

# Chromosome Fragments Possessing Only One Kinetochore Can Congress to the Spindle Equator

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**Abstract.** We used laser microsurgery to cut between the two sister kinetochores on bioriented prometaphase chromosomes to produce two chromosome fragments containing one kinetochore (CF1K). Each of these CF1Ks then always moved toward the spindle pole to which their kinetochores were attached before initiating the poleward and away-from-the-pole oscillatory motions characteristic of monooriented chromosomes. CF1Ks then either: (a) remained closely associated with this pole until anaphase (50%), (b) moved (i.e., congressed) to the spindle equator (38%), where they usually (13/19 cells) remained stably positioned throughout the ensuing anaphase, or (c) reoriented and moved to the other pole (12%). Behavior of congress-

ing CF1Ks was indistinguishable from that of congressing chromosomes containing two sister kinetochores. Three-dimensional electron microscopic tomographic reconstructions of CF1Ks stably positioned on the spindle equator during anaphase revealed that the single kinetochore was highly stretched and/or fragmented and that numerous microtubules derived from the opposing spindle poles terminated in its structure. These observations reveal that a single kinetochore is capable of simultaneously supporting the function of two sister kinetochores during chromosome congression and imply that vertebrate kinetochores consist of multiple domains whose motility states can be regulated independently.

**I**N vertebrates, each replicated chromosome possesses two small discrete structures, known as kinetochores, that are positioned on opposite sides of its primary constriction. During mitosis, these “sister” kinetochores become attached to the forming spindle by capturing dynamically unstable microtubules (Mts)<sup>1</sup> growing from the opposing spindle poles (reviewed in Rieder and Salmon, 1994; Wordeman, 1995). Once captured, these Mts become more stable and form a kinetochore fiber (K-fiber) that tethers the chromosome to the pole while also producing and/or transmitting the forces for poleward chromosome motion. After both kinetochores have attached to the spindle, the now “bioriented” chromosome undergoes a series of movements, termed congression (Darlington, 1937), that align it halfway between the two poles on the spindle equator or “metaphase plate.”

The molecular mechanism(s) that underlie congression remain largely unknown, but all models envision that it re-

quires two functional sister kinetochores and their associated K-fibers (for reviews see Mitchison, 1989a,b; Salmon, 1989; McIntosh and Hering, 1991; Rieder and Salmon, 1994). As a bioriented chromosome congresses towards the spindle equator, the “trailing” kinetochore moves away from its associated pole (away-from-the-pole [AP] motion) and its K-fiber elongates, while the “leading” kinetochore moves towards its pole (poleward [P] motion) and its K-fiber shortens. We know from microinjection studies that the elongation and shortening of K-fiber Mts on moving chromosomes take place primarily by the addition and removal of Mt subunits at the kinetochore (e.g., Mitchison et al., 1986; Wise et al., 1991). Recent video-enhanced light microscopic studies have also shown that kinetochores attached to the plus end of spindle Mts periodically switch between two distinctly different functional states (Skibbens et al., 1993; Khodjakov and Rieder, 1996). When in the P state, the kinetochore produces (and/or experiences) a force that moves it poleward. By contrast, when in the “neutral” state, it does not produce a force, but it can be pushed or pulled away from its associated pole by external forces (including a P-moving sister kinetochore; Khodjakov and Rieder, 1996; Waters et al., 1996). Periodic switches between these two functional states lead to the oscillatory motions characteristic of monooriented and bioriented chromosomes. For any one kinetochore, switching appears to be mediated by tension produced from the

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1. *Abbreviations used in this paper:* AP, away-from-the-pole; CF1K, chromosome fragment containing one kinetochore; IVEM, intermediate voltage electron microscope; K-fiber, kinetochore fiber; Mt, microtubule; MUG, mitosis with unreplicating genome; P, poleward.

activity of the proximal polar ejection force and, in the case of bioriented chromosomes, also by the opposing kinetochore (Skibbens et al., 1993, 1995; Rieder and Salmon, 1994).

When Chinese hamster cells are induced to enter mitosis during S-phase of the cell cycle (termed mitosis with unreplicated genome [MUG]), the unreplicated kinetochores detach from most of the underlying condensing chromatin (Brinkley et al., 1988) and fragment into smaller units that "curl" around the associated residual chromatin (Zinkowski et al., 1991). Electron microscopic analyses of random cell populations undergoing a MUG reveal that ~25% of these kinetochore fragments are positioned near the spindle equator (Christy et al., 1995). Of these, the great majority consist of two joined and opposing fragments, likely derived from the same (single) kinetochore, that are attached by Mts to the opposing spindle poles (Brinkley et al., 1988; Christy et al., 1995). Although a clear demonstration is lacking, it has also been suggested that just one kinetochore fragment can become similarly bioriented and positioned on the spindle equator (Brinkley et al., 1988). These data, which were obtained from fixed cells, clearly reveal that the kinetochores in vertebrates can be induced to fragment and that these fragments maintain their ability to attach to spindle Mts.

An important but yet-to-be-proven implication from the MUG studies is that when two joined and opposing pieces of the same kinetochore acquire an attachment to the opposing poles, the complex moves (i.e. congresses) to the spindle equator (see Brinkley et al., 1988; Christy et al., 1995). If this is true, it has important ramifications for how vertebrate kinetochores function. It would mean, for example, that the elongation and shortening of kinetochore Mts, as well as the P and neutral activity states of a kinetochore, are not bulk features of the kinetochore. Instead, individual kinetochores must consist of two or more independently regulated domains, each of which contains the complete molecular machinery for kinetochore function. However, since the behavior of kinetochore fragments cannot be observed in living cells undergoing a MUG, it remains to be determined whether single kinetochores (or their fragments) possess the ability to move to the spindle equator when attached to Mts derived from the opposing spindle poles. It is possible instead that bioriented fragments positioned near the spindle equator in cells, fixed while undergoing a MUG, are nonmotile, and that they were simply positioned roughly equal distances between the two poles at the time of spindle formation.

The goal of our study was to test the hypothesis that a single kinetochore possesses the capability of congressing to the spindle equator when it becomes attached to both poles. To do this, we used laser microsurgery to produce, from bioriented chromosomes in living prometaphase PtK<sub>1</sub> cells, two chromosome fragments each of which contained one kinetochore (CF1K). We have previously shown that the only kinetochore on a CF1K produced in this manner behaves normally, i.e., after the operation the CF1K moves towards the pole to which its kinetochore is attached and then initiates P and AP oscillatory motions indistinguishable from those of neighboring, nonirradiated monooriented chromosomes (Khodjakov and Rieder, 1996). In this report, we detail the behavior of these CF1Ks dur-

ing the latter stages of mitosis and demonstrate that they are capable of congressing and that the only kinetochore on a congressed CF1K is attached by bundles of Mts to both poles.

## Materials and Methods

### Cell Culture

PtK<sub>1</sub> cells (rat kangaroo kidney) were cultured as previously described (Rieder et al., 1994). In brief, stock cultures were maintained in 5% CO<sub>2</sub> in Ham's F12 medium supplemented with 10% FCS. For experiments, the stock cells were subcultured onto 25-mm<sup>2</sup> 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) that contained L-15 media supplemented with 10% FCS and 10 mM Hepes. These chambers were then placed on the stage of the laser-microscope system, where they were maintained throughout the experiments at 35–37°C with a custom built incubator described by Rieder et al. (1994).

### Laser Microsurgery and Video-Light Microscopy

Our laser microsurgery system has been described in detail by Cole et al. (1995) (see also Rieder et al., 1995; Khodjakov and Rieder, 1996). It is based on an inverted light microscope (model Optiphot 200; Nikon, Inc., Garden City, NY) equipped with de Sernamont differential-interference-contrast optics. This microscope is coupled to a motorized microscope stage (model MAC 2000; Ludl Electronics Ltd., Hawthorne, NY) and a nanosecond-pulsed YAG laser (model Continuum; Santa Clara, CA). For the studies reported here, the cells were illuminated with shuttered 546-nm light obtained from a Hg lamp and viewed using a 60× objective lens (NA = 1.4; Nikon, Inc.). Time-lapse images were captured every 2–4 s with a CCD camera (model 100; Palttek Imaging, Princeton, NJ) and routed into Image I (Universal Imaging Corp., West Chester, PA) for processing before storage on optical disks using a laser videodisk recorder (model LVR-3300M; Sony Corp. of America, Montval, NJ).

