Microinjection of Biotin-tubulin into Anaphase Cells Induces Transient Elongation of Kinetochore Microtubules and Reversal of Chromosome-to-Pole Motion

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Abstract. During prometaphase and metaphase of mitosis, tubulin subunit incorporation into kinetochore microtubules occurs proximal to the kinetochore, at the plus-ends of kinetochore microtubules. During anaphase, subunit loss from kinetochore fiber microtubules is also thought to occur mainly from microtubule plus-ends, proximal to the kinetochore. Thus, the kinetochore can mediate both subunit addition and loss while maintaining an attachment to kinetochore microtubules. To examine the relationship between chromosome motion and tubulin subunit assembly in anaphase, we have injected anaphase cells with biotin-labeled tubulin subunits. The pattern of biotin-tubulin incorporation was revealed using immunoelectron and confocal fluorescence microscopy of cells fixed after injection; chromosome motion was analyzed using video records of living injected cells. When anaphase cells are examined ∼30 s after injection with biotin-tubulin, bright "tufts" of fluorescence are detected proximal to the kinetochores. Electron microscopic immunocytochemistry further reveals that these tufts of biotin-tubulin–containing microtubules are continuous with unlabeled kinetochore fiber microtubules. Biotin-tubulin incorporation proximal to the kinetochore in anaphase cells is detected after injection of 3–30 mg/ml biotin-tubulin, but not in cells injected with 0.3 mg/ml biotin-tubulin. At intermediate concentrations of biotin-tubulin (3–5 mg/ml), incorporation at the kinetochore can be detected within 15 s after injection; by ∼1 min after injection discrete tufts of fluorescence are no longer detected, although some incorporation throughout the kinetochore fiber and into nonkinetochore microtubules is observed. At higher concentrations of injected biotin-tubulin (13 mg/ml), incorporation at the kinetochore is more extensive and occurs for longer periods of time than at intermediate concentrations. Incorporation of biotin-tubulin proximal to the kinetochore can be detected in cells injected during anaphase A, but not during anaphase B. Analysis of video records of microinjection experiments reveals that kinetochore proximal incorporation of biotin-tubulin is accompanied by a transient reversal of chromosome-to-pole motion. Chromosome motion is not altered after injection of 0.3 mg/ml biotin-tubulin or 5 mg/ml BSA. These results demonstrate that kinetochore microtubules in anaphase cells can elongate in response to the elevation of the tubulin concentration and that kinetochores retain the ability to mediate plus-end–dependent assembly of KMTs and plus-end–directed chromosome motion after anaphase onset.

D URING mitosis, the motion of chromosomes is mediated by kinetochore microtubules (KMTs),[1] which are attached at their plus, or fast growing, ends to the kinetochore (Euteneuer and McIntosh, 1981). During prometaphase, KMTs must increase in length during motion of the chromosome away from the spindle pole, and shorten during motion toward the spindle pole; during anaphase chromosomes move to the poles as KMTs shorten. In vivo experiments demonstrate that shifting the equilibrium between MT assembly and disassembly can induce changes in chromosome motion. For example, MT disassembly, induced by the treatment of metaphase cells with MT depo-

lymerizing agents such as cold or high hydrostatic pressure (Inoue, 1976; Salmon, 1976), has been shown to cause reversible motion of chromosomes toward an attached spindle pole. Additional experiments on anaphase cells reveal that anaphase chromosome motion can be reversed when MT assembly is induced by the addition of taxol (Bajer et al., 1982). Using these approaches, however, sites of MT assembly and disassembly could not be identified and correlated with chromosome motion.

To address this issue, several laboratories have performed experiments in which modified tubulin subunits have been injected into prometaphase and metaphase cells and subsequently localized in fixed cells. The results of these experiments demonstrate that an increase in length of the kineto-

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1. *Abbreviation used in this paper*: KMT, kinetochore microtubule.

chore fiber during motion of chromosomes away from the spindle pole occurs by addition of tubulin subunits at the plus-end of the kinetochore fiber microtubules, proximal to the kinetochore (Mitchison et al., 1986; Geuens et al., 1989; Wise et al., 1991). Controversy exists over the site of KMT subunit loss during prometaphase. Some experiments (Wise et al., 1991), indicate that loss of subunits can occur at the kinetochore during prometaphase. In contrast, photoactivation of modified tubulin has provided evidence for subunit loss at the pole or minus-ends of KMTs during metaphase in vivo (Mitchison, 1989). However, the rate of minus-end subunit loss calculated from these photoactivation experiments was significantly slower than the velocity of chromosome oscillations seen in the same cells. Thus, loss of tubulin subunits at the pole cannot fully account for normal oscillatory chromosome motion, and it is likely that both subunit addition and subunit loss can occur at the kinetochore in metaphase.

During anaphase, the motion of mitotic chromosomes is predominantly minus-end directed, although brief, asynchronous oscillations of chromosomes away from the pole during anaphase have been observed in diverse cells (Aist and Bayles, 1988; Bajer, 1982). Experiments conducted in vivo demonstrate that the majority of tubulin subunit loss during KMT shortening in anaphase is at the kinetochore (Gorbsky et al., 1988). Thus, the transition between metaphase and anaphase is accompanied by a change from bidirectional chromosome motion to predominantly minus-end-directed motion and from both addition and loss of tubulin subunits to predominantly loss of subunits at the kinetochore.

Despite its importance, the mechanism by which kinetochores remain attached to MTs during KMT length changes remains unknown. Recently, however, in vitro assays of kinetochore function have begun to examine the interaction between kinetochores and MTs under defined conditions. For example, MTs containing a stable, labeled minus-end have been shown to translocate away from an isolated kinetochore by insertion of tubulin subunits at the MT plus-ends, proximal to the kinetochore (Hyman and Mitchison, 1990; Mitchison and Kirschner, 1985a,b). Others (Koshland et al., 1988; Hyman and Mitchison, 1990; Coue et al., 1991), have further demonstrated that isolated kinetochores can remain attached to MTs that are induced to shorten by dilution of the tubulin subunit pool. Thus, kinetochores in vitro can provide useful models of in vivo kinetochore function.

In the experiments reported here, we have examined MT assembly and chromosome motion in anaphase cells following microinjection of biotin-labeled tubulin. We report that the increase in cytosolic tubulin concentration following injection can induce assembly of KMTs at their kinetochore proximal ends and transiently reverse anaphase chromosome-to-pole motion. Our experiments demonstrate that kinetochores in anaphase cells retain the ability to mediate chromosome motion away from the spindle pole. Furthermore, these results reveal for the first time that kinetochores do not prevent tubulin subunit addition at KMT plus-ends after anaphase onset. Our observations therefore reveal that KMT shortening in vivo is not accomplished simply by the inhibition of KMT polymerization as has been suggested (Hyman and Mitchison, 1990, 1991) and further suggest that tubulin

concentration may regulate kinetochore behavior during mitosis.

Materials and Methods

Preparation of Biotin Tubulin

Biotin-tubulin was prepared as described previously (Mitchison et al., 1986; Wadsworth et al., 1989). Small aliquots were stored at −70°C in injection buffer (20 mM sodium glutamate, 1 mM EGTA, and 0.5 mM MgSO4, pH 7.2). Before use, the protein solution was made 1 mM in GTP and centrifuged for 10 min at maximum speed in an Eppendorf microcentrifuge. The biotin-tubulin concentration was determined by a modification (Schacterle and Pollack, 1973) of the method of Lowry (Lowry et al., 1951).

Cell Culture and Microinjection

PtK1 cells were grown at 37°C in Ham's F-12 medium supplemented with 10% FBS, 10 mM Hepes, and antibiotics. Cells were plated on glass coverslips and allowed to grow for 36–48 h before use. Coverslips were then placed in a laboratory constructed microinjection chamber (Wadsworth et al., 1989), which was mounted on the stage of a Zeiss IM-35 microscope, and microinjected as described previously (Wadsworth et al., 1989). Temperature was maintained at 30°C using an Opti-Quip Red Beam Incubator calibrated with a YSI Telethermister. Cells analyzed for biotin-tubulin incorporation were photographed just after injection and again just after lysis using 35 mm film. For some experiments, in which cells were incubated longer than 30 s, the living cell was photographed after injection and just before lysis to record any change in chromosome position.

