Kinetochores Moving Away from Their Associated Pole Do Not Exert a Significant Pushing Force on the Chromosome

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Abstract. We used video-light microscopy and laser microsurgery to test the hypothesis that as a bioriented prometaphase chromosome changes position in PtK_1 cells, the kinetochore moving away from its associated pole (AP) exerts a pushing force on the centromere. When we rapidly severed congressing chromosomes near the spindle equator between the sister kinetochores, the kinetochore that was originally "leading" the motion towards a pole (P) always (17/17 cells) continued moving P whereas the "trailing" kinetochore moving AP always stopped moving as soon as the operation was completed. This trailing kinetochore then initiated motion towards the pole it was originally moving away from up to 50 s later. The same result was observed (15/15 cells) when we selectively destroyed the leading (P moving) kinetochore on a congressing chromosome positioned ≥ 3 μm from the pole it was moving away from. When we conducted this experiment on congressing chromosomes positioned within 3 μm of the pole, the centromere region either stopped moving, before switching into motion towards the near pole (2/4 cells), or it continued to move AP for 30–44 s (2/4 cells) before switching into P motion. Finally, kinetochore-free chromosome fragments, generated in the polar regions of PtK_1 spindles, were ejected AP and often towards the spindle equator at ~2 μm/min. From these data we conclude that the kinetochore moving AP on a moving chromosome does not exert a significant pushing force on the chromosome. Instead, our results reveal that, when not generating a P force, kinetochores are in a "neutral" state that allows them to remain stationary or to coast AP in response to external forces sufficient to allow their K-fiber to elongate.

During mitosis chromosomes become attached to the forming spindle by bundles of microtubules (Mts)[1], known as kinetochore fibers (K-fibers), that firmly tether their sister kinetochores to the opposing spindle poles. In vertebrates K-fibers are formed as the kinetochores capture and stabilize dynamically unstable Mts growing from the poles (for review see Kirschner and Mitchison, 1986) and they usually form asynchronously on sister kinetochores (reviewed in Rieder, 1990). As a rule the first kinetochore to attach is the one closest to and facing a pole at the time of nuclear envelope breakdown (Roos, 1976; Rieder and Borisy, 1981). As this kinetochore begins to assemble a K-fiber the chromosome becomes "monooriented" and moves towards the closest pole where it then initiates conspicuous oscillatory motions towards and away from the pole (Roos, 1976; Bajer, 1982; Alexander and Rieder, 1991). During these oscillations the attached kinetochore switches between persistent phases of poleward (P) and away-from-the-pole (AP) motion (Bajer, 1982; Rieder et al., 1986; Skibbens et al., 1993). The constant and autonomous switching of attached kinetochores between P and AP motilities is a ubiquitous feature of mitosis in vertebrates (and some other organisms; see Fuge, 1987) and has been termed kinetochore directional instability (Skibbens et al., 1993). Once attached to the spindle the motility of a kinetochore remains directionally unstable until mid-anaphase (Lewis, 1939; Bajer, 1982).

When the unattached kinetochore on a monooriented chromosome finally attaches to the far pole the now "bioriented" chromosome moves to the spindle equator by a process known as congression (Darlington, 1937). The mechanism of congression remains mysterious (reviewed in Mitchison, 1989*a*; Rieder and Salmon, 1994). We do know that when one kinetochore on a congressing chromosome moves towards the pole to which it is attached (P motion), its sister must move away-from-the-pole to which it is attached (AP motion), and that during this time the K-fiber Mts on the "leading" P moving kinetochore shorten while those on the trailing AP-moving kinetochore elongate (for review see Hyman and Mitchison,

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1. *Abbreviations used in this paper*: AP, away-from-the-pole; K-fiber, kinetochore fiber; LM, light microscopy; Mts, microtubules; P, poleward.

1990). We also know that this elongation and shortening of K-fiber Mts occurs primarily by the addition and removal of Mt subunits at the kinetochore (Mitchison et al., 1986; Mitchison, 1989*a*; Wise et al., 1991; Mitchison and Salmon, 1992).

Recently, Skibbens et al. (1993, 1995; see also Mitchison, 1989*a*; Rieder and Salmon, 1994) proposed a "push-pull" model of congression, based on the directionally unstable behavior of kinetochores, in which P motion is favored when a kinetochore is under low tension and AP motion is favored when it is under high tension. In this model, net displacements of a chromosome are generated primarily by differences in the duration and not the velocities of kinetochore P and AP movements. The level of tension on a particular kinetochore is envisioned to be determined by the motile behavior of its sister, transmitted through the centromere (Skibbens et al., 1995), and by the polar ejection forces that act along the chromosome to push it away from the closest pole (Rieder and Salmon, 1994; Murray and Mitchison, 1994; Cassimeris et al., 1994). Congression occurs because kinetochores are "smart" (Mitchison, 1989*a*), sensing their position on the spindle and then using this information to regulate the frequency with which they switch direction. Central to this model is the notion that the leading kinetochore on a congressing chromosome generates a P "pulling" force on the chromosome, while the trailing (sister) kinetochore generates an AP "pushing" force (see Mitchison, 1989*a*; Skibbens et al., 1993, 1995; Rieder and Salmon, 1994; Cassimeris et al., 1994).

It is clear from behavioral studies that kinetochores moving towards a pole are experiencing a P-directed force (e.g., McNeil and Berns, 1981; Bajer, 1982; Rieder et al., 1986; Skibbens et al., 1993). Current evidence indicates that this force is produced at or very near the kinetochore (Nicklas, 1989; Rieder and Alexander, 1990), and a number of possible force-producing mechanisms have been implicated including K-fiber Mt disassembly (for review see Desai and Mitchison, 1995; Inoue and Salmon, 1995), K-fiber Mt flux (Mitchison, 1989*b*; Mitchison and Salmon, 1992), and kinetochore-associated Mt minus-end motor molecules (for review see Thrower et al., 1995; Wordeman, 1995).

By contrast, although supported by in vitro (Hyman and Mitchison, 1991*a*,*b*) and immunolocalization (e.g., Wordeman and Mitchison, 1995) studies placing Mt plus-end motor proteins at the kinetochore and by data that elongating Mt plus ends can generate a pushing force in vivo (e.g., Bajer et al., 1982; Shelden and Wadsworth, 1992; for review see Inoue and Salmon, 1995), behavioral data from living cells that kinetochores moving AP exert a pushing force on the chromosome are weak. In fact the only in vivo support for this hypothesis comes from Skibbens et al.'s (1993; see also Cassimeris et al., 1994) video-enhanced light microscopic observation that the centromere region, adjacent to the kinetochore moving AP on monooriented and congressing chromosomes, sometimes becomes "flattened or punched" into the chromosome. However, when not attached to Mts, kinetochores are normally buried within the primary constriction of the chromosome (e.g., see Rieder, 1982; Rieder and Alexander, 1990). As a result, it is possible that any indentation of the centromere observed in response to an AP moving kinetochore is simply a manifestation of the kinetochore returning, on the ends of K-fiber Mts that are elongating in response to forces acting on the chromosome that are external to the kinetochore, to its least stretched position within the primary constriction. This idea is supported by the fact that pulling chromosomes in grasshopper spermatocytes away from the closest pole with a microneedle induces the K-fiber attached to that pole to elongate with a corresponding AP displacement of the kinetochore (Nicklas, 1988). Furthermore, when a monooriented chromosome moves AP its arms lead the motion, as if the AP force acts equally along the whole chromosome to push it backwards (Bajer, 1982; Rieder et al., 1985). In summary, there is no clear evidence from living cells that kinetochores can move AP without either being pulled by a P moving sister kinetochore (or microneedle) and/or pushed by forces acting along the chromosome external to the kinetochore.

To determine the contribution of the AP moving kinetochore to chromosome congression, we used laser microsurgery to rapidly separate the sister kinetochores, or to destroy the P-moving kinetochore on monooriented or congressing chromosomes in living PtK_1 cells. We found no evidence that kinetochores moving AP exert a pushing force on the chromosome. Instead our data are fully consistent with the hypothesis that kinetochores moving AP are in a neutral state that allows them to be displaced AP in response to forces external to the kinetochore sufficient to allow the K-fiber to elongate.

Materials and Methods

Cell Culture

PtK_1 cells were cultured as previously described (Rieder et al., 1994). In brief, stock cultures were maintained in 5% CO_2 in Ham's F12 medium supplemented with 10% FCS. For experiments the stock cells were subcultured onto 25-mm^2 coverslips lying in the bottom of Petri dishes. Mitotically active coverslip cultures were then mounted in Rose chambers, modified by milling for high resolution light microscopy (LM), which contained L-15 media supplemented with 10% FCS and 10 mM Hepes. These chambers were then placed on the stage of the LM-laser system, where they were maintained throughout the experiments at 35–37°C with a custom built incubator described in Rieder et al. (1994).

Light Microscopy and Laser Microsurgery

Our laser microsurgery system is detailed in Cole et al. (1995) and is based on an inverted Nikon (Garden City, NY) Optiphot 200 de Sernamont differential interference contrast (DIC) LM, which is coupled to a Ludl MAC 2000 (Ludl Electronics Ltd., Hawthorne, NY) motorized microscope stage and a Continuum (Santa Clara, CA) nanosecond pulsed YAG laser operated at 10 Hz. Time-lapse DIC images were captured every 2–4 s with a Paultek 100 CCD (Paultek Imaging, Princeton, NJ) camera, coupled to the video port of the LM, and were routed into the Image I (Universal Imaging Corp., West Chester, PA) for processing before storage on optical disks using a LVR-3300M Sony (Sony Corp. of America, Montval, NJ) laser videodisk recorder. Cells were illuminated with shuttered 546-nm light obtained from an Hg lamp.

