The Checkpoint Delaying Anaphase in Response to Chromosome Monoorientation Is Mediated by an Inhibitory Signal Produced by Unattached Kinetochores

Conly L. Rieder,*‡ Richard W. Cole,* Alexey Khodjakov,* and Greenfield Sluder§

*Division of Molecular Medicine, Wadsworth Center, Albany, New York 12201-0509; ‡Department of Biomedical Sciences, State University of New York, Albany, New York 12222; and §Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

Abstract. During mitosis in Ptk_1 cells anaphase is not initiated until, on average, 23 ± 1 min after the last monooriented chromosome acquires a bipolar attachment to the spindle—an event that may require 3 h (Rieder, C. L., A. Schultz, R. W. Cole, and G. Sluder. 1994. *J. Cell Biol.* 127:1301–1310). To determine the nature of this cell-cycle checkpoint signal, and its site of production, we followed PtK_1 cells by video microscopy prior to and after destroying specific chromosomal regions by laser irradiation. The checkpoint was relieved, and cells entered anaphase, 17 ± 1 min after the centromere (and both of its associated sister kinetochores) was destroyed on the last monooriented chromosome. Thus, the checkpoint mechanism monitors an inhibitor of anaphase produced in the centromere of monooriented chromosomes. Next, in the presence of one monooriented chromosome, we destroyed one kinetochore on a bioriented chromosome to create a second monooriented chromosome lacking an unattached kinetochore. Under this condition anaphase began in the presence of the experimentally created monooriented chromosome 24 ± 1.5 min after the nonirradiated monooriented chromosome bioriented. This result reveals that the checkpoint signal is not generated by the attached kinetochore of a monooriented chromosome or throughout the centromere volume. Finally, we selectively destroyed the unattached kinetochore on the last monooriented chromosome. Under this condition cells entered anaphase 20 ± 2.5 min after the operation, without congressing the irradiated chromosome. Correlative light microscopy/elctron microscopy of these cells in anaphase confirmed the absence of a kinetochore on the unattached chromatid. Together, our data reveal that molecules in or near the unattached kinetochore of a monooriented PtK_1 chromosome inhibit the metaphase–anaphase transition.

KINETOCHORE fibers (K-fibers)[1] are bundles of dynamic microtubules (MTs) formed in animal somatic cells as kinetochores capture growing centrosome-nucleated MTs. These fibers tether chromosomes to the poles and act as force-production scaffolds for kinetochore-based chromosome motion (reviewed in Rieder, 1990; McIntosh, 1994; Desai and Mitchison, 1994). During spindle formation in animal cells K-fibers form asynchronously on sister kinetochores. As a result forming spindles in these cells typically contain a variable number of "monooriented" chromosomes that are attached to and positioned near one spindle pole. Because MT capture at the unattached kinetochore of a monooriented chromosome is a stochastic process, and because this kinetochore is positioned a variable distance from the pole to which it must ultimately attach, the time required for all monooriented chromosomes to achieve biorientation is highly variable (Rieder et al., 1994). Thus, a high incidence of chromosome non-disjunction would occur if the time of anaphase onset was determined by an invariant timing mechanism. To avoid this many cells have evolved a feedback mechanism, or checkpoint control (concepts reviewed in Hartwell and Weinert, 1989; Murray, 1994), that delays anaphase until the last monooriented chromosome acquires a bipolar attachment (Rieder et al., 1994).

The checkpoint pathway that delays anaphase in response to monooriented chromosomes is presumably active by the time of nuclear envelope breakdown when the sister kinetochores on each chromosome begin to interact with centrosome-nucleated MTs to form the spindle. It is then turned off or "relieved" after the last monooriented

Address correspondence to C. L. Rieder, Lab of Cell Regulation, Wadsworth Center for Labs and Research, P.O. Box 509, Albany, New York 12201-0509. Tel.: (518) 474-6774.Fax: (518) 486-4901. E-mail: RIEDER@WADSWORTH.ORG.

1. *Abbreviations used in this paper*: av., average; DIC, differential interference contrast; EM, electron microscopy; K-fiber, kinetochore fiber; LM, light microscopy; MTs, microtubules.

chromosome becomes bioriented, a process that may require as many as several (≥3) hours in PtK_1 cells (Rieder et al., 1994). As argued by McIntosh (1991) this checkpoint is probably based on a negative feedback control that monitors monooriented rather than bioriented chromosomes; in the presence of numerous bioriented chromosomes, it is easier for the cell to distinguish one monooriented chromosome rather than to detect relatively small changes in the percentage of chromosomes attached in a bipolar fashion. Since the difference between a mono- and bioriented chromosome involves the attachment of its previously unattached kinetochore to spindle MTs, it is also assumed that the checkpoint delaying anaphase in response to monoorientation monitors an activity (or lack of activity) associated with centromere region of the chromosome that contains the sister kinetochores (e.g., McIntosh, 1991; Earnshaw et al., 1991; Gorbsky and Ricketts, 1993; Tomkiel et al., 1994; Rieder et al., 1994; Li and Nicklas, 1995; Jang et al., 1995). However, as emphasized by Earnshaw and MacKay (1994) the concept that the centromere region of a monooriented chromosome produces any kind of checkpoint signal, yet alone an inhibitory one, has never been directly tested. Indeed, recent experimental observations on sea urchin zygotes suggest that the delay in anaphase caused by monoorientation is positively controlled by an activity asssociated with those chromosomes attached in a bipolar fashion (Sluder et al., 1994; see also Murray, 1994).

