

Chromosomes Move Poleward in Anaphase along Stationary Microtubules That Coordinately Disassemble from Their Kinetochore Ends

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Abstract. During the movement of chromosomes in anaphase, microtubules that extend between the kinetochores and the poles shorten. We sought to determine where subunits are lost from these microtubules during their shortening. Prophase or prometaphase cells on coverslips were injected with fluoresceinated tubulin and allowed to progress through mitosis. Immediately after the onset of anaphase, a bar-shaped beam of laser light was used to mark a domain on the kinetochore fibers by photobleaching a band, $\sim 1.0 \mu\text{m}$ wide, across the spindle. In different cells, spindles were photobleached at varying distances from the chromosomes. Cells were allowed to continue in anaphase until the chromosomes had further separated. They were then lysed, fixed, and prepared for double-label immunofluorescence with an antibody to fluorescein that does not bind appreciably to bleached fluorescein, and with an anti-

body to tubulin. Photobleached domains of microtubules appeared as bands of reduced fluorescence in the anti-fluorescein image. However, the anti-tubulin labeling revealed that microtubules were present and continuous through the photobleached domains. In all cases, the chromosomes approached and invaded the bleached domain while the bleached domain itself remained stationary with respect to the near pole. These results demonstrate that the chromosomes move along stationary kinetochore microtubules and that depolymerization of these microtubules during anaphase takes place at the kinetochore. In contrast to the generally accepted older view that chromosomes are passive objects pulled by "traction fibers," we suggest that the kinetochore is an active participant in generating the motive force that propels the chromosome to the pole.

THE kinetochore is the structural component of the chromosome at which microtubules of the spindle attach (2, 3, 17, 22). During the poleward movement of chromosomes in anaphase, the microtubules connecting the kinetochore to the pole lose subunits and shorten (11, 16). Numerous proposals have been suggested to account for this movement but decisive experimental evidence has been lacking (1, 6, 9, 10, 19). With certain notable exceptions anaphase theories have generally advocated a pulling force acting on spindle fibers to drag the passive chromosomes to the poles (1, 6, 9, 10). Often cited to support this type model are ultraviolet microbeam experiments in insect spermatocytes showing that areas of reduced birefringence move to the pole synchronously with the chromosomes during anaphase (4). In contrast, based in large part on observations of living diatoms, Pickett-Heaps et al. (19) suggested that kinetochores might actively move on the microtubules of the spindle.

These two views on the role of the kinetochore could be distinguished, in principle, by determining whether the microtubules that run from the pole to the kinetochore are moved poleward in anaphase accompanying the movement of the chromosomes. If kinetochores served merely as struc-

tural linkages and if chromosomes were passively dragged by traction forces applied to the kinetochore microtubules, then these microtubules would be expected to move poleward during anaphase, and they would be depolymerized at the pole. In contrast, if kinetochores traveled along the microtubules, then kinetochore microtubules might be expected to act as stationary tracks over which the chromosomes moved. In this case, the kinetochore microtubules would depolymerize at the kinetochore end during or just after passage of the chromosome.

Recently, using colloidal gold immunocytochemistry of microinjected, biotinylated tubulin, Mitchison et al. (15) provided evidence that tubulin subunits incorporated into microtubules near the kinetochore during metaphase were lost when the cells entered anaphase. These authors concluded that shortening of kinetochore microtubules occurred primarily through loss of subunits at the kinetochore but their conclusions were drawn from analyses of populations of cells and were necessarily statistical in nature.

To investigate these questions more definitively, we have attempted to obtain direct evidence of relative motions of chromosomes and their kinetochore microtubules. Using the

technique of photobleaching we have marked segments of kinetochore microtubules in living cells just after the onset of anaphase. Then, to analyze the movement of these marked segments, we used the method of hapten-mediated immunocytochemistry (26). Here we describe the relative motions of kinetochores, kinetochore fibers, and poles during the movement of the chromosomes to the poles in anaphase.

Materials and Methods

Cell Culture

The cell line LLC-PK, derived from porcine kidney, was obtained from the American Type Culture Collection (Rockville, MD). Cells were grown in DME (Gibco, Grand Island, NY) supplemented with 10% fetal bovine serum (HyClone Laboratories, Logan, UT), 20 mM Hepes, and antibiotics. Coverslips, on which cells were grown for experiments, were prepared as follows. 400 mesh locator grids (Ted Pella Inc., Tustin, CA) were placed on No. 1.5, 22-mm × 22-mm glass coverslips. These were placed in a vacuum evaporator and shadowed with carbon. After removing the grids, a carbon locator pattern was left on the coverslips. The coverslips were baked at least 1 h at 160°C to adhere the carbon strongly to the coverslips. Coverslips were mounted with silicone grease (Dow Corning Corp., Midland, MI) into plastic 35-mm dishes, the bottoms of which contained a 19-mm hole. The dishes were then sterilized with ultraviolet light. Cells were plated into these dishes and allowed to grow for 2–3 d until ½ to ¾ confluent.

Microinjection

Porcine brain tubulin labeled with dichlorotriazinylaminofluorescein (DTAF) was prepared and characterized as previously described (23, 26). Fluorescein-tubulin at 8 mg/ml in 100 mM Pipes, 1 mM EGTA, 0.1 mM MgCl₂, 1 mM GTP, pH 6.94, was stored in small aliquots in liquid nitrogen. Just before use an aliquot was thawed and spun at 20,000 g for 30 min to remove particulates. Micropipettes with 1–2 μm diameter tips were back-loaded with 0.25–0.5 μl of DTAF-tubulin. Microinjection was carried out at room temperature according to the general procedures of Kreis and Birchmeier (7) by means of a Leitz micromanipulator (E. Leitz, Inc., Rockleigh, NJ) and a Zeiss IM-35 microscope (Carl Zeiss, Inc., Thornwood, NY) or a Nikon Diaphot microscope (Nikon Inc., Garden City, NY).

Cells in prophase or pro-metaphase were located on the carbon grid pattern and their positions were recorded on an enlargement of the pattern produced by photographing a locator grid. Cells were injected with ~5% of their total cellular volume. After injection of several cells, the medium in the dish was replaced and overlaid with mineral oil. The dish was then returned to the incubator to allow cells to progress through mitosis.