The 1,064-nm output of the YAG laser was frequency doubled to 532 nm, filtered, attenuated, and routed into the Optiphot via its epi-port. When passed through our 1.4 NA 60× objective the laser beam has a waist of ~0.5 μm at focus, and its position within the video field was determined and marked daily by firing several laser pulses into a dried film of red blood cells (see Cole et al., 1995). Laser microsurgery was conducted by passing that region of the chromosome to be irradiated through the stationary laser beam with the motorized stage. Although the mechanism is unclear (see Cole et al., 1995), nanosecond pulses of 532-nm laser light can

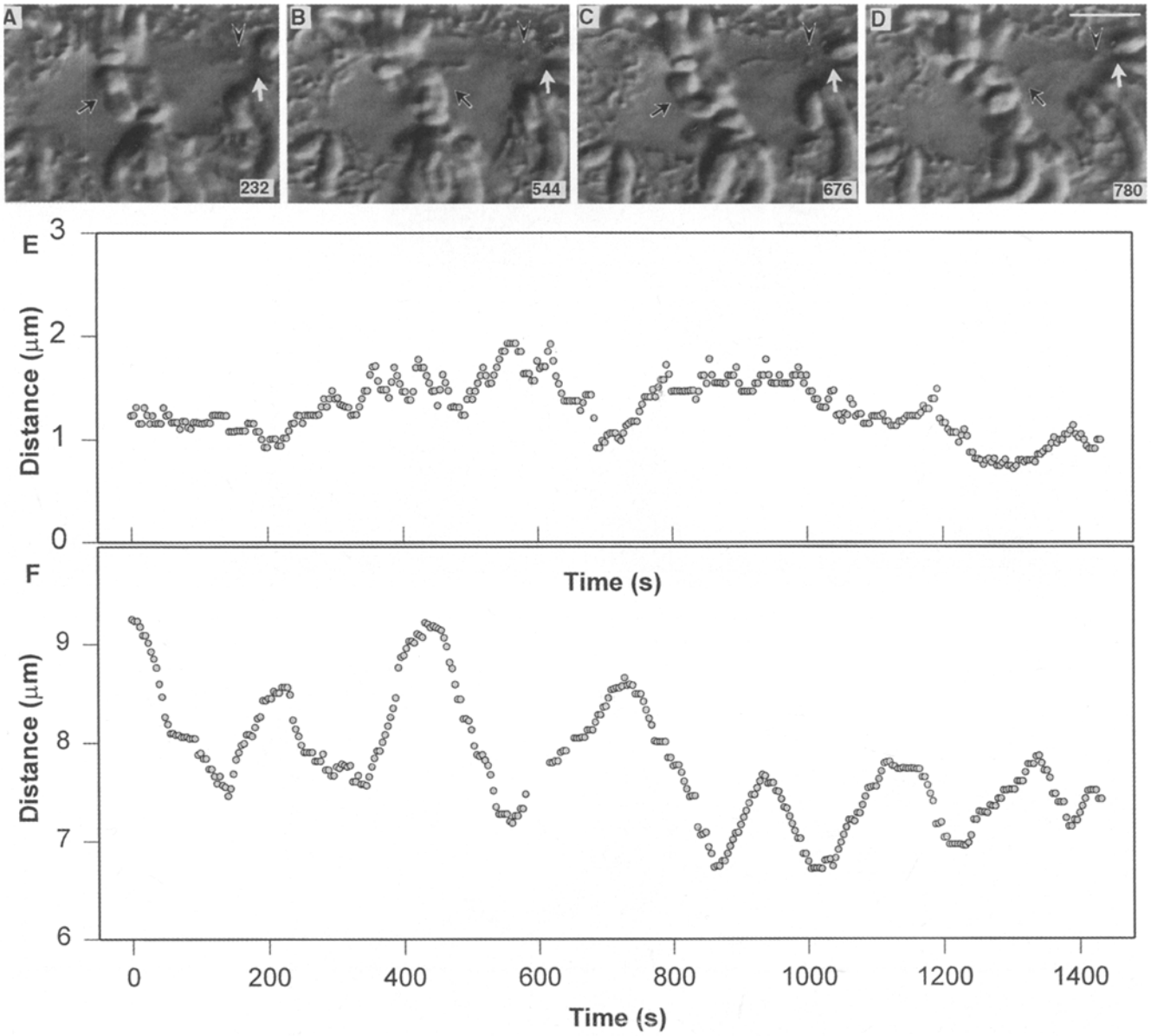

Figure 1. (*A–D*) Selected frames from a prometaphase PtK_1 cell depicting the behavior of a fully congressed oscillating chromosome (black arrow in *A–D*) and monooriented chromosome positioned behind or on the side of a spindle pole (e.g., white arrow in *A–D*). Black arrowhead in *A–D* notes the centrosome from which all distances were measured. (*E*) Plot showing changes in position of the attached kinetochore region, relative to the centrosome, on the monooriented chromosome noted by the white arrow in *A–D*. (*F*) Similar plot of the bioriented chromosome noted by the black arrow in *A–D*. Time (in seconds) on each frame corresponds to the time axis on each plot. Bar in *D*, 5 μm.

be used to rapidly and selectively destroy chromatin and chromosome-associated organelles (e.g., nucleolar organizers and kinetochores) without disrupting the progress of mitosis in many cell types (McNeil and Berns, 1981; Rieder et al., 1986; Hays and Salmon, 1990; Rieder et al., 1995; Khodjakov et al., 1996).

In some cells a chromosome was either severed between its kinetochore regions with the laser, or the kinetochore moving towards its associated pole was selectively destroyed. During the severing operation the chromosome was passed through the stationary laser microbeam using the motorized stage (see Cole et al., 1995 for details). During this process the laser beam was maintained as close as possible to the longitudinal chromosome axis (i.e., between the sister kinetochores) by constantly adjusting the direction of stage motion. Poleward moving kinetochores on congressing chromosomes were selectively destroyed by positioning the kinetochore just in front of where the laser would hit, and then opening the shutter to the laser for 1–2 s (10–20 pulses) as soon as the kinetochore moved into the "kill" zone (see Rieder et al., 1995). In other cells we created monooriented chromosomes lacking an unattached kinetochore (i.e., monocentric chromosomes) by cutting one of the kinetochore regions from bioriented chromosomes. Finally, in some cells we generated "acentric" chromosome fragments lacking kinetochores from monooriented chromosomes by simply severing the arms of a chromosome near its centromere.

Data Analysis

Plots of time vs kinetochore region, or chromosome fragment distance from the closest pole, were generated in two ways. Initially, all of the data were analyzed manually using the Image I system, which calculates the distance between two moveable cursors. For these analyses, time-lapse video disk images were rerouted through a time-base corrector (FOR.A