Immunofluorescence

After microinjection, cells were lysed for ∼50 s in lysis buffer containing 80 mM Pipes, 5 mM EGTA, 1 mM MgSO4 and 0.5% Triton X-100, pH 6.8 (Cassimeris et al., 1986). Cells were fixed for 25 min in 2% paraformaldehyde and 0.1% glutaraldehyde in PBS, pH 7.3, and rinsed in PBS containing 0.1% Tween and 0.02% azide (PBS-Tw-Az) and for 5 min in 1% sodium borohydride. Antibody incubations were performed in humid chambers at room temperature. Cells were incubated first with rabbit anti-biotin antibodies (Enzo Biochemicals, New York, NY) at a dilution of 1:50 in PBS-Tw-Az containing 1% BSA for 30 min, rinsed in PBS-Tw-Az, and incubated with fluorescein goat antirabbit and then fluorescein rabbit antigoat antibodies. Cells were then incubated in a mouse monoclonal antitubulin antibody for up to 3 h, followed by rhodamine goat antimouse antibodies. All fluorescent secondary antibodies were purchased from Cappel Research Reagents, Organon Teknika Corp (Durham, NC) and used at a final dilution of 1:50 in PBS-TW-Az containing 1% BSA, for 30 min at room temperature. Stained cells were mounted in 0.1% n-phenyldiamine in glycerol and sealed with nail polish.

Microscopy

For confocal microscopy, a laser scanning head (model MRC500; BioRad Laboratories; Cambridge, MA) mounted on a Nikon optiphot microscope equipped with a 60× 1.4 NA objective lens was used. The confocal microscope was operated with the pinhole set for a section thickness of ∼0.9 μm (Shelden and Wadsworth, 1990). Through-focus series of the rhodamine and fluorescein staining patterns were collected independently for each cell. 30–50 scans of the specimen were averaged to produce each image. Through-focus series of the rhodamine and fluorescein staining patterns were collected independently for each cell. Images were then digitally rescaled by making the lowest non-zero value black (a value of 0) and the highest pixel value white (a value of 255). This technique (contrast stretching) enhances the appearance of images by using the full range of grey-scale values to display each image. Multiple optical sections obtained from a through focus series were combined by displaying only the highest pixel value in the series at each screen location in the final image.

Pseudocolor Analysis of Images

A computer generated color look-up table was used to examine the changes in staining pattern at short and long times postinjection. The look-up table was generated on a Masscomp computer using locally written software and shows low pixel intensities in purple and blue, intermediate values in green

and yellow, and high pixel values in red. Images were contrast stretched before applying this look-up table. To further reveal changes in immunofluorescent labeling intensity, pixels in some images were vertically displaced to a new location based on intensity value (intensity mapping). Using this technique, regions of different intensities could be easily identified, as brighter pixels were displaced further vertically than less-bright pixels. Linear interpolation was used to fill any spacing which resulted from neighboring pixels being vertically displaced to nonneighboring locations in the new image. Finally, the look-up table described above was applied to these new images.

Electron Microscopy

The procedure used to prepare specimens for immunolocalization of biotin-labeled tubulin has been published previously (Wadsworth et al., 1989). Thin sectioned material was observed on a JEOL TEM100S operated at 60 kV.

Video Recording of Injection Experiments

Cells used for video analysis of chromosome motion during injection were held in a Rose chamber (Rose et al., 1958) modified as follows. Coverslips containing PtK$_1$ cells were placed between two sheets of parafilm in which holes corresponding to the opening of the Rose chamber had been cut. The chamber was filled with medium and placed on a Zeiss IM 35 inverted microscope. Temperature was maintained at 30°C using an Optiquip red beam incubator and a Nickolson Precision Instruments Air Stream Incubator to which flexible hoses were added to allow even heating of the injection chamber (McKenna and Wang, 1989). The red-beam incubator was shut off just before injection and the stage heated only from below using the air stream incubator. This technique reduced evaporation losses from the open injection chamber during the experiment. Temperature was measured using a YSI temperature monitor after injection. No cooling of the injection chamber was detected during these short (2–3 min) injection experiments. Microinjections were made while observing cells with a Zeiss 63× 1.4 NA oil immersion lens using phase contrast optics. The specimen was illuminated with a Hoffman Optics 0.5 NA (25 mm working distance) phase condenser and a 100 W mercury arc light source. Both heat and interference filters were placed between the illumination source and the cells. Although efforts were made to reduce evaporation losses during the course of observation, cells held in the injection chamber for long periods of time appeared to suffer light and/or heat stress during our initial experiments. Only cells which had been found, observed, and injected within a few minutes of being placed upon the microscope stage could be used for further analysis. To alleviate these problems, the chamber was subsequently modified by the addition of a cover fashioned from clear acetate. This cover was carefully removed before injection and replaced immediately after injection. This technique significantly extended the time over which cells could be observed on the microscope stage. Video sequences were recorded using a Dage MTI Newvicon video camera (model 67M). Images were background subtracted and contrast stretched using a Hughes Aircraft Co. image processor (model 794; Hughes Aircraft Co., Carlsbad, CA) before being stored on 3/4 inch video tape using a Sony video recorder (model VO-5800H; Sony Corporation, Park Ridge, NJ).

Data Analysis

Video sequences were processed using a three frame running average, contrast enhanced, and digitized in real time using a Masscomp computer. For cells in which a reversal of chromosome-to-pole motion could be detected, rates of chromosome motion were measured before reversal, during the period of reversal, and after recovery of chromosome-to-pole motion. For cells in which no reversal could be detected, rates of chromosome motion were measured before injection, for 30 s after injection, and for up to two min following this 30 s postinjection interval. Mouse button commands were used to freeze individual frames from the video sequence. The time at which each image was obtained was automatically recorded in a data file. A mouse driven cursor was placed on the screen and used to mark the kinetochore region of two sister chromatids. The distance in pixel units between these two points was recorded by the computer in the same data file as the time measurements. This procedure was repeated successively for the length of the video sequence. The video sequence was then restarted and the procedure repeated for the next pair of sister chromatids, and so on. Lengths were converted from pixel units to microns using pixel/micron ratios obtained by imaging a slide micrometer. The resulting data sets were

graphed with a Macintosh computer. Linear regression analysis of these data was used to determine rates of chromosome motion.

Small changes in the motion of individual chromosomes are underestimated using the technique described above, as chromosome motion is measured as a function of the distance between pairs of chromosomes. In addition, both sister chromatids rarely remained in the plane of focus during the entire observation period, making the accurate localization of more than one chromosome throughout the video sequence extremely difficult. To address these problems, the motion of individual chromosomes was quantified using a second method. For this analysis, images were displayed and recorded as described above. The first point recorded was a reference point chosen to lie both behind the chromosome being analyzed and on a line containing the spindle pole and the kinetochore region. Instead of measuring the distance between separating chromosomes, the location of an individual kinetochore region was then recorded in the data file. A computer program calculated the distance between the original reference point and the position of the kinetochore region. This approach enabled nearly twice as many measurements of chromosome location to be made as the first method, as only one point per image was marked. Furthermore, this method was much more sensitive to small changes in chromosome motion, and could more accurately measure rates of motion for individual chromosomes. Unfortunately, the reference point could not be changed during the course of a video sequence, so only cells in which the spindle did not rotate or change position could be analyzed using this method.

The mitotic stage of injected cells was determined as described previously (Shelden and Wadsworth, 1990). The number of kinetochore proximal tufts of incorporation was determined for cells incubated for 40 s or less postinjection by displaying each image of a cells' through focus series on a video monitor. Clear acetate sheets were placed over the video monitor, and the location of any tufts of incorporation were marked on the acetate sheet. The number of tufts/cell for anaphase A (mid and late) and anaphase B cells was counted, and the average number of tufts/cell for each cell stage was then compared using a two-tailed t test.

Results

Incorporation of Biotin-Tubulin Into Anaphase Cells

When anaphase PtK$_1$ cells are microinjected with biotin-tubulin (3.5–5.5 mg/ml in the injection pipette) and immunostained to localize biotin-tubulin and total tubulin, an unexpected biotin-tubulin staining pattern is observed (Fig. 1). When examined at ~30 s postinjection, bright segments of biotin-tubulin fluorescence are detected proximal to the kinetochore—presumably the site of subunit loss in anaphase cells (Gorbsky et al., 1988). Comparison of the biotin-tubulin and total tubulin staining patterns (Fig. 1, a and b, respectively) reveals that these "tufts" of antibiotin stained microtubules colocalize with the plus-ends of KMTs (arrows, Fig. 1 a). Antibiotin-stained microtubule segments are also detected in the spindle aster and interzone.

