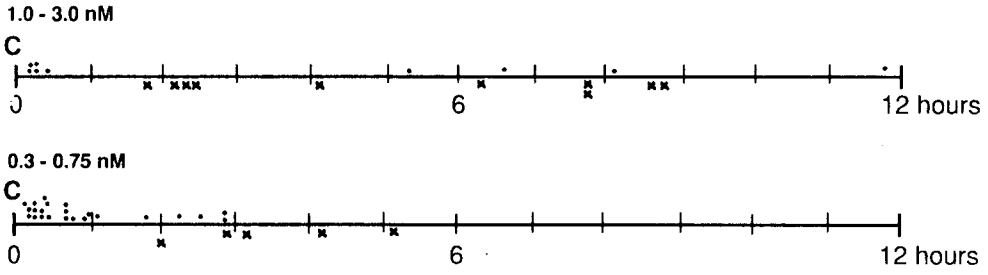


Figure 7. Hours between congression of the last monooriented chromosome (*C*) and anaphase onset for cells treated with two ranges of Taxol concentrations. Individual cells were perfused with Taxol immediately after the last monooriented chromosome completed congression. The points above the time axes show the time of anaphase onset for all individuals that went through the metaphase-anaphase transition while being filmed. The Xs below the time axes show the time at which filming was terminated for individuals that were still in mitosis (i.e., that had not initiated anaphase). The 126 untreated cells followed in this study initiated anaphase between 9 and 48 min (average 23 min) after the last monooriented chromosome initiated congression.

anaphase. Finally, the remaining five cells did not enter anaphase by the time filming was terminated between 2 and 5 h after addition of Taxol. In cells where Taxol delayed anaphase onset the spindle maintained its fusiform bipolar shape, and the chromosomes remained in an equatorial alignment (Figs. 5 and 6), until anaphase (Fig. 5) or until these relationships became obscured by cell rounding several hours into the treatment.

Discussion

Anaphase Onset in PtK_1 Is Controlled by a Checkpoint That Detects Unattached Kinetochores

We have shown that the mean interval from NEB to anaphase onset in PtK_1 cells is 50 ± 2 min (n = 126) under standard culture conditions. However, as graphically shown in Fig. 3, it is important to note that this mean masks a significant variation for the duration of mitosis at the individual cell level (23–198 min). Thus, the interval between NEB and anaphase onset in PtK_1, and likely other somatic cells, is neither consistent nor predictable as was previously suggested (Snyder et al., 1982; Vandre and Borisy, 1989), and mean values are of little use in predicting when a particular PtK_1 cell will enter anaphase.

In a previous study on PtK_1, Snyder et al., (1982) reported that the time from NEB to anaphase onset was 30 ± 2 min for a sample size of 20 cells. The reasons for the large (20 min) discrepancy in the duration of spindle formation in PtK_1, between our study and that of Snyder et al. (1982), are unknown. However, when compared to those used by Snyder et al. (1982) our PtK_1 cells remained much flatter throughout spindle formation which, based on the mechanism of kinetochore fiber formation (see Introduction), should be expected to prolong the time it takes for monooriented chromosomes to become bioriented.