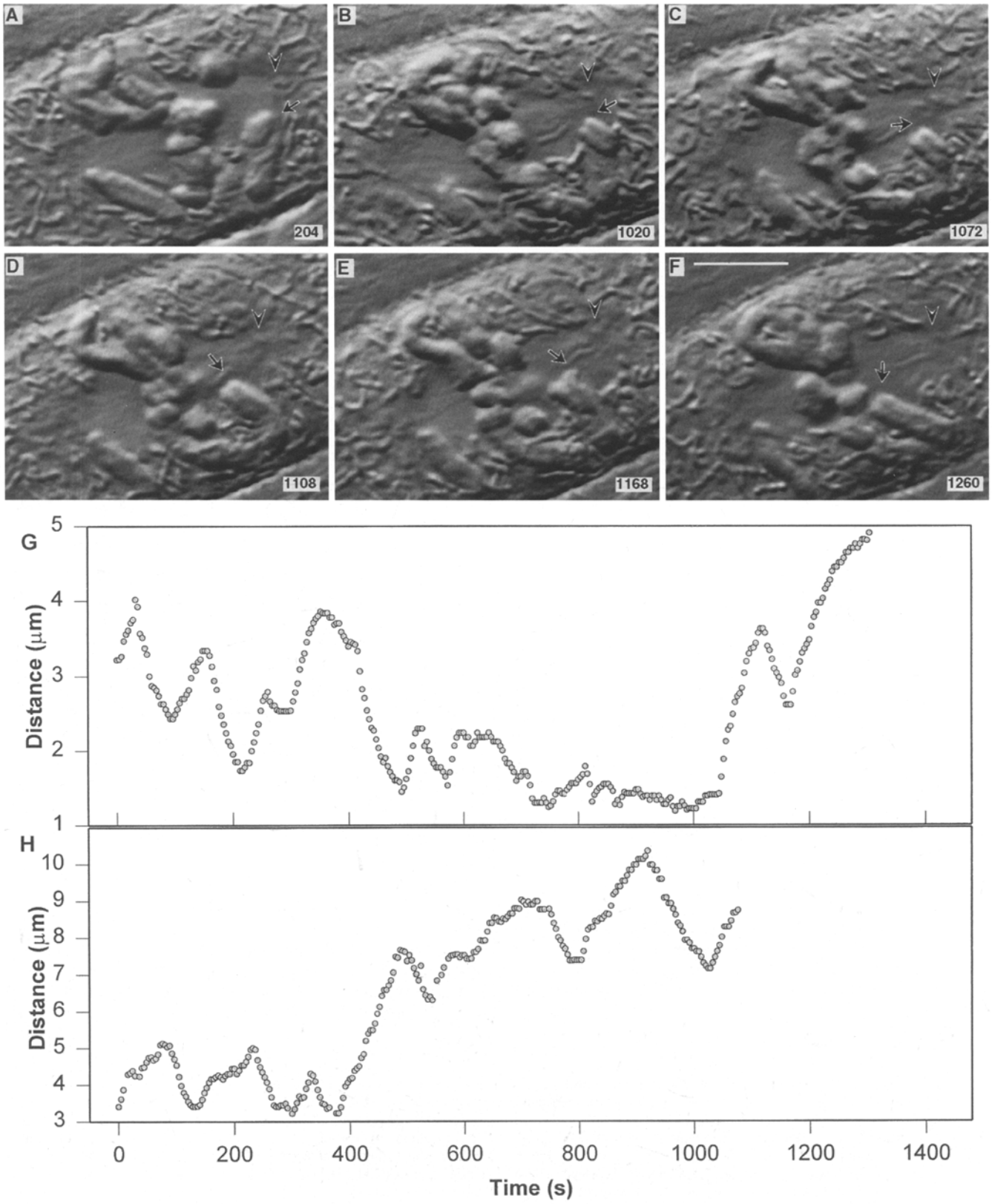

Figure 2. (*A–F*) Selected frames from a prometaphase PtK_1 cell in which an oscillating monooriented chromosome (black arrow in *A*) biorients (*B*) and then congresses (*C–F*). Black arrowhead notes the centrosome from which distances were measured. (*G*) Plot showing positional changes of the attached (and then trailing) kinetochore region on the congressing chromosome indicated by the black arrow in *A–F*. Note that its oscillations diminish ~250 s before biorientation, which occurred near the 1,000-s mark, and that the trailing kinetochore exhibited one oscillation (also pictured in *E*) during congression. (*H*) Similar plot from another cell depicting the behavior of an oscillating monooriented chromosome before (0 –390 s), during (390 to ~780 s) and after (≥780 s) congression. In this example the only attached kinetochore oscillated until biorientation, and then exhibited another oscillation while congressing. Time (in seconds) in *A–F* corresponds to the time axis on *G*. Bar in *F*, 5 μm.

Corp., Natick, MA) into the Image I. For each frame one cursor was superimposed over the closest centrosome while the other was placed over the region on the chromosome being followed. Since the kinetochores themselves were not visible in our video records, their positions were defined for tracking purposes as the leading edge of the primary constriction. In those experiments in which the sister kinetochores were separated by the laser, the distance between both sister kinetochore regions and the same (always the closest) pole (centrosome) were calculated.

Selected sequences (including all of the figures shown) were also analyzed using a semi- automatic tracking program contained in the ISEE (Inovision Corp., Durham, NC) software package, which we ran on a SUN Sparc 10 workstation. This software is based on algorithms described by Gelles et al. (1988) and very similar to that used by Skibbens et al. (1993; see also Salmon et al., 1991) to track the motion of kinetochore regions with respect to spindle poles in newt lung cells. This tracking system can detect lateral displacements in DIC images of 1 pixel, which under our optical conditions equates to 60–80 nm. For this procedure a 14×14 pixel box was placed manually over the kinetochore region to be tracked. The program then read the pixel values within the frame, recorded the coordinates, and advanced to the next frame, where it searched an area 15 pixels in both directions from the previous coordinates for the same matrix. If the program could not locate the same structure with a correlation coefficient of $\geqslant$0.9, then it stopped for operator assistance. The same process was then used to track the closest spindle pole. For practice, operator as-

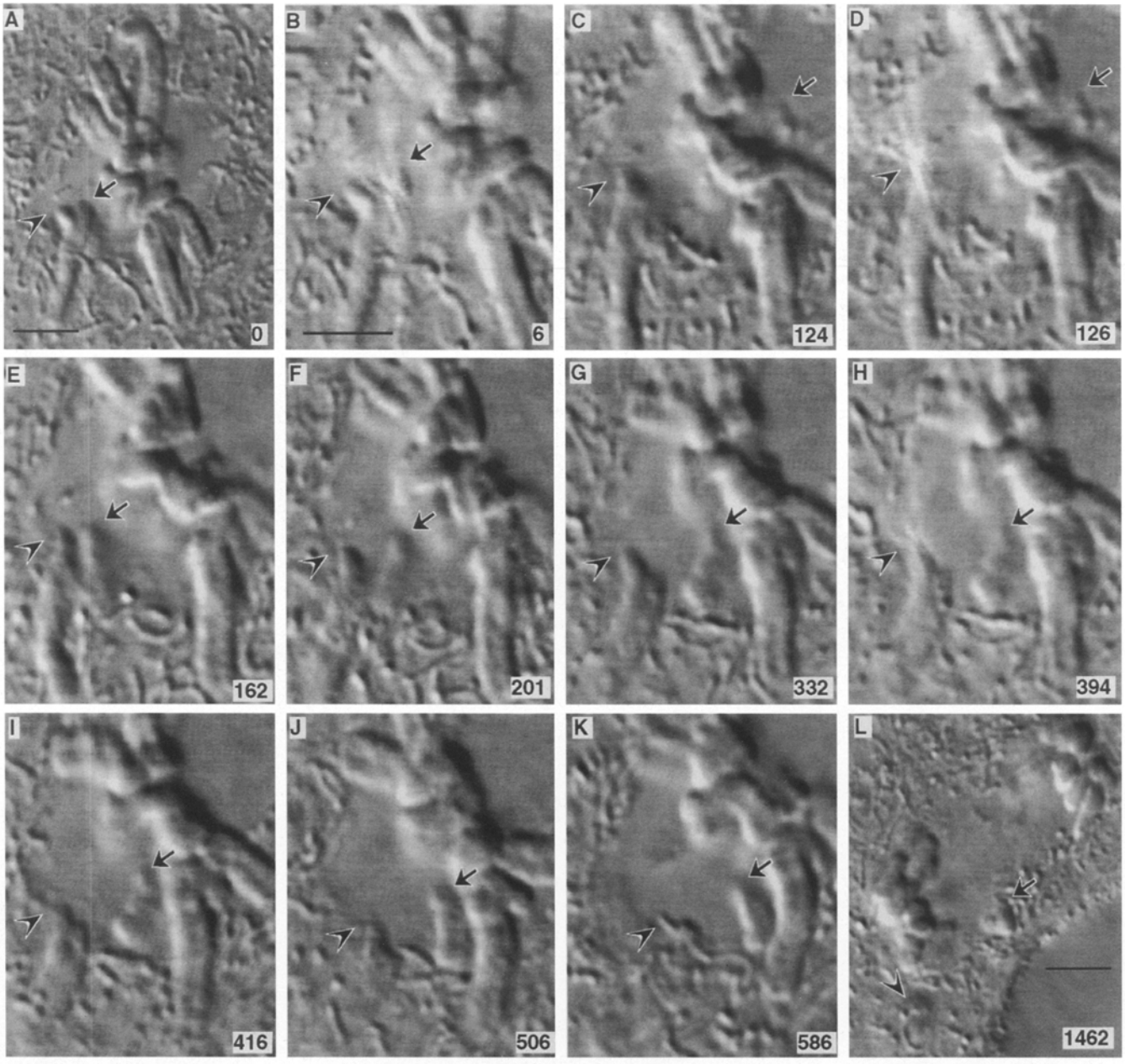

Figure 3. (*A–L*) Selected frames from a prometaphase PtK_1 cell containing a single monooriented chromosome that had just initiated congression. In this example the two well separated kinetochore regions are visible in *A* (*arrow* and *arrowhead*). In *B,* one kinetochore region (*arrow*) is severed from the chromosome to produce a large monocentric chromosome positioned near the pole (black arrowhead in *B–G*) and a small kinetochore-containing chromosome fragment (arrow in *B–D*). The large monocentric chromosome was then severed near its kinetochore region (*D*), and the resulting acentric fragment (arrow in *E–L*) was ejected to the spindle equator (cf. Fig. 4). When the kinetochore region on this chromosome fragment was finally destroyed (arrowhead in *H*), it too was ejected away from the pole into the cytoplasm (arrowhead in *I–K*). Bars: (*A*, *B*, and *L*) 5 μm.

sistance was required only during those portions of the video sequence when the stage moved (e.g., during cutting) or when focus changed abruptly. The pixel values for both the chromosome region and the closest pole were then exported into Quattro Pro 6.0 (Novell, Orem, UT) for distance calculations. In all cases the plots produced by this semi-automatic tracking system led to the same conclusions as those produced manually with the IMAGE 1 system. Finally, regardless of the tracking method, at least 3 video frames (6 s) were required to determine whether a kinetochore had switched direction.

Results

Centromere Behavior on Monooriented and Congressing PtK_1 Chromosomes

The centromeres of monooriented chromosomes behind or on the side of an aster were usually positioned ≤2 μm from the centrosome, and they rarely exhibited oscillatory motions (Fig. 1, *A–E*). By contrast the centromeres of monooriented chromosomes located on the front side of the aster, i.e., towards the forming metaphase plate, were usually positioned ≥2 μm from the pole and routinely exhibited conspicuous oscillatory behavior (Fig. 2, *A–H*). During these oscillations the centromere moved towards the pole (P) and then away from the pole (AP) with similar velocities ($\bar{X}$ = 2.3 ± 0.2 μm/min, N = 14; $\bar{X}$AP = 2.0 ± 0.2 μm/min; N = 14), and, although the switch from P to AP (and AP to P) motion could be abrupt (≤6 s), it often required ≥8 s (Fig. 2, *G* and *H*). Similarly, although kinetochores generally switched from P to AP (and AP to P) motion every 75–100 s, this frequency could vary considerably, and this variability led to differences in the amplitudes of sequential oscillations (e.g., Figs. 2, *G* and *H* and 5 *G*).