Photobleaching

The photobleaching apparatus was assembled according to the methods of Petersen et al. (18) and has been described in detail (23a). The 488-nm beam from a Spectra Physics model 2020 argon ion laser (Spectra-Physics Inc., Mountain View, CA) was imaged through a 300-mm focal length lens and a 200-mm focal length cylindrical lens to produce a bar-shaped beam focused in the specimen plane. The laser was operated at 200 mW and the beam attenuated by a 0.9 OD neutral density filter. The laser beam was channeled into the epi-illumination system of a Zeiss IM-35 microscope. When focused through a neofluar 100×, 1.3 NA, phase 3 objective the beam produced a bleached band ~1 μm wide in the spindle of a typical injected cell.

Approximately 15 min after return to the incubator following microinjection, culture dishes were transferred to the stage of the IM-35. The stage and culture dish were maintained at 34–35°C by means of an air curtain incubator (Nicholson Precision Instruments, Bethesda, MD). A cell was observed until the onset of anaphase and was then photobleached once or twice with a 100–300-ms pulse. Light emitted by the fluorescein molecules excited

by the laser light during the photobleaching was collected onto TRI-X film. Simultaneously a weak phase image of the cell was recorded on the same negative by allowing an attenuated amount of green-filtered light from the tungsten source to illuminate the specimen during photobleaching. Normally exposed phase images were taken before and after photobleaching to record the progress of the cell through mitosis.

Cell Lysis and Fixation

After photobleaching, anaphase was allowed to continue for some time. Then, the culture dishes were rapidly rinsed with two changes of 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, and 2 mM MgCl₂, pH 6.95 (PHEM) at room temperature and the cells were lysed for 90 s with a solution consisting of 0.5% Triton X-100 in PHEM containing 1 μg/ml taxol. The time between removal of the dish from the stage until the addition of lysis solution was 5–10 s. Cells were then fixed for 20 min in 5 mM ethylene glycol bis-(succinic acid *N*-hydroxy succinimide ester) (EGS) (Sigma Chemical Co., St. Louis, MO) in PHEM with 1 μg/ml taxol. EGS was prepared as a 100-mM stock in DMSO. Immediately before use, 100 μl of the stock was added to 1.9 ml of PHEM with taxol. This mixture was swirled and added to the cells. After fixation, cells were rinsed several times with 140 mM NaCl, 3 mM KCl, 10 mM PO₄, 3 mM Na₂SO₄, pH 7.3 (PBS). The fixative was then quenched with 0.1 M glycine, pH 7.0 for 10 min. Cells were rinsed with PBS and processed for immunolabeling immediately or stored overnight in PBS at 4°C.

Immunolabeling

Anti-fluorescein antibody was prepared in the following way. 50 mg of key-hole limpet hemocyanin (Calbiochem-Behring Corp., San Diego, CA) was dissolved in 5 ml of 0.1 M carbonate buffer, pH 9.0. To this was added 20 mg of FITC isomer I (Molecular Probes, Inc., Junction City, OR), and the solution was incubated overnight at room temperature. The next day, 1 ml of 1 M Tris buffer, pH 8.0, was added, and the solution was spun at 48,000 g for 15 min to remove large aggregates. The fluoresceinated hemocyanin was desalted into PBS without azide to remove free fluor.

To elicit antibodies, 800 μg fluorescein-hemocyanin (0.5 ml mixed 40:60 with complete Freund's adjuvant) was injected into a rabbit intradermally and intramuscularly. At 3-wk intervals, booster injections of 500 μg of protein with incomplete adjuvant were given. After the second boost, the rabbit serum showed a strong reaction with fluorescein-BSA as judged by Ouchterlony double diffusion.

Before use, the anti-fluorescein serum was pre-extracted as follows. 1 ml of serum was added to 500 mg of porcine liver acetone powder (Sigma Chemical Co.), incubated 1 h at room temperature and at 4°C overnight. The serum was centrifuged at 20,000 g for 1 h, filtered through a 0.22-μm Millex-GV filter unit (Millipore Corp., Bedford, MA), aliquoted, and stored at –70°C. Rat monoclonal anti-tubulin antibody was from Accurate Chemical & Scientific Corp. (Westbury, NY); affinity-purified fluorescein-anti-rabbit IgG was from Kirkegaard & Perry Laboratories, Inc. (Gaithersburg, MD); and affinity-purified Texas red-anti-rat IgG was from Jackson Immunoresearch (Avondale, PA).

For labeling, cells on coverslips were first treated with 25% normal goat serum (Gibco) for 30 min at 37°C to block nonspecific binding sites. They were then rinsed in PBS and labeled for 45 min at 37°C with anti-fluorescein serum diluted 1:1,000 in PBS with 10% goat serum. This diluent was used for all antibodies. Coverslips were washed in three changes of PBS for a total of 30 min and then, to stabilize the antibody, were refixed for 15 min at room temperature with 2.5 mM EGS in PBS. After rinsing in PBS, coverslips were treated with 0.1 M glycine and washed in PBS. Samples were then labeled with fluorescein-anti-rabbit IgG at 27 μg/ml for 45 min at 37°C. The coverslips were again washed in PBS and treated for 45 min at 37°C with rat anti-tubulin ascites fluid diluted 1:500. After washing in PBS, Texas red-anti-rat IgG (20 μg/ml) was applied for 45 min at 37°C. Coverslips were washed in PBS, rinsed in distilled water, and mounted in 10% polyvinyl alcohol containing 2 mg/ml paraphenylene diamine.

Cells were observed with a Zeiss Universal microscope equipped with epifluorescence optics. All photographs of live cells and some immunofluorescence micrographs were taken on Tri-X film and developed in HC-110, dilution A. Some fixed and labeled cells were photographed with hypersensitized technical Pan 2415 film (Lumicon, Livermore, CA) and developed in D-19. Except as noted, exposures were adjusted to best bring out details in the microtubule bundles between the poles and the chromosomes and suppress the less intense fluorescence of the astral microtubules.

1. *Abbreviations used in this paper:* DTAF, dichlorotriazinylaminofluorescein; EGS, ethylene glycol bis-(succinic acid *N*-hydroxy succinimide ester); PHEM, 60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM MgCl₂, pH 6.95.

