Chromosome Fiber Dynamics and Congression Oscillations in Metaphase PtK₂ Cells at 23°C

Dwayne Wise, Lynne Cassimeris, Conly L. Rieder, Patricia Wadsworth, and Edward D. Salmon

Department of Biological Sciences, Mississippi State University (D.W.); Department of Biology, University of Pennsylvania, Philadelphia (L.C.); Department of Biology, University of North Carolina, Chapel Hill (E.D.S.); Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany (C.L.R.); Department of Zoology, University of Massachusetts, Amherst (P.W.)

A bioriented chromosome is tethered to opposite spindle poles during congression by bundles of kinetochore microtubules (kMts). At room temperature, kinetochore fibers are a dominant component of mitotic spindles of PtK₂ cells. PtK₂ cells at room temperature were injected with purified tubulin covalently bound to DTAF and congression movements of individual chromosomes were recorded in time lapse. Congression movements of bioriented chromosomes between the poles occur over distances of 4.5 μm or greater. DTAF-tubulin injection had no effect on either the velocity or extent of these movements. Other cells were lysed, fixed, and the location of DTAF-tubulin incorporation was detected from digitally processed images of indirect immunofluorescence of an antibody to DTAF. Microtubules were labeled with an anti-beta tubulin antibody. At 2–5 minutes after injection, concentrated DTAF-tubulin staining was seen in the kinetochore fibers proximal to the kinetochores; a low concentration of DTAF-tubulin staining occurred at various sites through the remaining length of the fibers toward the pole. Kinetochore fibers in the same cell displayed different lengths (0.2 to 4 μm) of concentrated DTAF-tubulin incorporation proximal to the kinetochore, as did sister kinetochore fibers. Ten minutes after injection, the lengths of DTAF-containing chromosomal fibers were greater than expected if incorporation resulted solely from the lengthening of kinetochore microtubules due to congression movements of the chromosomes. Besides incorporation as a result of chromosome movement, two other mechanisms might explain the length of the DTAF-containing segments: 1) a poleward flux of tubulin subunits (Mitchison, 1989) or 2) capture of DTAF-containing nonkinetochore microtubules.

Key words: mitosis, microtubules, tubulin incorporation

INTRODUCTION

Chromosomal fibers tether kinetochores to spindle poles and the forces for polar movement are generated either within the kinetochore (chromosomal) fiber complex, or by the kinetochore itself [McIntosh and Koonce, 1989; Salmon, 1989; Mitchison, 1989; Koshland et al., 1988]. At metaphase, sister kinetochores are attached to opposite spindle poles, but in many cell types, such as PtK₂, chromosomes oscillate to and fro between the spindle poles with concurrent lengthening and shortening of opposing chromosomal fibers. In anaphase, poleward

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Address reprint requests to Dwayne A. Wise, Dept. of Biological Sciences, P.O. Drawer GY, Mississippi State, MS 39762.

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movement of the kinetochores occurs along with chromosomal fiber shortening and the disassembly of the microtubules which span the distance between the kinetochore and the poles.

Chromosomal fibers appear to be a complex of kinetochore and non-kinetochore microtubules. The spindle is formed from overlapping arrays of polar microtubules extending from opposite spindle poles. Polar microtubule assembly is nucleated at the poles and growth occurs by subunit addition to their plus (distal) ends [Mitchison et al., 1986]. Kinetochore microtubules (KMTs) are those attached to kinetochores and these are oriented with their plus ends proximal to the kinetochore. The formation of a chromosome fiber appears to occur by the binding of the plus (growing) ends of polar microtubules to sites on the kinetochore [McIntosh and Vigers, 1987; reviewed in Salmon, 1989].