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 the 1.4 NA 60× objective, the waist of the laser beam is ~0.5 μm at focus (Cole et al., 1995). During microsurgery, that region of the chromosome to be irradiated was passed through the stationary laser beam with the motorized stage.

### Immunofluorescence Microscopy

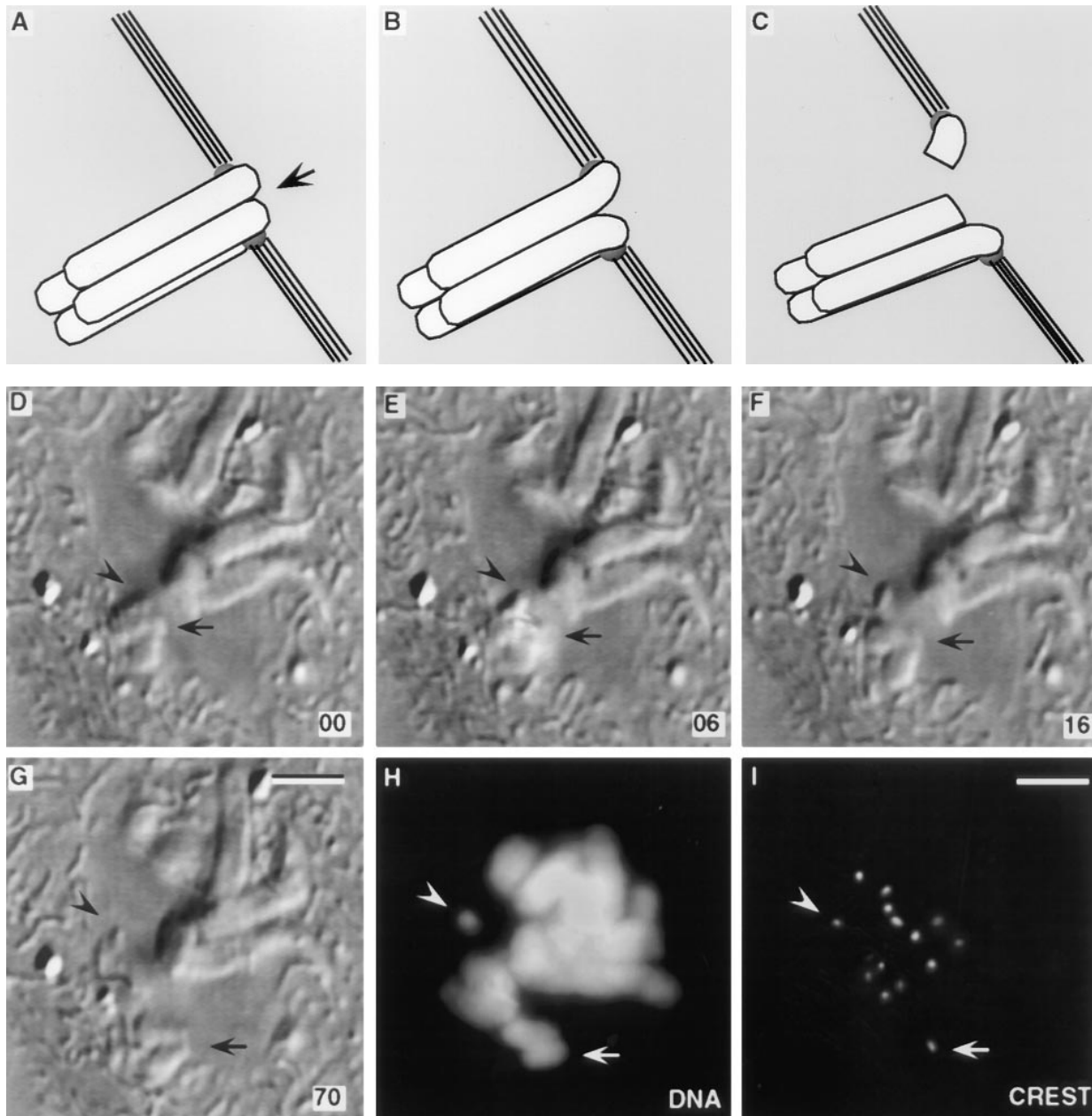
Selected mitotic cells were subjected to laser microsurgery in Rose chambers as described above. These were then followed on the stage of the laser microscope until fixation by perfusion with 3% paraformaldehyde in PBS. After 10 min, the chamber was disassembled and the culture-containing coverslip was treated with 1% Triton X-100 in PBS for an additional 10 min. The cultures were then processed for immunofluorescence light microscopy using CREST-serum (a kind gift from Dr. W.R. Brinkley, Baylor College of Medicine, Houston, TX) diluted 1:400 and a goat anti-human TRITC-conjugated secondary antibody (Sigma Chemical Co., St. Louis, MO). Cells followed *in vivo* were then relocated and imaged with a cooled CCD (model KAF-1400; Photometrics Ltd.; Tucson, AZ) camera run by ISEE (Inovision Corp., Durham, NC) software on a SGI workstation (Silicon Graphics, Inc., Mountain View, CA).

### Data Analysis

Plots of distance versus time were generated using the semiautomatic tracking program contained in ISEE software, which we also run on a SUN Sparc 10 workstation (Sun Microsystems, Inc., Mountain View, CA). This system is described in detail elsewhere (Khodjakov and Rieder, 1996), and we use it to (semi) automatically plot the distance between a given kinetochore region and its associated pole. Since kinetochores themselves were not visible in our video records, their positions were defined for tracking purposes as the leading edge of the primary constriction.