Upon biorientation the centromere initiated sustained motion towards the spindle equator with an initial velocity of 2.2 μm/min ($\bar{X}$AP = 2.2 ± 0.3 μm/min N = 9; Fig. 2, *G* and *H*). During this time the chromosome arms trailed the centromere as the newly attached kinetochore led the motion towards the distal pole while its sister moved away from the closer pole (Fig. 2, *B–F*). Congressing centromeres underwent at least one and usually several short oscillations before reaching the spindle equator (Fig. 2, *G* and *H*). Once near the equator they continued to oscillate about this position until anaphase onset (Fig. 1, *A–D* and *F*). During these oscillations the duration of sister kinetochore P and AP motions were approximately equivalent as were their amplitudes ($\bar{X}$AP = 1.7 ± 0.2 μm/min, N = 14; $\bar{X}$P = 1.9 ± 0.2 μm/min, N = 14).

The Polar Regions of PtK_1 Spindles Exert an Away-from-the-Pole Force on the Chromosomes

It has been proposed that chromosome positioning in ver-

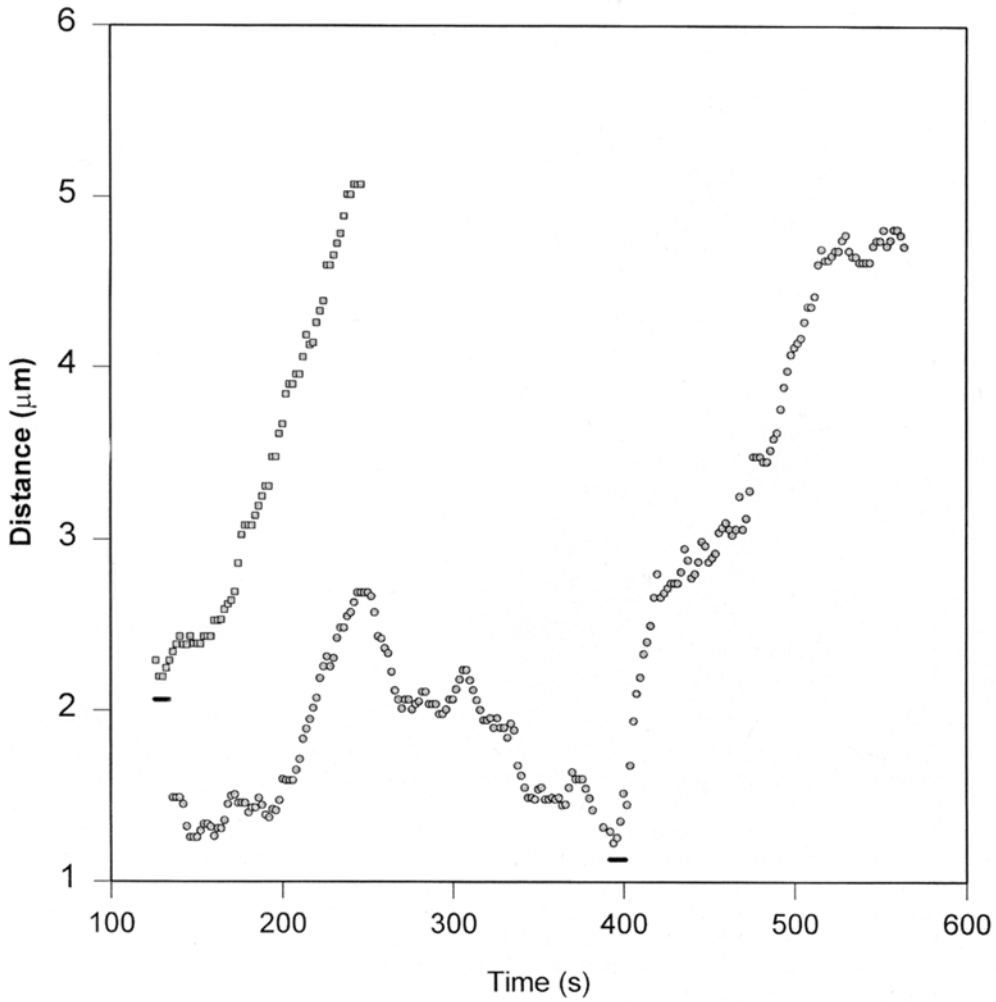

Figure 4. Plots depicting the behavior of the acentric chromosome fragments generated in Fig. 3. The boxes track the motion of the initial fragment created in Fig. 3 *D* (arrow in *E–H*), as it is ejected to the spindle equator. The circles track the motion of the other kinetochore-containing fragment (arrowhead in *B–L*) before (126–393 s) and after (≥394) destruction of its kinetochore. Solid bars above the time axis represent the points and duration of laser irradiation.

tebrate somatic cells is mediated partly by expulsion forces associated with each half-spindle that act on the chromosome arms to expel them AP (for review see Rieder and Salmon, 1994). To determine whether these "polar ejection forces" are a feature of PtK_1 spindles, we severed one of the kinetochore regions from a bioriented chromosome to create "monocentric" chromosomes containing only one kinetochore that were positioned near a pole (e.g., Fig. 3, *A–C*). We then generated a large kinetochore-free acentric chromosome fragment by either cutting the monocentric chromosome through its primary constriction without damaging the kinetochore (as evidenced by the fact that it remained attached to the pole and continued to exhibit normal oscillations; Figs. 3, *D–E* and 4), and/or by destroying the only attached kinetochore on this chromosome (Fig. 3 *H*). Under both conditions the chromosome fragment was always immediately transported AP (arrow in Figs. 3, *D–G*; arrow in *H–K*; Fig. 4), and often to the spindle equator (Fig. 3, *D–G*), with an average velocity of 2.16 ± 0.33 μm/min (N = 12; Fig. 4). From these experiments, we conclude that the polar regions of PtK_1 spindles support the production of an AP force that acts along the length of the chromosome.

Behavior of Sister Kinetochore Regions on Bioriented Chromosomes after Separation by Laser Microsurgery

To determine whether the trailing kinetochore on a moving bioriented chromosome is exerting a pushing force, we used laser microsurgery to "split" small bioriented congressing or oscillating chromosomes along their long axis, between the sister kinetochores, and then tracked the subsequent motion of the resultant kinetochore-containing chromosome fragments (Figs. 5 and 6). This surgical procedure required 4–6 s (2–3 video frames) and produced two similarly sized monocentric chromosome fragments, each of which was connected to an opposing spindle pole (Figs. 5, *A–F* and 6, *A–F*). After the operation each monocentric fragment moved towards the pole to which its kinetochore was connected and then initiated oscillatory mo-