It has long been apparent that the kinetochore fiber of a bipolarly oriented chromosome moving toward a spindle pole must shorten and its sister fiber must lengthen. Therefore, almost any conceivable hypothesis about mitotic force production must take into account exchange of tubulin subunits with microtubules of the kinetochore fiber [Forer, 1986; Inoué and Ritter, 1975; Margolis et al., 1978; Pickett-Heaps et al., 1982; Mitchison et al., 1986; Salmon, 1989]. The exact way in which kinetochore fibers assemble and disassemble, especially at anaphase, has long been controversial [Schaap and Forer, 1984; Inoué and Sato, 1967; Gorbsky et al., 1987]. Recently, new ultrastructural evidence has suggested that subunit incorporation occurs at the kinetochore during metaphase [Mitchison et al., 1986; Mitchison, 1989; Geuens et al., 1989].

In this paper, we examine the organization and dynamics of the cluster of non-kinetochore and kinetochore microtubules which compose the chromosomal fibers in PtK2 cells cultured at 23°C instead of their normal (37°C) temperature. Cells dividing at room temperature lose about 50% of their non-kinetochore microtubules and thus show greatly increased contrast of the KMTs. This allows for improved visualization of the kMt bundles. We have used these cells for microinjection of derivatized tubulin in order to study the dynamics of tubulin incorporation into kMTs as the kinetochore fibers lengthen and shorten during chromosome oscillations between the poles in prometaphase and metaphase.

**MATERIALS AND METHODS**

**Cell Culture**

Male rat kangaroo (PtK2) cells were grown at 37°C in Ham’s F12 medium (GIBCO) with glutamine, glucose, 10% fetal calf serum, antibiotics, and bicarbonate buffer. These cells were the kind gift of Dr. Greta Lee. For injection, cells were grown on glass coverslips in petri dishes until about 80% confluent, were transferred to F12 medium with HEPES buffer (Sigma), and were kept at room temperature (22–24°C) for at least 15 minutes before injection. Other cells were grown in Rose chambers and injected at room temperature.

**DTAF-Tubulin and Antibodies to DTAF**

Tubulin was purified from pig brain and covalently linked to 5-(4,6-dichlorotiazin-2-yl) amino fluorescein (DTAF) according to the technique of Leslie et al. [1984]. This DTAF-tubulin has been shown to behave as native tubulin in vitro and in vivo [Salmon, 1984]. DTAF-tubulin at approximately 10 mg/ml in 20 mM sodium glutamate, pH 6.9, 1 mM EGTA, and 0.2 mM MgSO4 was stored at −90°C in 30 µl aliquots until use for microinjection. Antibody to DTAF was raised in New Zealand white rabbits by injection of fluorescein isothiocyanate (FITC)-keyhole limpet hemocyanin (Sigma) complex in Freund’s complete adjuvant [Luby-Phelps et al., 1984]. Booster injections were given at monthly intervals for 4 months. Hyperimmune sera were collected by ear puncture and were tested for cross-reactivity to DTAF by the immunoperoxidase dot-blot technique. Anti-FITC antisera were also the kind gift of Dr. Edward Voss (Department of Immunology, University of Illinois, Urbana). All rabbit anti-fluorescein antibodies were found to contain contaminating antibodies and were purified by affinity column chromatography (Pharmacia) [Jarvis et al., 1982]. The column fractions were tested for reaction to FITC, DTAF, bovine serum albumin (BSA), tubulin, and DTAF-tubulin by polyacrylamide gel electrophoresis (PAGE) followed by Western blotting and probing with anti-rabbit IgG and peroxidase. To check for binding of the anti-DTAF antibody to whole microtubules, DTAF-tubulin was polymerized in vitro, spun onto coverslips, and stained with the purified anti-DTAF antibody followed by fluorescent secondary and tertiary antibodies. All commercially prepared antibodies were from Cooper Biomedical (Malvern, PA).

**Microinjection**

Microinjection was accomplished by the method of Wadsworth and Salmon [1986].