### Electron Microscopy

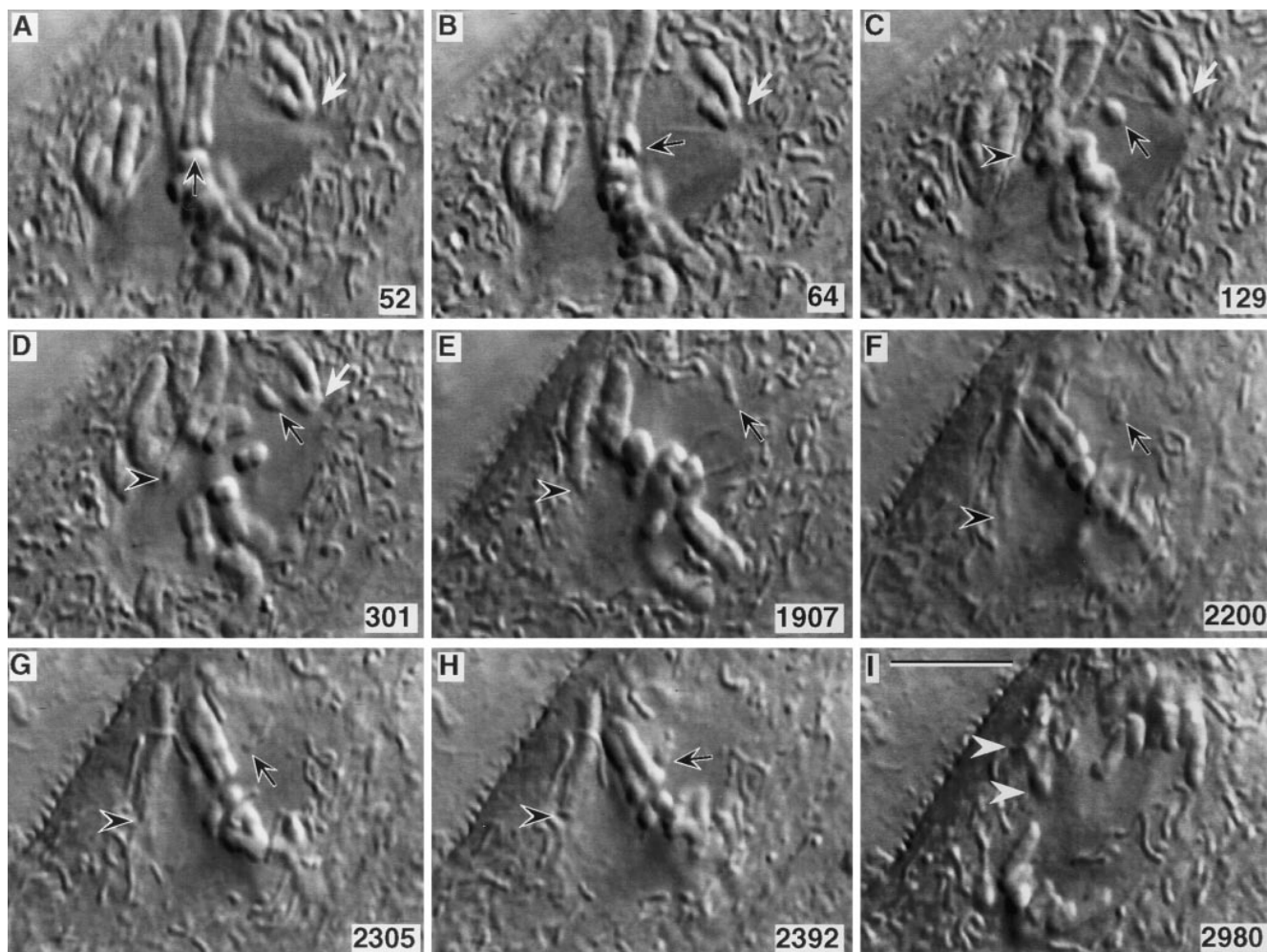
Experimental cells were fixed at selected times within the Rose chambers



**Figure 1.** (A–C) Diagram of how two different size CF1Ks can be created from a bioriented chromosome. (D–I) Video micrographs of a prometaphase cell in which the laser was used to sever the region between two kinetochores on a congressed chromosome (E, black arrow, arrowhead) to produce two CF1Ks (F, black arrow, arrowhead) that moved towards their respective polar areas (G). In PtK<sub>1</sub>, as in most animals, large metaphase chromosomes are usually folded at their primary constriction so that their arms lie on top of one another (A and D). The centromere region on these chromosomes is positioned on the surface of the spindle, and the axis between its associated and opposing sister kinetochores is parallel to the spindle long axis (A). When both kinetochore regions stretch poleward, the area between them can be cut with the laser without damage to either kinetochore (B and E). Then, as the kinetochore regions continue to move towards their respective poles, a small section that contains a single kinetochore can be loped from the bulk of the chromosome (C and F). The cell followed in D–G was fixed shortly after G and processed for the fluorescent localization of DNA (H) and kinetochores (I). A comparison of G, H, and I clearly reveals that both CF1Ks produced by this operation (H, white arrow, arrowhead) contain a single kinetochore (I, white arrow, arrowhead). Time in seconds is noted in the bottom right corner of A–G. Bars, 5  $\mu$ m.

by perfusion with 2.5% glutaraldehyde in 0.1 M Millonig's phosphate buffer, pH 7.3. 30 min later, the coverslip culture was removed from the chamber, washed twice in phosphate buffer, and then postfixed in 2% aqueous OsO<sub>4</sub> for 60 min at 4°C. After three washes in buffer, the cells were treated with 0.15% tannic acid (in buffer) for 1 min, washed once in buffer, and then twice in distilled H<sub>2</sub>O. Next, they were stained en bloc in 1% uranyl acetate (4°C; 60 min), washed in distilled H<sub>2</sub>O, dehydrated in a graded series of ethanols, and flat-embedded in Epon (for review see

Rieder, 1981). Cells previously followed *in vivo* were then relocated and serially thick-sectioned (0.25  $\mu$ m). Ribbons of sections were mounted in the center of formvar-coated slot grids, on which 25-nm colloidal gold had been lightly deposited to facilitate subsequent micrograph alignment. After staining in uranyl acetate and lead citrate, the sections were viewed and photographed in an intermediate voltage electron microscope (IVEM) (model JEM 4000 FX; JEOL U.S.A., Inc., Peabody, MA) equipped with a computer-controlled tilt/rotation specimen holder. In some cases, three-



**Figure 2.** (A–I) A bioriented chromosome (A, black arrow) is cut between its sister kinetochores (B, black arrow) to produce two CF1Ks (C, black arrow, arrowhead) that then moved into their respective polar areas (D–E). The small CF1K (C–E, black arrow) initiated congression in F, and was fully congressed by the time of anaphase onset in H. This CF1K then segregated to one of the poles during anaphase. The larger CF1K (C–I, black arrowhead) remained monooriented until anaphase onset, at which time it disjoined into a kinetochore-containing chromatid that moved into the pole and two smaller acentric fragments (I, white arrowheads). The white arrow in A–D notes a nonirradiated monooriented chromosome, the congression behavior of which is plotted in Fig. 3 (curve 2). Time in seconds is at lower right corner of each frame. Bar, 10  $\mu$ m.

dimensional reconstructions were generated from stereo pairs of serial section images using Stereon (for review see Marko and Leith, 1996). In other cases, the biology within a selected thick section was reconstructed by IVEEM tomography as detailed by McEwen et al. (1993).

## Results

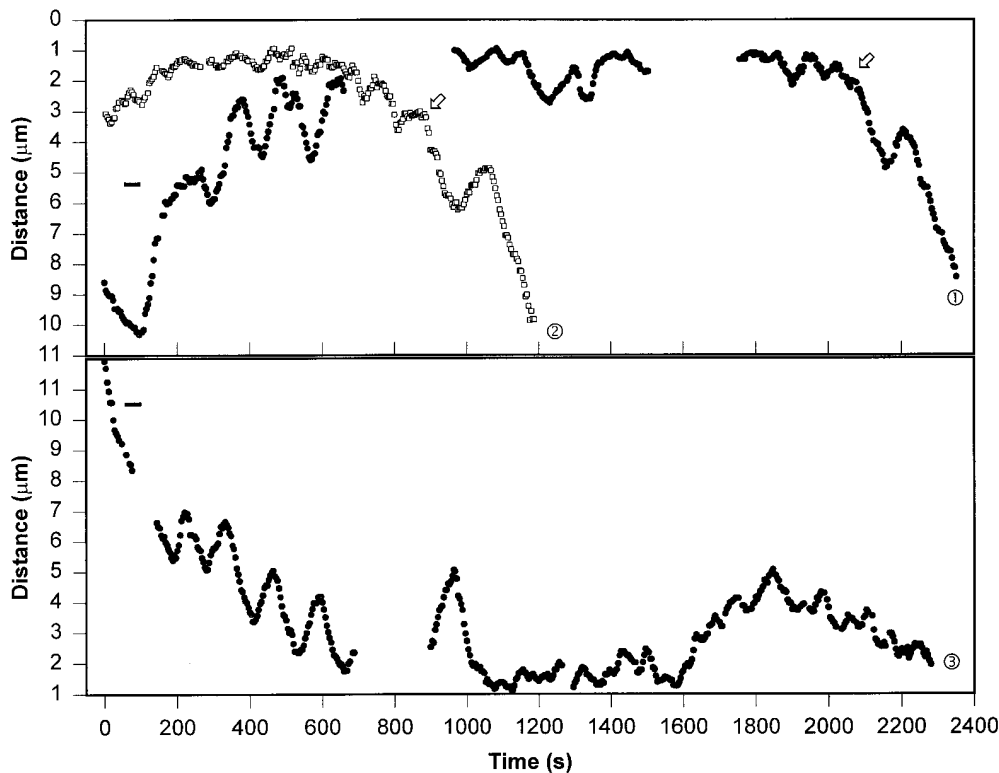
### Generating CF1Ks by Laser Microsurgery

