# Microtubule Depolymerization Promotes Particle and Chromosome Movement In Vitro

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***Abstract.*** We have developed a system for studying the motions of cellular objects attached to depolymerizing microtubules in vitro. Radial arrays of microtubules were grown from lysed and extracted *Tetrahymena* cells attached to a glass coverslip that formed the top of a light microscope perfusion chamber. A preparation of chromosomes, which also contained vesicles, was then perfused into the chamber and allowed to bind to the microtubule array. The concentration of tubulin was then reduced by perfusing buffer that lacked both tubulin and nucleotide triphosphates, and the resulting microtubule depolymerization was observed by light microscopy. A fraction of the bound objects detached in the flow and washed away, while others stabilized the microtubules to which they were bound. Some of the particles and chromosomes, however, moved in toward the *Tetrahymena* ghost as their associated microtubules shortened. The mean speeds for particles and chromosomes were 26 ± 20 and 15 ± 12 μm/min, respectively. These motions occurred when nucleotide triphosphate levels were very low, as a result of either dilution or by the action of apyrase. Furthermore, the motions were unaffected by 100 μM sodium orthovanadate, suggesting that these forces are not the result of ATP hydrolysis by a minus end–directed mechanoenzyme. We conclude that microtubule depolymerization provided the free energy for the motions observed. All the objects that we studied in detail moved against a stream of buffer flowing at ~100 μm/s, so that the force being developed was at least $10^{-7}$ dynes. This force is large enough to contribute to some forms of motility in living cells.

THE importance of microtubules (MTs)[1] in the movements of cytoplasmic organelles and chromosomes has been established by studies that correlate structure with function, studies that perturb MTs with drugs or changes of environmental variables, and studies that mutate the genes for tubulin or its associated proteins (reviewed in Inoue, 1981; Dustin, 1984; McIntosh and Koonce, 1989; Raff, 1984). The mechanisms by which MTs contribute to these motile processes is, however, less clear. Three ideas have received the most attention over the past years: (*a*) MTs are skeletal elements, and movements relative to them are achieved by cytoplasmic muscle proteins (reviewed in Forer, 1985); (*b*) MTs bind and activate their own mechanoenzymes, e.g., dynein and kinesin, and serve as tracks for the movements generated by these motor molecules (reviewed in McIntosh and Porter, 1989); and (*c*) MTs polymerize and depolymerize to push or pull on objects attached at or near their ends (reviewed in Inoue, 1981). The first possibility is not well supported by the evidence available so far, but the second now appears to be at least a part of the truth. Both kinesin (Vale et al., 1985; Schroer et al., 1988*a*) and cytoplasmic dynein (Paschal and Vallee, 1987; Lye et al., 1987; Schroer et al., 1988*b*) have been shown to generate life-like motions of objects in vitro, suggesting strongly that they contribute to cytoplasmic mechanics.

The possibility that polymer dynamics could generate mechanochemical forces in cells has been considered for many years. Before MTs were characterized, Ostergren (1949) described a model in which the spindle was said to be composed of tactoid liquid crystals. Chromosome movements were suggested to result from the dynamic exchange of individual tactoid particles between chromosomal fibers and other fibers of the spindle. Inoue (1952) was the first to present the idea that the polymerization and depolymerization of labile protein filaments could do mechanical work during mitosis. This idea was expanded and refined through observations and experiments on living cells into an explicit model for several aspects of mitotic movements (Inoue, 1959; 1964; Inoue and Sato, 1967). A wealth of experimental work shows that some movements during mitosis in vivo are associated with either MT polymerization (Inoue, 1952; Tilney and Porter, 1967; Salmon, 1975*a*) or depolymerization (Inoue, 1952; Inoue and Sato, 1967; Salmon, 1975*b*;

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1. *Abbreviations used in this paper*: DIC, differential interference contrast; EGS, ethylene glycol-(bis) succinimidyl succinate; MT, microtubule.

Fuseler, 1975; Spurck and Pickett-Heaps, 1987), but the complexity of cytoplasm and the number of reactions that might contribute to the motions seen has made it impossible to conclude rigorously that the polymerization reaction itself is either the motor or the source of energy for the movements seen.

In the last ten years there has been a resurgence of interest in polymerization reactions as sources of motive force. Several theoretical treatments have examined the thermodynamics of this possibility (Hill, 1981, 1987; Hill and Kirschner, 1982). Experimental work in vitro, has demonstrated that MT polymerization within liposomes can deform the surrounding membrane (Miyamoto and Hotani, 1988; Hotani and Miyamoto, 1990), providing compelling evidence that polymerization alone can transduce the chemical energy of MT formation into the mechanical energy necessary to distend a membrane. The possibility that depolymerization can do work has been examined by Koshland et al. (1988) who developed the first appropriate experimental system. They polymerized stable MTs from labeled tubulin and elongated these seeds at their plus ends (the fast growing ones) to make polymers with visible markers for structural polarity. These MTs were bound to isolated chromosomes and then diluted in buffers lacking both ATP and GTP and containing successively lower concentrations of tubulin to promote MT depolymerization. As the MTs shortened, samples of the preparation were centrifuged onto coverslips for fluorescence microscopy. At successive times after dilution, the mean distance from the chromosomes to the minus ends of their associated MTs decreased, suggesting that MT depolymerization in the absence of soluble nucleotide triphosphate could perform the mechanical work of pulling the labeled end of the MT closer to the chromosome.

This exciting conclusion has important implications for cell motility in general and mitosis in particular. There are, however, several features of the data that raise concerns about the utility of the model system for understanding the mechanisms of cytoplasmic movements in vivo. The average number of MTs attached to each chromosome decreases along with the mean distance from the chromosome to the minus end of the MTs still bound, so the system may be based on an unstable association between the polymers and their anchors. This raises questions about the amount of force this in vitro system can generate. Since the movement seen was that of a single MT relative to its associated chromosome, the force generated might have been very small. No force measurements have yet been made in this system, and such measurements may be technically difficult. Furthermore, all of the graphs depicting the distance from the chromosome to the MT minus end as a function of time are concave up, suggesting that the movement slowed with time. This is not a characteristic of vesicle or chromosome motions in cells (reviewed in Rebhun, 1972; Nicklas, 1988), nor is it a characteristic one would expect for an end-dependent reaction, like polymer disassembly.