**Immunofluorescence Microscopy**

PtK2 cells growing on glass coverslips and injected with DTAF-tubulin were lysed for 30 seconds in PEM buffer (80 mM PIPES, 5 mM EGTA, 1 mM MgCl2, pH 6.8), with 0.5% Triton X-100. They were then fixed for 10 minutes in 2% paraformaldehyde, 0.1% glutaraldehyde (pH 7.3) in PBS, rinsed in PBS, and blocked for nonspecific antibody binding with 1% BSA, 0.1% Tween in PBS for 15 minutes at room temperature. Cov-
erslips were then rinsed in PBS and inverted on a drop of anti-DTAF antibody at 1:10 dilution and incubated at 37°C for 30 minutes. Cells were then rinsed and incubated at 37°C with appropriate dilutions of secondary (rhodamine-labeled goat anti-rabbit IgG) and tertiary (rhodamine-labeled rabbit anti-goat IgG) antibodies. Coverslips were finally mounted in Gelvatol (Monsanto Corp.) for viewing in the fluorescence microscope. Total microtubule polymer was revealed by treatment with a monoclonal mouse anti-tubulin antibody followed by FITC-labeled goat anti-mouse IgG.

**Electron Microscopy**

Cells were grown at 37°C and then placed at 23°C for 25 minutes before fixation. They were fixed for 30 minutes in 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7, postfixed in 1% osmium tetroxide in buffer for 15 minutes on ice, and stained, en bloc, in 1% aqueous uranyl acetate overnight. Cells were then embedded in Epon-Araldite and cross sections were cut 1–2 μm from the metaphase plate [Rieder et al., 1985].

**Analysis of Chromosome Movements**

Cells growing at 37°C in Rose chambers were placed at room temperature for at least 15 minutes before videotaping. Time-lapse videotapes were made using an upright phase-contrast microscope. The temperature in the room was measured periodically and was found to vary between 22°C and 24°C. Chromosome movements were analyzed by making acetate tracings from the videotapes.

**Analysis of DTAF Incorporation**

Cells prepared for immunofluorescence were viewed with a 1.4 NA, 63 ×, oil-immersion lens using an inverted microscope equipped for fluorescence observation at two different wavelengths and for phase-contrast microscopy. Images were collected by a silicon-intensified target (SIT) video camera. These were digitized and a rolling average, generated by an image analysis computer, of 8 seconds of the image in each focal plane through the cell was stored on videotape. The through-focus series of the phase contrast image, the fluorescein image, and the rhodamine image was collected in this way. For three-dimensional reconstruction of the fluorescent kinetochore fibers, the digitized image from 8 seconds in each focal plane was summed by the computer and acetate tracings of the summed image were made. These tracings were overlaid to generate the 3-D image of the cell in the fluorescein and rhodamine channels and in phase-contrast. In addition, the summed images in each focal plane were recorded from the video monitor onto 35 mm film. Sites of new microtubule assembly in the kinetochore fibers of fixed cells were identified as fluorescent segments in the rhodamine image. Because the data from the rhodamine channel were collected first for any given cell, and because of a small amount of "bleedthrough" from the rhodamine into the fluorescein channel, pictures of total tubulin polymer in cells are slightly faded. Data on the sites and extent of DTAF-tubulin incorporation (new assembly) were collected as follows. Sets of sister kinetochore fibers were identified by overlaying the 3-D phase-contrast and fluorescein (total tubulin) images of the same cells. Sites of DTAF-tubulin incorporation were identified and measured by then superimposing the image of the cell in the rhodamine channel. New assembly was measured only in matched sets of sister kinetochore fibers; all others were rejected. The data were normalized for differences in apparent total kinetochore fiber length by calculating the sum of the distances—pole 1 to kinetochore 1, kinetochore 1 to its sister kinetochore (2), and kinetochore 2 to pole 2—and expressing this total distance as 100%. DTAF-containing segments were then scored as percents of this length.