To gain insight into the mechanism that delays anaphase in response to chromosome monoorientation in somatic animal cells we have sought to directly determine whether the checkpoint pathway works through a positive or negative feedback control, and whether it involves the centromere. Our approach has been to use a laser microbeam to selectively destroy specific areas on monooriented and bioriented chromosomes in living PtK_1 cells.

Materials and Methods

Cell Culture

Stock cultures of PtK_1 cells (2N = 12) were grown in 75 cm^2 T-flasks at 37°C in MEM supplemented with nonessential amino acids and 10% fetal calf serum. For study the cells were enzymatically removed from the flasks and pipetted into Petri dishes containing Hepes-buffered L-15 medium with 10% fetal calf serum and 25 mm^2 glass coverslips (Rieder et al., 1994). After a 1–2-d incubation at 37°C the coverslips were mounted in Rose chambers filled with L-15 medium. These chambers allow cells to be viewed at high resolution yet contain enough media to support continued growth of the culture for several days.

Laser Microsurgery and Video-enhanced Light Microscopy

Rose chamber cultures of PtK_1 cells were mounted on a NIKON Diaphot 200 inverted microscope (Nikon, Garden City, NY) equipped with a Ludl MAC 2000 (Ludl Electronics Ltd., Hawthorne, NY) motorized stage. The cultures were maintained at 35–37°C with a Rose chamber heater (Rieder et al., 1994), and selected cells were followed by time-lapse differential interference contrast (DIC) video light microscopy (LM) using framing rates of 15–60 frames/min. The illumination, provided by a 100 W high pressure mercury arc lamp, was filtered with Nikon GFI 546 ± 20 nm, Omega KG5 (Omega Optical, Brattleboro, VT), and Omega GG400 filters and was shuttered between frames with a Uniblitz shutter controlled by IMAGE 1 software. Cells were viewed with a 60× DIC objective (NA = 1.4) and a 0.85 NA condenser. Video images, obtained by integrating two video frames directly on a Paultek P100 CCD chip (Paultek, Princeton, NJ) were routed through an IMAGE 1 (Universal Imaging Corp., West Chester, PA) image processor prior to storage on a Panasonic TQ 2028 optical memory disk recorder. Electronic and optical noise within the system was eliminated by background subtraction, and recording an eight frame jumping average.

The laser-based microscopic cutting system used in our study was similar to that developed by Berns and colleagues (reviewed in Berns, 1978; Berns et al., 1980, 1991; Liang et al., 1994) and used DIC optics. In brief, the 1,064 nm output from a pulsed (5 nanosecond) Neodymium-YAG (yttrium-aluminum-garnet) laser (Surelite II; Continuum, Santa Clara, CA) was frequency doubled to 532 nm and filtered to remove stray 1,064 nm light. This beam was then steered into the epiport of the Diaphot 200 where it was reflected, via a custom-made Omega dichroic mirror, through the Wollaston prism and onto the back aperture of the Nikon 60× (NA = 1.4) objective. The objective then focused the beam to a diffraction limited spot. The original diameter of the laser beam was 7 mm, but in order to completely fill the back aperture of the objective it was increased to 10 mm by running it several meters along the optical bench. After passing through the specimen the laser light was blocked in the condenser assembly by an Omega filter that reflected all wavelengths below 540 nm.

Theoretically the waist of the laser beam at the focal point of our objective lens can be approximated by the Bessell equation:

$$\text{waist} = 1.22\,\lambda/\text{NA} \quad (1)$$

(where waist = diameter of the laser beam at focus; λ = the wavelength of laser light in micrometers (i.e., 0.532 μm); and NA = the numerical aperture of the objective (i.e., 1.4). This equation predicts that the spot size should be approximately 0.5 μm, and indeed direct measurements of the central spot in the airy disk pattern formed when the objective lens focuses the laser beam confirms that the beam diameter is approximately 0.5 μm (data not shown; see also Schneider and Webb, 1981).

In our system the focused laser spot at the specimen plane was set near the center of a video screen, and its exact position was determined daily by irradiating a dried film of red blood cells (see Berns, 1978). Once located, the position of the laser on the video screen was marked with cross-hairs. Cutting was then achieved by using the Ludl motorized stage to pass the specimen through the fixed laser beam path, using the cross-hairs as a reference mark for the laser beam. Optimal chromosome cutting with our system was produced by operating the laser at 10 Hz with 5 ns pulses, each of which contained ~400 nJ of power as measured at the focal point of the objective lens. In practice it took ~1–2 s, or 10–20 laser pulses, to completely sever a PtK_1 chromosome across its short axis.

Electron Microscopy

Cells followed in vivo were fixed for electron microscopy by rapidly removing the Rose chamber from the microscope stage and exchanging the media with 2.5% glutaraldehyde in 0.1 M Millonig's phosphate buffer (pH 7.3). This procedure took no longer than 15 s to complete, after which the culture was placed back on the microscope stage and the cell relocated, circled with an objective scribe, and photographed for future reference. After 30 min the chamber was disassembled and the cell-containing coverslip was washed in phosphate buffer. After postfixation in 2% OsO_4 for 1 h at 4°C, the cells were dehydrated up to 70% ethanol. They were then left in 70% ethanol containing 2% uranyl acetate for 2–12 h, prior to completing the dehydration and flat embedding steps (see Roos, 1973). Cells followed in vivo were then relocated, excised, and serially thin sectioned (see Rieder, 1981 for details). The ribbons of sections were collected on Formvar-coated slot grids and subsequently stained by uranyl acetate and lead citrate. The pertinent areas of each section were then photographed with a Zeiss 910 EM operated at 80 kV, and the sequential photographs stacked into a three-dimensional volume and rendered using STERECON software (Marko et al., 1988).