Results

Experimental Design

The LLC-PK line consists of large flat cells that generally remain extended during mitosis. Microinjection of DTAF-tubulin was carried out on prophase or prometaphase cells to facilitate the homogeneous incorporation of label into spindles. The use of carbon-patterned coverslips (see Materials and Methods) permitted us to easily relocate injected cells without exposure of them to blue light. This was important because we found that cells microinjected with fluorophore were hypersensitive to blue irradiation, presumably because of excitation of the fluorescein. Exposure of an entire injected cell to blue light from a 100-W mercury arc source for ~ 2 s caused arrest of mitosis whereas non-injected cells were not blocked by an exposure of 30 s. In the protocol followed, only the fluorescein molecules in the laser light path were excited at any time in the living cells and then only for the brief period of the photobleaching pulse. To avoid damage to the cells, no attempt was made to completely bleach all the fluorophore in the laser light path. Instead we chose conditions of intensity and duration of photobleaching near the minimum necessary to provide a clear bleached zone after antibody labeling. Thus, some proportion of the fluorophore in the laser light path remained unbleached. Of 47 cells microinjected with fluorescent tubulin and photobleached at the onset of anaphase, 46 of them progressed in anaphase at rates comparable to non-injected, non-irradiated cells up to the moment of lysis. The one cell that was arrested in anaphase received two 300-ms pulses of irradiation. Therefore, at the intensities used in our experiments, there was no significant effect of laser photobleaching on the progression of cells through anaphase.

The progress of cells in mitosis was recorded by phase-contrast microscopy on 35-mm film. Because chromosomes in anaphase move rapidly it was important to record their position at the exact moment of photobleaching. Therefore, we simultaneously exposed film to a weak phase-contrast image and to the light emitted by the injected fluorescein tubulin excited by the laser. The band of laser-induced emitted light captured on film appeared to be ~ 3 μm wide. However, due to the Gaussian profile of the beam, only the central region of the laser beam was of sufficient intensity to cause bleaching. Thus bleached areas were generally ~ 1 μm wide as determined by later measurements on anti-fluorescein-stained cells.

Previous photobleaching studies of microtubule dynamics in mitotic cells have relied upon continuous or intermittent recording of direct emission from fluorescein-tubulin injected in living cells (23, 24, 30). However, significant information about microtubule dynamics can be obtained if domains of microtubules can be labeled at one time point and reidentified at a second time point after a significant event, such as the movement of chromosomes, has taken place. Our first time point was the moment of photobleaching. Our second time point was the moment of cell lysis. Lysis also allowed improvement of the signal-to-noise ratio of our injected tracer (27). Lysis reduced noise by removing DTAF-tubulin not incorporated into the spindle, and indirect immunolabeling amplified the signal of unbleached fluorescein present along microtubules that incorporated DTAF-tubulin.

An important precondition for use of the anti-fluorescein antibody in photobleaching experiments is that it have a very low affinity for bleached fluorescein. We discovered this condition was met when we used the antibody to label DTAF-tubulin-injected cells that had already been exposed to mercury-arc illumination. The binding of the antibody was greatly reduced in those regions of cells that had been bleached by the mercury-arc light (data not shown). Similarly the antibody failed to bind laser-bleached domains of microtubules in injected interphase cells (23a). A further improvement in our protocol involved choice of fixative. Glutaraldehyde induced a high background fluorescence that was only partially eliminated by sodium borohydride treatment. Formaldehyde proved inadequate to fix microtubules. As an alternative we have used EGS. This fixative had little effect on fluorescein and preserved microtubules well. EGS has been used previously for fixation of microtubules *in vitro* (8).

Experimental Results

Fig. 1 shows a sequence of micrographs for a cell photobleached early in anaphase. The bleached zone was placed $\sim 2/3$ the distance from the chromosomes to the poles (Fig. 1 *b*). The cell was lysed and fixed (Fig. 1 *d*) 120 s later after significant movement of the chromosomes had occurred. While some of this movement was due to the separation of the poles, anaphase B, most resulted from the decrease in the distance of the chromosomes to the pole, anaphase A, and in this paper we will focus on events in anaphase A. The bleached zone was clearly revealed as a lack of labeling in the anti-fluorescein image (Fig. 1 *e*). The anti-tubulin label, however, showed that microtubules were present and continuous through the photobleached region (Fig. 1 *f*). Careful inspection revealed that the distance between the bleached zone and the pole had not detectably changed, whereas the distance between the kinetochores and the bleached zone had significantly diminished.

A second observation was that the bleached zone remained nearly devoid of fluorescent fibers. Only a few very weakly fluorescent fibers were present in the bleached zone. These few fibers may represent incompletely bleached microtubules, some slight recovery of microtubules in the bleached zone, or a minor change in shape of the spindle during anaphase causing the bleached domains of microtubules to become slightly out-of-register. However, the images show that relatively little fluorescence is present in the bleached zone compared to the intense staining of the prominent fibers that are seen to extend from the poles and end at the chromosomes in phase and fluorescent micrographs of the lysed cell (Fig. 1, *d-f*). Clearly, within these fibers that run exclusively between the poles and the chromosomes, the bleached zone remained stationary with respect to the near pole. Moreover, unbleached domains of the kinetochore bundles did not invade the bleached zone accompanying the movement of the chromosomes.

These observations suggested that the disassembly of microtubules in the half spindle must have occurred somewhere between the bleached zone and the kinetochores and not between the bleached zone and the pole. Disassembly along the length of the microtubules between the bleached zone and the kinetochore is unlikely to account for the obser-