**RESULTS**

**Antibody Specificity**

The anti-FITC antibody purified by affinity chromatography reacted with DTAF-tubulin, DTAF-BSA, and FITC-BSA but did not recognize either unlabeled tubulin or unlabeled BSA. When purified DTAF-tubulin dissolved in injection buffer was injected into interphase PtK₂ cells kept at room temperature for 10 minutes and then processed according to our immunofluorescence protocol (see "Materials and Methods"), many heterogeneous lengths of labeled microtubules were seen in the cytoplasmic microtubule complex, as expected for DTAF-tubulin incorporation at the plus ends of the microtubules [Soltys and Borisy, 1985; Schulze and Kirschner, 1986]. Uninjected cells in mitosis showed no fluorescence when processed with the anti-DTAF antibody; however, commercially prepared secondary antibodies used to detect the DTAF-tubulin cross-reacted with intermediate filament-like arrays in dividing and non-dividing cells. Dividing cells treated with these secondary antibodies only showed no fluorescence of the spindle, so the antibodies were used in the procedure. These results indicate that our DTAF-tubulin was assembly-competent and that the anti-fluorescein antibody detected only DTAF-containing regions of microtubules.

**Metaphase Spindle Structure at 23°C**

When PtK₂ cells cultured at 37°C were transferred to room temperature (22–24°C) for 15 minutes or longer, many of the non-kinetochore and most of the astral microtubules of the mitotic spindle were lost and kMt bun-
Fig. 1. Comparison of spindle anti-tubulin fluorescence between PtK₂ cells dividing at 37°C (A) and at 23°C (B). A: These cells were fixed before lysis and processing for immunofluorescence; therefore, we believe that microtubules were not lost because of disassembly in the lysis buffer. B: 37°C. Abundant astral and non-kinetochore microtubules are evident. B: 23°C. kMt bundles are clearly resolved, and the population of non-kinetochore and astral microtubules is greatly diminished. Bar represents 10 μm.

Fig. 2. A tracing of microtubule profiles from a high-voltage electron micrograph of a cross section through a region 1–2 μm from the equator in a PtK₂ cell fixed at room temperature. Microtubule profiles occur in discrete bundles (circles)—these probably represent the chromosomal fibers. Bar represents 1 μm.

dles ("fibers") became readily visible (Fig. 1). Previous studies have shown that PtK cells can form a normal spindle and can complete mitosis at 23°C [Rieder, 1981b]. Two metaphase cells, kept at room temperature for 25 minutes, were fixed and processed for electron microscopy. Microtubules were counted in serial, thick cross sections 1–2 μm from the metaphase plate. This region contained 767 and 745 microtubules, respectively, in the two cells. PtK₂ cells grown at 37°C and 23°C contain the same number of kinetochore microtubules; 24 per kinetochore [Rieder, 1982; Cassimeris et al., 1990]. Assuming an average of 24 microtubules attached to each kinetochore and 13 kinetochores per half spindle in PtK₂ [Walén and Brown, 1962], then there were 312 kinetochore microtubules, leaving 455 to 433 non-kinetochore microtubules near the kinetochores. This contrasts with about 1,500 microtubules in the same half-spindle region in PtK₁ cells fixed at 37°C [Brinkley and Cartwright, 1971, 1975; reviewed in McIntosh et al., 1975]. Therefore, the ratio of non-kMts:kMts is 1.4:1 at 23°C and 3.8:1 at 37°C. Clearly, maintenance for 15 or more minutes at room temperature greatly diminishes the number of non-kMt; however, it should be pointed out that, assuming a uniform distribution of the remaining non-kMt, each kinetochore has available for capture about 34 neighboring non-kMt. Presumably most of these would have their plus ends proximal to the kinetochore.