Results

Severing Chromosome Arms Does Not Affect the Timing of Anaphase Onset Relative to Biorientation of the Last Monooriented Chromosome

PtK_1 do not enter anaphase in the presence of monooriented chromosomes and initiate anaphase 23 ± 1 min after

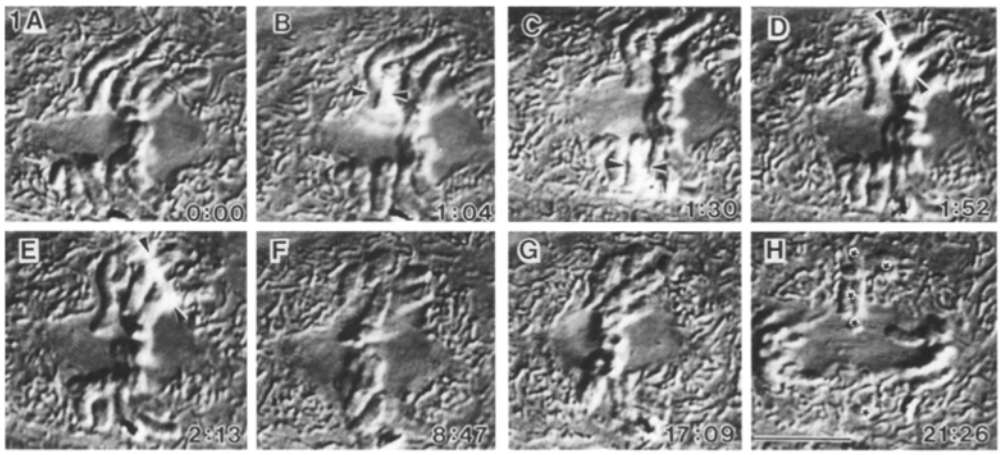

Figure 1. (*A–H*) Selected video frames of a prometaphase PtK_1 cell proceeding through anaphase after the arms of 4 chromosomes (*B–E, arrowheads*) were severed with the laser over a 1-min period. The last monooriented chromosome (*A* and *B*, *white arrows*) initiated congression between frames *B* and *C*, and the cell entered anaphase ~16 min later (*G*). A number of severed chromosome arms can be seen between the separating groups of anaphase chromosomes (*H*, *asterisk*). Time in min/sec at lower right corner of each frame. Bar, 10 μm.

the last monooriented chromosome biorients at 35–37°C — an event that may require several hours (Table I; Rieder et al., 1994). To determine if laser microsurgery on chromosomes influences the duration of this "metaphase" stage of mitosis, we severed one or both chromosome arms from one or more monooriented or bioriented chromosomes in prometaphase cells containing monooriented chromosomes (Fig. 1). Under these conditions the cells entered anaphase within a normal period of time after the last monooriented chromosome initiated congression (22 ± 2 min; Table I). Thus, laser microsurgery on prometaphase or metaphase chromosomes outside of the centromere region does not effect the timing or progression of prometaphase cells into anaphase.

Irradiating Near A Kinetochore Does Not Disrupt Chromosome Motion

Hays and Salmon (1990) reported that irradiating a K-fiber near its kinetochore in grasshopper spermatocytes with pulsed 532 nm laser light does not effect chromosome behavior. To determine if this is the case for PtK_1 cells we irradiated K-fibers for 1–2 s ~0.50 μm in front of one of the kinetochores on an oscillating bioriented chromosome in prometaphase/metaphase cells. Such irradiation did not produce monoorientation or inhibit chromosome motion (data not shown).

On average PtK_1 chromosomes are 1.3 ± 0.1 μm (N = 20; range = 1.5–1.1 μm) wide at the primary constriction, and 2.0 ± 0.20 μm (N = 70; range = 2.7–1.5 μm) wide outside of this region. To clearly define the functional radius of damage caused by the laser in chromatin we followed oscillating bioriented chromosomes for several minutes and then used the laser to cut through the centromere, along the chromosome long axis. Under this condition the laser beam had to have approached one of the kinetochores within ≤0.35 μm (i.e., the width of the primary constriction [1.25 μm] minus the diameter of the laser [0.50 μm] divided by 2). Using this approach we could create two various sized kinetochore-containing chromosome fragments which quickly began to move towards their respective poles (Fig. 2). When the centromere region just under one kinetochore was cut, the kinetochore remained tethered to the bulk of the chromosome by thin compliant chromatin strands (see also Skibbens et al., 1995). Importantly, this kinetochore remained functional after the cut as evidenced by the fact that it moved poleward and then, after adopting a new average position, began to undergo the same directionally unstable behavior seen on attached kinetochores on monooriented chromosomes (Fig. 2).