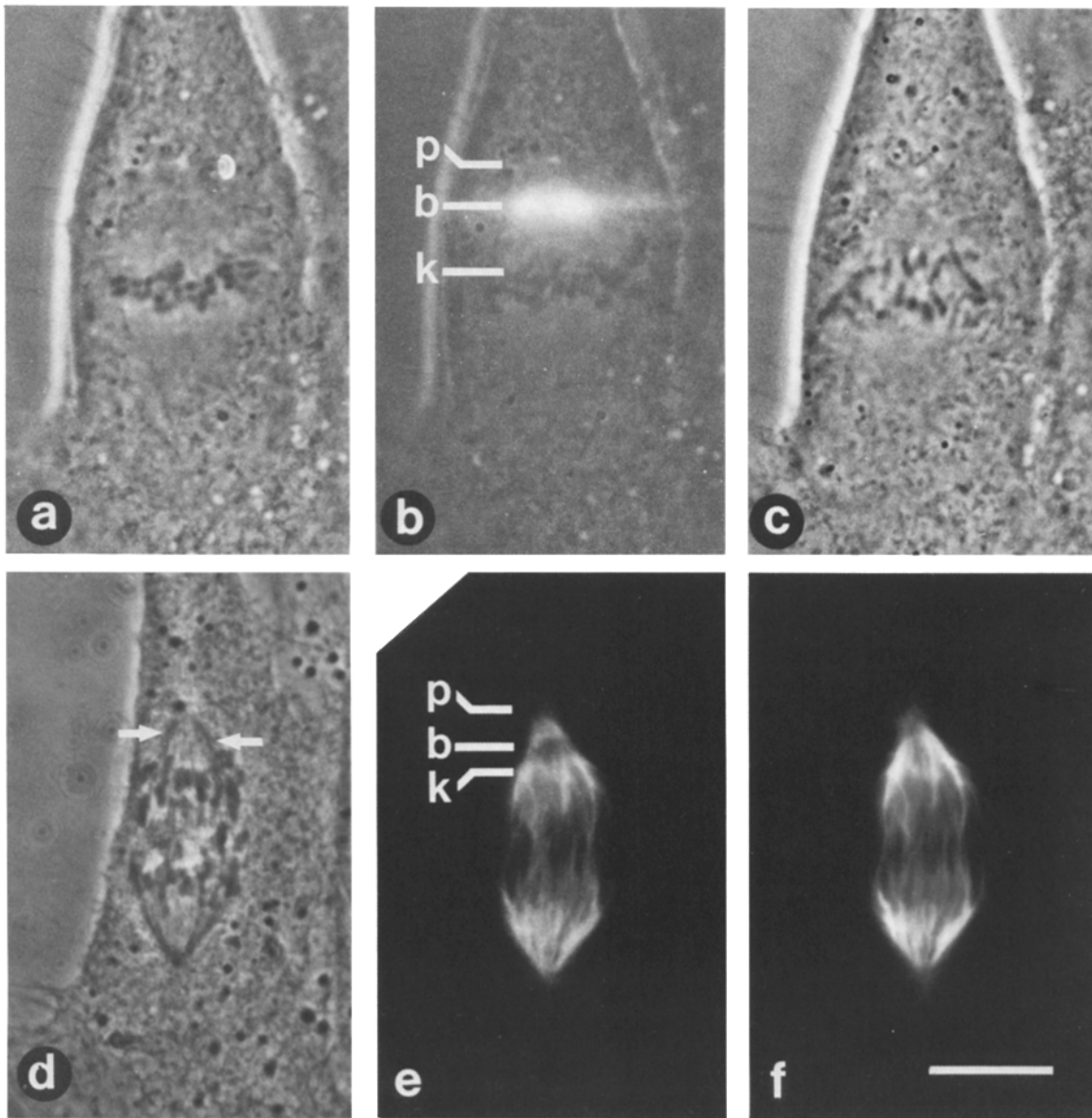


Figure 1. LLC-PK cell microinjected with DTAF-tubulin and photobleached immediately after anaphase onset. (a) Phase image of living cell before photobleaching. Chromatids have just begun to separate. (b) Cell during photobleaching with a 200-ms pulse. Simultaneous exposure of weak phase image and of fluorescence due to excitation of injected DTAF-tubulin by laser light beam. Indicated in the upper half spindle are the positions of the pole (*p*), the bleaching beam (*b*), and the average position of the kinetochores (*k*). (c) Phase image of living cell 70 s after photobleaching. (d) Phase image of cell lysed and fixed 120 s after photobleaching. Chromosomes are well separated. Note the phase-dense kinetochore fibers that originate at the poles and end at the chromosomes (*arrows*). (e) Anti-fluorescein immunofluorescence. The bleached zone has remained stationary with respect to the pole. The distance between the bleached zone and the chromosomes has decreased considerably. (f) Anti-tubulin immunofluorescence showing that fibers are present and continuous in the photobleached region. Bar, 10 μ m.

variations since it would be difficult to imagine why such disassembly would not also take place between the bleached zone and the pole. Therefore, this single class of experiment already strongly suggested that disassembly of microtubules during anaphase A occurs predominantly if not exclusively at or very near the kinetochore.

Nevertheless, we wished to submit the idea of kinetochore-mediated disassembly to further test. Kinetochore disassembly predicts that a bleached zone placed near the kinetochores in early anaphase should be encroached upon and finally overrun by the advancing chromosomes. To test this hypothesis we bleached across the spindles of anaphase cells

near the kinetochores and lysed the cells at progressively longer intervals. Fig. 2 shows the sequence for a cell bleached and then lysed after 47 s. A few but not all the chromosomes have reached the bleached zone. In the anti-fluorescein image, a number of small stubs of microtubule bundles extend between the bleached zone and the chromosomes (Fig. 2 *e*). The anti-tubulin image reveals that these stubs are continuous with fibers extending from the poles (Fig. 2 *f*).

Fig. 3 shows the sequence of micrographs for a cell bleached near the kinetochores and lysed 90 s later. Here all the chromosomes have invaded deeply into the bleached zone. Note, however, in the anti-fluorescein image, that some