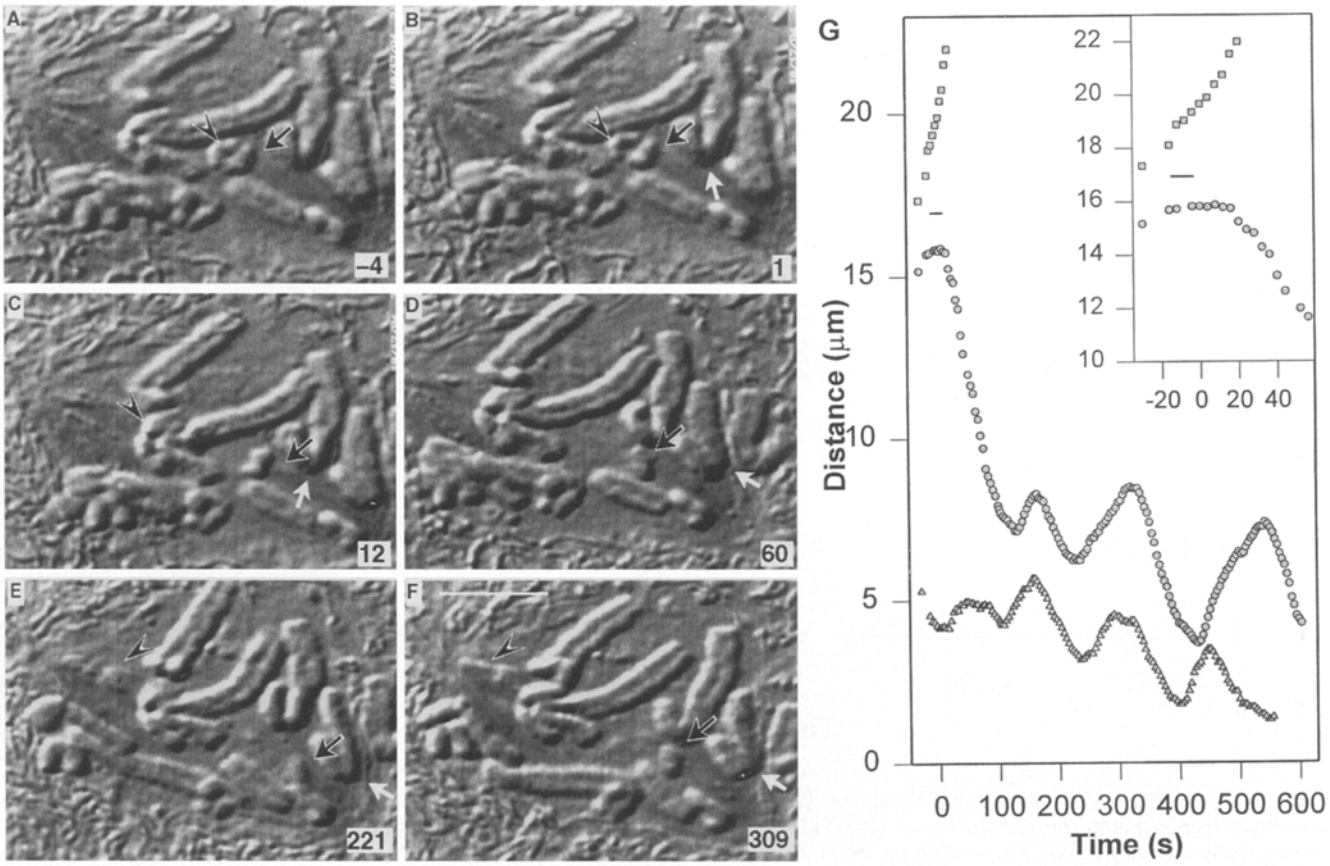

Figure 5. (*A–F*) Selected frames from a prometaphase PtK_1 cell in which a small bioriented chromosome is bisected between its kinetochores by the laser (*A–B*, *arrow* and *arrowhead*) as it moves away from the right-hand pole (from which distances were subsequently measured). Upon separation the two monocentric chromosome fragments move toward opposite poles (arrowhead and arrow in *C–F*). In *C* and *D* the left hand fragment is transiently lost as it moves under another chromosome (near arrowhead in *C*), and then becomes highly stretched as it reaches the polar region (arrowhead in *E* and *F*). (*G*) Plot depicting the kinetic behavior of both monocentric chromosome fragments (*squares* and *circles*), noted respectively by the black arrowhead and arrow in *B–F*. The triangles note the motion of a natural monooriented chromosome (white arrow in *B-F*). After moving into its polar region the monocentric chromosome fragment (circles on plot; black arrowhead in *A–F*) initiated oscillations that were similar to that of the adjacent naturally monooriented chromosome (*triangles*). Insert in *G* depicts an expanded scale analysis of how the two kinetochore regions behaved before, during (*solid bar*), and after severing. Note that the poleward moving kinetochore region continued to move poleward but that the trailing kinetochore ceased its motion at the time of the operation. It remained motionless for 16 s before initiating motion towards its pole. Bar in *F*, 5 μm.

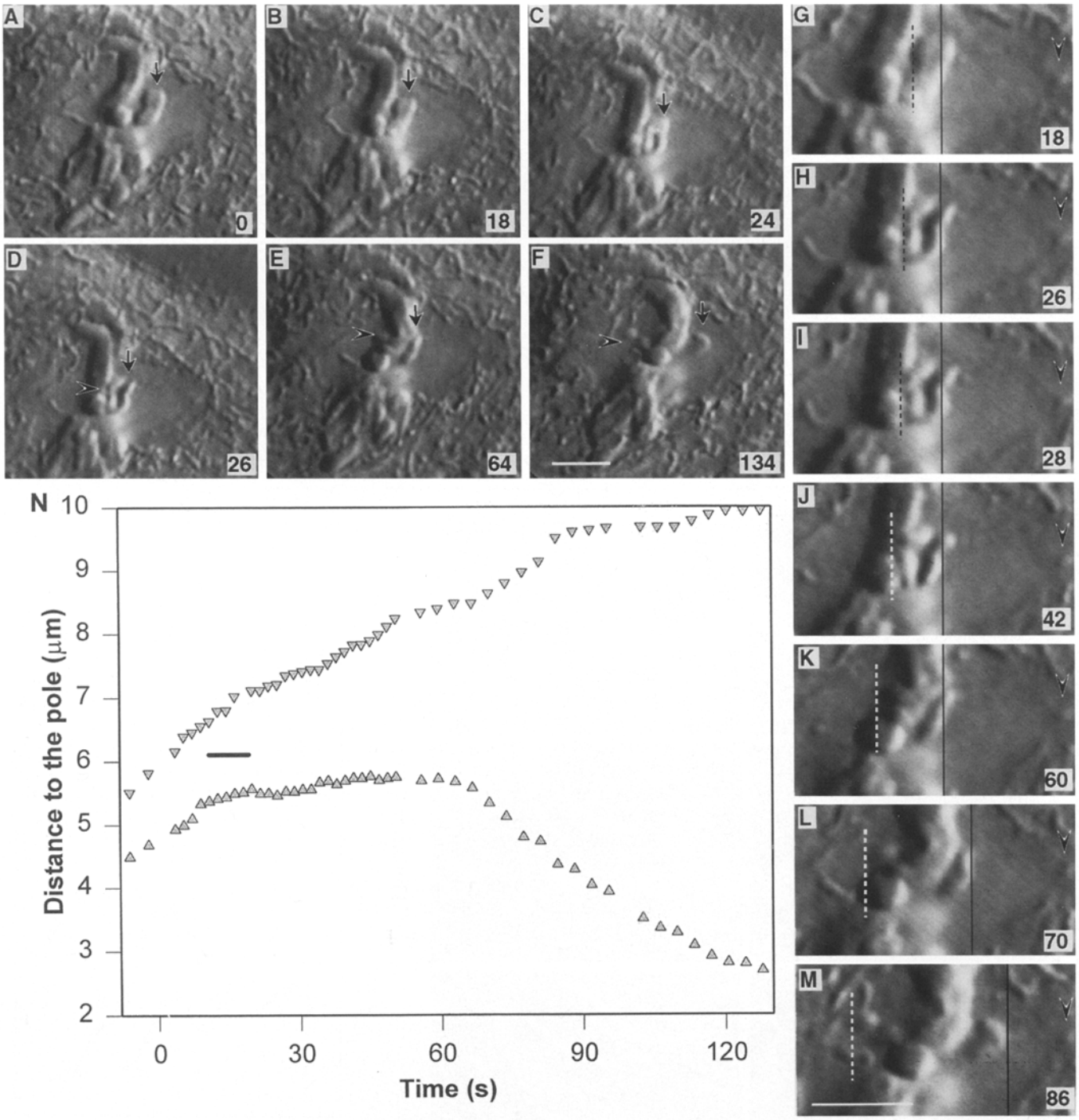

Figure 6. (*A–F*) Selected frames from a metaphase PtK_1 cell in which a small oscillating bioriented chromosome (arrow in *A*) was bisected between its kinetochores by the laser (*B–C*) as it was moving away from the right hand centrosome (from which distances were subsequently measured). After formation the two monocentric fragments subsequently moved towards their respective polar regions (*D–F*). (*G–M*) Selected frames showing the formation and behavior of the two monocentric fragments pictured in *A–F*. The solid vertical line represents the position of the trailing kinetochore region just before (*B* and *G*) and after (*H–M*) severing the chromosome. The dashed vertical line represents the position of the poleward moving kinetochore region during and after the operation. Note that the fragment containing the leading kinetochore (*dashed line*) continued to move poleward during and after the operation. By contrast, the fragment containing the trailing kinetochore stopped moving as soon as the operation was completed (*H*), and then remained stationary for 42 s (*H–K*) before initiating poleward motion (*L–M*). (*N*) Plot depicting the motion of both kinetochore regions, relative to the right hand centrosome (arrowhead in *G–M*), before, during (*horizontal black bar*), and after separation. Bar in *F*, 5 μm.

tions that were indistinguishable from those of adjacent monooriented chromosomes (Fig. 5 *G*). The fact that both kinetochores exhibited normal behavior after the operation reveals that neither was significantly damaged by our protocol.

We split 17 bioriented chromosomes that were positioned near and moving towards the spindle equator. In every case the leading kinetochore region moving towards its pole at the time of the operation remained in a P state of motion during and after the operation (Figs. 5 *G* and 6 *N*). By contrast the trailing kinetochore region, which was moving AP at the time of the operation, always ceased moving when the operation was completed. In 4 of these cells this kinetochore region then initiated motion towards the pole to which it was connected ≤6 s after the operation was completed. However, in the other 13 cells it remained stationary (i.e., it exhibited a maximum positional change of ≤0.2 μm) for 20 ± 4 s (range = 8–50 s) before initiating P motion (Figs. 5 *G* and 6 *N*). This result strongly suggests that, during the motion of a bioriented chromosome, the AP-moving trailing kinetochore does not push on the chromosome.

Centromere Behavior after Destroying Just the Poleward Moving Kinetochore on Congressing Chromosomes

It is possible that our splitting approach, which required that both sister kinetochores be passed very near the stationary laser microbeam (≤0.35 μm; see Cole et al., 1995), transiently and selectively inhibited the ability of kinetochores to produce an AP but not a P force. To evaluate this possibility, we used the microbeam to selectively destroy just the P moving kinetochore on 15 congressing PtK_1 chromosomes after they had moved ≥3 μm from the nearest pole (e.g., Fig. 7, *A–F*). In these cells the laser beam impacted the chromosome ≥1.25 μm from the AP moving kinetochore, and at this distance any potential effects of the laser on the AP moving kinetochore would be greatly reduced.