For a thorough study of depolymerization-dependent motions one would like a system that permits real-time viewing, so sampling and fixation are not necessary, and where one can directly measure the force being generated. This paper describes our effort to develop such a system and the results we have obtained with it.

## Materials and Methods

### Isolation of the Assay Components

Phosphocellulose-purified tubulin was prepared from bovine brain according to Williams and Detrich (1979). Mitotic chromosomes were isolated from CHO cells arrested with 10 μg/ml vinblastine sulfate overnight by the procedure of Mitchison and Kirschner (1985). *Tetrahymena* (strain SB 255) were grown to mid-log phase in 2% proteose peptone, 30 μg/ml sequestrine, and 50 μg/ml penicillin/streptomycin. Cells were collected by centrifugation and washed first with fresh medium, then with cold PME buffer (100 mM Pipes, 1 mM $MgCl_2$, 2 mM EGTA, pH 6.9) containing protease inhibitors (1 μg/ml leupeptin, 1 μg/ml pepstatin, 10 μg/ml aprotinin, 0.1 mg/ml soybean trypsin inhibitor, and 0.1 mg/ml benzamidine). Cells were lysed with 0.25% NP-40 in PME (with protease inhibitors) and deciliated using a Sorvall Omni-mixer for three 10-s pulses at setting four. The pellicles were rinsed by pelleting and resuspending them three times in PME buffer without detergent. When necessary, the detergent extraction was repeated until no cytoplasmic streaming could be detected inside the cell ghosts by differential interference contrast (DIC) microscopy. The pellicles were tested for the ability of their basal bodies to nucleate microtubules. Successful preparations were divided into aliquots, frozen in liquid $N_2$, and stored at −70°C.

### ATP Assays

To determine the concentrations of ATP in the components of our in vitro system we used a luciferin/luciferase bioluminescent assay (Sigma Chemical Co., St. Louis, MO), which can detect picomolar levels of ATP. The reactions were followed with a luminometer (monolight 2000; Analytical Luminescence Labs, Inc., San Diego, CA) capable of measuring light emission within milliseconds after sample injection.

### Perfusion Chamber

All of the movement assays were performed in a perfusion chamber on a microscope stage at 36 ± 1°C. Stage temperature was maintained with an air curtain incubator (Sage Instruments, Cambridge, MA). A 6 × 22 mm chamber was constructed from a 22 × 22 mm coverslip separated from a microscope slide by two streaks of vacuum grease. The chamber was sealed on two edges with Valap (vaseline, lanolin, and paraffin at 1:1:2). The ends of the chamber were kept open and 25-μl wells were formed on the slide with Valap to allow liquids to be passed through quickly.

Many of our experiments required a change of the solution in the perfusion chamber. We assessed the efficiency of such changes by loading a chamber with a sample containing $^{35}$S-methionine, then diluting the label with successive chamber volumes of isotope-free buffer. By comparing the specific activities of the solution in the chamber before and after each dilution we determined that each buffer exchange achieves approximately a 10-fold dilution.

We looked for gross heterogeneities in the flow during perfusion (which may have resulted from eddies created around pellicles) by passing dye through the chamber. In the central portion of the coverslip, the area used in all movement assays, no spatial variation in dye concentration during perfusion could be detected.

### Assay for Particle and Chromosome Movement

*Tetrahymena* pellicles were diluted 10-fold in PME buffer and a 15-μl sample was adsorbed to a coverslip for 3 min before the perfusion chamber was assembled as described above. The chamber was washed thoroughly by passing through several chamber volumes of PME buffer, leaving behind only those pellicles that were firmly attached to the coverslip. 30 μM phosphocellulose-purified tubulin in PME buffer containing 1 mM GTP was then introduced into the chamber. Incubation at 36°C for 15 min resulted in a dense array of MTs emanating from the basal bodies of the pellicles. The preparation of mitotic chromosomes was diluted 10-fold into PME buffer containing 23 μM phosphocellulose-purified tubulin, 1 mM GTP, 0.1 mM spermidine, and 0.05 mM spermine, and then flowed into the chamber. This mixture was allowed to incubate for 5 min before the coverslip was scanned with DIC optics to identify particles and chromosomes bound to the MTs. Once a particular pellicle was selected for observation, the MTs were induced to depolymerize by flowing in PME buffer that contained polyamines, but lacked both tubulin and nucleotides. To determine

whether a bound object was a chromosome, tubulin dilution was preceded by a wash with the buffer used for capture containing 1 μg/ml of the DNA dye, Hoechst 33258. The chromosomes could then be identified with fluorescence optics.

In some assays, the chromosome preparation was incubated for 3 min before perfusion with both 10 U/ml of apyrase, to reduce the levels of any possible ATP contamination, and 100 μM sodium orthovanadate, to inhibit the ATPase activity of the known minus-end directed mechanoenzymes (Paschal and Vallee, 1987; Walker et al., 1990). Subsequent perfusions also contained the same concentrations of apyrase and vanadate.

### Microscopy

Perfusion chamber preparations were viewed with a Zeiss Universal microscope or a Zeiss Standard microscope (Carl Zeiss, Oberkochen, West Germany). Each microscope was equipped with a plan 100×/1.25 NA objective lens, a 1.4 NA condenser and DIC optics. The light from a 100-W Hg arc lamp was passed through a heat filter and a 546-nm interference filter. The Zeiss Standard microscope was equipped with a fiber optic light scrambler (Technical Video, Ltd., Woods Hole, MA) to improve the brightness and to fill the back focal plane with uniform illumination (see Ellis, 1985 in Inoue, 1986). Video images were taken from the Zeiss Standard microscope with a DAGE MTI 68 video camera (Dage Inc., Michigan City, IN). Images from the Zeiss Universal were taken with a DAGE 67 camera, and this microscope was also equipped for epifluorescence microscopy. All video images were recorded with a Super-VHS video cassette recorder (Mitsubishi International Corp., New York, NY), and photographs were taken from the screen of a Tektronix 634 flat-faced monitor (Tektronix, Inc., Beaverton, OR), using a Canon camera with a Canon 50-mm Macro lens and Kodak Technical Pan Film (no. 2415; Eastman Kodak Co., Rochester, NY).

To examine the fine structure of MT initiation at the basal bodies, pellicles were incubated at 70 μM tubulin at 37°C for 15 min, collected by centrifugation (12,000 *g* for 3 min), and processed for EM as described in Heidemann and McIntosh (1980).