To produce a large CF1K that could be clearly followed throughout the duration of mitosis, we used the scheme summarized in Fig. 1. For this approach, we first located a bioriented chromosome positioned near the spindle equator in which both sister kinetochores were in a P state (which stretched the primary constriction in a plane parallel to the interpolar spindle axis; Fig. 1, A and D). We then started cutting the centromere between the stretched kinetochore regions (Fig. 1, B and E). That our operation was separating the sister kinetochores could be easily assayed on a functional basis because, during a successful opera-

tion, the two kinetochore regions continued uninterrupted motion towards their poles (Fig. 1, B, E–G). This operation was then continued until we had lopped off a small piece of the chromosome that contained one of the kinetochores (Fig. 1, C and F). Corresponding immunofluorescent analyses revealed that each of the CF1Ks produced by this operation contained just a single kinetochore (Fig. 1, G–I). Over time the smaller CF1K usually became progressively stretched and unrecognizable, but the larger CF1K remained clearly visible throughout mitosis (unless it moved back into the mass of chromosomes already positioned on the forming metaphase plate—see below).

### CF1Ks Can Become Bioriented and Stably Positioned on the Spindle Equator

Once generated from a bioriented chromosome, CF1Ks always moved towards their respective poles. After nearing the polar region, they then initiated P and AP oscillatory motions that were indistinguishable from those of



**Figure 3.** Time-versus-distance plots depicting the behavior of the two CF1Ks noted by the black arrow and arrowhead in Fig. 2, as well as the naturally monooriented chromosome noted by the white arrow in this figure. Plot 1 (top, solid circles) represents changes in distance between the right-hand pole and the kinetochore region on the CF1K (Fig. 2, C–H, black arrow), while plot 2 (top, open squares) depicts changes in distance between the right-hand pole and the nonirradiated control chromosome (Fig. 2, A–D, white arrow). Note that both the CF1K and the control chromosome exhibited low amplitude oscillatory motions until they initiated congression (open arrows) and that each underwent a single oscillation at about the same point during the congression period. The bottom part of this figure depicts the behavior of the larger CF1K (Fig. 2, black arrowhead) relative to its (i.e., the left-hand) pole. This CF1K remained monooriented until anaphase onset. The black bar at about 100 s represents time of laser surgery (corresponding to Fig. 2 B).

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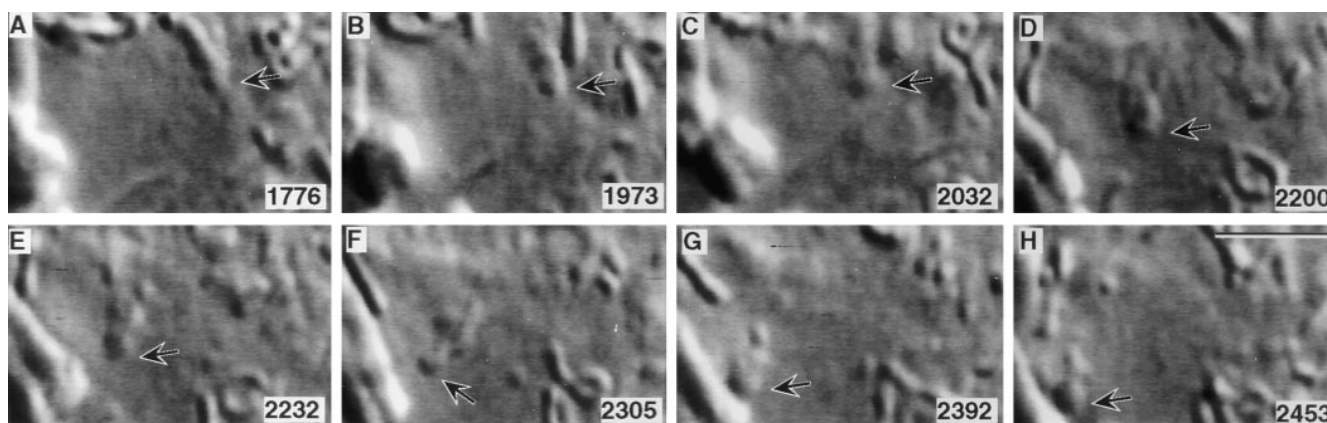
neighboring, nonirradiated monooriented chromosomes (Khodjakov and Rieder, 1996; Figs. 2 and 3). As a rule, when a CF1K moved AP during an oscillation, the kinetochore region remained nearest the pole, i.e., the whole CF1K appeared to be pushed away from the pole with its kinetochore region trailing (Fig. 2, C–G, black arrowhead; Fig. 3, curve 3; see also Rieder et al., 1986).

In our study, half (25/50) of the CF1Ks that could be followed until anaphase onset remained associated with the pole to which they were originally attached and behaved as a monooriented chromosome. The other half ultimately moved onto the metaphase plate. Importantly, during this motion the CF1Ks exhibited behavioral changes that are characteristic of the biorientation and congression of nonirradiated chromosomes. First, as during congression of untreated chromosomes containing two kinetochores, when a CF1K congressed the kinetochore region led the motion towards the spindle equator (Fig. 4, arrow). This contrasts sharply with the behavior of the kinetochore region on CF1Ks and monooriented chromosomes moving AP during a normal oscillation, where the kinetochore region usually trails the motion (see above). Second, the AP motion of an oscillating monooriented chromosome is always followed, some reasonable time thereafter, by a corresponding P motion of the chromosome (so that the average kinetochore-to-pole distances remains about the same—see Skibbens et al., 1993; Khodjakov and Rieder, 1996). By contrast, more often than not (see below) CF1Ks that moved onto the metaphase plate remained stably positioned on the spindle equator until or even throughout anaphase. Third, as in the newt (e.g., see Fig. 4

B in Skibbens et al., 1993), the AP congression motion of an untreated biorienting PtK<sub>1</sub> chromosome towards the spindle equator is always interrupted by at least one oscillation toward the proximal pole (Khodjakov and Rieder, 1996; Fig. 3, curve 2), and CF1Ks exhibited the same behavior as they moved onto the spindle equator (Fig. 3, curve 1). Finally, when initiating congression from a position near the pole, CF1Ks and normal chromosomes both covered the 6–10  $\mu$ m distance in about 5 min (Fig. 3, curves 1 and 2), and during this motion the ratio of the AP and P distances moved in relation to the proximal pole was always much higher (often approaching 5) than that exhibited when monooriented chromosomes or CF1Ks undergo a normal oscillatory cycle (where the ratio is usually about 1).

Of the 25 CF1Ks that moved to the spindle equator, six returned, after a variable period of time but before anaphase, to the same pole to which they were originally oriented (data not shown). After reaching the spindle equator, six other CF1Ks ultimately moved through the metaphase plate and into the opposing half spindle until they reached the opposing spindle pole, where they began to oscillate normally (Fig. 5). These CF1Ks, which had undergone “reorientation” (for review see Nicklas and Ward, 1994), then remained associated with this pole until anaphase.