Table I. Duration between Biorientation of the Last Monooriented Chromosome or Laser Irradiation and Anaphase Onset in PtK_1 Cells

	Number of cells	Average and S.E. of mean	Range
		min	*min*
Controls			
No laser irradiation*	126	23 ± 1	9–48
Arm(s) severed from ≥1 chromosomes‡	11	22 ± 2	14–31
Experimental			
When the centromere on last monooriented chromosome is completely destroyed	11	17 ± 1	11–22
When one Kinetochore on a congressing chromosome is destroyed in the presence of a naturally monooriented chromosome§	24	24 ± 1.5	15–42
After destruction of the unattached kinetochore on the last monooriented chromosome	12	20 ± 2.5	7–42

*Duration between biorientation of the last monooriented chromosome and anaphase onset (from Rieder et al., 1994).
‡From bioriented and/or monooriented chromosomes, at least one of which was cut in the presence of ≥1 monooriented chromosomes. Duration represents time between biorientation of the last monooriented chromosome and anaphase onset.
§Timed, in the presence of the laser-generated monooriented chromosome, from biorientation of the last naturally occurring monooriented chromosome to anaphase onset.

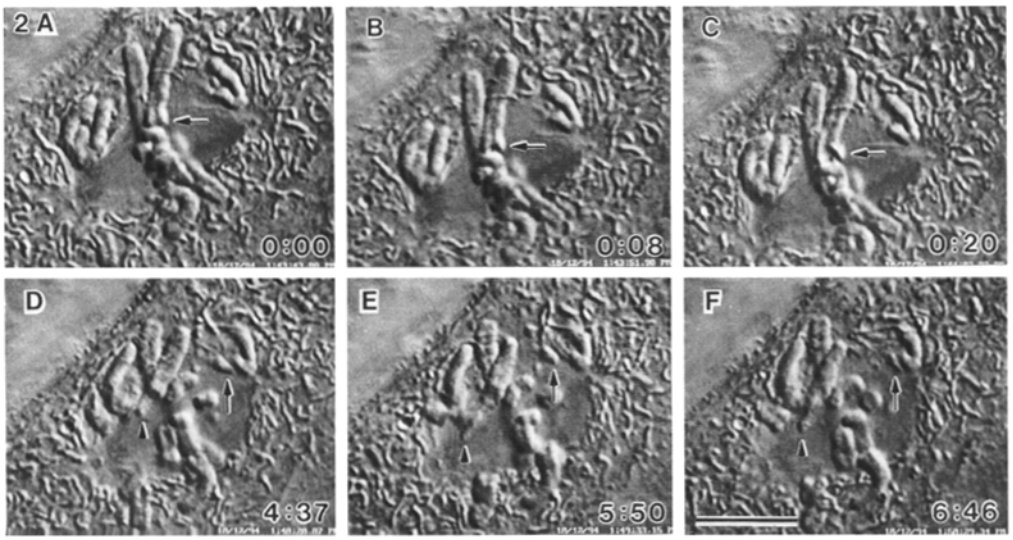

Figure 2. (*A–F*) Selected video frames of a prometaphase PtK_1 cell in which the centromere of one bioriented chromosome (*A* and *B*, *arrow*) was severed (*C*, *arrow*) close to one of its kinetochores. The focused (*green light*) laser is not visible in this sequence as it cuts through the centromere and chromosome arms in *C*. As a result of this operation two kinetochore-containing chromosome fragments were produced. One possessed most of the (stiff) centromeric region and monooriented to the right pole (*D–F*, *arrow*) where it began to undergo normal oscillatory motions. The kinetochore on the other chromosomal fragment (*D–F*, *arrowhead*) contained much less associated centromere material but remained tethered to the bulk of the chromosome by thin compliant strands of chromatin. It also moved towards its associated pole where it began to oscillate normally (*D–F*, *arrow*). Note that the initial laser cut (*C*, *arrow*) was very close to this kinetochore. Time in min/sec at lower right corner of each frame. Bar, 10 μm.

This functional assay reveals that laser-induced damage to chromatin is restricted to the width of the beam spot size.

The Checkpoint Delaying Anaphase Is Relieved by Destroying the Centromere on the Last Monooriented Chromosome

We have defined the centromere, for all of the experiments reported here, to be that region in the primary constriction of the chromosome that lies between and includes the sister kinetochores. If the delay in anaphase onset effected by the last monooriented chromosome is associated with an inhibitory activity within its centromere, then anaphase should start on average ≤23 min after this region is destroyed by the laser. For this experiment we located cells containing a single monooriented chromosome and then completely severed the chromosome through its centromere (Fig. 3). This operation took 1–2 s, and produced two acentric chromosome fragments that were no longer attached to the spindle. Under this condition anaphase was initiated on average 17 ± 1 min (see Table I) after the centromere on the last monooriented chromosome was destroyed. In most cases each of the two chromosome fragments produced by the laser microsurgery disjoined into two separate chromatid fragments at anaphase onset. The results of this experiment demonstrate clearly that the signal transducers delaying anaphase in response to monoorientation are located in the centromere of monooriented chromosomes and that they produce an inhibitor of the metaphase–anaphase transition.

Anaphase Onset Is Not Delayed by Monooriented Chromosomes with Largely Intact Centromeres That Lack Unattached Kinetochores

If the checkpoint that delays anaphase in response to chromosome monoorientation monitors and activity associated with the unattached kinetochore on a monooriented chromosome, then the presence of a monooriented chromosome lacking an unattached kinetochore should not delay anaphase onset. Conversely, if the checkpoint monitors something produced by the only attached kinetochore, stretching within the centromere caused by biorientation (McIntosh, 1991), or unequal numbers of K-fibers in opposing half spindles, then a laser-generated monooriented chromosome with no distal (unattached) kinetochore should continue to inhibit anaphase onset.