### Velocity Measurements

The velocities of particles and chromosomes were measured by transferring individual frames of sequences from Super-VHS tape to an optical disc recorder (model TQ 2028F; Panasonic Corp., Japan). The trajectories of particular objects were tracked from one disc-stored image to the next, using an analogue position detector (Model 271; Colorado Video Inc., Boulder, CO) interfaced to an AT Personal Computer (Dell Inc., Austin, TX) by G. W. Hannaway and Associates (Boulder, CO). Displacement versus time was computed and plotted, and mean speeds were determined by linear regression from the data that described a particular motion.

### Estimating the Force for Movement

The force exerted on a moving object by the flow of medium through the perfusion chamber was estimated from the Stokes' equation for spherical particles. For calculations involving data from chromosomes we used the extension of this equation to prolate ellipsoids, as described by Tanford (1961). A buffer viscosity of one centipoise was assumed. The velocity of the fluid was determined from the rate at which free particles in the same focal plane as a moving object attached to the pellicle-bound MTs flowed past the pellicle.

### Determining the Site of MT Depolymerization

We obtained evidence for the site of MT depolymerization in this system by stabilizing the pellicle-initiated MTs at their minus ends. First, MTs were polymerized from the basal bodies of pellicles bound in the perfusion chamber, using 40 μM tubulin to assure a dense MT growth. Then, after growth to 6–10 μm, the MTs were fixed with ethylene glycol-(bis)succinimidyl succinate (EGS) for 5 min. This reaction was quenched by perfusing a solution that contained 10 mM glutamate, 50% sucrose, and 0.5 mM β-mercaptoethanol in PME and incubating for 30 min. PME buffer was then perfused into the chamber to rinse the pellicles, followed by 30 μM tubulin in PME plus 1 mM GTP to elongate the stable MT stubs. These MTs were used for a typical movement assay, and the behavior of the bound objects was observed with DIC optics.

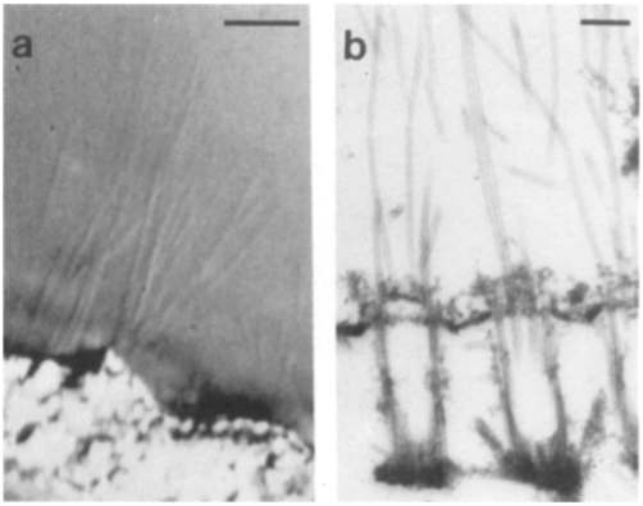


*Figure 1.* *a* is a DIC image of part of a pellicle that has been incubated in 30 μM tubulin for 15 min at 37°C. These conditions promote the formation of a moderately dense array of pellicle-initiated MTs with essentially no growth of free MTs in solution. *b* is a transmission electron micrograph that demonstrates that single MTs are initiated from the basal bodies that are lodged within the pellicles. Bars: (*a*) 5 μm; (*b*) 0.2 μm.

## Results

### The In Vitro Assay

The number and length of the MTs per pellicle depended on the tubulin concentration, temperature, and time of incubation. We chose conditions for MT polymerization that would optimize subsequent chromosome attachment: a moderate density of MTs in the array, an average MT length of ~15 μm, and no spontaneous nucleation of MTs in solution (Fig. 1 *a*). EM showed that the MTs emanating from the pellicles were initiated by the basal bodies embedded in the pellicles of the *Tetrahymena* ghosts and that the MTs from one basal body are not simply confined to a single bundle (Fig. 1 *b*) (see also Heidemann and McIntosh, 1980).

Once stuck, the pellicles remained attached to the coverslip surface throughout an experiment, in spite of the frequent and rapid perfusions of buffer. Their free surfaces faced down from the coverslips into the perfusion chamber, so most of the pellicle-initiated MTs and anything attached to them remained suspended in solution, unbound to the coverslip. As the different buffers were perfused through the chamber, the pellicle-associated MTs were observed to bend with the flow.

Light microscopy with DIC and fluorescence optics revealed that the preparations of chromosomes used for these experiments were heterogenous. Chromosomes were identified on the basis of size, shape, and staining by Hoechst 33258. The preparations also contained small, round, phase-dense particles (diameter = 0.5–1.0 μm) and membranous vesicles that were sometimes free and sometimes associated with the chromosomes. These objects did not bind the DNA-specific dye. On average, five to ten objects (particles or chromosomes) became associated with the MTs growing from each pellicle (Fig. 2 *a*).