A tracing of microtubule profiles from a high-voltage electron micrograph of a 0.25 μm section 1–2 μm from the metaphase plate of a PtK₂ cell fixed at room temperature is shown in Figure 2. Note that the microtubule profiles show prominent bundles (circles in Fig. 2) of 30–50 microtubules [Brinkley and Cartwright, 1971]. Since an average of 24 of these are attached to the kinetochore, then it seems clear that the kinetochore fiber consists of a composite of non-kMts and kMts. These results imply that, in the cells injected at room temper-
nature and processed for immunofluorescence, the decrease in non-kMts number was due to the lowered temperature, not to disassembly in the lysis buffer.

**Metaphase Chromosome Oscillations Between the Spindle Poles**

Prometaphase movements of chromosomes in PtK$_2$ cells dividing at room temperature were recorded by time-lapse video microscopy and were analyzed for direction of movement and velocity. Movements of chromosomes in cells injected with DTAF-tubulin were recorded in the same way. These results are shown in Figure 3. During 10 minutes of mid-late prometaphase, chromosomes did not move beyond approximately the midpoint of the half-spindle and to-and-fro movements were common. The average chromosome velocity in control cells was 0.89 μm/min, with a range of 0.20–2.05 μm/min. The average chromosome velocity in injected cells was 0.65 μm/min, with a range of 0.19–1.35 μm/min (Fig. 3). Both of these rates were considerably slower than PtK$_2$ chromosome velocities at 37°C [2.5 μm/min, McNeill and Berns, 1981; 3.0 μm/min, Roos, 1976], but were not different in any other regard. Therefore, injection of tubulin had no significant effect on chromosome velocity in cells dividing at room temperature.

**Analysis of Tubulin Incorporation Into Chromosomal Fibers**

A total of seven cells was injected and incubated at room temperature for 2 minutes, lysed, fixed, and processed for immunofluorescence. One of these cells is shown in Figure 4. Several of the kinetochore fibers show significant lengths of label interstitially in the fiber and some show small, bright tufts of DTAF-containing microtubules proximal to the kinetochore. Inspection of Figure 4 shows that the sister kinetochore fibers were not labeled symmetrically.

Phase-contrast, fluorescein, and rhodamine images of two cells fixed and processed 5 minutes after injection of DTAF-tubulin are shown in Figure 5. Once again, some kinetochore fibers show extensive amounts of kinetochore-proximal label, significant interstitial labeling, and heterogeneous sister kinetochore labeling.

Figure 6 shows a cell in late prometaphase fixed and processed for immunofluorescence 10 minutes after injection. Most of the chromosomal fibers show DTAF-tubulin incorporation through most of their lengths; however, some kinetochore-proximal tufts of fluorescence are apparent and a few unlabeled segments are seen.

A sample of the DTAF-tubulin incorporation data from fixed cells is shown diagrammatically in Figure 7. Lengths and intensities of DTAF-containing segments were determined by making tracings of serial optical sections through the spindle as described in "Materials and Methods." The data were normalized as follows: the sum of the kinetochore-to-pole distances and the interkinetochore distance is shown as 100% and each labeled segment is shown as a percent of this distance. Qualitatively estimated differences in fluorescence intensity are shown as three different thicknesses of the labeled segments, the brightest label indicated by the thickest line. Fifty percent marks the position of the equator and kinetochores are represented as open circles. Only matched
sets of sister kinetochore fibers are shown. A pattern of DTAF-tubulin incorporation through time can be seen. At 2 minutes, most kinetochores had proximal, bright tufts and a few kinetochore fibers were extensively labeled interstitially. At 5 minutes, the pattern of label was not remarkably different, although more kinetochore fibers appeared to have incorporated DTAF-tubulin. At 10 minutes after injection, a major difference in the label pattern appeared. Kinetochore-proximal tufts had all but disappeared and most fibers had long, heavily labeled segments, most of which were contiguous with the kinetochore.