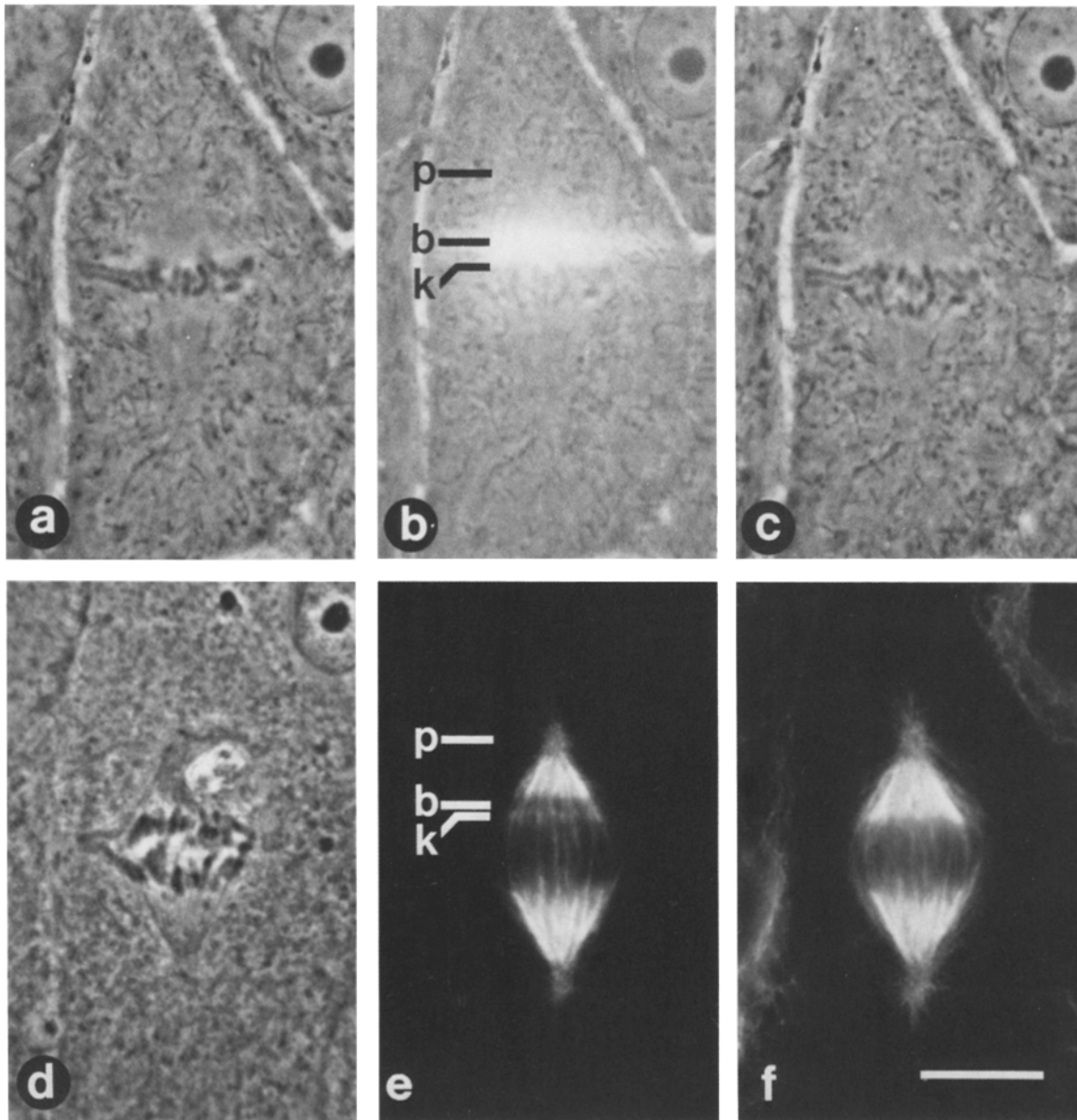


Figure 2. Cell microinjected with DTAF-tubulin and photobleached close to the kinetochores after anaphase onset. (a) Phase image of living cell before photobleaching. (b) Cell during photobleaching with a 100-ms pulse. (c) Living cell 8 s after photobleaching. (d) Phase image of cell lysed and fixed 47 s after photobleaching. (e) Anti-fluorescein immunofluorescence showing that chromosomes have moved closer to the bleached zone. (f) Anti-tubulin immunofluorescence. Note that this cell received a relatively short pulse (100 ms) of laser light. Hence the bleaching was relatively less complete than in the other cells depicted. *p*, Pole; *b*, bleached zone; *k*, average position of kinetochores. Bar, 10 μm .

interzonal (nonkinetochore fibers) penetrate the bleached zone (arrowhead, Fig. 3 *e*). These interzonal fibers have apparently formed or moved into the bleached zone during the interval between bleaching and lysis.

The cell shown in Fig. 4 was bleached twice. First a bleached zone was placed near the upper pole. 15 s later, a second bleaching of equal intensity and duration to the first was performed on the lower half spindle near the kinetochores. The cell was lysed 135 s later. Anti-fluorescein labeling revealed that, as expected, the first bleached zone (*b*) did not move with respect to the pole (*p*) although the kinetochores (*k*) moved with respect to the bleached zone (Fig. 4

g). The second bleached zone could not be detected. However, this was expected since the kinetochores (*k'*) had moved past the point on the spindle where the second bleaching (*b'*) had been placed. Presumably, the domains of microtubules present in the second bleached zone depolymerized as the chromosomes moved past. Because the first bleached zone (*b*) was produced 15 s earlier than the second and clearly persisted for the length of the experiment, the disappearance of the second bleached zone (*b'*) cannot be accounted for by a recovery process such as microtubule turnover which would be expected to operate on both bleached zones.