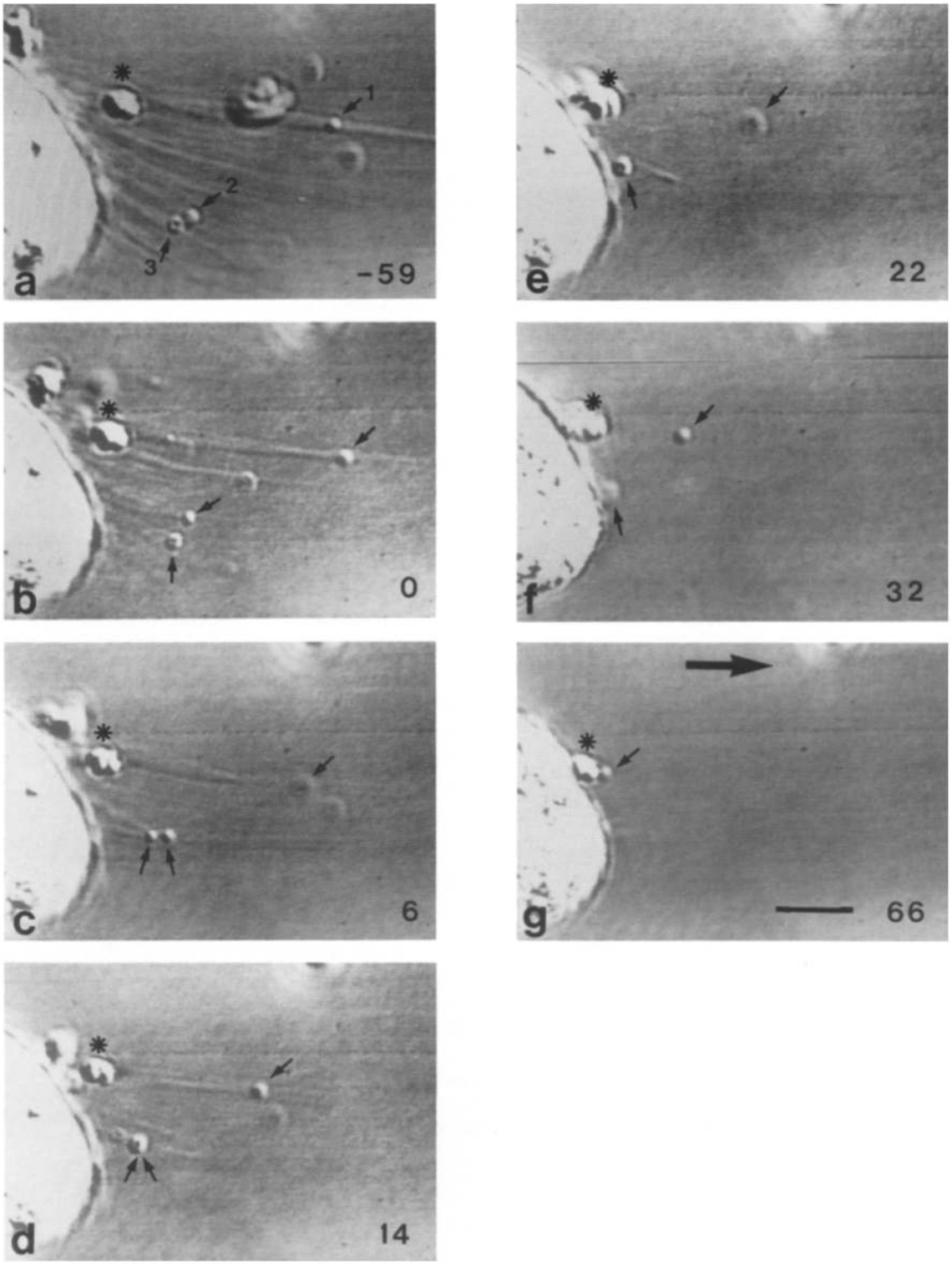


*Figure 2.* A chromosome (*) and three particles (*arrows*), which are bound to MTs, move in toward the pellicle as the MTs depolymerize. The MTs initially appear to extend beyond the bound objects (*a*). Particles begin to move 57 s after the onset of tubulin dilution (t = 0) (*b*). *c–g* are frames taken 6, 14, 22, 32, and 66 s after movement began. Each moving particle is indicated by an arrow. Two particles that were initially attached to different MT fibers merge into a single object (*c*) and move as one thereafter (*d–f*). A chromosome (*) bound close to the pellicle also moved a short distance. The particle and chromosome motions occurred against a continuous flow of buffer moving in the direction indicated by the large arrow in *g*. Bar, 5 μm.