While the immunofluorescent staining pattern reveals that biotin-tubulin can incorporate at the kinetochore proximal end of kinetochore fibers in anaphase, it is not possible, given the resolution limit of the light microscope, to determine if labeled MT segments are continuous with preexisting KMTs, or instead represent new nucleation of MTs at unoccupied sites on the kinetochore plate. To resolve this issue, cells have been injected with 5.5 mg/ml biotin-tubulin and processed for electron microscopic immunocytochemistry. Examination of injected cells reveals that the biotin-tubulin-containing KMT segments are continuous with unlabeled KMT segments (Fig. 2). Thus, kinetochore proximal incorporation of biotin-tubulin does not occur by nucleation at unoccupied sites at the kinetochore but represents elongation at the plus-ends of existing KMTs.

We have used immunofluorescent staining of cells injected with biotin-tubulin to further characterize the conditions un-
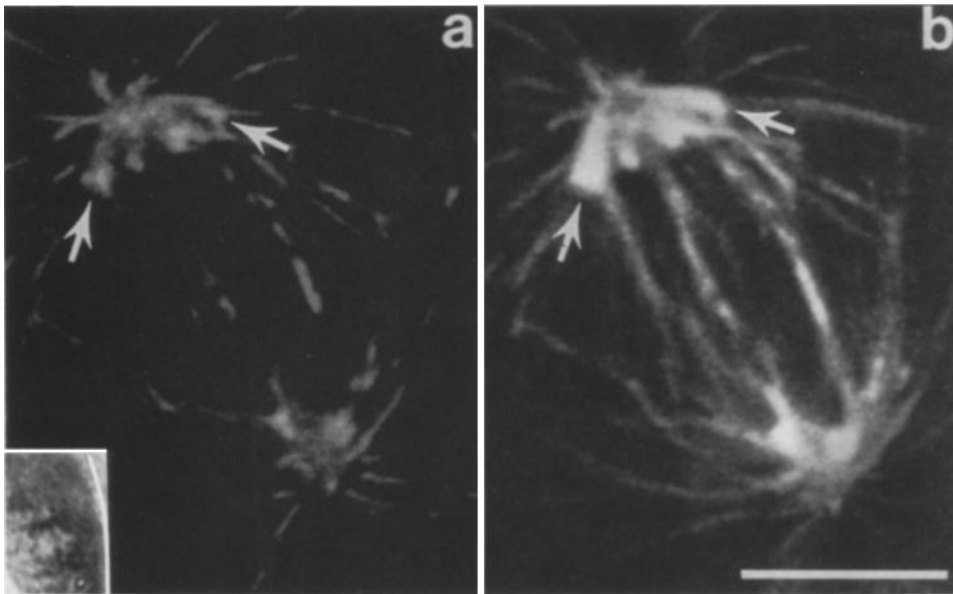
Figure 1. Biotin-tubulin incorporates at the plus-ends of KMTs after injection into anaphase cells. A PtK₁ cell injected with 5.5 mg/ml biotin-tubulin and incubated at 30°C for 22 s after injection is shown. The cell has been stained with (*a*) antibiotin; (*b*) antitubulin antibodies. Assembly of biotin-tubulin (*a, arrows*), is detected at the kinetochore proximal or plus-ends of kinetochore fiber microtubules (*b, arrows*). Phase-contrast image was recorded just after injection (*inset*). Bar, 10 μm.

der which biotin-tubulin incorporates into kinetochore fiber microtubules at their plus-ends during anaphase. First, the incorporation of biotin-tubulin proximal to the kinetochore in mid-anaphase cells has been examined as a function of the concentration of injected biotin-tubulin. When cells are injected with low concentrations of biotin-tubulin (0.3 mg/ml), little or no staining of KMTs is detected at 30 s postinjection (Fig. 3 *a*), although dim staining of non-KMTs is occasionally observed (not shown). Higher concentrations of injected biotin-tubulin (3–30 mg/ml) produce prominent kinetochore proximal "tufts" of biotin-tubulin incorporation after similar postinjection intervals (Fig. 3, *b* and *c*). The intensity of kinetochore proximal staining was somewhat greater in cells injected with biotin-tubulin at concentrations higher than
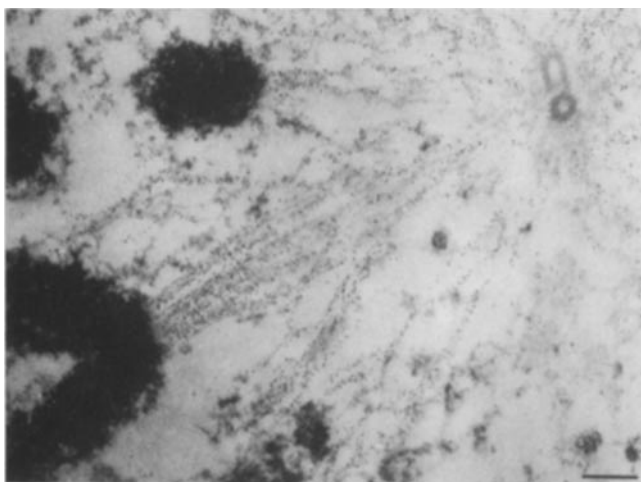

Figure 2. Antibiotin staining at the distal or plus-ends of kinetochore fiber microtubules is continuous with unlabeled kinetochore fiber microtubules. Electron microscopic immunocytochemistry of an anaphase cell injected with 5.5 mg/ml biotin-tubulin and incubated for 39 s after injection. The cell was stained with antibiotin antibodies and 5-nm gold–labeled secondary antibodies. Bar, 0.5 μm.

3 mg/ml when examined after similar postinjection intervals (Fig. 3 *c*, 30 mg/ml). In general, the number of kinetochore proximal tufts/cell and the overall pattern of incorporation were similar in cells injected with 3 or 30 mg/ml biotin-tubulin and examined 20–40 s postinjection (for example, compare Fig. 3, *b* and *c*).

The time course of biotin-tubulin incorporation proximal to the kinetochore has been examined in cells injected with 3–5.5 mg/ml of biotin-tubulin. Kinetochore-proximal tufts are detected by ~15 s postinjection (the minimum amount of time required to inject, photograph and lyse a cell) (Fig. 3 *d*), but are less pronounced than tufts in cells incubated for ~30 s (Fig. 3, *b* and *e*). However, when cells injected with 3–5.5 mg/ml biotin-tubulin are examined after 1 min of postinjection incubation, discrete kinetochore-proximal tufts of incorporation are no longer detected. Rather, kinetochore fibers appear uniformly labeled using light microscopy, and increased labeling of nonkinetochore MTs can be detected (Fig. 3 *f*, 65 s). It is important to contrast these results with those obtained from cells injected with higher concentrations of tubulin (see Fig. 7 *d*). Higher concentrations of injected biotin-tubulin result in incorporation at the kinetochore for extended periods of time. Pronounced kinetochore-proximal tufts of incorporation are still present 1 min after injection with 13 mg/ml biotin-tubulin, but are not detected at similar times in cells injected with 3 mg/ml biotin-tubulin (compare Fig. 7 *d*, with Fig. 3 *f*). Given the limitations of the light microscope, we did not determine if incorporation was also more rapid at higher concentrations of injected tubulin.