We created monooriented chromosomes with largely intact centromeres by selectively destroying one of the kinetochores on a bioriented chromosome (Fig. 4). We conducted this experiment on mid-to-late prometaphase cells containing one or more monooriented chromosomes so that we could determine whether the laser-generated monooriented chromosome delayed anaphase onset relative to the 23 min average period required to initiate anaphase after biorientation of the last naturally monooriented chromosome. For this experiment we positioned the laser beam in front of the most highly stretched kinetochore region on a bioriented chromosome and irradiated for 1–2 s while slowly moving the chromosome towards the beam with the motorized stage. After this kinetochore was destroyed the chromosome immediately changed its direction of motion and began moving away from the laser beam and towards the pole to which its undamaged kinetochore was attached (Fig. 4; see also Brenner et al., 1980; McNeil and Berns, 1981). Once near the pole it began to undergo normal oscillatory motions which, in some cells, carried the chromosome very close to the metaphase plate. Anaphase onset occurred 24 ± 1.5 min (see Table I) after the last non-irradiated monooriented chromosome initiated congression, and in the presence of the laser generated monooriented chromosome. During anaphase one intact chromatid of the laser-generated monooriented chromosome remained associated with the pole while the other either remained associated with the attached chromatid or

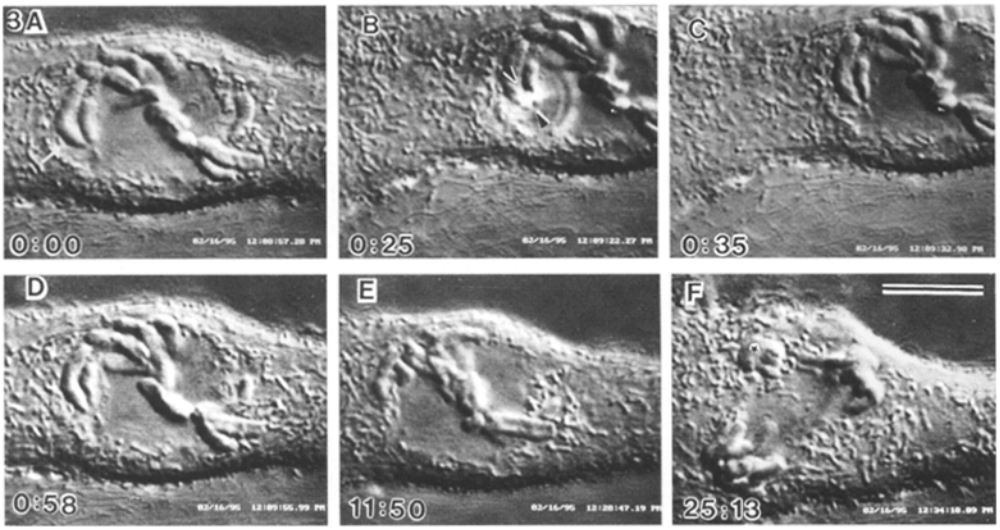

Figure 3. (*A–F*) Selected video frames of a prometaphase PtK_1 cell in which the kinetochore/centromere complex on the last monooriented chromosome (*A*, *white arrow*) was destroyed with the laser by cutting the chromosome through its primary constriction perpendicular to the chromosome long axis (*B*, *arrowheads*). The two acentric chromosome fragments produced by this procedure remained at the periphery of the spindle (*C* and *D*) until the cell entered anaphase 11 min later (*E*). They were then found in the cytoplasm between the separating groups of anaphase chromosomes (*F*, *asterisk*). Time in min/sec at lower left corner of each frame. Bar, 10 μm.

floated away into the cytoplasm. In many cases the centromere of the experimental chromosome was ≥50% intact as evidenced by the fact that the unattached chromatid was not broken after chromatid disjunction (Fig. 4). The results of this experiment reveal that the checkpoint does not delay anaphase in response to unequal numbers of K-fibers in opposing half-spindles. It also demonstrates that the checkpoint does not monitor a signal produced by the attached kinetochore on a monooriented chromosome or throughout the majority (≥50%) of the centromere volume.

Destroying the Unattached Kinetochore on the Last Monooriented Chromosome Relieves the Checkpoint

In our final experiment we sought to destroy the unattached kinetochore on the last monooriented chromosome in late prometaphase cells. For this study we irradiated that region of the last monooriented chromosome where the unattached kinetochore was predicted to be for 1–2 s, and then followed the cell (Fig. 5). One of two outcomes were then observed: in 60% of the cells (N = 18) the chromosome bioriented after a highly variable period of time and moved to the spindle equator. Since biorientation requires two functional kinetochores, and since laser irradiation near a kinetochore does not affect its behavior, we concluded that in these cases the unattached kinetochore was not destroyed. By contrast in 40% of the cells (N = 12) the chromosome remained associated with the pole to which it was monooriented and the cell entered anaphase 20 ± 2.5 min after the irradiation (see Table I).