The other 13 CF1Ks that congressed remained positioned on the equator until anaphase (e.g., Figs. 2 and 6). Of these, four segregated to one of the poles during the ensuing anaphase, but the other nine remained stably positioned midway between the groups of separating anaphase chromosomes (e.g., Fig. 6). At anaphase onset, the larger

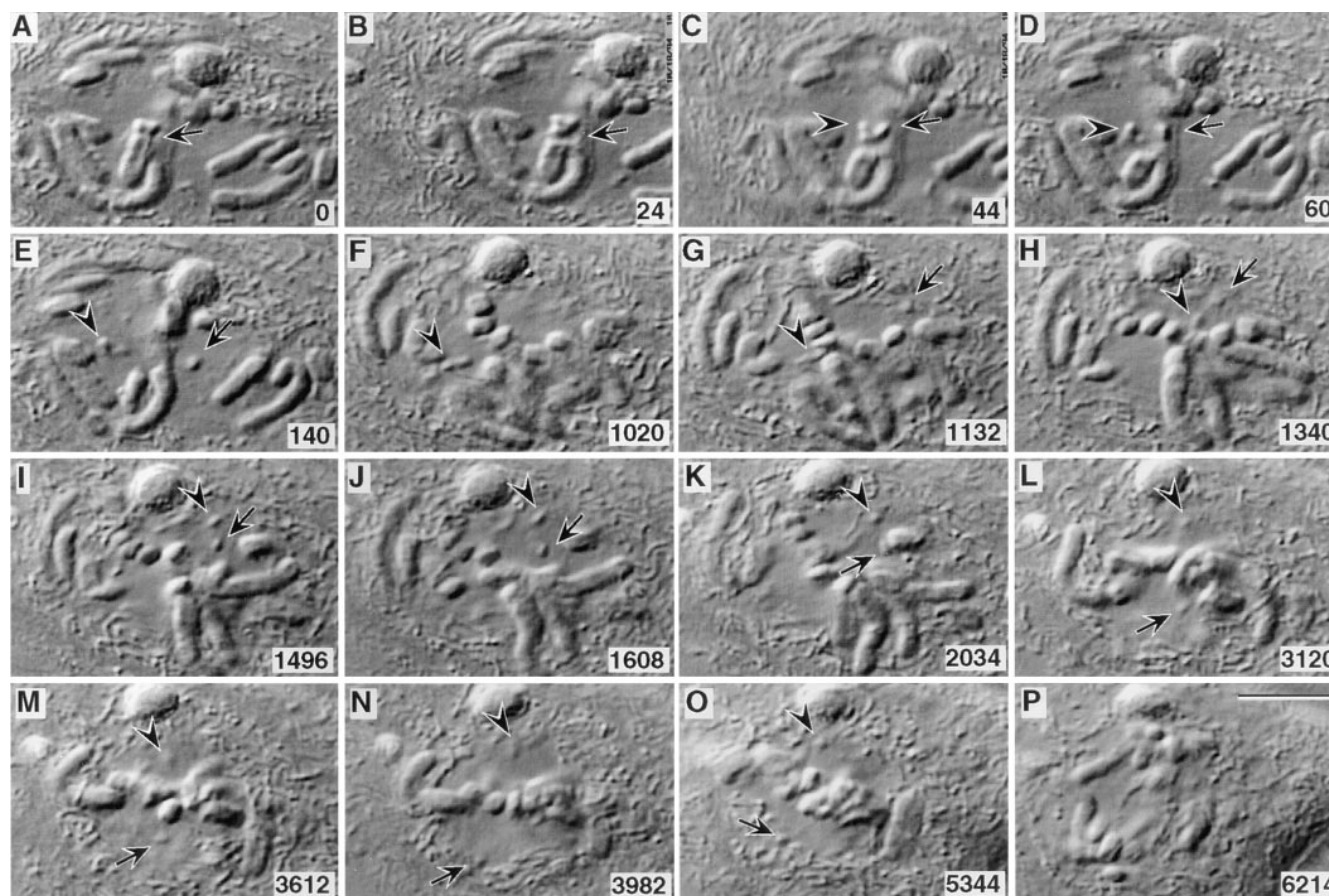


**Figure 4.** (A–H) Highly magnified selected images of the small CF1K noted by the black arrow in Fig. 2 as it congresses. Note that once congression is initiated (between B and C), the kinetochore region (*black arrow*) leads in motion towards the spindle equator. Bar, 5  $\mu$ m.

CF1Ks always disjoined into a single chromatid and two non-kinetochore-containing chromosome fragments (e.g., Fig. 2 I, *white arrowheads*; see also Khodjakov and Rieder, 1996). As subsequently determined by three-dimensional IVEM analyses, the single chromatid contained one kineto-

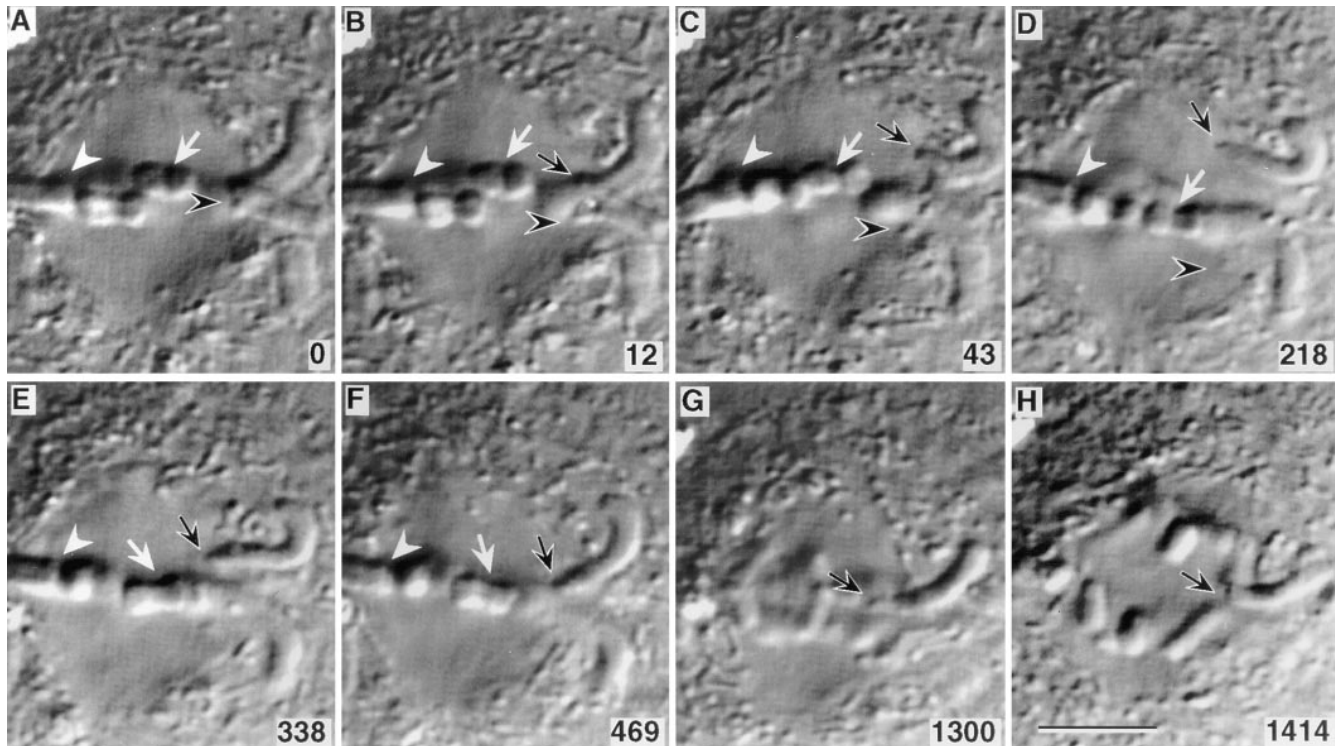
chore that was connected via Mts to both of the poles (see below).

Once a CF1K moved onto the metaphase plate, its ensuing motions were difficult to determine with certainty. However, in one cell (Fig. 6, *black arrow*) we were able to



**Figure 5.** (A–P) Selected frames from a video series showing the formation of two CF1Ks and their reorientation. In this cell, the arms were first separated from the centromere region of a large chromosome (compare *arrows* in A and B). The resultant fragment was then split along its long axis (C) to create two CF1Ks similar in size (D–O, *arrow*, *arrowhead*). The CF1K noted by the arrowhead in C moved towards its pole (D and E), and then towards the spindle equator (F and G). This fragment then ultimately crossed the equator (H) and became permanently associated with the other spindle pole (I–O). After moving into its pole (C–H, *arrow*) the other CF1K also reoriented (H–J, *arrow*) and moved through the spindle equator (K–L, *arrow*) until it reached the other pole (M–O, *arrow*). Bar, 10  $\mu$ m.