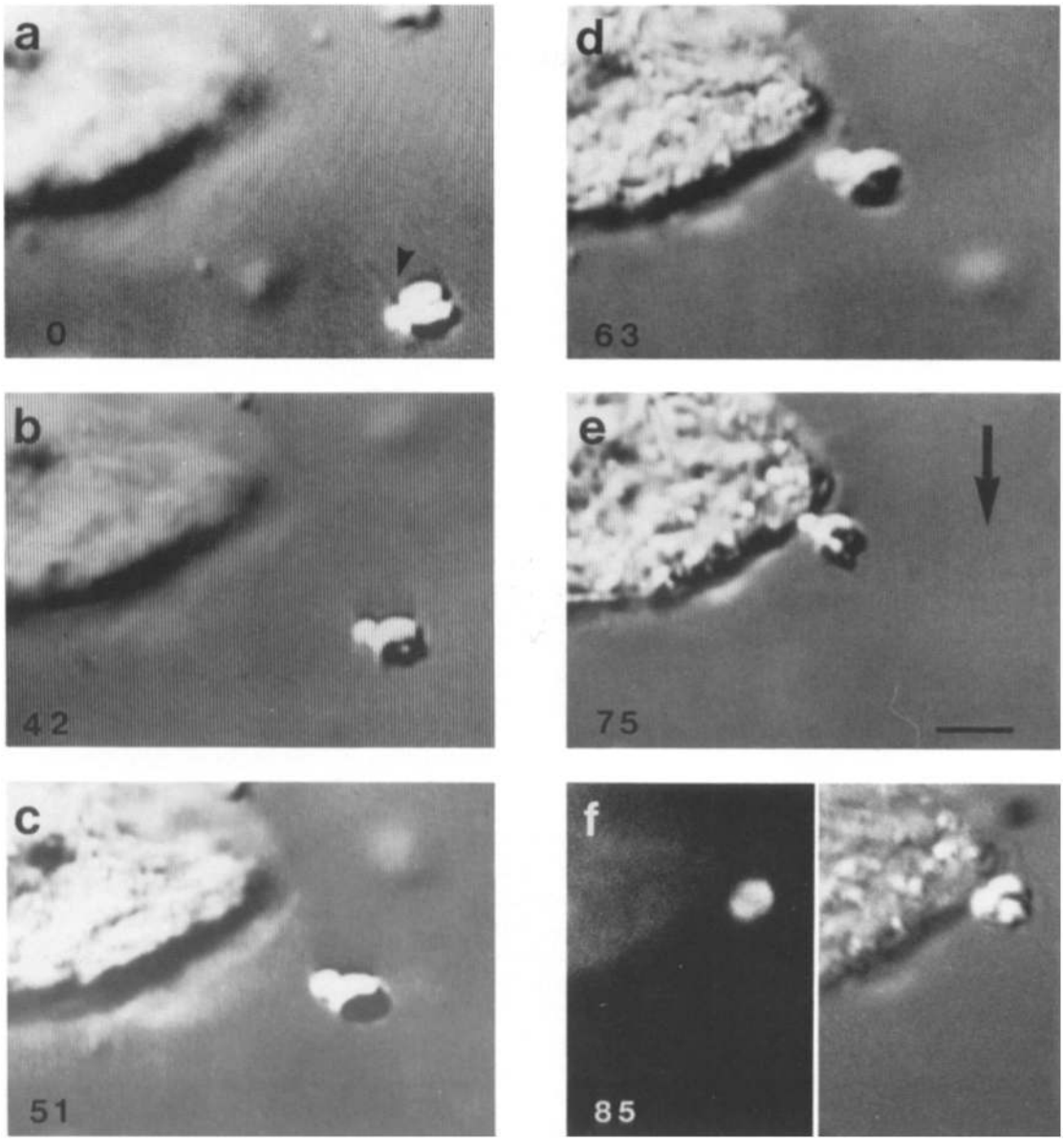


*Figure 3.* Identification of a moving chromosome by staining with Hoechst 33258. Once the chromosome had bound to the pellicle-initiated MTs, it was stained by perfusing PME buffer containing tubulin, GTP, and 1 μg/ml of the DNA dye, Hoechst 33258. The soluble tubulin in the chamber was then diluted to initiate MT depolymerization and chromosome movement. *a* shows the beginning of movement (t = 0), which occurred 60 s after the onset of dilution. The chromosome is associating with a single visible MT fiber through what appears to be its primary constriction (*arrowhead*). *b–e* were taken at 42, 51, 63, and 75 s after movement began, respectively. *f* shows the Hoechst staining of the chromosome in parallel with its DIC image, recorded 10 s after the end of movement (t = 85). The direction of buffer flow is indicated by an arrow in *e*. Bar, 5 μm.

The precise nature of the interaction between pellicle-initiated MTs and the objects attached to them was not easy to define, partly because of the limited resolution of DIC optics and partly because of the structural complexity of the objects involved. The visualization of MTs in solution is limited by the narrow depth of field in a DIC image and the random orientation of the MTs relative to both the optical axis and the direction of DIC shear. Our images indicated, however, that when particles or chromosomes first attached to the MTs, they would often interact with the MT wall. MTs frequently appeared to make lateral contact with and extend beyond the bound objects (Figs. 2 *a* and 4 *a*). The particles usually interacted with a single visible fiber, which may have been composed of one or several MTs (Fig. 2 *a*). The chromosomes, on the other hand, tended to make several types of contacts with MTs, binding at both the primary constriction (Fig. 3 *a*) and the arms (Fig. 4 *a*).

### *Particle and Chromosome Movement in Association with the Depolymerization of MTs*

Shortly after the flow of tubulin-free buffer was introduced into the perfusion chamber, the MTs started to depolymerize. The velocity of buffer flow was measured by the speed of the particles that continually detached from the pellicles

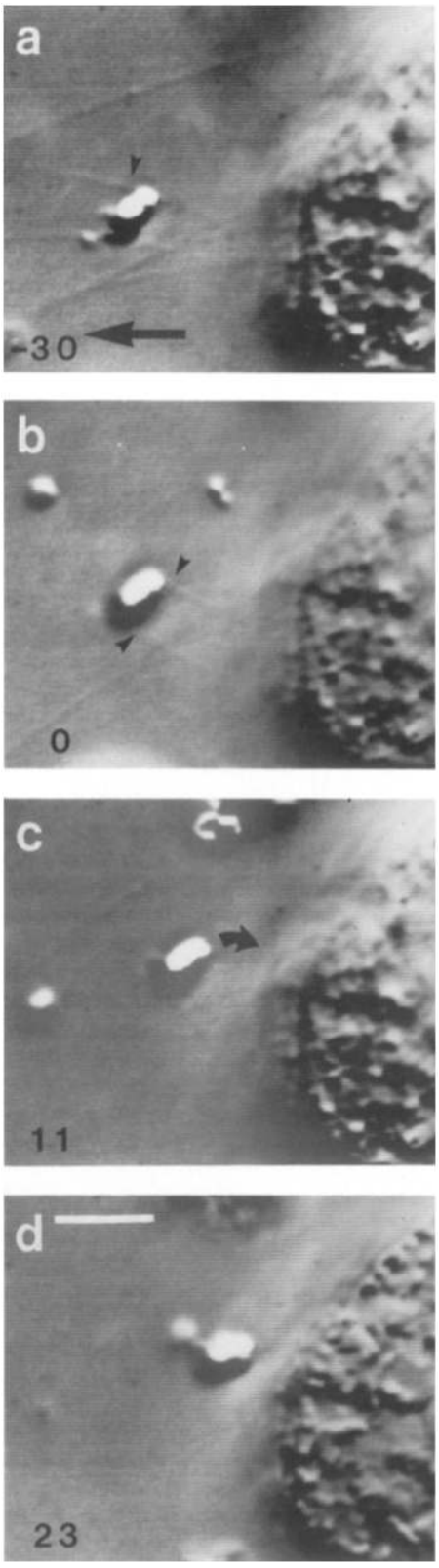


*Figure 4.* Chromosome movement in association with the depolymerization of MTs that are bound to the arms of a chromosome. *a* shows the preparation before dilution of the soluble tubulin. The arms of the chromosome appear to associate laterally with at least two MT fibers that extend beyond the chromosome (*arrowheads*). Movement starts in *b* (t = 0) when the depolymerizing ends of the MT fibers reach the chromosome. The upper MT fiber appears to depolymerize faster than the lower and thereby dominate in establishing the direction of movement. This results in a tilting of the chromosome during movement (*c* and *d*). The time in seconds is shown on each panel. The direction of the buffer flow is indicated by a large arrow in *a*. Bar, 5 μm.

and streamed past the field of view. Flow speeds ranged from 33 to 116 μm/s in different experiments. Approximately 30% of the objects captured by pellicle-initiated MTs were released and washed away in this flow, while the rest remained bound to the MTs. This binding either stabilized the MTs against subsequent depolymerization (~20%) or resulted in the movement of the bound objects toward the pellicle as the MTs shortened (~50%) (Figs. 2–4). In the absence of added polyamines, a fraction of the chromosomes (~60%) in some of our preparations lost their MT attachment during movement.