To further examine the pattern of incorporation of biotin-tubulin into KMTs at short and long times post-injection in cells injected with 3.0–5.5 mg/ml biotin-tubulin, the images shown in Fig. 3 (*e* and *f*) have been examined for pixel brightness using computer generated false color and pixel mapping (see Materials and Methods). After a 30-s postinjection incubation, antibiotin staining of the kinetochore fiber is highly nonuniform; a peak in labeling intensity is clearly detected at the plus-end of KMTs, proximal to the kinetochore (Fig. 4, *a* and *c*). At this time, relatively little
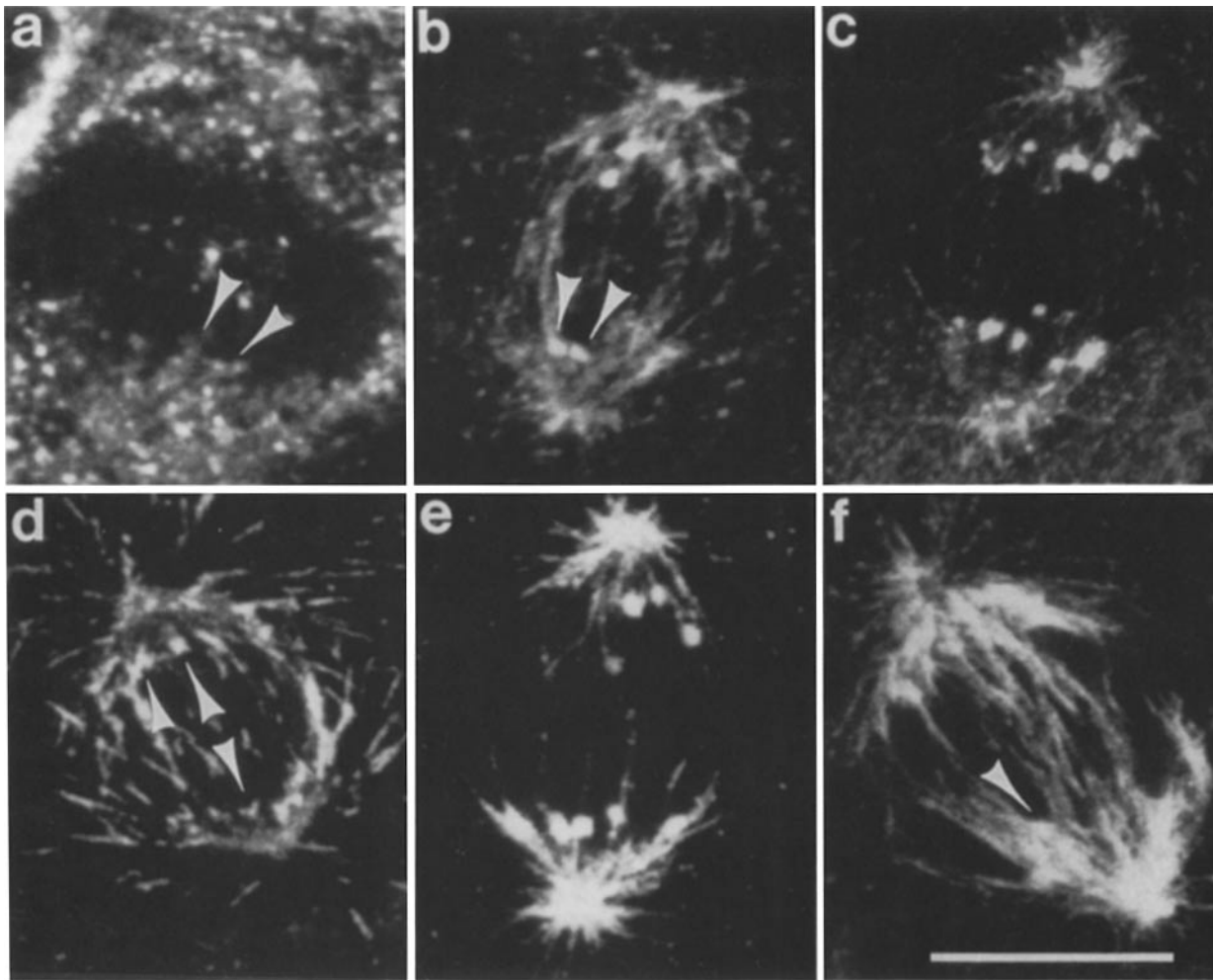
Figure 3. Incorporation of biotin-tubulin at KMT plus-ends in anaphase cells is dependent on the concentration of biotin-tubulin and the postinjection interval. Kinetochore fibers are indicated by arrowheads in *a*, *b*, *d*, and *f*. Antibiotin staining is shown in each panel. (*a*) 0.3 mg/ml biotin-tubulin, 28 s postinjection, two optical sections; (*b*) 3.0 mg/ml biotin-tubulin, 28 s postinjection, five optical sections; (*c*) 30.0 mg/ml biotin-tubulin, 26 s postinjection, eight optical sections. Plus-end–dependent incorporation of injected tubulin into KMTs is seen at high (*b* and *c*), but not low (*a*) concentrations of injected biotin-tubulin. (*d*) 5 mg/ml, 18 s postinjection, four optical sections; (*e*) 3.0 mg/ml injected tubulin, 27 s postinjection two optical sections; (*f*) 3 mg/ml, 65 s postinjection, three optical sections. Plus-end–dependent incorporation of biotin-tubulin is more pronounced at 27 s postinjection than at 18 s postinjection. At 67 s postinjection, incorporation is detected throughout the spindle. Bar, 10 μm.

incorporation of biotin-tubulin has occurred within the kinetochore fiber or in the spindle asters. When kinetochore fibers are examined following longer postinjection intervals, no peak of antibiotin labeling at the plus-ends of KMTs is detected (Fig. 4, *b* and *d*). Rather, label is more evenly distributed along the kinetochore fiber. Further, more biotin-tubulin is detected in the astral region than along the kinetochore fiber at these longer times postinjection. This increase in antibiotin staining of the astral region presumably reflects the rapid turnover of non-KMTs (Salmon et al., 1984; Mitchison et al., 1986). Thus, a qualitative difference in the antibiotin staining pattern is clearly seen when cells that have been injected with 3–5.5 mg/ml biotin-tubulin are examined after short and long postinjection intervals. It should be noted, however, that these images have been contrast-stretched before this analysis, and therefore no quantitative comparison between cells should be made.

Changes in the pattern of biotin-tubulin incorporation at short and long times postinjection can also be detected at the resolution of the electron microscope. At short time points postinjection, discrete lengths of biotin-labeled microtubules are detected proximal to the kinetochore using immunoelectron microscopy (Fig. 2). At longer time points after injection with a similar concentration of biotin-tubulin, no clearly defined segments of biotin-tubulin incorporation can be seen using immunoelectron microscopy (see Wadsworth et al., 1989). However, incorporation of biotin-tubulin is detected in a relatively small fraction of KMTs in cells examined at longer times postinjection. This incorporation is detected along the length of the kinetochore fiber, rather than specifically at the kinetochore-proximal ends of KMTs. In addition, both labeled and unlabeled MTs can be seen to contact the kinetochore. Thus, a distinct change in the pattern of incorporation can be seen between cells incubated for short and long times postinjection using either immunofluorescent or electron microscopic observation.