**Figure 6.** (A–H) Same conditions as in Fig. 2 except that after congression, the large CF1K produced by laser microsurgery in this cell (B–H, black arrow) remained stably bioriented on the spindle equator. In this example, the small CF1K (B–D, black arrowhead) moved out of the focal plane about 3.5 min after it was generated. However, the larger CF1K, after moving towards its associated pole (B–D, black arrow), moved back to the spindle equator (E and F, black arrow), where it remained until mid anaphase (G–H, black arrow), at which time the cell was fixed for a subsequent three-dimensional EM analysis. The white arrow in A–F notes a small oscillating bioriented chromosome positioned near the top surface of the spindle, while the white arrowhead notes a larger bioriented chromosome positioned opposite that of the congressed CF1K (see Fig. 7). Bar, 10  $\mu$ m.

clearly follow the behavior of a CF1K well after it had become bioriented and stably positioned on the spindle equator (Fig. 7, curve 1). On this CF1K, the oscillatory motions of the (single) kinetochore region appeared to become progressively damped once it achieved a position on the spindle equator (Fig. 7, curve 1). The significance of this observation is, however, unclear since at the same time and in the same cell some of the other congressed but nonirradiated chromosomes could be undergoing similar low amplitude oscillations (Fig. 6, white arrowhead; Fig. 7, curve 2) while others were undergoing high-amplitude oscillatory motions (Fig. 6, white arrow; Fig. 7, curve 3).

### **Structural Analyses of CF1Ks Stably Positioned on the Spindle Equator**

Thus far, our behavioral data reveal that CF1Ks can congress to the spindle equator. In some cases, these CF1Ks subsequently moved into one of the polar regions before or during anaphase. However, 36% (9/25) of the time a congressed CF1K remained stably positioned on the metaphase plate throughout anaphase.

Initially, we sought to determine the distribution of Mts on and around the single kinetochore of CF1Ks as soon as they had moved onto the metaphase plate. However, once on the spindle equator, CF1Ks were extremely difficult to differentiate from other chromosomes positioned on the

metaphase plate. Moreover, we had no way of knowing at the time of fixation whether the experimental chromosome was stably bioriented or whether it was in the process of losing a connection to one of the poles, as during reorientation and unstable congression (e.g., see Ault and Nicklas, 1989; Nicklas and Ward, 1994). To eliminate these concerns, we followed cells containing congressed CF1Ks into anaphase and then fixed those that contained a non-segregating intact chromatid between the groups of separating chromatids in early anaphase (e.g., Fig. 6).

For this part of our study, we used Stereon to generate three-dimensional reconstructions from serial 0.25- $\mu$ m-thick sections cut from three congressed CF1Ks that remained positioned on the spindle equator after anaphase onset. In all of these reconstructions, the CF1K was found to consist of just one chromatid and a single highly distorted kinetochore. In all three cases, this kinetochore was connected to each of the opposing spindle poles by conspicuous bundles of K-fiber Mts (not shown; see below).

To more carefully determine the distribution of Mt plus ends and their relationship to the single kinetochore on these CF1Ks, we used tomography to compute high-resolution three-dimensional volumes from thick sections through the heart of the centromere region on all three congressed CF1Ks. An analysis of sequential 3-nm-thick slices through these tomographic volumes (e.g., Fig. 8, A–C) revealed that the kinetochore was highly distorted,

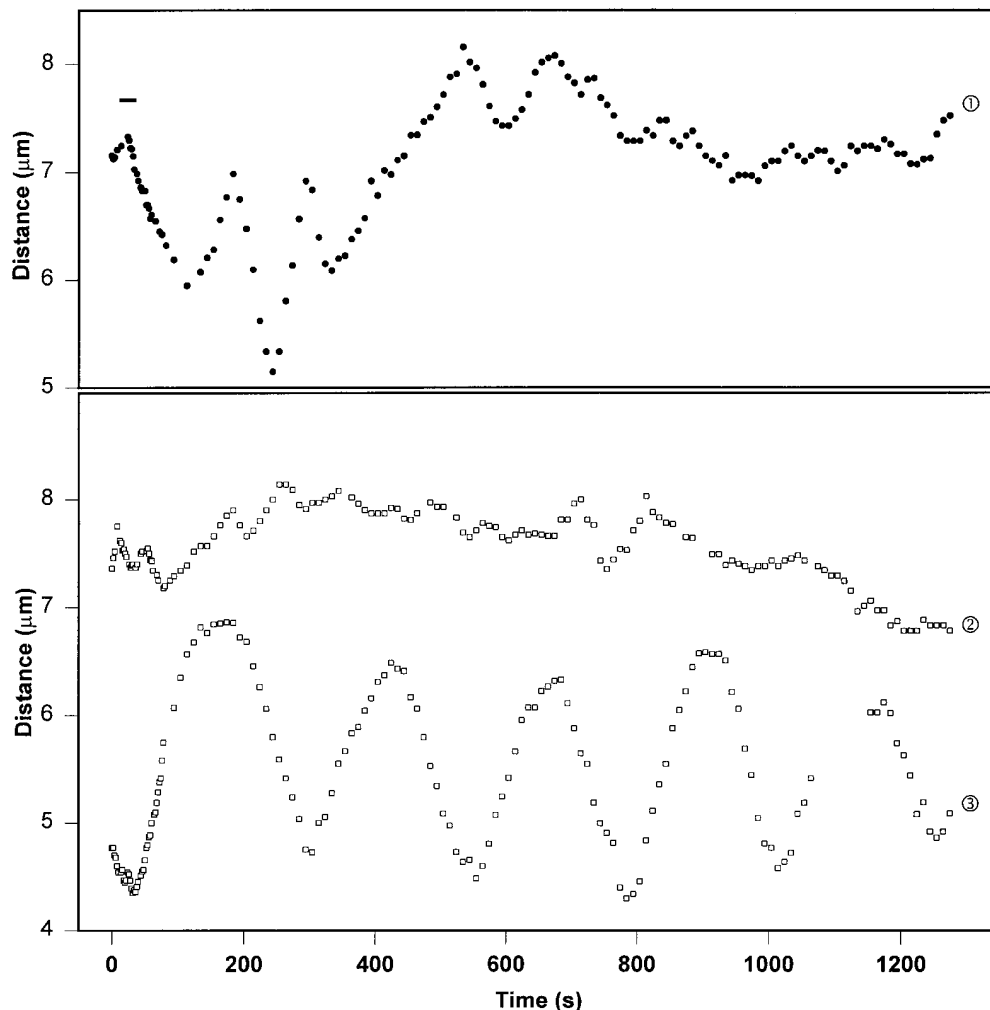


Figure 7. Time versus distance from the pole plots of the large CF1K shown in Fig. 6 (black arrow), as well as the two bioriented metaphase chromosomes noted in this figure (Fig. 6, white arrow and arrowhead). The top curve (1, solid circles) depicts the behavior of the kinetochore region on the large CF1K (Fig. 6, black arrow), which was tracked relative to the top pole. Once created (solid bar at time 0), this CF1K exhibited oscillatory motions that favored a net displacement towards its associated pole (0–220 s). It then initiated congression (near the 220-s timepoint), and during this process, it exhibited one oscillation before reaching the spindle equator (near the 550-s timepoint; compare with Fig. 3). Once positioned on the spindle equator, the amplitude of its oscillations became dampened (compare with plot 1). The middle curve (2, open squares) depicts the behavior of a large nonirradiated metaphase chromosome (Fig. 6, white arrowhead), while the bottom curve (3, open squares) follows the small bioriented nonirradiated metaphase chromosome (Fig. 6, white

arrow). The kinetochore region on both of these chromosomes was tracked relative to the bottom pole. Note that the amplitudes of the oscillations exhibited by these two “control” chromosomes varies widely.

appearing stretched and/or fragmented, and that it was connected to opposing spindle poles by numerous Mts. The distribution of these Mts, their plus ends, and their relationship to each other could be more clearly visualized by Stereon reconstructions made from stacking the pertinent information contained within these 3-nm-thick slices. In all three reconstructions, two bundles of 6–12 parallel Mts, running in a plane parallel to the spindle long axis, terminated on opposite sides of the kinetochore region (Fig. 8 D).