In all of these cells the centromere region abruptly stopped moving as soon as the P moving kinetochore was destroyed (Fig. 7, *G–K, N*). In 8 of these cells (8/15) the kinetochore originally moving AP initiated motion towards its pole ≤6 s later. However, in the other 7 cells (7/15) the centromere remained stationary after the operation for 21 ± 5 s (range = 12–48 s; Fig. 7 *N*) before the kinetochore originally moving AP initiated motion towards its associated pole (e.g., Fig. 7, *G–N*).

We also selectively destroyed just the P moving kinetochore on 4 congressing chromosomes while they were still ≤3 μm from the pole they were moving away from (e.g., Fig. 8, *A–F*). In two of these cells the centromere region stopped moving immediately after the operation, and subsequently initiated motion towards the closest pole. However, in the remaining two cells (e.g., Fig. 8), the centromere continued moving away from the closest pole for up to 44 s after the P moving kinetochore was destroyed, before switching into motion towards the pole. The fact that both of these chromosomes remained monooriented until anaphase onset (e.g., Fig. 8, *E–F*) demonstrates that the P moving kinetochore was destroyed by our operation.

Discussion

For the most part our observations on the behavior of centromeres in untreated PtK_1 cells are consistent with those reported by Skibbens et al. (1993) for newt cells. As in newts, we found that, once attached to the spindle, the direction that a kinetochore moves in PtK_1 is generally unstable. We use the term "generally" because not all attached kinetochores exhibit the oscillatory motions diagnostic of directional instability. Included here are the kinetochores on monooriented chromosomes positioned behind or on the side of an aster that often remain relatively motionless for extended periods (Fig. 1 *E*; see also Cassimeris et al., 1994 for chromosomes on monopolar spindles). As reported by Skibbens et al. (1993), we also found that switches between P and AP (and AP to P) motility could be abrupt (in ≤6 s). However, we note that in PtK_1, these switches just as often require more than 8 s, and sometimes kinetochores switch from a motile state into a relatively stationary state of an extended duration (Fig. 2 *G*).

Kinetochores Moving Away from Their Associated Poles Do Not Exert a Significant Pushing Force on the Chromosome

From our data we conclude that kinetochores moving AP do not exert a significant pushing force on the chromosome. Our evidence for this is that the AP moving trailing kinetochore on a congressing chromosome always ceased its AP motion immediately after we severed the chromosome between its sister kinetochore, and then, after a variable period (6–48 s), switched into motion towards its pole. We know that this behavior was not due to the laser approaching the AP moving kinetochore too closely, and temporary destroying putative AP force produces, because in 17/19 cases the same outcome was observed when we destroyed just the P moving kinetochore on a congressing chromosome, which is positioned ≥1.25 μm from the AP moving kinetochore.

In two experiments the congressing centromere continued to transiently move AP after we destroyed or removed the P moving kinetochore. Although it is possible that this AP motion was generated by the AP moving kinetochore, we think this highly unlikely since this outcome was observed on only 2 of 36 chromosomes, and only on the two chromosomes that were positioned at the time of the operation closest to the poles they were moving away from. A straightforward explanation for why these centromeres continued to move AP, after the P moving kinetochore was destroyed, is that the chromosomes and their centromere regions were pushed AP by the proximal polar ejection force, which we show here to be a component of PtK_1 spindles.

If the polar ejection force in PtK_1 cells is strong enough to transport large chromosome fragments lacking K-fibers from near the pole to the spindle equator (Figs. 3 and 4), and to transiently sustain the AP motion of centromeres lacking a P moving kinetochore that are positioned near a pole, why didn't it push all of the centromeres AP after we destroyed or removed the P moving kinetochore? The most logical explanation for this is that the strength of the ejection force is not sufficient by itself (i.e., in the absence of a P force acting on the opposing sister kinetochore) to

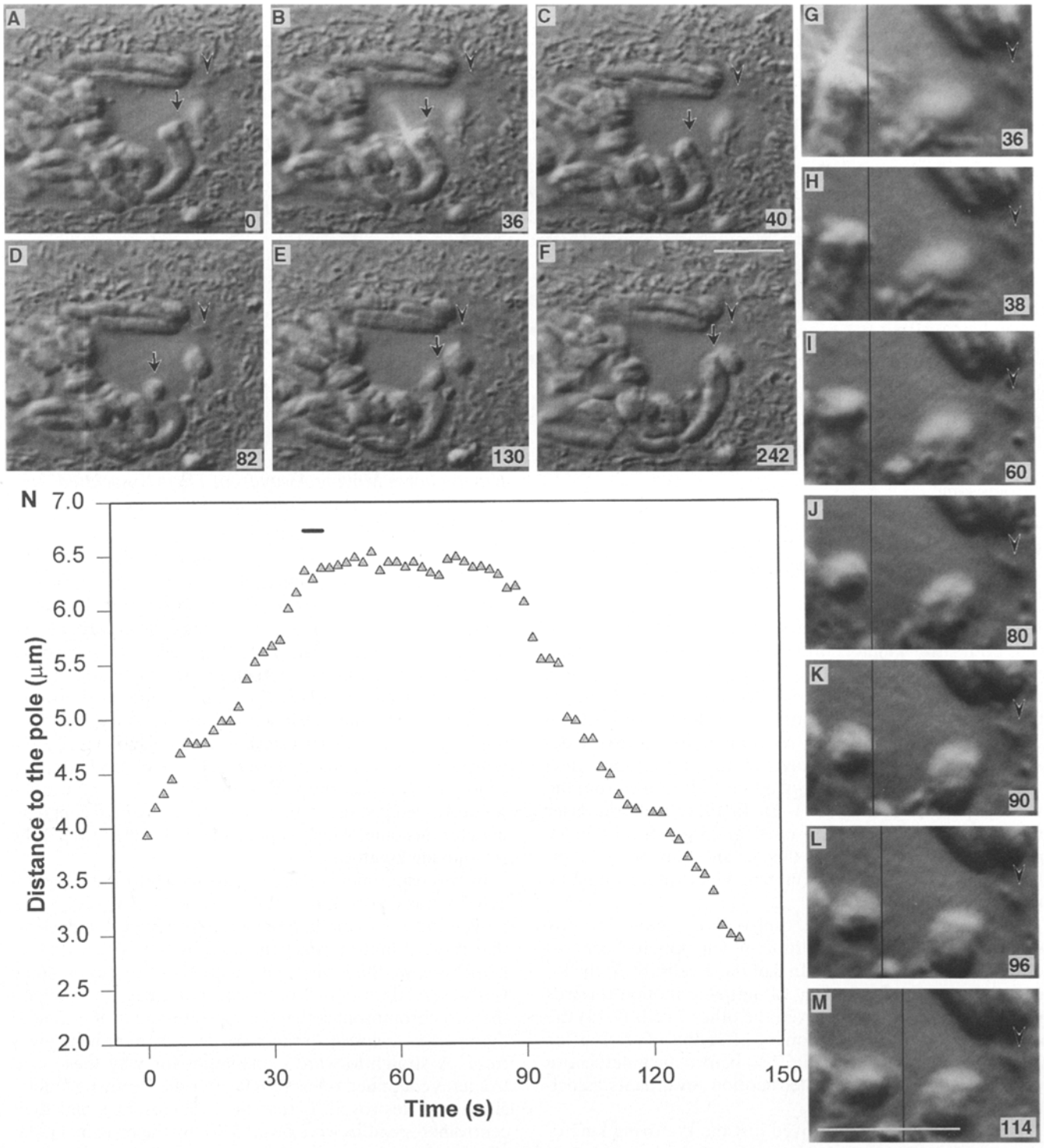

Figure 7. Same format as in Fig. 6, except that in this prometaphase cell the poleward moving kinetochore on a congressing chromosome (arrow in *A*) is destroyed (*B*) as the chromosome is moving away from the near pole (arrowhead in *A–M*), which is about 6 μm away. This operation produced a monocentric chromosome that then moved toward the pole (*arrow*, *C–F*). The behavior of this chromosome at a higher magnification is shown in *G–M* where the vertical black line represents the position of the trailing kinetochore at the time of (*G*), and after (*H–M*), the operation. Note that the trailing kinetochore region of this chromosome stopped moving after its poleward moving kinetochore was destroyed (*G*), which then remained stationary for 52 s (*H–K*) before initiating poleward movement (*L–M*). (*N*) Plot depicting the behavior of the trailing kinetochore region before, during (*horizontal black bar*), and after destruction of the poleward moving kinetochore. Bar in *F*, 5 μm.