While cells injected in anaphase A incorporate biotin-tubulin into kinetochore-proximal tufts, few or no tufts of in-
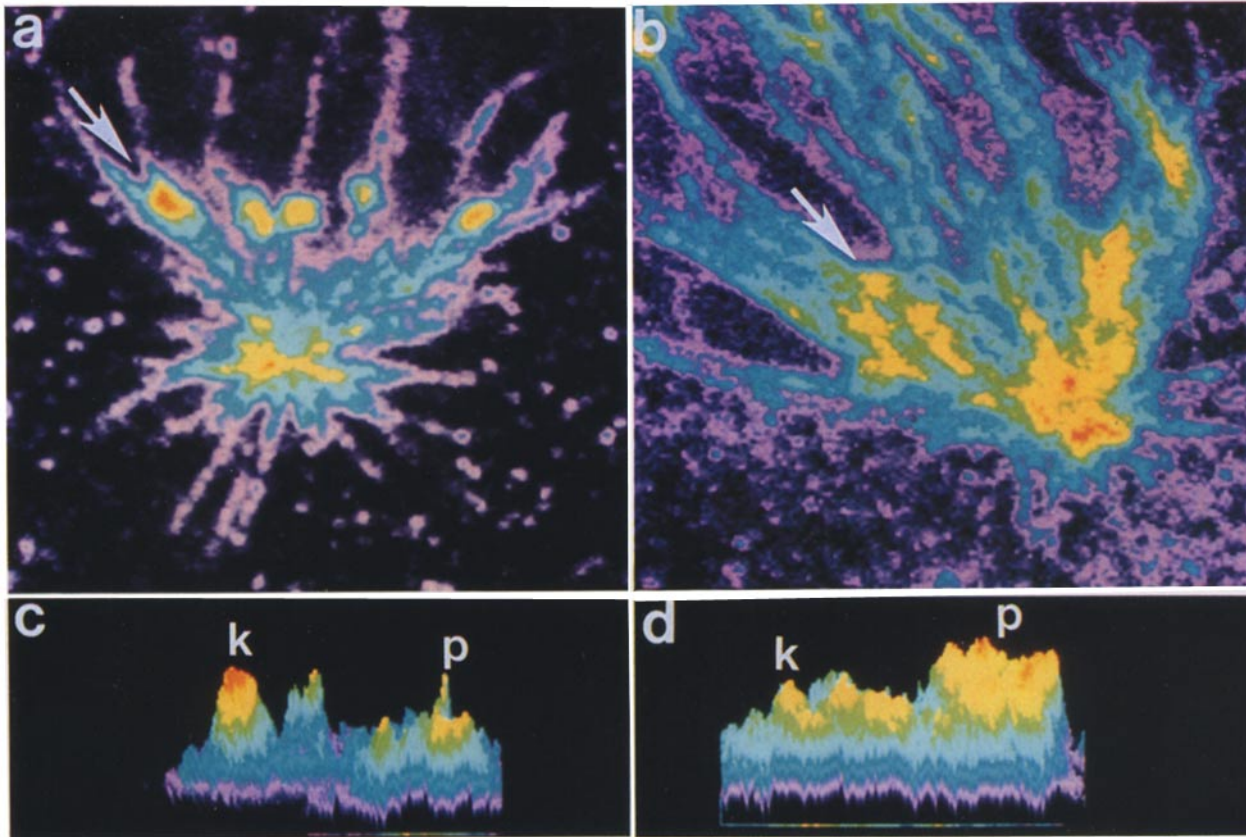
Figure 4. Comparison of antibiotin tubulin staining patterns for cells incubated for short and long times postinjection. Pseudo-color analysis of the lower half spindle of cells shown in Fig. 3. (*e* and *f*). (*a*) When examined after 27 s postinjection incubation, labeling of kinetochore fibers is highly nonuniform, and KMT plus-ends are as bright or brighter than the region of the spindle pole. (*b*) At 65 s postinjection, labeling along KMTs is more uniform and less bright than labeling in the region of the spindle pole. (*c* and *d*) Pixel mapping of individual kinetochore fibers (*arrows*, *a* and *b*). The high intensity of staining proximal to the kinetochore (*k*) seen at short time points postinjection is not detected at longer time-points; more staining is detected near the pole (*p*) at long time points postinjection than at shorter time-points.

corporation can be detected when cells are injected after the completion of chromosome-to-pole motion and examined after similar post-injection intervals (compare Fig. 5 *a*, mid-anaphase with Fig. 5 *b*, late anaphase). To examine further these cell cycle–dependent changes in KMT plus-end incorporation of biotin-tubulin, we have quantified the number of

tufts detected in cells injected with 3–5.5 mg/ml biotin-tubulin from complete through-focus series obtained using confocal microscopy (see Materials and Methods). Our data demonstrate that the number of tufts/cell decreases as cells progress through anaphase (Table I). Cells injected during early-mid anaphase A have significantly more tufts than cells
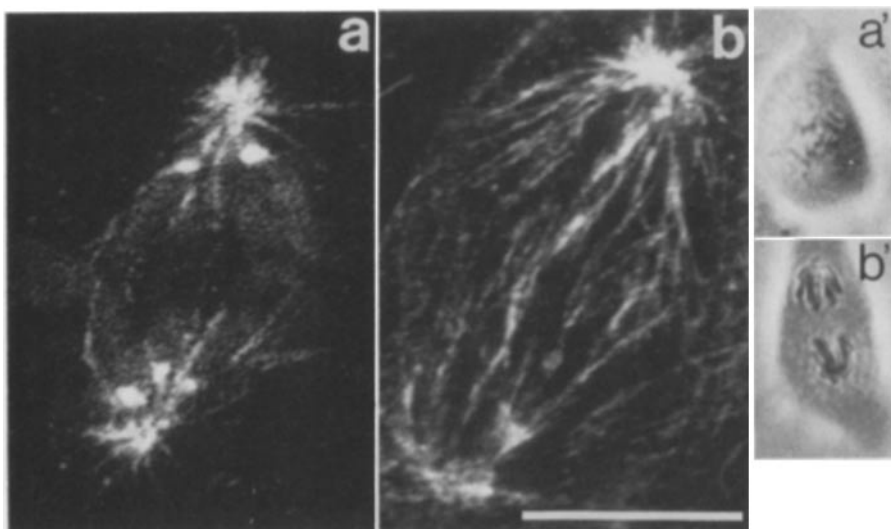

Figure 5. Incorporation of biotin-tubulin proximal to the kinetochore in anaphase cells occurs during anaphase A, but not anaphase B. (*a*) Antibiotin immuno-fluorescence of a cell injected in mid-anaphase with 3 mg/ml biotin-tubulin and incubated for 24 s postinjection. Numerous bright tufts of fluorescence are seen proximal to the kinetochores. (*b*) A cell injected in late anaphase with 3 mg/ml biotin-tubulin and examined 28 s postinjection. Astral and interzonal MTs are labeled, but no tufts of biotin-tubulin fluorescence are detected. Phase-contrast micrographs (*a′* and *b′*) of the cells shown in *a* and *b*. The cells are shown before (*a′*), and after (*b′*) lysis. Bar, 10 μm.

Table I. The Number of Kinetochore Proximal Tufts/Cell

Mid-Anaphase A	18.3 ± 6.0 ($n = 11$)
Late Anaphase A	11.7 ± 3.4 ($n = 10$)
Anaphase B	0.4 ± 1.0 ($n = 9$)

The number of kinetochore proximal tufts were counted from confocal sections of cells stained with antibodies to biotin-tubulin. Cells were injected with 3.0 mg/ml or higher biotin-tubulin and incubated 16 to 41 s postinjection.

injected at the end of anaphase A ($P < 0.05$) or during anaphase B ($P < 0.01$) (Table I; Shelden and Wadsworth, 1990). Unfortunately, we cannot accurately determine what percent of the total kinetochore fibers are labeled in these cells for two reasons. First, the cells used for this analysis were not all double labeled for antitubulin. Second, not every kinetochore fiber can be unambiguously detected in these cells. It is clear, however, that the number of labeled kinetochore fibers decreases as anaphase progresses. Together, these data strongly suggest that progression through anaphase influences the incorporation of biotin-tubulin proximal to the kinetochore.

Analysis of Chromosome Motion in Injected Cells

To determine if incorporation of microinjected biotin-tubulin during anaphase is accompanied by changes in chromosome motion, video records of injection experiments have been analyzed. In the first series of experiments, cells were injected with either 5.5 or 0.5 mg/ml of biotin-tubulin or 5 mg/ml BSA. The rate of chromosome motion was measured before injection, for ~30 s immediately postinjection, and following the 30-s postinjection interval (recovery). Measurements of sister chromatid separation in cells injected with 5.5 mg/ml biotin-tubulin (see Materials and Methods) revealed that chromosomes paused or transiently reversed direction in 9 of 10 cells examined (data not shown). To more critically examine these changes in chromosome motion, the motion of individual chromosomes was also examined in these cells (see Materials and Methods). Because these measurements were made relative to a fixed location on the video monitor, only chromosomes in spindles which were relatively stationary during the experiment could be accurately measured. For nine chromosomes in five cells analyzed using this method, the measured rates of chromosome motion before injection, during motion away from the pole, and after the recovery of chromosome-to-pole motion were 1.0

± 0.5, −0.5 ± 0.4, and 0.9 ± 0.2 μm/min (Table II, example, see Fig. 6 a). The minus sign indicates motion away from the pole. These measurements reveal that chromosomes can reverse their direction of motion in cells injected with 5.5 mg/ml biotin-tubulin. However, since poleward motion resumes at preinjection rates after the reversal of chromosome-to-pole motion, this reversal does not result from a permanent alteration of the kinetochore.