The movements of MT-associated objects in our system began 30–60 s after solute dilution by perfusion. By this time two to three chamber volumes of buffer without tubulin and nucleotide triphosphate had passed through the chamber. Our isotope dilution experiments suggest that by this time the concentrations of solutes that were absent from the rinse buffer had been reduced 100–1,000-fold in the chamber (see Materials and Methods). For example, after washing with two chamber volumes the soluble tubulin concentration should have been $\leq 0.23$ μM. The initial lag in movement that we observed probably represented the time necessary to lower the tubulin concentration enough to promote MT depolymerization. In the cases where MTs extended beyond the object bound to them, this lag probably also reflected the time necessary for the shortening MT end to reach the object.

Most of the particles and chromosomes that moved traveled distances of several micrometers, usually all the way to the pellicle surface. Once they reached the pellicle, most (~70%) of the objects either bound to the pellicle surface or remained very close, presumably attached to the pellicle by stable MT stubs too short to detect. Some objects (~30%) lost their MT attachment after moving to the pellicle and washed away in the flow.

Although each object moved at an approximately constant rate for a distance of several micrometers (Figs. 5 and 6), the speed varied considerably from object to object. The mean velocity of particles was 26 ± 20 μm/min, while chromosomes moved 15 ± 12 μm/min (see Fig. 7 for the velocity histograms). Objects attached to the same pellicle generally moved at similar rates (Fig. 5). Some objects (36% of those that moved) paused during movement for 1–30 s (e.g., Figs. 5 *b*, 6, *a* and *b*). When particles resumed movement after a pause, their speed was either the same as that before the pause or slightly slower. 25% of the moving objects changed speed without a detectable pause at some time during their movement (e.g., Fig. 6 *a*).

### *Concentration of Soluble ATP during Movement*

Although the movements described above were all seen without the addition of ATP, it was possible that the components of our assay contained sufficient ATP to contribute to the activities of motor enzymes that may be present in the preparations. We, therefore, measured the ATP concentration of each component of the system with a bioluminescent ATP as-

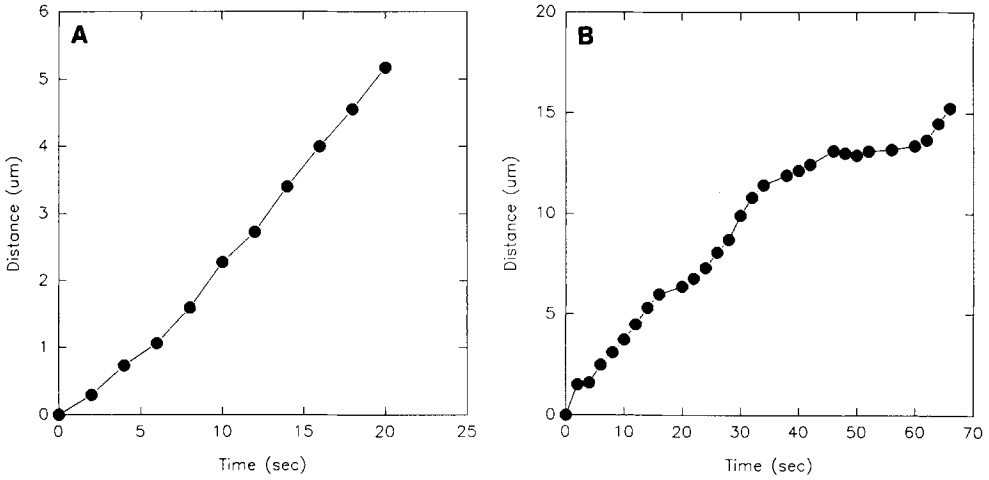


*Figure 5.* Graphs depicting the movement of two particles. The distances traveled for particles 1 and 3 in Fig. 2 were plotted versus time. The speed of particle 1 (*a*), as determined by linear regression with these data, was 15.9 μm/min (r = 0.991). Particle 3 moved the first 12 μm at 18.5 μm/min (r = 0.986), then paused for 15 s before continuing toward the pellicle at a rate of 19.3 μm/min (r = 0.970).

say. The GTP used to promote MT polymerization was contaminated with 0.05% ATP, so the ATP levels in the perfusion chamber at the time of object attachment were ~0.5 μM. ATP was not detectable in the pellicle preparations with the bioluminescent assay, so this component of the assay would have contributed only picomolar levels of ATP (or less) before tubulin dilution. Four out of five chromosome preparations contained measurable amounts of ATP when assessed prior to diluting them to the chromosome concentrations used in the assay. The highest measured ATP concentration in a chromosome preparation was 100 nM, so as added to the pellicles in our system, this preparation would have contained 10 nM ATP. We asked whether this ATP was tightly bound to the chromosomes, or whether it would have been diluted in the perfusion washes, by sedimenting the chromosomes from their isolation buffer at 12,000 *g* for 8 min at 4°C, resuspending them in PME buffer, and measuring the ATP in both the supernatant and the resuspended pellet. After centrifugation, all of the ATP was recovered in the supernatant, suggesting that this nucleotide was free to equilibrate with the perfusion buffers. Therefore, at the time of particle and chromosome movement the highest ATP concentration present in our experiments was probably between 0.5 and 5.0 nM.

To obtain evidence on whether these low levels of ATP could be contributing to the activities of motor enzymes in our system, we compared the movements described above for particles and chromosomes with those seen after treatments designed to specifically remove ATP. In seven experiments, we observed that the addition of 10 U/ml of apyrase had no effect on the velocity and frequency of particle and chromosome motions (Fig. 8, *a* and *b*). This concentration of apyrase reduces the ATP concentration from 100 μM to below the sensitivity of the bioluminescent assay ($10^{-12}$ M) in <10 s. Since we incubated the chromosome preparation in apyrase for 3 min before adding it to the system and apyrase was present in the PME buffer during tubulin dilution, the ATP concentration was probably well below that needed for the action of known motor enzymes. In five of the experiments described above in which apyrase was added, we also examined the effects of sodium orthovanadate, a potent inhibitor of MT minus end-directed motor enzymes, on the motions seen with our system. There was no detectable effect on either the rate or the frequency of particle and chromosome movements (Fig. 8, *a* and *b*) after we incubated the chromosome preparations for 3 min in PME buffer containing 100 μM sodium orthovanadate and kept the same concentration of vanadate in the dilution buffer during movement.