The most significant finding of our study is that delays in the attachment of a single kinetochore in PtK_1 produce corresponding delays in anaphase onset. Importantly, we also found that the average interval from the attachment of the last kinetochore to the spindle and anaphase onset is essentially constant whether the cell contained unattached kinetochores less than 20 min or greater than 40 min after NEB. Together these findings confirm Zirkle's (1970) original contention, elaborated by Hartwell and Weinert (1989), that the metaphase-anaphase transition in vertebrate somatic cells is subject to a checkpoint control pathway that monitors kinetochore attachment, and delays anaphase until all monooriented chromosomes are properly bioriented. Due to the stochastic nature of kinetochore fiber formation on sister kinetochores, chromosome monoorientation is a normal facet of spindle assembly in vertebrates. Thus, this checkpoint must become operational at the time of NEB and, as we have observed, it leads to substantial variability in the duration of mitosis between cells. Our results obtained from vertebrate somatic cells are clearly different from those reported by Sluder et al. (1994) for sea urchin zygotes. They demonstrated that anaphase onset occurs at the normal time when 50% of the chromosomes are unattached or are monooriented to one spindle pole. Clearly, although sea urchin zygotes possess a checkpoint that delays anaphase in response to the disruption of spindle structure and/or the complete loss of any bipolar chromosome attachment (reviewed in Sluder, 1988), they do not possess a kinetochore attachment checkpoint control that detects the presence of a single unattached kinetochore as vertebrate somatic cells do. Since a high degree of synchrony appears to be required during the early cleavage stages of development, this checkpoint may be generally lacking in zygotes because of its tendency to promote asynchronous cleavage which would alter important specific spatial arrangements between blastomeres.

As outlined by Hartwell and Weinert (1989) checkpoint controls can be distinguished from extrinsic (e.g., substrate-product) controls if conditions are found that allow "a late event to occur even when an early, normally prerequisite event, is prevented." In the case of the kinetochore-based checkpoint such a relief of dependence can be demonstrated by preventing spindle formation with Colcemid. Although anaphase is delayed 6–10 h under these conditions, it ultimately occurs in the presence of a full complement of unattached kinetochores in PtK_1 (our data) and other vertebrates (reviewed in Kung et al., 1990; Rieder and Palazzo, 1992). These findings clearly demonstrate that PtK_1 cells allocate a finite period of time to the mitotic portion of the cell cycle, and enter anaphase after this time expires regardless of whether the criteria for passage through the kinetochore-based checkpoint is satisfied.

In PtK_1 cells the kinetochore-based checkpoint appears to accelerate the onset of anaphase which, in the absence of a spindle, would occur hours later. Given this condition it is possible that the kinetochore-based checkpoint pathway works in conjunction with a background "oscillator" that is normally entrained with, but can work independently of, the kinetochore attachment checkpoint control. During mitosis the influence of such an oscillator may build slowly over time or be constant, while the strength of the kinetochore checkpoint control may remain the same or become progressively weaker. Also, the relative strengths of the two systems may vary considerably between cells. For example, anaphase onset appears to be controlled solely by the oscillator in some embryonic cells (reviewed in Li and Murray, 1991). Alternatively, those vertebrate cells such as HeLa strains that are permanently inhibited from entering anaphase when spindle assembly is perturbed may have weak or nonfunctional oscillators (see Kung et al., 1990; Murray, 1992).

What Event or Process Is Monitored by the Kinetochore-Based Checkpoint Control?

What aspect of chromosome attachment does the kinetochore-based checkpoint control monitor given that it can detect a single unattached kinetochore against a background of 23 attached kinetochores and a normal spindle structure? The most obvious candidate is the acquisition of MTs by the last unattached kinetochore, which attaches it to the spindle. However, the results of several studies suggest that the presence of MTs at the kinetochore per se is not the event monitored by this checkpoint. In response to low concentrations of Taxol (Jordan et al., 1993) or microinjected centromeric proteins (e.g., Bernat et al., 1990; Tomkiel et al., 1994), HeLa cells do not enter anaphase even though they form normal looking spindles with fully congressed chromosomes. Under these conditions the attachment of all kinetochores to spindle MTs is not sufficient by itself to initiate anaphase. That MT attachment is not the event monitored is also indicated by Callan and Jacobs (1952) finding that univalent chromosomes, which have only one kinetochore, block untreated *Mantis religiosa* (mantid) spermatocytes from entering anaphase I even though all kinetochores in the cell are attached to the spindle. Thus, unless the kinetochore attachment checkpoint monitors the number of MTs associated with each kinetochore, and that this number is reduced on univalents and in experimentally treated HeLa cells, it is doubtful that attachment of MTs at a kinetochore is the event monitored by the checkpoint.