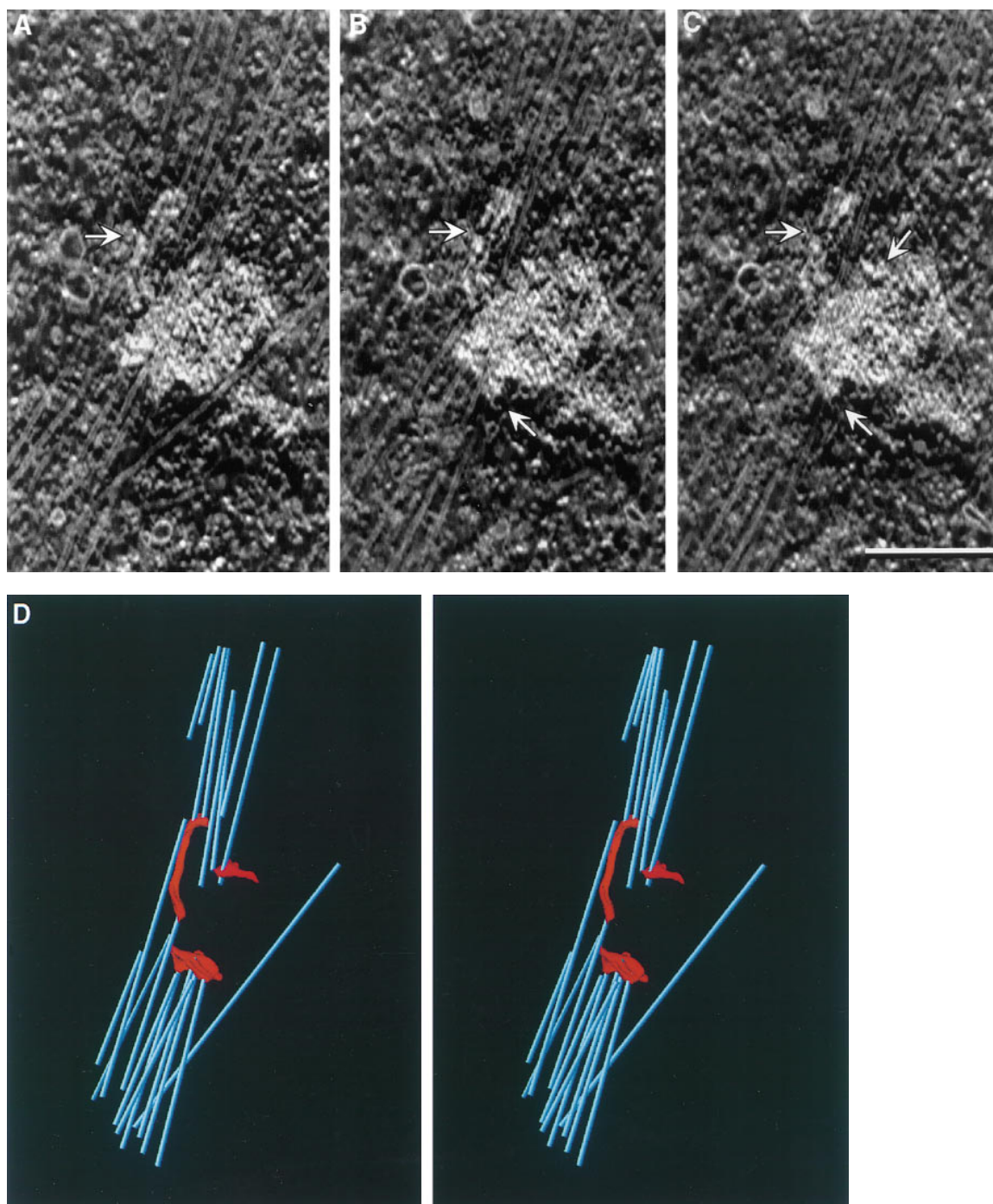
## Discussion

Once attached to the spindle, kinetochores in vertebrate cells exhibit a directionally unstable behavior that is characterized by rapid, periodic switches between P and AP states of motion. Although these two states were originally attributed to rapid switches between kinetochore-generated P “pulling” and AP “pushing” forces (Skibbens et al., 1993), we now know that kinetochores moving AP do not exert a significant pushing force on the chromosome (Khodjakov and Rieder, 1996; Waters et al., 1996). Instead, during AP motion the kinetochore is in a “neutral”

state in which it is coasting AP on the tips of kinetochore Mt plus ends elongating in response to external forces. A major implication of these recent findings is that the source of the force for moving a chromosome AP differs between mono- and bioriented chromosomes. On mono-oriented chromosomes, the force responsible for AP motion appears to be generated solely by the proximal polar ejection force (for review see Rieder and Salmon, 1994), whereas on bioriented congressing chromosomes, it is generated primarily by the P motion of the attaching “distal” sister kinetochore (Khodjakov and Rieder, 1996). That is, the chromosome must become attached via Mts to both poles before congression can be initiated, and this “biorientation” results in the production of a P force that acts on the kinetochore attaching to the distal pole. As a result, the attaching kinetochore leads the motion of the chromosome to the spindle equator during congression.

We have previously demonstrated that the damage created in chromosomes by our laser system is restricted to the 0.5- $\mu$ m diameter irradiated area. Indeed, when one set of arms is severed from a large chromosome, 0.25–0.50  $\mu$ m from the centromere, the chromosome behaves like a normal chromosome throughout the duration of mitosis





**Figure 8.** Three-dimensional structure of the kinetochore region on the congressed CF1K noted by the black arrow in Fig. 6 *H*. (*A–C*) Selected 3-nm-thick slices from the tomographic volume generated from a thick (0.25- $\mu\text{m}$ ) section through this region. Note that a number of microtubules impact and terminate on both sides of the kinetochore/chromatin complex. White arrows note structural differentiations that resemble pieces of the kinetochore outer plate, which is highly distorted. (*D*) Color-coded stereo volume generated from stacking all of the pertinent information found in sequential slices of the tomogram shown in *A–C*. Recognizable portions of the kinetochore outer plate are red and associated microtubules are blue. In this example, several Mts derived from the upper pole in Fig. 6 terminate in the upper part of the kinetochore plate, while those from the bottom pole terminate in the bottom half. Mts from both poles also appear to be connected to another region of the plate that is stretched between the poles. Bar, 0.25  $\mu\text{m}$ .

(Rieder et al., 1995; Khodjakov and Rieder, 1996). Furthermore, when the region between the sister kinetochores is severely weakened or severed with the laser without completely separating the kinetochore regions from the chromosome, the sister kinetochores behave normally

throughout the remainder of mitosis, with the exception that their respective motilities are no longer coordinated (Skibbens et al., 1995). The single kinetochore on a CF1K created by our operation also behaves the same when it is monooriented as the only attached kinetochore on a natu-

rally monooriented chromosome (Khodjakov and Rieder, 1996; also Fig. 3 of the present study). That is, it moves towards the pole to which it is attached and then begins to oscillate normally. Since the velocity and amplitude of these P and AP motions are about the same, the kinetochore on a CF1K, as that on a monooriented chromosome, usually maintains a relatively constant average position with respect to its pole as long as it is monooriented (Fig. 3; see also Skibbens et al., 1993, 1995; Khodjakov and Rieder, 1996). Together, these observations strongly support the contention that our laser microsurgery protocol does not damage either of the kinetochores during the production of CF1Ks.