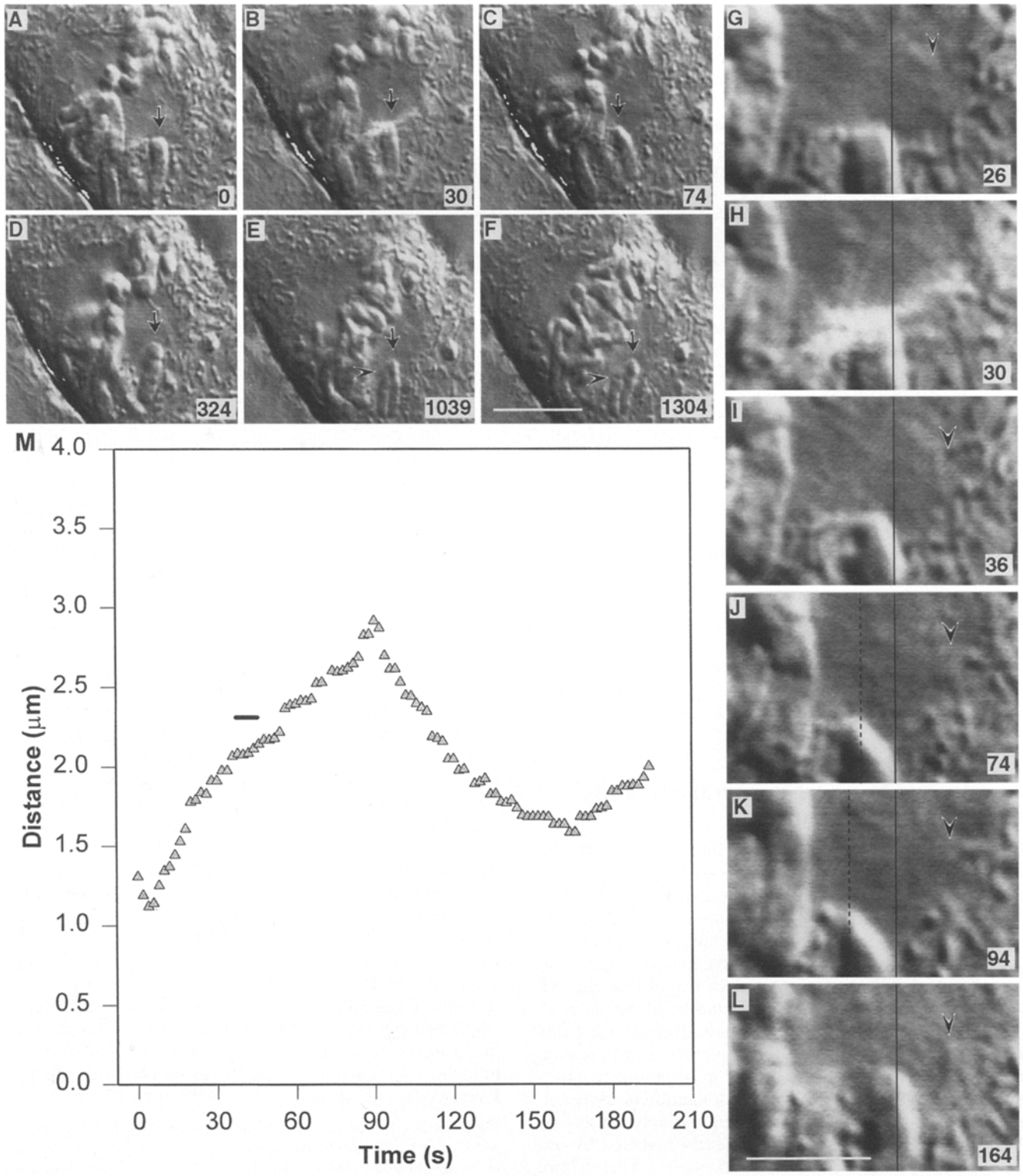

Figure 8. Same conditions as in Fig. 7, except that in this cell the congressing chromosome (arrow in *A*) was <2.5 μm from the proximal pole at the time when its leading kinetochore was destroyed (*B* and *H*). After the operation the monocentric chromosome continued to move away from the pole for 44 s (*H–J*) before the trailing kinetochore switched into poleward motion (*K–L*). The solid vertical line in *G–L* represents the position of the trailing kinetochore region before (*G*) and during (*H*) the operation. The vertical dashed line in *J–K* notes the position of the leading kinetochore region just before its destruction (*H*). Note that this monocentric chromosome remained mono-oriented to the closest pole until anaphase onset (*E* and *F*). (*M*) Plot depicting the behavior of the trailing kinetochore region before, during (*horizontal black bar*), and after destruction of the poleward moving kinetochore. Bar in *F*, 5 μm.

sustain the elongation of K-fiber Mts on the AP-moving kinetochore attached to the proximal pole, if it is ≥3 μm from that pole. This argument is consistent with the fact that the strength of the ejection force appears to be positively correlated with Mt density (Ault et al., 1991; Cassimeris et al., 1994), which is highest immediately adjacent to the poles. It is also supported by Nicklas' (1988) conclusion that in grasshopper spermatocytes, ~1,000 more force is needed to move an attached kinetochore away from its pole (i.e., to induce K-fiber elongation), than is required to move an unattached chromosome at the speeds observed during mitosis. However, regardless of the explanation, our data clearly reveal that once a congressing PtK_1 centromere has moved ~3 μm away from the closest spindle pole, the AP motion of its trailing kinetochore immediately ceases after the P force acting on its leading kinetochore is abrogated. From this finding, we conclude that the force for moving a kinetochore AP is not generated by the AP-moving kinetochore and, thus, that AP moving kinetochores do not exert a significant pushing force on the chromosome.

Our data support a hypothesis in which kinetochores moving AP are simply coasting on the tips of elongating K-Fiber Mts, growing in response to tension generated by the sister kinetochore and/or the proximal polar ejection force, which then allows the chromosome/centromere to move AP on the tips of growing Mts. In this model the kinesin-like proteins associated with the kinetochore (e.g., Wordeman and Mitchison, 1995; Walczak et al., 1996) are not force-producers for chromosome motion, but moving tethers that secure the plus ends of elongating kinetochore Mts to the kinetochore (see also Desai and Mitchison, 1995).

When Not in a Forward (Poleward) Motility Mode, Kinetochores Are in Neutral

Skibbens et al. (1993) concluded that attached kinetochores exist predominantly in P or AP motility phases, both of which were viewed as power-production states. However, these authors noted that attached kinetochores can also exist in "an 'indeterminate' phase of no or confused motion that [is] usually brief compared to the duration of P and AP phases." Our data reveal that the AP phase of kinetochore motility reported by Skibbens et al. (1993) is not a power production phase. Instead it appears to be a neutral phase in which the kinetochore can remain motionless or, more often, coast AP in responses to external forces strong enough to induce elongation of its associated K-fiber Mts to elongate. Although usually masked by AP motion, this neutral phase is clearly revealed by our experiments. When we suddenly uncouple a kinetochore from the external force that is moving it AP, it stops moving and remains motionless (before switching into P motion) for up to 50 s even though it is attached to the plus ends of K-fiber Mts. Neutral is also manifested during normal oscillations as a pause of variable duration, sometimes ≥10 s, as the chromosome switches between AP and P motion (e.g., see Figs. 1 *F*, 2, *G* and *H*, and 5 *G*). Finally, the kinetochores on monooriented chromosomes positioned behind or on the side of the aster, which remain relatively motionless, may be "resting" in this neutral position (Fig. 1 *E*; see also Cassimeris et al., 1994). However, it is possible that in these instances the kinetochores are undergoing normal periodic switches between P and neutral states, but, because they possess extremely short K-fibers, switches into the P states are not converted into noticeable P motion. Similarly, switches into the N states may not be converted into AP motion because the chromosome resides in an area where the polar ejection forces are too weak to allow and maintain K-fiber elongation (see above; also Cassimeris et al., 1994).

The length of time that an attached kinetochore spends in neutral appears to vary. On chromosomes oscillating in an uninterrupted fashion it usually lasts between 75–100 s, or all of the time that the kinetochore is not in a P state (e.g., Figs. 1 *F*, 2, *G* and *H*, and 5 *G*). Under our experimental conditions we could generate stationary kinetochores, tethered to the plus ends of K-Mts that remained in neutral for as along as 50 s (Figs. 6 and 7) before abruptly shifting into P motion. Unfortunately, because the oscillation periodicity of mono- and bioriented PtK_1 chromosomes is so variable (e.g., Figs. 1 *F* and 2, *G* and *H*), it was not possible to determine whether the stationary kinetochores created in our experiments switched into P phases at the time they normally would have had their sisters not been removed. Regardless, it is clear from our data that attached kinetochores spend a considerable portion of their time in a nonforce producing phase which we term neutral.

Skibbens et al. (1993, 1995) concluded that switches between kinetochore AP and P phases of motion occur abruptly (≤6 s), and that they are mediated by tension on the kinetochore with decreasing tension favoring AP to P switches. Our data, however, reveal that these switches really represent switches between a P force producing phase and a nonforce producing neutral phase. This being the case, it is possible that preparations for switching from neutral into the P-phase start early and require the duration of neutral (often 75–100 s) to complete. Although tension is likely involved in regulating the switching event, it does not appear to be the only factor since kinetochores can remain motionless for many (up to 50) seconds after the tension on them is rapidly and drastically reduced (Figs. 6 and 7). This finding raises the possibility that kinetochores must periodically spend some time in neutral, whether it be stationary or coasting AP, before they can shift into P regardless of the tension level. If true, such periodic switches into neutral may not only allow for changes in chromosome position, but they may also enable the kinetochore to reacquire components, depleted during the P phase, that are necessary for regenerating P motion.

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Note Added in Proof. It has been shown that centromeres on oscillating chromosomes in newt lung cells are stretched ~90% of the time and un-

der compression <6% of the time (Waters et al., 1996). This clearly indicates that most of the time the trailing AP moving kinetochore does not produce any pushing force, sufficient to release the centromere stretching.

References

Alexander, S.P., and C.L. Rieder, 1991. Chromosome motion during attachment to the vertebrate spindle: initial saltatory-like behavior of chromosomes and a quantitative analysis of force production by nascent kinetochore fibers. *J. Cell Biol.* 113:805–815.

Ault, G.J., A.J. DeMaco, E.D. Salmon, and C.L. Rieder. 1991. Studies on the ejection properties of asters: astral microtubule turnover influences the oscillatory behavior and positioning of mono-oriented chromosomes. *J. Cell Sci.* 99:701–710.