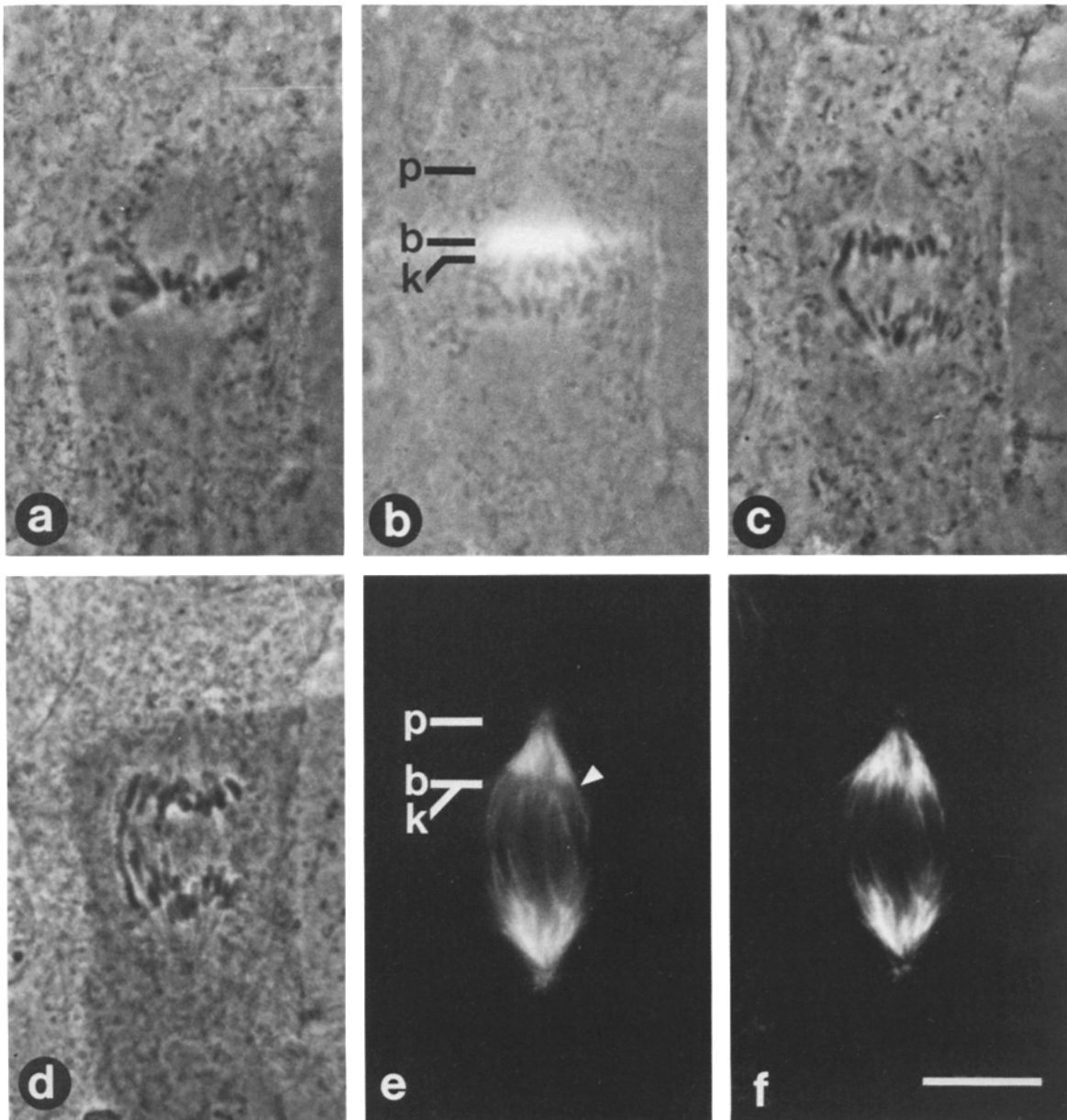


Figure 3. Cell showing chromosomes invading the bleached region. (a) Living metaphase cell before photobleaching. (b) Cell during photobleaching with a 200-ms pulse. Chromosomes have already separated slightly. Bleached zone is placed close to kinetochores. (c) Living cell 70 s after photobleaching. (d) Phase image of cell lysed and fixed 90 s after photobleaching. (e) Anti-fluorescein immunofluorescence showing that the chromosomes have progressed well into the photobleached zone. Arrowhead indicates an interzonal fiber that penetrates the bleached zone. This fiber was assembled or recruited after the moment of photobleaching. (f) Anti-tubulin immunofluorescence. *p*, Pole; *b*, bleached zone; *k*, average position of kinetochores. Bar, 10 μ m.

Discussion

We sought to determine the movement of kinetochores relative to their attached microtubules and the site of subunit loss from kinetochore microtubules during anaphase because this information would provide important insights about the mechanism generating the force for chromosome translocation. Photobleaching pre-existing fluorescent microtubules with a bar of light marked a domain on kinetochore microtubules in living mitotic cells. The mark served as a reference point by which we could assay the movement of kinetochores relative to a microtubule domain and the movement of this domain relative to the poles (Fig. 5). Relocating the marked

domain later, after lysis and fixation, allowed interpretation of microtubule movements in the living cells from the time of bleaching to the time of lysis.

Three possible outcomes of these experiments might be considered. If shortening of kinetochore fibers in anaphase occurred by the loss of subunits all along the length or from both ends of the microtubules, then a bleached zone placed between the pole and chromosomes at early anaphase would appear closer to both pole and chromosomes at late anaphase. If subunit loss occurred exclusively at the poles, then the bleached zone would progress toward the pole at the same rate as the chromosomes. Finally, if subunit loss took place