We found that a CF1K can move from a polar region to the spindle equator and that once on the equator, it can remain stably positioned throughout the ensuing anaphase. That this motion to the equator is true congression and not just an exaggerated AP motion due to a normal oscillation is strongly supported by several different lines of evidence. First, during congression CF1Ks behave the same as normal congressing chromosomes. In fact, without prior knowledge, it is not possible to distinguish the congression motions of a CF1K from that of a biorienting chromosome with two kinetochores (see Results). Second, this behavior culminates in a CF1K that, more often than not, is stably positioned on the metaphase plate, which is the end product of congression. Finally, when we examined the structure of the only kinetochore region on CF1Ks stably positioned on the spindle equator during anaphase, it was always seen to be connected to both opposing spindle poles by bundles of Mts, a feature which is a hallmark of a bioriented chromosome and a requirement for congression (see above). That its trilaminar structure was not clearly evident around the attenuated primary constriction is not surprising considering the fact that the kinetochore was experiencing P (anaphase) forces at the time of fixation that were directed in two opposing directions. As noted by Roos (1973) (see also Rieder and Borisy, 1981), even the P forces acting on a monooriented PtK<sub>1</sub> chromosome can distort the attached kinetochore to the point where its trilaminar structure is not recognizable. However, although the kinetochore region on fully congressed CF1Ks lacked a clearly discernible trilaminar structure, the fact that it led the motion of the chromosome during congression and was the termination point for bundles of Mts derived from the opposing poles clearly signals that it contained the kinetochore. The alternative explanation, that the congressed CF1Ks we followed contained one complete and one partial kinetochore that became attached to the opposing poles, is not defensible. Under this condition, CF1Ks would never be expected to remain stably positioned at the spindle equator during anaphase because as for untreated chromosomes, chromatid disjunction at anaphase onset would separate the opposing kinetochore regions and allow them to move poleward.

Since kinetochores in PtK<sub>1</sub> cells do not exert a significant pushing force against the chromosome (Khodjakov and Rieder, 1996; see also Waters et al., 1996) and since the proximal polar ejection force is not sufficient by itself to keep a monooriented CF1K at the spindle equator (Khodjakov and Rieder, 1996), how does a CF1K congress? Based on our data, we propose the following scheme for

this process. When created from a bioriented chromosome, CF1Ks move towards the pole to which their kinetochore is attached by P forces acting on (or generated by) the kinetochore (McNeil and Berns, 1981; Rieder et al., 1995). As the chromosome moves progressively towards the centrosome, poleward progress becomes impeded and the kinetochore initiates the P and AP oscillatory motions characteristic of normal monooriented chromosomes that are positioned within a dense array of Mts (e.g., see Cassimeris et al., 1994). During AP motions, the whole chromosome fragment is pushed AP by the proximal polar ejection force so that the kinetochore region trails the movement (see above). If given enough time before anaphase, the kinetochore encounters, and then binds, one or more Mts growing from the distal pole. This induces it to initiate motion towards the spindle equator and the chromosome arms begin to trail this motion as the kinetochore approaches the metaphase plate (see Fig. 4). Under conditions in which the kinetochore maintains a stable attachment to both poles, it moves to the spindle equator, where it remains until anaphase onset or even through anaphase (Figs. 6–8). However, because Mts bound to and/or terminating in the kinetochore turnover relatively rapidly (for review see Zhai et al., 1995) and because the stability of these Mts is deleteriously compromised by distortions in the structure of the kinetochore (Nicklas and Ward, 1994) produced by the opposing P directed forces acting on it, some bioriented kinetochores ultimately lose their attachment to one of the poles. When this occurs, the kinetochore either returns to the pole it was originally attached to or it completes a successful reorientation to the other pole (Fig. 5). This scheme, as outlined, is not only consistent with our data but also with the existing structural data on how kinetochores on bivalents reorient during meiosis (see Ault and Nicklas, 1989; Nicklas and Ward, 1994) and mitosis (Ault and Rieder, 1992; McEwen, B.F., and C.L. Rieder, unpublished data).

To our knowledge, the attachment of a single kinetochore to both spindle poles has not previously been documented during the course of a normal bipolar mitosis, although it commonly occurs during the reorientation of bivalents during meiosis (e.g., Ault and Nicklas, 1989; Nicklas and Ward, 1994). It must occur during mitosis, at least on a transient basis, when spindle pole separation is delayed until well after nuclear envelope breakdown. Under this condition, kinetochores facing the single polar region are exposed, and likely attach, to Mts growing from both of the closely spaced centrosomes. As for these transient monopolar spindles, it is not unusual for one kinetochore to also be stably attached to two poles during a multipolar division when both poles face the kinetochore (Heneen, 1975). However, because of the steric considerations inherent in the back-to-back positioning of sister kinetochores on mitotic chromosomes, considerations which ensure that when one faces one pole the other faces the opposite pole, the biorientation of a single kinetochore is likely to be a rare event when a bipolar spindle forms between two well-separated centrosomes (see Nicklas, 1971), which is the prevalent route of spindle formation in animal cells (Aubin et al., 1980; Waters et al., 1993).

Why does removing one kinetochore by laser microsurgery enhance biorientation of the remaining kinetochore?

A logical explanation is that attenuating the primary constriction with the laser allows the remaining kinetochore to encircle more completely the remainder of the primary constriction, which makes it readily "visible" to Mts growing from both poles. This explanation is consistent with observations on the structure of kinetochore fragments in cells undergoing a MUG (Zinkowski et al., 1991; Christy et al., 1995), which reveals that the outer Mt-binding plate of the vertebrate kinetochore is under an internal elastic force that tends to curl it around the primary constriction of the chromosome (see also McEwen et al., 1993; Thrower et al., 1996).

From our data we conclude that the single kinetochores on CF1Ks can become attached to Mts from both poles and that this biorientation then leads to congression. This means that different parts of a kinetochore can exist, at the same time, in different functional states, i.e., while one part is generating or experiencing a P force and its associated Mts are shortening, another part can be in neutral and be displaced AP while its Mts elongate. For this to occur, a single kinetochore must be composed of multiple associated domains, and the P and AP motility states of these domains can be regulated independently of one another. This conclusion strongly supports the Zinkowski et al. (1991) hypothesis that vertebrate kinetochores are constructed of multiple identical subunits, and it extends this hypothesis to the functional level.

Since different regions of a single kinetochore can switch independently into different motility states, the motility state of a kinetochore is not regulated at the level of the whole structure. Rather, at any point in time, the overall behavior of a kinetochore is determined by the cumulative behavior of multiple, independently regulated sites that may or may not all be working in concert. It is likely that the behavior of these sites is normally coordinated by tension since switches between bulk kinetochore P and AP (neutral) activity are correlated with this parameter, i.e., increasing tension on the kinetochore favors net switching from P to neutral state, whereas diminishing tension favors neutral to P switches (Skibbens et al., 1993, 1995). This tension, which on a CF1K is generated across the kinetochore when those parts attached to the two opposing poles are in a P state, likely also stabilizes some of the Mts associated with the kinetochore (e.g., those that terminate perpendicular to the plate structure; see Nicklas and Ward, 1994).

We thank Ms. G. Osorio for her expert assistance with serial sectioning, Ms. A. Heagle for help with tomography, and Mrs. C. Hughes for maintaining and growing PtK<sub>1</sub> cells. We also thank Drs. M. Koonce, R. Sloboda (Dartmouth College), E.D. Salmon (University of North Carolina, Chapel Hill, NC), and K. Bloom (University of North Carolina, Chapel Hill, NC), for stimulating discussions.

This work was supported by National Institutes of Health grant GMS 40198 (to C. Rieder), National Science Foundation grant MCB 9420772 (to B. McEwen), and National Institutes of Health grant NCRR/BTP P41-01219, which partly supports the Wadsworth Centers Biological Microscopy and Image Reconstruction (BMIR) Facility as a National Biotechnological Resource. The video light microscopy component of the BMIR is also supported by the Wadsworth Center as a Core facility.

Received for publication 9 August 1996 and in revised form 11 October 1996.

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