While not every chromosome in cells injected with 5.5 mg/ml biotin-tubulin could be observed to reverse its direction of motion after injection, several factors make small changes in chromosome motion difficult to detect. First, small displacements of the kinetochore region cannot be detected unless the microscope is focused directly on the kinetochore of a chromosome under observation. Second, the three-dimensional nature of the spindle also makes accurate measurements of chromosome motion difficult. Motion of a chromosome which is not in the plane of focus will be only partially detected, and thus the measured rate of chromosome motion will be an underestimate of the actual rate of motion.

Chromosome motion in control cells injected with either low concentrations (0.5 mg/ml) of biotin-tubulin, or 5.0 mg/ml BSA have also been analyzed using these methods of quantification. Although small changes in the average rates of chromosome motion can be detected following injection in these control experiments, these changes are much more subtle than those seen after injection of higher tubulin concentrations (data not shown). When individual chromosomes are analyzed (Fig. 6 b and Table II), no evidence of a reversal of chromosome-to-pole motion could be detected postinjection in these cells.

In the experiments described above, cells were allowed to recover normal chromosome-to-pole motion following injection, so it was not possible to determine if incorporation of biotin-tubulin at KMT plus-ends had occurred during the reversal of chromosome-to-pole motion. Furthermore, the reversals of chromosome-to-pole motion detected following injection with 5.5 mg/ml biotin-tubulin are small and resemble the normal oscillations of anaphase chromosomes seen in some cells (see Discussion). To more clearly demonstrate that injection induces the reversal of chromosome motion, and that this reversal is accompanied by subunit addition at the plus-ends of attached KMTs, a second series of experiments was performed. For these experiments, cells were injected with 13 mg/ml biotin-tubulin to increase our ability to detect reversal of chromosome-to-pole motion. Video records of injected cells were made before injection and up to 90 s postinjection, after which the cells were lysed, fixed, and stained to reveal the sites of biotin-tubulin incorporation. At this higher concentration of injected biotin-tubulin, the motion of chromosomes away from the pole occurs over a longer period of time than at lower concentrations and can be more clearly observed postinjection (Fig. 7; see arrows, b and c and asterisks, f and g). Examination of the biotin-tubulin staining pattern (Fig. 7, d and h) further reveals that incorporation of biotin-tubulin into KMTs is more extensive and occurs over a longer period of time than in cells injected with lower concentrations (compare Fig. 7 d, 13 mg/ml and 63 s postinjection with Fig. 4, b and d, 5 mg/ml and 65 s postinjection). In some cases, tufts of incorporation can be seen at the plus-ends of kinetochore MTs attached to chro-
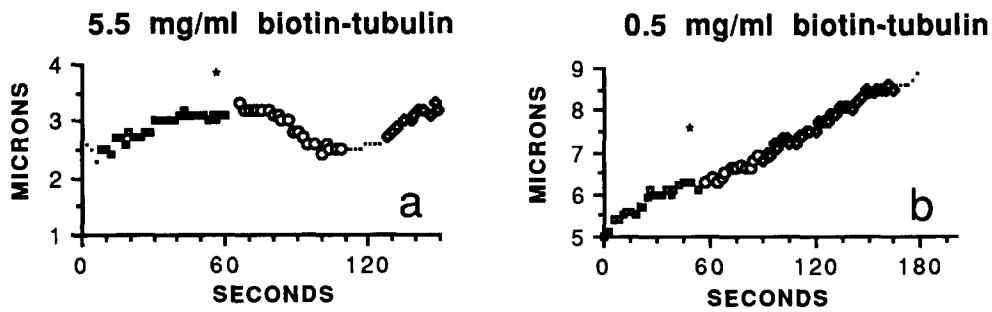
Table II. The Rate of Motion of Individual Chromosomes in μm/min

Concentration	Before injection	30 s postinjection	Recovery
mg/ml			
5.5 Tb. (5*)	1.0 ± 0.5	−0.5 ± 0.4	0.9 ± 0.2
	$n = 9‡$	$n = 9$	$n = 9$
0.5 Tb. (4)	1.4 ± 0.2	0.9 ± 0.2	1.2 ± 0.2
	$n = 8$	$n = 8$	$n = 8$
5.0 BSA (2)	1.9 ± 0.1	1.1 ± 0.4	1.3 ± 0.2
	$n = 2$	$n = 4$	$n = 4$

The rate of individual chromosome motion in injected cells measured from a stationary reference point.
* The number of cells analyzed for each experiment.
‡ The number of chromosomes analyzed for each interval.

5.5 mg/ml biotin-tubulin

0.5 mg/ml biotin-tubulin

Figure 6. Analysis of chromosome motion in anaphase cells following injection of 5.5 mg/ml biotin-tubulin (*a*), or 0.3 mg/ml biotin-tubulin (*b*). (*a*) The motion of an individual chromosome in a cell injected with 5.5 mg/ml biotin-tubulin. Transient reversal of chromosome-to-pole motion is clearly seen postinjection. (*b*) No reversal of chromosome-to-pole motion can be detected in a cell injected with 0.5 mg/ml biotin-tubulin. Squares represent points recorded before injection, circles represent points recorded for 30 s postinjection interval or until recovery of chromosome-to-pole motion, and diamonds represent points recorded as chromosome-to-pole motion resumed postinjection. Asterisk marks the time of injection.

mosomes which were clearly observed to reverse their direction of motion during the injection experiment (Fig. 7 *c*, *arrows*).

These observations strongly suggest that plus-end–dependent assembly of KMTs during anaphase and reversal of anaphase chromosome-to-pole motion are induced by the injection of biotin-tubulin through the action of the same mechanism. First, both can be induced by the injection of high, but not low concentrations of biotin-tubulin. Second, the time course of KMT plus-end assembly corresponds closely with the time course of the reversal of anaphase chromosome-to-pole motion. Motion of chromosomes away from the pole is initiated immediately after injection of biotin-tubulin, and tufts of incorporation are detected at the
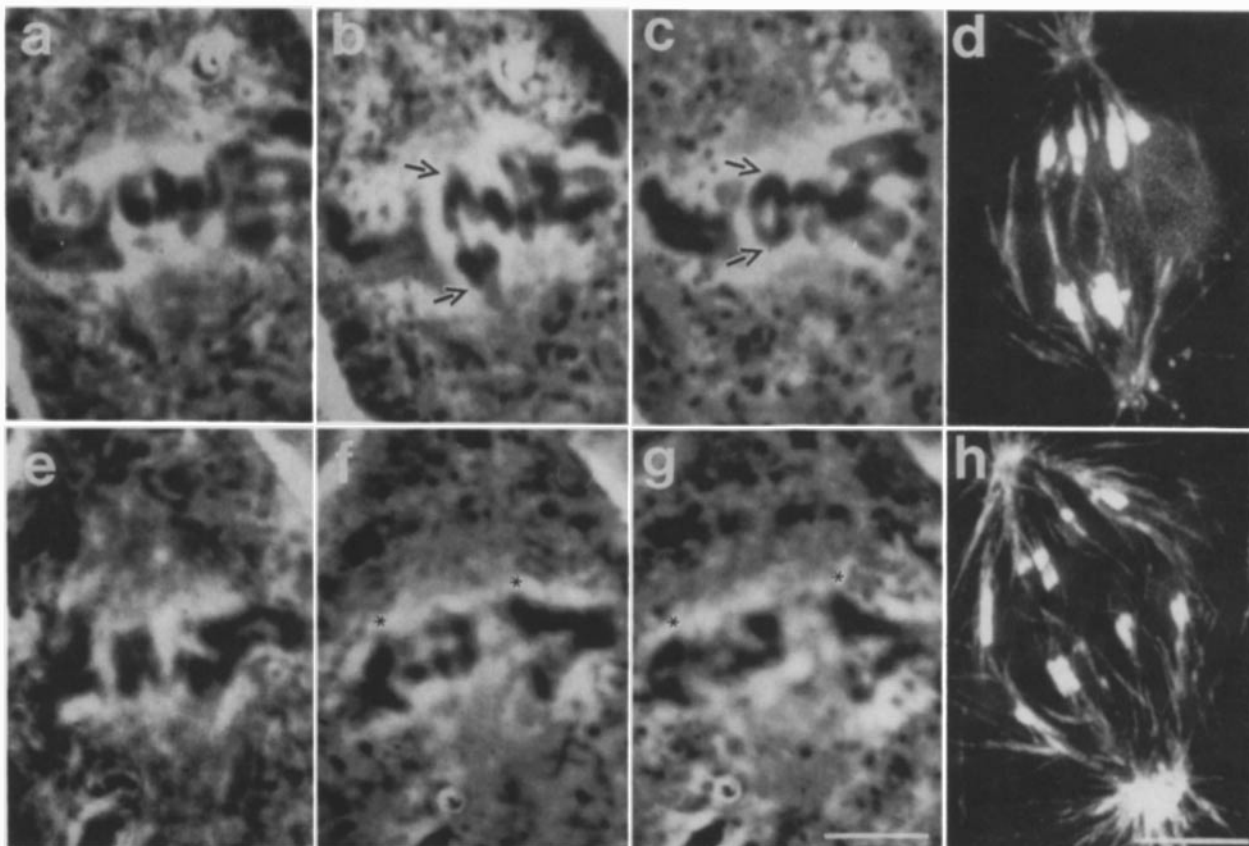