The DTAF-tubulin incorporation data from all injected fixed cells are analyzed statistically in Table I. For the 46 sets of sister kinetochore fibers from 7 different cells injected and fixed 2 minutes later, the mean length...
of labeled kinetochore fiber was 35.54% (range = 7–67%). For cells fixed 5 minutes after injection, based on a similar sample size, the mean length of labeled kinetochore fiber was 28.62% (range = 0–71%) and for cells fixed 10 minutes after injection the mean labeled fiber length was 51.05% (range = 14–72%). The t-test for differences of means indicated that the 2 minute cells contained significantly more label than did 5 minute cells. However, cells fixed 10 minutes after injection contained significantly more label than either 2 or 5 minute cells. The large range of labeled length in each case should be pointed out. This apparently means that non-sister kinetochore fibers incorporated new tubulin on a very heterogeneous basis and independently of each other.

In order to further analyze the pattern of tubulin incorporation proximal to the kinetochore, we analyzed statistically that subset of labeled segments which was attached to a kinetochore, disregarding all other label. These results are shown in Table II. When only kinetochore-proximal incorporation was considered, cells fixed 2 minutes after injection had a mean length of labeled kinetochore fiber of 18.78% (range = 5–68%), cells fixed 5 minutes after injection had a mean labeled length of 9.56% (range = 0–43%), and cells fixed 10 minutes after injection had a mean labeled length of 23.32% (range = 0–58%).

These data show that cells fixed 2 minutes after injection had incorporated significantly more DTAF-tubulin than cells fixed 5 minutes after injection. Cells fixed 10 minutes after injection showed significantly more label than 5 minute cells, but not more than 2 minute cells.

In order to answer the question whether or not sister kinetochores incorporated tubulin subunits symmetrically (at the same rate), we analyzed for symmetry the fraction of DTAF-containing microtubule segments proximal to the kinetochore (Table III). This was done by setting, for each set of sister fibers, the fiber with the shorter length of labeled segment as the “left” kinetochore and then ordering all the sets of sister fibers accordingly. We calculated the Student’s t-test for significance of the difference of the means between the “left” and “right” populations of kinetochore fibers. For all three classes of cells (those fixed 2, 5, and 10 minutes after injection of DTAF-tubulin) the amount of label incorporated proximal to the kinetochore between sister kinetochores was very asymmetrical (see the “t” values in Table III). The data strongly imply that the two sister kinetochores did not incorporate tubulin subunits at the same rate during mid-late prometaphase in living cells.

**DISCUSSION**

Our data support those of Mitchison et al. [1986] and confirm that tubulin subunits are added to kinetochore fibers proximal to the kinetochores, not at the poles. The results are not consistent with a detectable
rate of tubulin subunit treadmilling down the fiber [Margolis and Wilson, 1981; Gorbsky and Borisy, 1989]; however, a treadmilling rate less than the average rate of chromosome movement (0.6 μm/min) would probably be obscured by the oscillatory movements of the chromosomes and would not be detected in these experiments. The asymmetry of labeling between sister kinetochores fibers could result from these oscillatory movements, but only in vivo labeling experiments in which chromosome movement can be directly correlated with label accumulation in the fiber can answer this question.