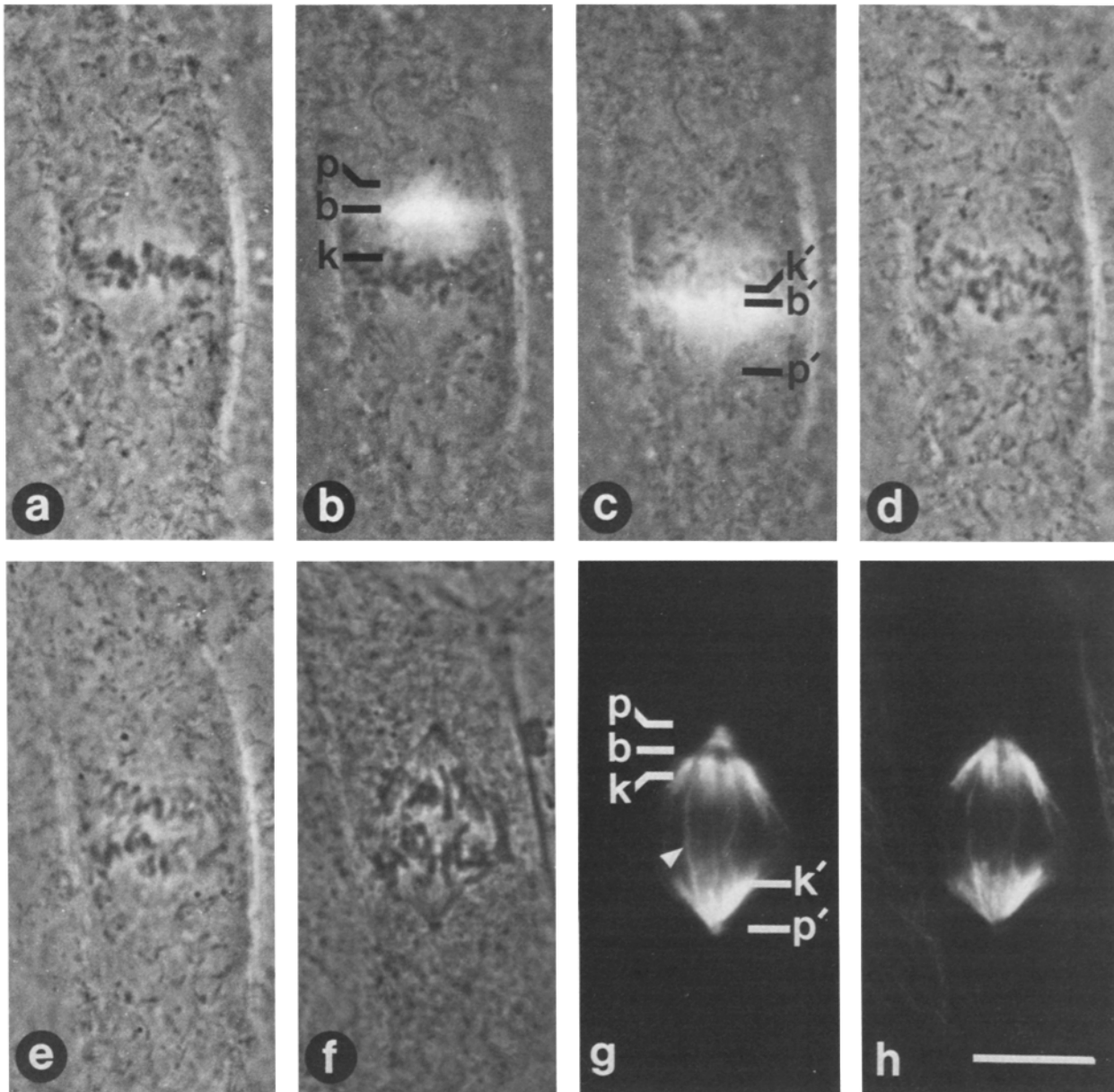


Figure 4. Movement of chromosomes past a bleached zone in the anaphase spindle. (a) Living cell before photobleaching. (b) First photobleaching, 200-ms pulse, near upper pole. (c) Second photobleaching, 200-ms pulse, 15 s later in lower half-spindle near the kinetochores. (d) Living cell 60 s after first photobleaching. (e) Living cell 105 s after photobleaching. (f) Phase image of cell lysed and fixed 150 s after first photobleaching. (g) Anti-fluorescein immunofluorescence. Chromosomes in the upper half-spindle have moved closer to the bleached zone. Chromosomes in the lower half spindle have moved past the bleached region. No evidence of second bleached zone is evident. Some unbleached interzonal fibers (*arrowhead*) traverse the region of the second bleached zone suggesting that they were assembled or recruited after the bleaching. (h) Anti-tubulin immunofluorescence. *p*, Upper pole; *b*, first bleached zone; *k*, average position of kinetochores in upper half-spindle; *p'*, lower pole; *b'*, second bleached zone; *k'*, average position of kinetochores in lower half-spindle. Bar, 10 μ m.

only at the kinetochore, then chromosomes would move toward the bleached zone while the zone itself remained stationary with respect to the pole.

Consistent with the last model, we observed that a zone of bleached microtubules in the anaphase spindle was successively approached, invaded, and passed by the chromosomes. In addition, within the limits of our ability to precisely localize poles in the living cell, we found that bleached zones remained stationary with respect to the near pole and accompanied that pole during pole-pole separation. All 46

cells successfully photobleached behaved in a similar manner. These observations indicate that microtubule disassembly during anaphase occurs at the kinetochore accompanying or following closely the passage of the chromosomes.

It might be argued that kinetochore fibers are indeed translocated toward the poles and that our stationary bleached zone represented only interzonal microtubules. This interpretation is unlikely to be valid for two reasons. First, kinetochore fibers in mammalian cells contain a high proportion (~50%) of microtubules that run continuously from

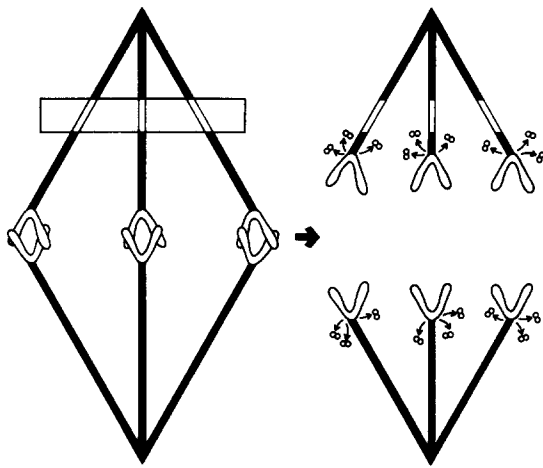


Figure 5. Schematic interpretation of the disassembly of kinetochore fiber microtubules during the movement of chromosomes to the pole in anaphase. Filled fibers represent microtubules with incorporated DTAF-tubulin. The rectangular box in the early anaphase spindle at left depicts the photobleaching beam. Bleached domains of microtubules are represented by open fibers. At right chromosomes have advanced along the kinetochore fiber microtubules toward the bleached zone which itself has remained stationary with respect to the pole. Kinetochore fibers shorten by releasing tubulin protomers at the kinetochores. For simplicity, the separation of the poles from each other, which also takes place in anaphase, is not depicted.

pole to chromosome (21). If kinetochore microtubules did, in fact, move poleward during anaphase, then unbleached domains of all the continuous microtubules of the kinetochore fiber as well as the noncontinuous ones between the bleached zone and the kinetochores should enter the bleached zone ahead of the chromosomes. However, no such invasion of the bleached zone by fluorescent kinetochore bundles was observed. Second, fluorescence of kinetochore fibers appears to predominate that due to astral and interzonal microtubules. Fluorescence micrographs such as those presented in Figs. 1–4 that are printed to most clearly resolve the pole-to-chromosome fibers showed the greatest detail in the bleached zone, while the astral and unbundled interzonal fibers were only weakly visualized. Presumably the predominance of the fluorescence of the pole-to-chromosome fibers is due to the close bundling of microtubules that comprise the kinetochore fibers.

In contrast, when photographic conditions are arranged to optimize visualization of astral and interzonal fibers, then the bleached zone is more difficult to detect (Fig. 6). The lack of clear definition of the photobleached zone in Fig. 6 can be partially attributed to incomplete bleaching and to light saturation of the photographic emulsion by the bright fluorescence of the kinetochore fiber region. However, it may also reflect the fact that rapid recovery of astral and interzonal microtubules tended to obscure the bleached zone. The enhanced persistence of the bleached zone within the kinetochore fibers supports the idea that the kinetochore microtubules underwent less reassembly than nonkinetochore microtubules over the course of the experiments.