Figure 7. Incorporation of biotin-labeled tubulin accompanies the reversal of chromosome-to-pole motion in injected anaphase cells. A cell is shown ~1 min before injection (*a*), immediately after injection (*b*), and 48 s after injection (*c*). A central pair of chromosomes which reverse their direction of motion is clearly detected (*arrows*, Fig. 7 *b* and *c*). At 63 s postinjection the cell was lysed, fixed, and processed for immunofluorescence observation. Antibiotin immunofluorescent staining reveals that biotin-tubulin has been incorporated into KMTs attached to these and other chromosomes (*d*). A second cell (*e–h*) is shown before injection (*e*), immediately after injection (*f*), and 55 s after injection (*g*), and was lysed and fixed ~65 s postinjection. A decrease in the distance between opposing chromosomes can be detected postinjection (compare *f* and *g*). Note that in this more rounded cell, the chromosomes also move laterally during reversal, increasing the lateral separation of chromosomes in the same half spindle (*asterisks, f* and *g*). Antibiotin-tubulin immunofluorescence staining reveals that biotin-tubulin has been incorporated proximal to most kinetochores in this cell (Fig. 7 *h*). Bars, 5 μm.
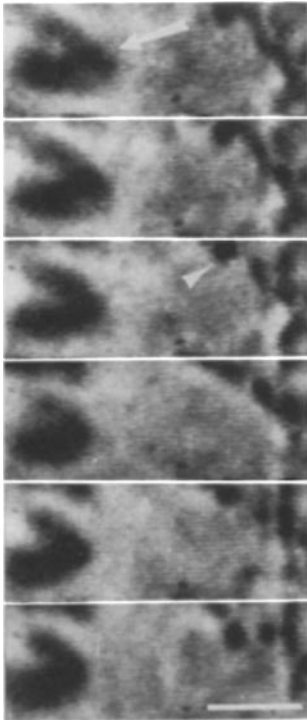
1416

Figure 8. Changes in chromosome shape and phase density accompany the reversal of anaphase chromosome-to-pole motion. Phase-contrast images of a chromosome (*arrow*) taken from video records of an injection experiment; photographs were taken at ~10-s intervals. The cell was injected just before the first image. The chromosome shown was located in the half-spindle opposite the site of injection, thus, the chromosome moved against the flow from the injection pipette. The kinetochore region of the chromosome becomes more flattened as the reversal of chromosome-to-pole motion occurs. Inspection of the trailing chromosome arms reveals that motion of the kinetochore precedes, and is more extensive than the motion of the arms. A stationary phase-dense organelle is indicated (*arrowhead*). Bar, 2 μm.

earliest time points that can be assayed. In cells injected with 5.5 mg/ml tubulin, the duration of motion away from the pole occurs over 40 ± 20 s, and kinetochore-proximal tufts are most pronounced in cells examined at the same postinjection interval. Recovery of anaphase chromosome-to-pole motion occurs on average 40 s postinjection in cells injected with 5.5 mg/ml biotin-tubulin. Similarly, kinetochore-proximal tufts of incorporation cannot be detected in cells injected with this concentration of biotin-tubulin and examined for biotin-tubulin incorporation at time points later than 60 s postinjection. Furthermore, the length of kinetochore-proximal biotin-tubulin incorporation detected using EM techniques is similar to the distance of anaphase chromosome-to-pole motion reversal documented using video microscopy. Finally, both incorporation of biotin-tubulin at KMT plus-ends and the reversal of chromosome-to-pole motion are more extensive and occur over a longer period of time in cells injected with 13 mg/ml biotin-tubulin than in cells injected with 5.5 mg/ml biotin-tubulin. Together, these results strongly indicate that the assembly of biotin-tubulin at the plus-ends of KMTs is required for the reversal of chromosome-to-pole motion seen in these experiments.

Careful analysis of video records of chromosome reversal in cells injected with 5.5 mg/ml biotin-tubulin indicates that the reversal of motion seen in these experiments is produced by a force acting directly at the kinetochore. First, stationary phase dense objects can be seen in cells near chromosomes undergoing motion away from the pole (Fig. 8, *arrowhead*). Thus, motion away from the pole is not caused by nonspecific relaxation of the cytoplasmic matrix. Second, motion of chromosomes away from the pole can be detected in half spindles both proximal and distal to the site of injection. Therefore, the reversal of chromosome motion is not caused by the pressure of injection and can in fact occur against any force generated by the injection. Third, the reversal of chro-

mosome motion is of much longer duration than the displacement of the cytoplasm caused by injection. Chromosomes, and other phase-dense objects displaced by the force of injection, returned to their original location within a few seconds postinjection. In contrast, chromosome motion away from the pole occurred over a 40 ± 20-s interval after injection of 5.5 mg/ml biotin-tubulin. Finally, examination of chromosome morphology during the reversal of chromosome-to-pole motion indicates that the force involved is acting directly upon the kinetochore. Chromosomes undergoing a reversal of chromosome-to-pole motion could be seen to change shape as they moved, becoming more flattened at the site of the kinetochore (Fig. 8). In addition, motion of the chromosome arms is delayed with respect to the motion of the kinetochore region in some instances (Fig. 8).

Discussion

In the experiments reported here, we have examined the relationship between KMT dynamics and chromosome motion in living anaphase PtK₁ cells following injection of biotin-labeled tubulin. Our results demonstrate that tubulin subunit addition can occur at the plus-ends of KMTs in anaphase cells, as has been previously demonstrated in prometaphase and metaphase cells (Mitchison et al., 1986; Wise et al., 1991). Analysis of video records of injection experiments further demonstrates that chromosomes in anaphase can be induced to transiently reverse their direction of motion in response to an elevation of the intracellular tubulin concentration. From these observations we conclude that the transition from metaphase to anaphase does not irreversibly alter the interaction between the kinetochore and KMTs with respect to its ability to allow plus-end dependent KMT polymerization.

The results of our experiments demonstrate that reversal of anaphase A chromosome motion and plus-end–dependent assembly of KMTs can be induced by an elevation in the concentration of intracellular tubulin. Injection of low concentrations of biotin-tubulin (0.5 mg/ml) did not alter chromosome motion, while injection of concentrations greater than or equal to 5.5 mg/ml biotin-tubulin reproducibly induced the transient reversal of chromosome-to-pole motion. Similarly, high, but not low, concentrations of injected tubulin induced assembly at the KMT plus-ends at the majority of kinetochores in mid-anaphase cells. Because a limited number of injection concentrations were tested, the minimum concentration which must be reached to induce KMT plus-end assembly and reversal of motion in anaphase has not been determined. However, concentrations of 3.0 mg/ml biotin-tubulin or greater reproducibly induced the formation of kinetochore proximal tufts of incorporation. If we estimate that 10% of the cell volume is injected (Saxton et al., 1984; Schultze and Kirschner, 1986; Mitchison et al., 1986; Guens et al., 1989) and that the cellular concentration of tubulin is ~2 mg/ml (Pfeffer et al., 1976; Hiller and Weber, 1978) of which roughly 50% is polymerized (Hiller and Weber, 1978), then injection of a solution of 3 mg/ml tubulin will increase the concentration of free subunits by ~33%. While these values are only estimates, they suggest that the kinetochore is sensitive to increases of tubulin in the physiological range. Finally, we note that during anaphase B, plus-

end assembly of KMTs is not detected in cells injected with biotin-tubulin, although incorporation is detected in interzonal MTs during this same period (Shelden and Wadsworth, 1990). Thus, KMT plus-ends, in cells which have completed anaphase A, apparently do not respond to the elevation in the level of cytosolic tubulin which results from the injection of biotin-tubulin. This observation further indicates that KMT plus-end assembly is not induced indiscriminately in these injection experiments, and, together with the demonstration that KMTs turnover occurs in early but not late anaphase (Wadsworth et al., 1989), suggests that a fundamental change in the attachment of the kinetochore to the KMTs occurs at the completion of anaphase A, the nature of which remains unknown.