In general, new tubulin subunits are added proximal to the kinetochore and, with time, the labeled segment extends toward the pole. Somewhat puzzling is the fact that, at 2 minutes after injection, more labeled tubulin was incorporated proximal to the kinetochore than in cells fixed 5 minutes after injection. This may come about because of the relatively high concentration (10 mg/ml) of tubulin being injected. This could promote transient assembly at the kinetochore, followed by adjustment of the dynamic equilibrium as the bolus of tubulin diffuses through the spindle. This would account for the slight but significant decrease in labeled subunits in the 5 minute cells. Alternatively, disassembly at the kinetochore due to chromosome oscillations between the moment of injection and the 5 minute time point could account for the decrease in label between the 2 minute and 5 minute times. In any case, models which invoke either tubulin subunit exchange at the kinetochore exclusively, or addition of subunits at the kinetochore coupled with loss at the poles, do not account for the total amount of DTAF-tubulin incorporated into kinetochore fibers. More DTAF-tubulin was incorporated than would accrue by either of these models. One reasonable explanation is that pre-labeled microtubules are captured by the kinetochore [Huhtorel and Kirschner, 1988]. These results are completely consistent with those of Cassimeris et al. [1988] obtained from fluorescence redistribution after photo-bleaching (FRAP) studies and from high-resolution polarization microscopic analysis of living cells. They found that assembly of microtubules within the kinetochore fiber was dynamic during prometaphase-metaphase and that there was no detectable poleward
flux of subunits within the fiber. They also presented evidence of transient lateral associations between kinetochores and non-kinetochore microtubules. As shown by our ultrastructural results, microtubules are bundled in cells dividing at room temperature, which lends further support to the possibility of a cycle of capture and release of microtubules by the kinetochore and/or recruitment of microtubules all along the length of the fiber. The notion of a capture-release cycle by the kinetochore would seem to contradict the well-documented stability of kinetochore microtubules. A number of workers have shown that kMTs are stable to a variety of agents which cause disassembly of non-kMTs [Rieder, 1981a; Brinkley and Cartwright, 1975; Salmon, 1975, 1984; Cassimeris et al., 1990]. Cassimeris et al. [1990] have recently shown that in PtK cells at 23°C, kMTs are much more stable to nocodazole than are non-kMTs but, nevertheless, the former do turn over with a half-time of 7.5 minutes. This means that in the present study, also with PtK cells at 23°C, we might expect that at the 10 minute time point, most of the 24 kMTs could have been replaced. Therefore, we believe that it is reasonable to consider a cycle of capture and release as one means to explain the present date. We consider the consequence of this cycle of microtubule capture and release in Figure 8.

In this discussion, we ignore the effect of chromosome movement on kinetochore-proximal incorporation to illustrate the point that significant label can be accumulated simply by capture and release by the kinetochore. Add to this the incorporation due to chromosome oscillations and the possible recruitment of labeled microtubules all along the length of the fiber and the result becomes impossible to illustrate. It may be that all three mechanisms operate in vivo and together account for the large amount of label that was incorporated.

According to our ultrastructural results, at the time of DTAF-tubulin injection in a cell at room temperature, there exist in the vicinity of each kinetochore about 34 free plus ends of non-kMTs. Immediately after injection, these begin to grow by adding DTAF-tubulin subunits at the rate of about 1–2 μm/min at 23°C [Schulze and Kirschner, 1986]. This means that within seconds after injection, many labeled plus ends are available for capture by the kinetochore and the length of these non-kinetochore labeled ends increases with time. If a cycle of capture and release by the kinetochore exists, then, clearly, microtubules captured 10 minutes after injection might be completely labeled. This would account for both the fact that DTAF-tubulin incorporation occurs proximal to the kinetochore and that the amount of DTAF-tubulin incorporated increases with time. It should be pointed out that our data do not distinguish between the two models of labeled subunit acquisition by the kinetochore discussed above. In fact, we cannot rule out the possibility that incorporation of new tubulin subunits into kinetochore fibers occurs at the kinetochore at the same time that capture of pre-labeled microtubules occurs. Only electron microscopy analysis of labeled tubulin acquisition by the kinetochore will clarify the issue. One of the most interesting aspects of the data is the appearance of interstitial labeling at early times after injection of labeled tubulin. The spindle poles were abundantly flu-
TABLE I. Statistical Analysis of the Total Percent of Kinetochoore Fiber Labeled in PtK₂ Cells Lysed and Fixed for Immunofluorescence 2, 5, and 10 Minutes After Injection

<table>
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<tr>
<th>Cells</th>
<th>N (cells)</th>
<th>N (chms)</th>
<th>Range</th>
<th>Mean</th>
<th>s.d.</th>
<th>t²</th>
<th>d.f.</th>
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<tr>
<td>5 min</td>
<td>8</td>
<td>42</td>
<td>0–71</td>
<td>28.62</td>
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<td>1.9646</td>
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<td>1.6338</td>
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<td>10 min</td>
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<td>14–72</td>
<td>51.05</td>
<td>14.88</td>
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<td>2.6528</td>
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*Only clearly identifiable sister kinetochoore fibers have been analyzed. In order to use Student’s t-test for significance of the difference of population means, the data were first normalized by the arcsin transformation.
*Computed value of t.
*Degrees of freedom.
*Critical value of t at the 1% confidence level.