The slow recovery we have observed in LLC-PK cell spindles in anaphase is in some contrast to previous photobleaching studies of spindles in sea urchin embryos (23) and in cultured cells of amphibian and mammalian origin (30). We

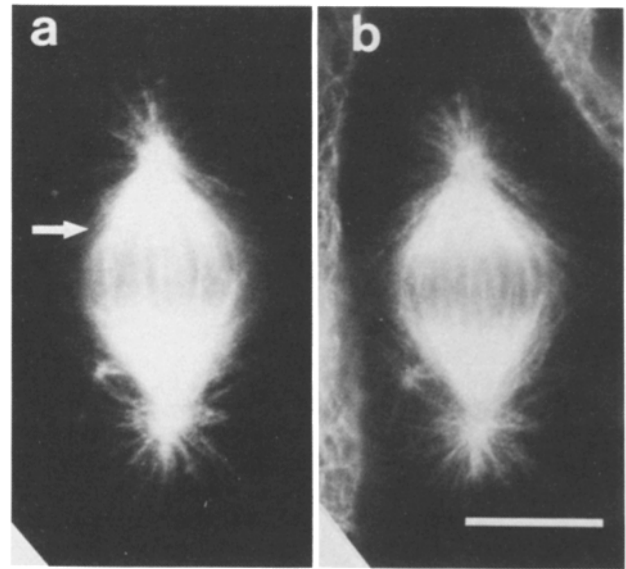


Figure 6. Anti-fluorescein (a) and anti-tubulin (b) images of a bleached cell photographed and printed to reveal astral and interzonal microtubules. This cell is the same cell depicted in Fig. 2. Under these photographic conditions the bleached zone (arrow) is only faintly visible. Note that astral and interzonal fibers are well preserved by our lysis and fixation procedure. Bar, 10 μ m.

observed clear persistence of bleached zones in anaphase kinetochore bundles as long as 150 s after photobleaching whereas Wadsworth and Salmon (30) found a recovery half-time of 37 s for mammalian cultured cells in metaphase. However, these same authors reported that $\sim 25\%$ of the fluorescence recovers at a much slower rate. They suggested that this slower component could represent the more stable kinetochore fibers. Rapid turnover of nonkinetochore microtubules might also explain the rapid fluorescence recovery of spindles in sea urchin embryos which have a relatively small proportion of kinetochore microtubules (23). Alternatively, the difference in the recovery rates in our study compared to previous work might reflect the different polymerization states of the kinetochore microtubules in metaphase versus anaphase spindles. Kinetochore microtubules in metaphase spindles are apparently in steady-state whereas those in anaphase spindles are in the process of breaking down. If recovery of fluorescence occurs by continuous breakdown and reformation of microtubules (12, 23, 25, 26), then slower recovery would be expected in anaphase kinetochore microtubules because their reformation is inhibited and their net reaction is breakdown. Recently, McIntosh et al. (12) also reported that in cultured cells, fluorescence recovery of microtubules is slower in anaphase than in metaphase.

Some direct evidence from our images suggests that the fluorescence redistribution of interzonal fibers is considerably more rapid than that of kinetochore bundles. Once the chromosomes have passed the region where a bleached zone was earlier placed, thus clearing away the kinetochore fibers, the bleached zone could no longer be detected in the interzonal fibers (Fig. 4 g). This observation suggests that interzonal microtubules assemble or move into the interzone during anaphase.

Our results have implications for the mechanism of chromosome translocation during anaphase A. Since we find that

chromosomes move poleward along stationary microtubules, the force driving this poleward motion is not likely to be transmitted through the microtubules to passive chromosomes from force generators located elsewhere. Rather, the results imply that force is applied directly at the kinetochore. These results are in general agreement with the conclusions of Mitchison et al. (15), who suggested the loss of subunits occurs primarily, though not exclusively, at the kinetochore. These authors also invoked a second, slower depolymerization at the pole to account for some of their observations. While we have seen no evidence for depolymerization at the pole during anaphase, a slow depolymerization (10–20% of the rate at the kinetochore) might be undetectable in the experiments presented here. However, such slow depolymerization could not contribute significantly to the shortening of microtubules during the rapid chromosome motion of anaphase.

How might the kinetochore be an active participant in generating force? One might consider two kinds of models, one in which translocation and disassembly are functionally linked and the other in which the two processes are independent. Multisite binding of kinetochores to microtubules would theoretically allow a chromosome to follow the end of a depolymerizing bundle of microtubules (5). Subunits would be lost from unbound sites while attachment would be maintained through bound sites. In this model, the force for chromosome-to-pole movement would arise from controlled disassembly of kinetochore microtubules beginning at the kinetochore. In this example, translocation and disassembly would be necessarily linked.

Alternatively, a force-generating molecule might actively drive chromosome movement. Such a molecule could be part of the kinetochore or be present along the length of the microtubule and function when in contact with the kinetochore. If the translocator molecule were an integral component of the kinetochore, then the kinetochore would act as a self-contained engine running on a microtubule track. If the translocator were distributed along the microtubule track, then the kinetochore would be moved by interaction with successive translocator molecules. In both of these models, disassembly of kinetochore microtubules would accompany but not necessarily drive chromosome motion.

Nevertheless, it is a fact that kinetochore microtubules shorten as chromosomes move poleward. The coordinate disassembly of kinetochore microtubules could be accounted for easily in terms of known factors governing microtubule stability. Microtubules are dynamically unstable (13) but can be stabilized by attachment of their plus ends to a kinetochore (14). Translocation of the kinetochore toward the minus end would uncap the plus ends, leaving behind unstabilized microtubule domains which would rapidly depolymerize to the point of attachment. The translocator models predict the existence of conditions under which translocation and disassembly may be decoupled.

Two force-generating molecules, dynein (31) and kinesin (28), are known to interact with microtubules and both have been found in mitotic spindles of echinoderm eggs (20, 25). However, dynein may be present only because it serves as a precursor for the cilia formed later in development. Kinesin is unlikely to be the anaphase translocator because when associated with microtubules it propels particles toward the plus ends (28) whereas in anaphase, the chromosomes move

toward the minus ends. The retrograde translocating activity observed in squid axoplasm (29) has the requisite polarity of action but has yet to be characterized, identified, and localized in spindles. Independent of the molecular identity of the translocator, our results suggest that the kinetochore is an active participant in generating the force propelling the chromosome to the pole.

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