Aside from the attachment of spindle MTs to sister kinetochores, a factor that distinguishes mono- from bioriented chromosomes in somatic cells is the repeated presence of transient tension across the centromere (Skibbens et al., 1993). Indeed, tension has been shown to play an important role in stabilizing the attachment of MTs to kinetochores during meiosis (reviewed in Nicklas, 1989). In this context McIntosh (1991) proposed that each centromere region contains stretch sensitive enzyme(s) that produce a diffusible inhibitor of anaphase when its associated sister kinetochores are not under tension. Once the last chromosome acquires a bipolar attachment, its centromeric enzymes become stretched and inactivated by the directionally unstable motions of opposing sister kinetochores. Over time a proposed cytoplasmic activity then degrades the inhibitor, which is now no longer being produced, until its concentration falls below that required to inhibit anaphase.

At face value our data, and that of others, do not appear to support a centromere-based tension model for the kinetochore-mediated checkpoint control. The metaphase-anaphase transition is delayed in HeLa and PtK_1 cells, in response to Taxol (our data; also Jordan et al., 1993) or microinjected centromere antibodies (Bernat et al., 1990; Tomkiel et al., 1994), even though all the centromeres become engaged by poleward forces sufficient to congress the chromosomes and, in the case of Taxol, later move them poleward during anaphase (our results). However, the force needed to move a chromosome throughout mitosis is substantially less than that normally generated by the spindle (e.g., Nicklas, 1988). Thus, a centromere-based, tension-mediated checkpoint may remain in effect under these experimental conditions if the centromeres experience less than normal stretching. This possibility is consistent with our finding that the number and amplitude of kinetochore oscillatory motions is significantly diminished in Taxol-treated PtK_1 cells.

Given that tension between sister kinetochores remains an attractive possibility for the event monitored by the checkpoint mechanism, observations on meiotic systems suggest that the explicit form of the model (McIntosh, 1991) may have to be modified. Chromosomes are rarely held together at their centromeres during most first meiotic divisions. Instead, the bivalents are usually held together at the chromosome ends by terminalized chiasmata, and any tension generated by biorientation will act primarily through the chromosome arms on the kinetochore, and not through the centromere. Should the checkpoint control monitoring chromosome attachment be the same in mitotic and meiotic cells, it may detect the increase in tension or strain between the kinetochore itself and its attached MTs, generated immediately after biorientation by the antagonistic (out of phase) poleward motilities of sister kinetochores (see Skibbens et al., 1993; Rieder and Salmon, 1994). Such a change in tension could produce changes in checkpoint-related signaling molecules located, not within the centromere, but in each kinetochore. In this respect it is noteworthy that PtK_1 kinetochores contain one or more phosphorylated sites that are lost or shed upon attachment (Gorbsky and Ricketts, 1993). Perhaps, the absence of sufficient tension, even at a single kinetochore in an untreated cell, could allow it to be detected by the checkpoint pathway. Such a checkpoint control mechanism offers an explanation for why attached univalents block anaphase in spermatocytes of certain mantids that do not normally contain them (Callan and Jacobs, 1952). An attached univalent is not subjected to an antagonistic tension-producing force generated by its sister. It also provides an explanation for why antibody-induced alterations of kinetochore/centromere organization in HeLa (Bernat et al., 1990; Tomkiel et al., 1994), or mutational and deletional manipulations of centromeric DNA sequences in yeast (Spencer and Hieter, 1992), delay anaphase. Such structural alterations may effect the degree of tension experienced by attached kinetochores. Conversely, dampening MT plus-end dynamics by substoichiometric doses of vinblastine (Wendell et al., 1993) or Taxol (this report; Jordan et al., 1993) would modify the motile behavior of kinetochores on the plus ends of their associated MTs, with a corresponding reduction in the tension experienced at the MT-kinetochore junction.

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