TABLE II. Statistical Analysis of the Percent of Kinetochoore Fiber Labeled Proximal to the Kinetochoore in PtK₂ Cells Lysed and Fixed for Immunofluorescence 2, 5, and 10 Minutes After Injection With DTAF-Tubulin

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<tr>
<td>5 min vs. 10 min</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>3.6256</td>
<td>59</td>
<td>2.6699</td>
</tr>
</tbody>
</table>

*Only clearly identifiable sister kinetochoore fibers have been analyzed. The data have been normalized and tested for significance of the difference of means by the t-test.
*Computed value of t.
*Degrees of freedom.
*Critical value of t at the 1% confidence level.

TABLE III. Statistical Analysis of the Asymmetry of the Percent of Kinetochoore Fiber Labeled Proximal to Sister Kinetochores in PtK₂ Cells Lysed and Fixed for Immunofluorescence 2, 5, and 10 Minutes After Injection With DTAF-Tubulin

<table>
<thead>
<tr>
<th>Cells</th>
<th>N (chms)</th>
<th>Mean</th>
<th>s.d.</th>
<th>t²</th>
<th>d.f.</th>
<th>.01'</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 min—left</td>
<td>46</td>
<td>4.37</td>
<td>3.72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min—right</td>
<td>46</td>
<td>17.30</td>
<td>12.19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min—left</td>
<td>34</td>
<td>2.68</td>
<td>4.04</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 min—right</td>
<td>34</td>
<td>15.32</td>
<td>12.77</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min—left</td>
<td>21</td>
<td>13.00</td>
<td>10.19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 min—right</td>
<td>21</td>
<td>29.14</td>
<td>13.89</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 min left vs. 2 min right</td>
<td></td>
<td></td>
<td></td>
<td>7.7791</td>
<td>90</td>
<td>2.6310</td>
</tr>
<tr>
<td>5 min left vs. 5 min right</td>
<td></td>
<td></td>
<td></td>
<td>7.3610</td>
<td>66</td>
<td>2.6540</td>
</tr>
<tr>
<td>10 min left vs. 10 min right</td>
<td></td>
<td></td>
<td></td>
<td>4.1308</td>
<td>40</td>
<td>2.7040</td>
</tr>
</tbody>
</table>

*The data have been ordered such that the “left” kinetochoore always had the smaller percent labeled of the pair of sister kinetochores. The mean percent labeled for “left” and “right” kinetochoore was then computed and the significance of the difference of these means was tested using the t-test. The data were normalized as detailed above.
*Computed value of t.
*Degrees of freedom.
*Critical value of t at the 1% confidence level.

Orescent even at the 2 minute time point, which implies that the non-kMs are highly dynamic, even at 23°C. If newly polymerized non-kMs can be recruited anywhere along the fiber, then the observed pattern of interstitial label accumulation at 2 and 5 minutes after injection can be rather easily explained. Add to this the accumulation of new tubulin subunits as a result of chromosome movement and it is possible to envision the tufts of label proximal to kinetochores seen at the early time points. What is abundantly clear is that most, or perhaps all, of the label observed to accumulate in the chromosomal fiber with time could not come about solely by tubulin...
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subunit exchange at the kinetochore. Surely, the dynamics of chromosomal fibers in vivo are more complicated than many of us have imagined.

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REFERENCES