In one cell, not included in Table I, we destroyed the unattached kinetochores on two different monooriented

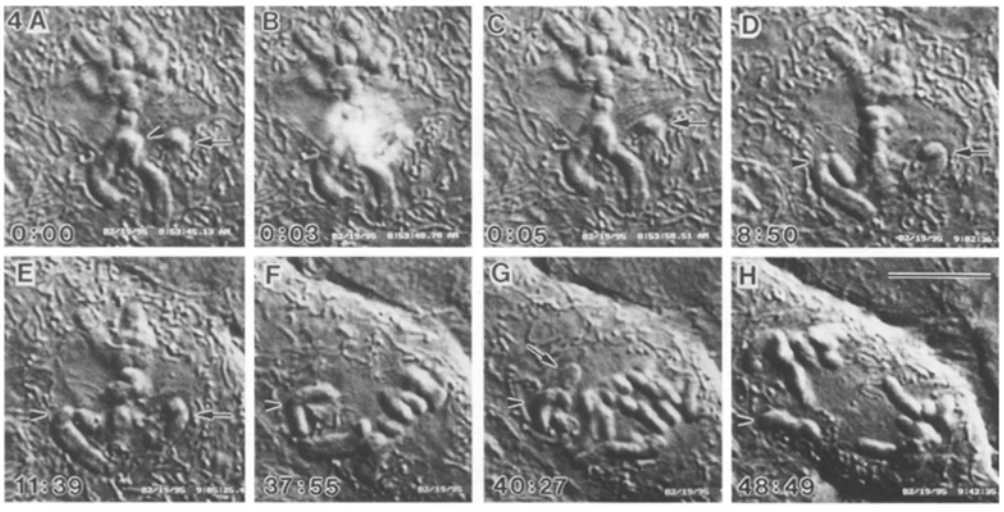

Figure 4. (*A–H*) Selected video frames of a prometaphase PtK_1 cell in which one of the kinetochores of a bioriented chromosome (*A*, *arrowhead*) is selectively destroyed by the laser (*B*) in the presence of a single naturally occurring monooriented chromosome (*A–E*, *arrows*). After its right kinetochore was destroyed the chromosome monooriented to the left pole (*D–F*, *arrowhead*) and remained associated with this pole until anaphase onset (*F*) which occurred ~26 min after the last naturally occurring monooriented chromosome (*A–D*, *arrows*) initiated congression (*E*). During anaphase one of the chromatids of the experimentally produced monooriented chromosome remained associated with the pole (*G*, *arrow*) while the other (*G* and *H*, *arrowheads*) drifted free into the cytoplasm. Note that the free chromatid is intact. Time in min/sec at lower right corner of each frame. Bar, 10 μm.

chromosomes. At the beginning of the observational period this cell contained one monooriented chromosome at each spindle pole. Initially we irradiated the area containing the unattached kinetochore on one of the chromosomes. Both chromosomes were still monooriented 69 min later. At this time we then irradiated the unattached kinetochore on the other monooriented chromosome. After this irradiation the cell entered anaphase 16 min later, and in the presence of both laser-irradiated monooriented chromosomes (data not shown).

One of the cells that entered anaphase in the presence of a laser-irradiated monooriented chromosome (Fig. 5) was fixed and processed for EM. A three-dimensional reconstruction from serial sections revealed that this chromosome possessed only one kinetochore (Fig. 6).

Discussion

The primary goals of our study were to determine if the signal that prevents anaphase in response to chromosome monoorientation in vertebrate somatic cells is a negative "wait anaphase" signal produced in the centromere of the monooriented chromosome, and if so whether it is generated throughout the centromere, and/or in the attached or unattached kinetochores. To achieve these goals we used a laser to destroy specific regions of chromosomes in living PtK_1 cells, and then followed the behavior of the cells by time-lapse video microscopy. As reported by Berns and others (e.g., Rattner and Berns, 1974; Brenner et al., 1980; McNeil and Berns, 1981; Rieder et al., 1986; Hays and Salmon, 1990; Liang et al., 1994; Skibbens et al., 1995) a pulsed Nd:YAG laser can be used to destroy chromatin and chromosome-associated organelles in living untreated cells without damage to other spindle components including MTs and centrosomes. Why chromatin is so sensitive to destruction with pulsed visible laser light remains to be determined (Berns et al., 1980).

Our control experiments confirm Hays and Salmon's (1990) finding that irradiating K-fibers near kinetochores on bioriented chromosomes with 532 nm laser light does not inhibit chromosome motion or lead to monoorientation. We also found that extensive laser microsurgery on the arms of monooriented or biorienting chromosomes does not affect the timing of anaphase onset after the last monooriented chromosome biorients (see Table I). Finally, using kinetochore behavior as a functional assay, our control work indicates that the damage created in the chromosome by the laser is restricted to the 0.5-μm diam irradiated area. We base this contention on our observations that sister kinetochore(s) exhibit normal behavior when one of the chromosome arms is severed 0.25–0.50 μm from the centromere (data not shown); that an unattached kinetochore on a monooriented chromosome can still attach when the chromosome is irradiated in its immediate vicinity (e.g., in those cases where the last monooriented chromosome congressed after we shot at but missed its unattached kinetochore); and that kinetochores exhibit normal behavior when the laser beam hits 0.35 μm from the kinetochore (Fig. 2).

The Checkpoint Monitoring Bipolar Chromosome Attachment in Vertebrate Somatic Cells Is Based on an Inhibitory Signal Produced by Monooriented Chromosome

The experiment in which we destroyed the centromere on the last monooriented chromosome allowed us to distinguish whether monooriented chromosomes produce an inhibitor of anaphase onset or whether bioriented chromosomes produce a promoter of anaphase. Untreated PtK_1 cells may contain one or more monooriented chromosomes 3 h after nuclear envelope breakdown, and as long as they do, anaphase is inhibited (Rieder et al., 1994). However, we found that the checkpoint was rapidly relieved after the centromere on the last monooriented chromosome was destroyed. The fact that the checkpoint was relieved by this operation clearly reveals that it is based on an inhibitory "wait anaphase" signal produced within the centromere of monooriented (or unattached) chromosomes.