### *Evidence for the Site of MT Depolymerization during Movement*

The site for MT depolymerization during movement was de-

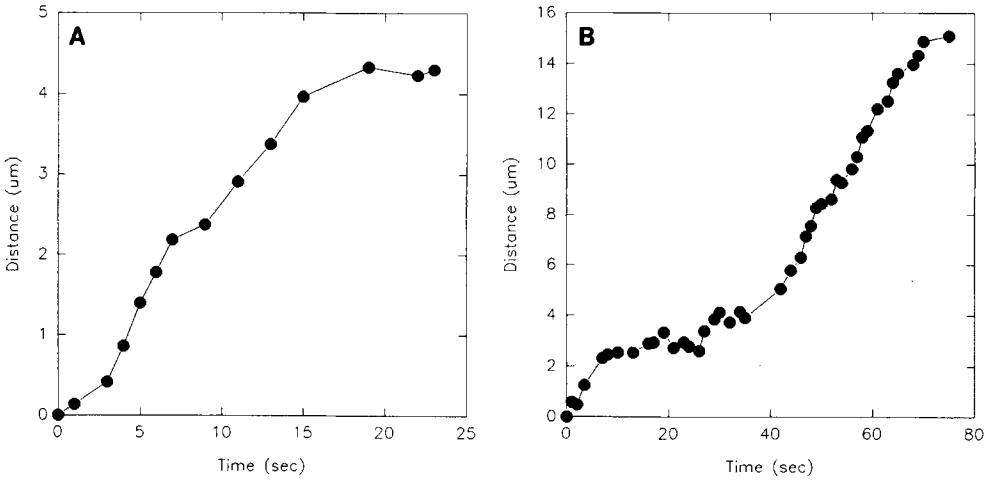


*Figure 6.* Graphs of chromosome movement. The chromosome shown in Fig. 4 moved linearly over a distance of 2 μm at a rate of 26 μm/min (r = 0.996), paused for 1 s, and resumed movement at a speed of 15.7 μm/min (r = 0.998), until it almost reached the pellicle (*a*). The chromosome in Fig. 3 was interrupted by a pause of ~30 s during movement (*b*). In this case, the speed from 0 to 8 s was 18.5 μm/min (r = 0.976) and from 42 to 75 s was 19.7 μm/min (r = 0.981).

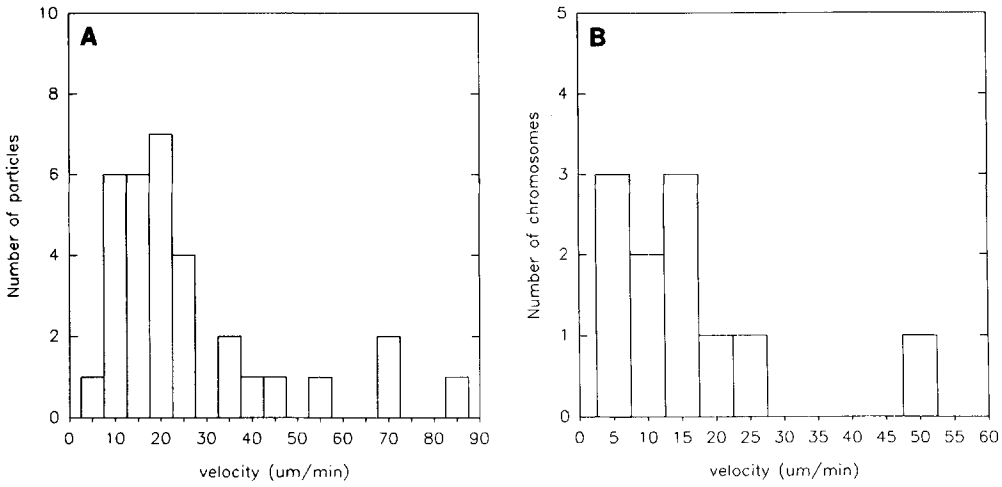


*Figure 7.* Histograms of particle (*a*) and chromosome (*b*) speeds. Movement of bound particles and chromosomes was induced by diluting the soluble tubulin with PME buffer plus 0.1 mM spermidine and 0.05 mM spermine at ~25°C. The mean velocity for particles was 26 ± 20 μm/min (N = 32) and for chromosomes was 16 ± 13 μm/min (N = 11). Under a different set of conditions, where the dilution buffer contained 0.75 mM spermidine and 0.375 mM spermine and its temperature was ~10°C, the mean velocities for particles and chromosomes were 52 ± 24 μm/min (N = 8) and 40 ± 25 μm/min (N = 29), respectively (data not shown).

termined indirectly. We used EGS to fix the short MTs that resulted from a brief exposure of pellicles to a high concentration of tubulin, as described in Materials and Methods. These MT stubs were elongated by the addition of more tubulin, and particles were then perfused into the chamber to bind to the hybrid MTs. When the tubulin was diluted to induce the depolymerization of the hybrid MTs, the particles moved normally, but they stopped when they reached the boundary between fixed and unfixed MTs (Fig. 9). Ultimately, all the objects that had moved to the EGS-stabilized MTs washed away with the flow. This experiment suggests both that MT depolymerization was necessary for motion and that the MT plus end was the active end for subunit loss.

## *Discussion*

We have described an in vitro system that allows the visualization of chromosome and particle movements in association with the depolymerization of MTs. By nucleating MT polymerization from an ordered array of basal bodies, we have obtained a set of MTs with known polarity (Heidemann and McIntosh, 1980) whose minus ends are mechanically fixed. With DIC optics we have been able to follow, in real-time, both the polymerization reactions of added tubulin and the subsequent attachment of objects prepared from mitotic cells. We have then watched the behavior of the bound objects during MT depolymerization. While some of the objects wash away in the buffer flow, many remain attached to the pellicle-initiated MTs and move as the polymers shorten. Such movement can occur even against a comparatively rapid flow of buffer, suggesting that the system is able to develop a substantial force for movement.

Several lines of evidence indicate that the in vitro particle and chromosome movements described above were a direct result of MT depolymerization. These movements occurred only after sufficient tubulin dilution to promote MT depolymerization. They occurred concomitant with the shortening of MTs, as seen by DIC microscopy. The attached objects did not move over EGS-fixed MTs, even under depolymerization conditions. Both particles and chromosomes moved at approximately constant speeds over distances of several micrometers, as would be expected for motions driven by an end-dependent reaction, like MT depolymerization.