Bajer, A.S. 1982. Functional autonomy of monopolar spindle and evidence for oscillatory movement in mitosis. *J. Cell Biol.* 93:33–48.

Bajer, A.S., C. Cyper, J. Mole-Bajer, and H. Howard. 1982. Taxol-induced anaphase reversal: evidence that elongating microtubules can exert a pushing force in living cells. *Proc. Natl. Acad. Sci. USA.* 79:6569–6573.

Cassimeris, L., C.L. Rieder, and E.D. Salmon. 1994. Microtubule assembly and kinetochore directional instability in vertebrate monopolar spindles: implications for the mechanism of chromosome congression. *J. Cell Sci.* 107:285–297.

Cole, R.W., A. Khodjakov, and C.L. Rieder. 1995. A differential interference contrast-based light microscopic system for laser microsurgery and optical trapping of selected chromosomes during mitosis in-vivo. *J. Microsc. Soc. Amer.* 1:203–215.

Darlington, C.D. 1937. Recent Advances in Cytology. 2nd Edition. Blakiston Co., Philadelphia, PA. 588 pp.

Desai, A., and T.J. Mitchison. 1995. A new role for motor proteins as couplers to depolymerizing microtubules. *J. Cell Biol.* 128:1–4.

Fuge, H. 1987. Oscillatory movements of bipolar-oriented bivalent kinetochores and spindle forces in male meiosis of *Mesostoma ehrenbergii*. *Eur. J. Cell Biol.* 44:294–298.

Gelles, J., B.J. Schnapp, and M.P. Sheetz. 1988. Tracking kinesin-driven movements with nanometre-scale precision. *Nature (Lond.).* 331:450–453.

Hays, T., and E.D. Salmon. 1990. Poleward force at the kinetochore in metaphase depends on the number of kinetochore microtubules. *J. Cell Biol.* 110:391–404.

Hyman, A.A., and T.J. Mitchison. 1990. Modulation of microtubule stability by kinetochores in vitro. *J. Cell Biol.* 110:1607–1616.

Hyman, A.A., and T.J. Mitchison. 1991*a*. Two different microtubule-based motor activities with opposite polarities in kinetochores. *Nature (Lond.).* 351: 206–211.

Hyman, A.A., and T.J. Mitchison. 1991*b*. Regulation of the direction of chromosome movement. *Cold Spring Harbor Symp. Quant. Biol.* 56:745–750.

Inoue, S., and E.D. Salmon. 1995. Force generation by microtubule assembly/disassembly in mitosis and related movements. *Mol. Biol. Cell.* 6:1619–1640.

Kirschner, M., and T.J. Mitchison. 1986. Beyond self-assembly: from microtubules to morphogenesis. *Cell.* 45:329–342.

Khodjakov, A., R.W. Cole, A.S. Bajer, and C.L. Rieder. 1996. The force for poleward chromosome motion in *Haemanthus* cells acts along the length of the chromosome during metaphase but only at the kinetochore during anaphase. *J. Cell Biol.* 132:1093–1104.

Lewis, W.H. 1939. Changes in viscosity and cell activity. *Science (Wash. DC).* 89:400.

McNeil, P.A., and M.W. Berns. 1981. Chromosome behavior after laser microirradiation of a single kinetochore in mitotic PtK_2 cells. *J. Cell Biol.* 45:515–527.

Mitchison, T.J., L. Evans, E. Schultze, and M. Kirschner. 1986. Sites of microtubule assembly and disassembly in the mitotic spindle. *Cell.* 45:515–527.

Mitchison, T.J. 1989*a*. Chromosome alignment at mitotic metaphase: balanced forces or smart kinetochores? *In* Cell Movement. Vol. 2. Kinesin, Dynein and Microtubule Dynamics. N.D. Warner and J.R. McIntosh, editors. Alan Liss, NY. pp. 421–430.

Mitchison, T.J. 1989*b*. Poleward microtubule flux in the mitotic spindle: evidence from photoactivation of fluorescence. *J. Cell Biol.* 109:637–652.

Mitchison, T.J., and E.D. Salmon. 1992. Poleward kinetochore fiber movement occurs during both metaphase and anaphase-A in newt lung cell mitosis. *J. Cell Biol.* 119:569–582.

Murray, A.W., and Mitchison, T.J. 1994. Kinetochores pass the IQ test. *Curr. Biol.* 4:38–41.

Nicklas, R.B. 1988. The forces that move chromosomes in mitosis. *Annu. Rev. Biophys. Biophys. Chem.* 17:431–449.

Nicklas, R.B. 1989. The motor for poleward chromosome movement in anaphase is in or near the kinetochore. *J. Cell Biol.* 109:2245–2255.

Rieder, C.L. 1982. The formation, structure and composition of the mammalian kinetochore and kinetochore fiber. *Int. Rev. Cytol.* 79:1–58.

Rieder, C.L. 1990. Formation of the astral mitotic spindle: ultrastructural basis for the centrosome-kinetochore interaction. *Electron Microsc. Rev.* 3:269–300.

Rieder, C.L., and Borisy, G.G. 1981. The attachment of kinetochores to the prometaphase spindle in PtK_1 cells: recovery from low temperature treatment. *Chromosoma.* 82:693–716.

Rieder, C.L., and S.P. Alexander. 1990. Kinetochores are transported poleward along a single astral microtubule during chromosome attachment to the spindle in newt lung cells. *J. Cell Biol.* 110:81–95.

Rieder, C.L., and E.D. Salmon. 1994. Motile kinetochores and polar ejection forces dictate chromosome position on the vertebrate mitotic spindle. *J. Cell Biol.* 124:223–233.

Rieder, C.L., E.A. Davison, L.C.W. Jensen, and E.D. Salmon. 1985. The distal kinetochore is not responsible for the oscillation of monooriented chromosomes in newt lung cells. *In* Microtubules and Microtubule Inhibitors. M. De Brabander, and J. De Mey, editors. Elsevier, Amsterdam. pp. 253–260.

Rieder, C.L., E.A Davison, L.C.W. Jensen, L. Cassimeris, and E.D. Salmon. 1986. Oscillatory movements of monooriented chromosomes, and their position relative to the spindle pole, result from the ejection properties of the aster and half spindle. *J. Cell Biol.* 103:581–591.

Rieder, C.L., A. Schultz, R. Cole, and G. Sluder. 1994. Anaphase onset in vertebrate somatic cells is controlled by a checkpoint that monitors sister kinetochore attachment to the spindle. *J. Cell Biol.* 127:1301–1310.

Rieder, C.L., R.W. Cole, A. Khodjakov, and G. Sluder. 1995. The checkpoint delaying anaphase in response to chromosome monoorientation is mediated by an inhibitory signal produced by unattached kinetochores. *J. Cell Biol.* 130:941–948.

Roos, U.-P. 1976. Light and electron microscopy of rat kangaroo cells in mitosis. III. Patterns of chromosome behavior during prometaphase. *Chromosoma.* 54:363–385.

Salmon, E.D., S. Magers, R. Skibbens, and N.R. Gliksman. 1991. Video-enhanced differential interference contrast (VE-DIC) light microscopy: a method for semiautomatic object tracking. *In* Proceedings 49th Annu. Meet. Elec. Microsc. Soc. Amer., G.W. Bailey, editor. San Francisco Press, San Francisco, CA. pp. 238–239.

Shelden, E., and P. Wadsworth. 1992. Microinjection of biotin-tubulin into anaphase cells induces transient elongation of kinetochore microtubules and reversal of chromosome-to-pole motion. *J. Cell Biol.* 116:1409–1420.

Skibbens, R.V., V.P. Skeen, and E.D. Salmon. 1993. Directional instability of kinetochore motility during chromosome congression and segregation in mitotic newt lung cells: a push-pull mechanism. *J. Cell Biol.* 122:859–875.

Skibbens, R.V., C.L. Rieder, and E.D. Salmon. 1995. Kinetochore motility after severing between sister centromeres using laser microsurgery: evidence that kinetochore directional instability and position is regulated by tension. *J. Cell Sci.* 108:2537–2548.

Thrower, D.A., M.A. Jordan, B.T. Schaar, T.J. Yen, and L. Wilson. 1995. Mitotic HeLa cells contain a CENP-E associated minus end-directed microtubule motor. *EMBO (Eur. Mol. Biol. Organ.) J.* 14:918–926.

Walczak, C.E., T.J. Mitchison, and A. Desai. 1996. XKCM1: A *Xenopus* kinesin-related protein that regulates microtubule dynamics during mitotic spindle assembly. *Cell.* 84:37–47.

Waters, J.C., R.V. Skibbens, and E.D. Salmon. 1996. Oscillating mitotic newt lung cells kinetochores are, on average, under tension and rarely push. *J. Cell Sci.* In press.

Wise, D., L. Cassimeris, C.L. Rieder, P. Wadsworth, and E.D. Salmon. 1991. Chromosome fiber dynamics and congression oscillations in metaphase PtK_2 cells at 23°C. *Cell Motil. Cytoskeleton.* 18:131–142.

Wordeman, L., and T.J. Mitchison. 1995. Identification and partial characterization for mitotic centromere-associated kinesin, a kinesin-related protein that associates with centromeres during mitosis. *J. Cell Biol.* 128:95-105.

Wordeman, L. 1995. Mechanisms of chromosome segregation in metazoan cells. *In* Progress in Cell Cycle Research. L. Meijer, S. Guidet, and H.Y.L. Tung, editors. Plenum Press, NY. 1:319–327.