We also found that PtK_1 cells entered anaphase significantly faster (with a 95% confidence level using the

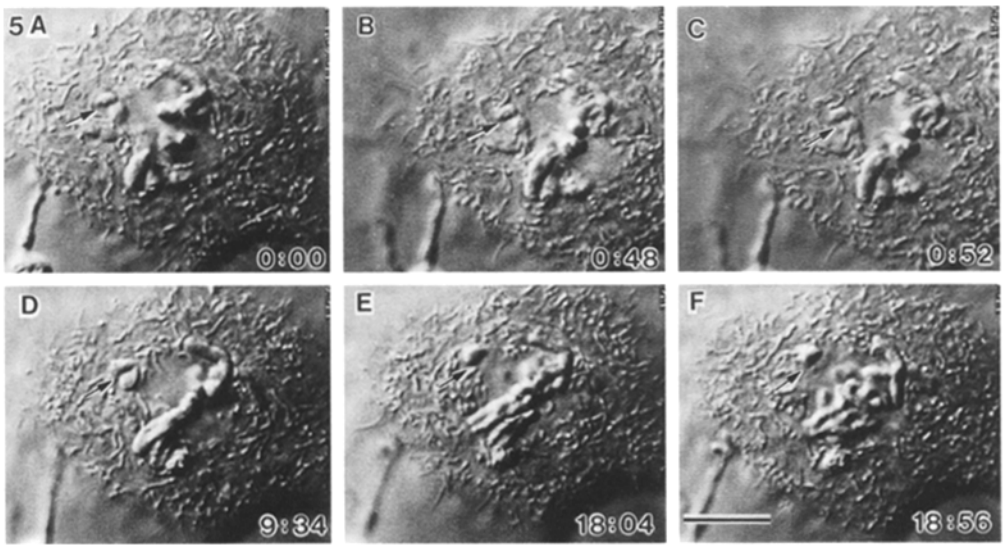

Figure 5. (*A–F*) Selected video frames of a prometaphase PtK_1 cell in which the unattached kinetochore on the last monooriented chromosome (*A*, *arrow*) was destroyed by the laser (*B*, *arrow*). As in Fig. 2 the focused laser is not visible in this sequence. The irradiated chromosome remained monooriented (*C–F*, *arrow*) until the cell entered anaphase (*E*) ~17 min after the laser operation. It was then fixed for a three-dimensional EM analysis immediately after F (see Fig. 6). Time in min:sec at lower right hand corner of each frame. Bar, 10 μm.

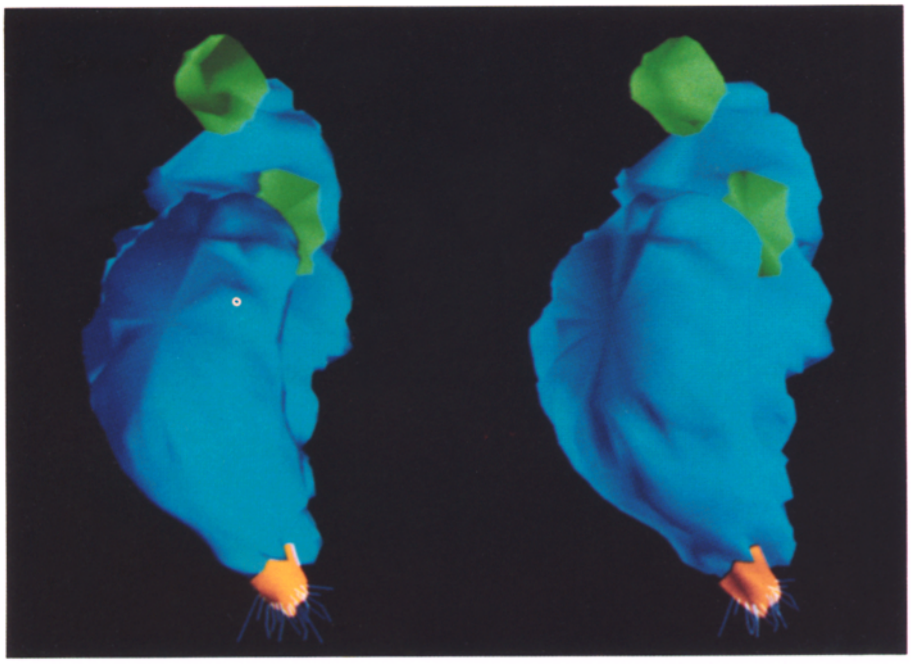

Figure 6. Stereo pair of a three-dimensional surface-rendered ultrastructural reconstruction, generated from serial thin sections, of the centromere region on the laser-irradiated monooriented chromosome pictured in anaphase in Fig. 5. In this reconstruction the chromatin is blue and the single (attached) kinetochore is orange. The two green patches are the nucleolar organizers that are positioned very near the centromere on the opposing chromatids of the small X chromosome (Shaw and Krooth, 1964). Only one kinetochore could be found in the complete serial series through this chromosome and it was attached to its associated pole by 16 microtubules. Bar, 1.0 μm.

Kruskal-Wallis one-way nonparametric ANOV) when the centromere on the last monooriented chromosome was destroyed (average [av.] 17 min) relative to when it was allowed to become naturally bioriented (av. 23 min; see Table I). A similar relationship was found when the unattached kinetochore on the last monooriented chromosome was destroyed by the laser. However, under this latter condition the time difference between the exerpimental (av. 20 min) and controls (av. 23 min) was not statistically significant because of a high standard error in the experimental data set (see Table I). This timing difference between our control and experimental cells indicates that the "wait anaphase" signal produced in the centromere is only gradually shut off once the chromosome becomes naturally bioriented.