Although chromosome motion during anaphase is predominantly directed toward the minus ends of the MTs, at the spindle poles, asynchronous oscillations, both toward and away from the poles, have been detected in diverse cells (Alexander and Rieder, 1991; Bajer, 1982; Rieder et al., 1986; Aist and Bayles, 1988). Natural oscillations occur with an average velocity which is similar to the values measured here for motion away from the pole induced by injection of 5.5 mg/ml biotin-tubulin (1.0 vs. 0.5 ± 0.4 μm/min) (Bajer, 1982). Furthermore, the duration of natural oscillations measured as chromosomes moved away from and then toward the pole (1.7 min; Bajer, 1982) is roughly twice the duration of motion away from the pole induced by injection of 5.5 mg/ml biotin-tubulin (0.6 ± 0.3 min). During oscillations away from the pole in anaphase, the kinetochore fiber elongates; our results clearly demonstrate that in cells injected with biotin-tubulin elongation of kinetochore microtubules occurs by subunit incorporation proximal to the kinetochore. The similarities between natural oscillations and the reversal of chromosome-to-pole motion induced by injection of biotin-tubulin suggest that incorporation also occurs at KMT plus-ends during natural oscillations. However, it is unlikely that the incorporation of injected tubulin into KMTs seen here occurs as a result of natural chromosome oscillations. In the experiments reported here all, or nearly all, of the kinetochore fibers in mid-anaphase cells incorporate injected biotin-tubulin proximal to the kinetochore. However, natural anaphase oscillations are highly asynchronous, and not all chromosomes reverse simultaneously. In addition, biotin-tubulin incorporation into KMTs and the reversal of chromosome motion are concentration dependent; incorporation and reversal are more extensive and occur over a longer period of time at 13 mg/ml injected biotin-tubulin than at 3-5 mg/ml injected tubulin. These observations demonstrate that the incorporation of biotin-tubulin into the kinetochore fiber, and the reversal of chromosome motion, are induced by the injection of biotin-tubulin.

Our observations of the reversal of chromosome motion indicate that force is applied at or near the kinetochore rather than along the chromosome arms. For example, compression of the kinetochore region can be detected during reversal (Fig. 8). In addition, motion of the trailing chromosome arms is delayed and less extensive than the motion of the kinetochore region. Chromosomes have been shown to stretch during the application of pulling forces at the kinetochore (Alexander and Rieder, 1991). In the experiments reported here, plus-end directed forces generated during KMT polymerization may allow recoil of the kinetochore

region before motion of the chromosome arms can be detected. Some observations of natural oscillations also indicate that movement in the plus-end direction is due to a pushing force at the kinetochore (Bajer, 1981), but in other cases oscillations are not accompanied by a detectable deformation of the kinetochore region (Bajer, 1982; Rieder et al., 1986; Rieder, 1991). These latter results, and the exclusion from the aster of chromosome fragments which lack a kinetochore, support the view that motion away from the pole results from the pushing of astral MTs along the length of the chromosome (Rieder et al., 1986; Rieder, 1991). In addition, experiments in which anaphase chromosome motion is reversed by taxol also demonstrate that chromosome arms can be pushed back toward the spindle equator (even to the point of breaking) while the kinetochore region remains stretched poleward (Bajer, 1982). Thus, our results can be distinguished from reversal induced by taxol, and from some instances of natural oscillations, by the differences in chromosome morphology observed during the reversal of chromosome-to-pole motion.

If MT polymerization at the kinetochore produces the force responsible for the plus-end-directed chromosome motion seen in these experiments, then the force generated by polymerization must either overcome or transiently turnoff the minus-end-directed force which produces normal chromosome-to-pole motion (Nicklas, 1983). Our results are most simply explained if the force for chromosome-to-pole motion is generated by KMT disassembly alone, as originally proposed by Inoue (Inoue and Sato, 1967; Coue et al., 1991). If this is the case, then a transition from disassembly to assembly of subunits at KMT plus-ends could itself prevent production of minus-end-directed forces. Such a transition could be induced by an elevation in the tubulin subunit concentration. The production of force by MT polymerization is consistent with both theoretical (Hill, 1981) and experimental evidence that MT polymerization can generate pushing forces (Bajer, 1982; Miyamoto and Hotani, 1988).

An alternative explanation for the reversal of motion observed in our experiments is that elongation of the anaphase kinetochore fiber activates plus-end-directed MT motors located at the kinetochore. For example, a model of kinetochore organization in which plus-end-directed motors are internal to minus-end-directed motors has recently been proposed (Huitorel and Kirschner, 1988). In our experiments, the elongation of KMT plus-ends may be sufficient to engage such internal plus-end-directed motors. In vitro experiments further reveal that kinetochores contain both plus-end- and minus-end-directed motor activity and that phosphorylation may regulate the direction and rate of motion (Hyman and Mitchison, 1991). However, movement of MTs relative to the kinetochore in these in vitro experiments is due to lateral interactions between the MTs and the kinetochore. The contribution of plus-end-directed motors to the motion of a chromosome which is attached end-on to a bundle of KMTs, as in normal anaphase, remains unknown.

Finally, the results presented here clearly demonstrate that important cellular events are perturbed by the injection of biotin-tubulin. Alterations in chromosome motion were reproducibly induced following injection of 3.0 mg/ml biotin tubulin. This concentration is within the range (0.15-30 mg/ml) of concentrations which have been utilized in a variety of experiments to examine MT dynamics (Saxton et al.,

1984; Mitchison et al., 1986; Schultze and Kirschner, 1986; Gorbsky et al., 1988; Geuens et al., 1989; Wadsworth et al., 1989; Shelden and Wadsworth, 1990; Wise et al., 1991). While it is beyond the scope of this paper to evaluate these previous reports, it is noteworthy that cell cycle–dependent changes in MT dynamic behavior have been observed using these injection methods (Saxton et al., 1984; Wadsworth et al., 1989; Shelden and Wadsworth, 1990; this report), suggesting that some of the mechanisms which regulate dynamics may function even when the level of tubulin is elevated. Other aspects of MT dynamics, such as the rate of MT elongation, however, may be more sensitive to perturbation (see Schultze and Kirschner, 1986). Finally, we demonstrate that the perturbation resulting from injection is transient; chromosome-to-pole motion resumes shortly after injection of 5.5 mg/ml biotin-tubulin, and a discrete tuft of biotin-tubulin proximal to the kinetochore is no longer detected. Together these observations indicate that measurement of MT dynamics in cells injected with low concentrations of tubulin analogues or after incubation periods significantly longer than 40 s, should accurately reflect endogenous MT activity (see Guens et al., 1989; Wadsworth et al., 1989), but that injection of high concentrations of tubulin and/or examination at short times postinjection should be used with great caution.

In summary, our experiments reveal that kinetochores retain the ability to mediate plus-end–dependent assembly of KMTs and the associated motion of chromosomes away from the spindle pole after the onset of anaphase, and that these events can be induced by microinjection of biotin-labeled tubulin subunits in a concentration-dependent manner. These results indicate that the transition from metaphase to anaphase chromosome motion is not accomplished through the permanent modification of the kinetochore, especially with respect to its ability to permit subunit incorporation. The reversal of chromosome motion observed here is transient, and is only observed for cells in anaphase A of mitosis. The sensitivity of anaphase chromosome motion to the intracellular tubulin concentration further indicates that the rate and direction of chromosome-to-pole motion may be regulated by the concentration of tubulin subunits at the kinetochore region of an actively moving anaphase chromosome.

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