The Signal Delaying Anaphase Onset Is Produced at or near the Unattached Kinetochore of a Monooriented Chromosome

The inhibitor of anaphase produced in the centromere of monooriented chromosomes could be generated between the sister kinetochores (e.g., McIntosh, 1991; Earnshaw et al., 1991; Bernat et al., 1991), in the unattached kinetochore (e.g., Gorbsky and Ricketts, 1993; Tomkiel et al., 1994; Campbell and Gorbsky, 1995), in the attached kinetochore, or in all three. By destroying one attached kinetochore on a bioriented chromosome we could generate monooriented chromosomes that separated at anaphase onset into two intact chromatids, only one of which contained a functional kinetochore. For these chromosomes to separate into two complete chromatids, and not one chromatid and two chromatid fragments, ≥50% of the original centromere had to be structurally intact. We found that these laser-generated monooriented chromosomes did not delay anaphase, thus demonstrating that the "wait anaphase" signal is produced primarily at or near the unattached kinetochore (which is missing from these chromosomes). This conclusion is supported by our subsequent finding that a monooriented chromosome which had never been bioriented does not delay anaphase onset after its unattached kinetochore is destroyed by the laser (Figs. 5 and 6).

The primary event associated with kinetochore attachment that gradually turns off the signal transducers inhibiting anaphase in somatic vertebrate cells remains to be determined. The most logical candidate is the acquisition of MTs by the kinetochore. In this context it is possible that the "wait anaphase" signal is produced by unoccupied MT binding sites within the kinetochore that become progressively filled with MTs as the nascent K-fiber matures over time. In mantid spermatocytes the failure of one X chromosome to pair as a XXY trivalent leads to a monooriented X univalent, lacking an unattached kinetochore, that checkpoints the cell in metaphase (Li and Nicklas, 1995). In this meiotic system anaphase is inhibited even though all of the kinetochores are attached to the spindle. The fact that anaphase can then be induced in these spermatocytes by pulling on the univalent X chromosome (Li and Nicklas, 1995) clearly reveals that the checkpoint is relieved when the kinetochore/chromosome junction on the X univalent is placed under sufficient tension. If the checkpoint in mantid spermatocytes is based on a "wait anaphase" signal produced in the kinetochore it is possible that tension, through its effect of stabilizing the attachment of MTs to the spermatocytes kinetochore (e.g., Ault and Nicklas, 1989; Nicklas and Ward, 1994), relieves the checkpoint by allowing the kinetochore to become fully saturated with MTs. However, the role of tension in relieving the checkpoint controling entry into anaphase is not universal since tension between homologous kinetochores during meiosis in *Drosophila* oocytes does not promote anaphase onset but instead leads to a metaphase arrest (Jang et al., 1995). The role of tension in the pathway

that monitors chromosome monoorientation in vertebrate somatic cells remains to be determined. Unlike mantid spermatocytes, monooriented chromosomes lacking unattached kinetochores in vertebrate somatic cells do not inhibit anaphase (our Figs. 4–6). In this respect it is possible that the tension experienced by the only and attached kinetochore on our experimentally created monooriented chromosomes is sufficient, e.g., due to the antagonistic action of kinetochore-based poleward pulling forces and away-from-the-pole aster ejection forces (see Rieder and Salmon, 1994), to turn off production of the "wait anaphase" signal. Alternatively, abrogation of the "wait anaphase" signal upon kinetochore attachment in vertebrate somatic cells may have more to do with structural changes induced in the kinetochore as it acquires MTs (e.g., condensation of the single large kinetochore plate into a smaller trilaminar structure; see Rieder, 1982; Cassimeris et al., 1990) than with tension.

Our conclusion that unattached kinetochores in vertebrate somatic cells inhibit anaphase onset until they become attached to the spindle provides an important criterion for identifying potential candidates for this signal transducer. It is, for example, consistent with Gorbsky and Ricketts (1993) contention that the phosphorylated epitope detected in PtK_1 kinetochores by the 3F2/3 antibody is involved in the checkpoint signaling pathway. This epitope is strongly expressed on unattached kinetochores, but its expression becomes progressively weaker as the chromosome biorients and moves to the spindle equator, and it is no longer detectable near the time of anaphase onset (Gorbsky and Ricketts, 1993). It is also consistent with our observation that the "wait anaphase" signal produced by unattached PtK_1 kinetochores becomes shut off only gradually after the kinetochore attaches to the spindle. Importantly, when microinjected into PtK_1 cells the 3F2/3 antibody does not block chromosome biorientation or congression to the spindle equator, but it does significantly delay both the disappearance of the epitope and anaphase onset (Campbell and Gorbsky, 1995).

The authors thank Ms. G. Osorio for assistance with the EM aspect of this study, Mrs. C. Hayes and Ms. M. K. Intino for cell culture and photographic assistance, and Mr. M. Marko and Ms. K. Buttle for help with STERECON.

Much of this work was conducted within the Wadsworth Center's Video-enhanced LM Core Facility, and was supported by National Institutes of Health grants GM 40198 (to C. L. Rieder), GM 30758 (to G. Sluder), and RR 01219 awarded by the Biomedical Research Technology Program, National Center for Research Resources (DHHR/PHS), to support the Wadsworth Center's Biological Microscopy and Image Reconstruction facility as a National Biotechnological Resource.

Received for publication 11 May 1995 and in revised form 15 May 1995.

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