The concentration of nucleotide triphosphates at the time

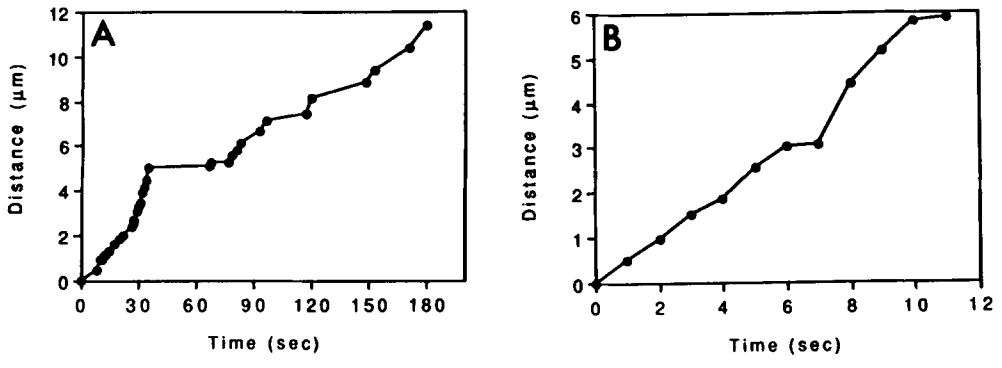


*Figure 8.* Graphs of particle and chromosome movement in the presence of apyrase and vanadate. The distances moved by a particle (*a*) and a chromosome (*b*) in the presence of 10 U/ml apyrase and 100 μM vanadate were plotted as a function of time. *a* depicts the movement of a particle that traveled for 35 s at a rate of 8.1 μm/min (r = 0.932), paused for 41 s, and then resumed movement towards the pellicle at a rate of 3.0 μm/min (r = 0.977). The chromosome used for the graph in *b* moved linearly to the pellicle surface at a rate of 34.0 μm/min (r = 0.981). With apyrase and vanadate added to the system, all objects moved at velocities within the range of those under normal conditions (see Fig. 7).

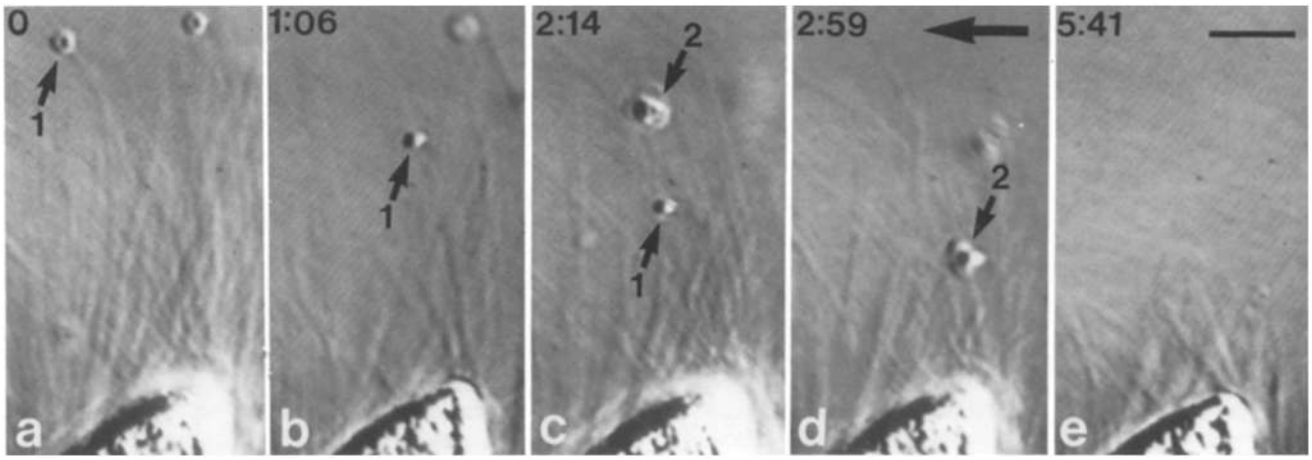


*Figure 9.* Motion of particles during the depolymerization of MTs with stabilized minus ends. Pellicles were decorated with short MTs and fixed with EGS, as described in Materials and Methods. These stubs were elongated with phosphocellulose-purified tubulin, and chromosomes and particles were perfused into the chamber and allowed to attach to the MT array. 74 s after the onset of tubulin dilution, particle 1 began to move (t = 0, Fig. 8 *a*). It moved to the EGS-fixed portion of the MTs (Fig. 8, *b* and *c*) and then washed away. Particle 2 began moving later (Fig. 8 *c*), moved to the region of stable MTs (Fig. 8 *d*), stayed there for 3 s, and then it too washed away (Fig. 8 *e*). The MTs that remain associated with the pellicle at 5:41 in Fig. 8 *e* represent the stable EGS-fixed population. Bar, 5 μm.

and place of movement is a critical issue in assessing the possibility that motor enzymes might contribute to the motions seen in our system. In all of our experiments, no ATP was added to the system at any time. 1 mM GTP was present both when the MTs were polymerized and as the chromosomes were added to the system, but it was absent from the buffer used to effect MT depolymerization. Since we perfused two to three chamber volumes to reduce the tubulin concentration, the measured dilution factor of ~10-fold per chamber suggests that the GTP concentration during movement was 1–10 μM. The two minus end–directed, MT-dependent motor enzymes currently known, dynein (reviewed in McIntosh and Porter, 1989) and "*claret* segregation protein", the product of the *Drosophila NCD* locus (Walker et al., 1990; McDonald et al., 1990), both require ATP for motility. The residual GTP is therefore unlikely to be of importance for the observed motility.

The measured levels of ATP in the various components of our assay system add up to 5 nM or less at the time that we observed movement. Considering that the Km for mammalian brain cytoplasmic dynein is 15 μM (Shpetner et al., 1988), it seems unlikely that this nucleotide contamination could provide the energy for the movement we have seen. Furthermore, the movements were unaffected by the presence of 10 U/ml of apyrase, an ATPase and ADPase that effectively reduces ATP concentrations from the micromolar range to below picomolar levels within seconds. One could argue that molecules of ATP may have been bound to the attached objects extremely tightly, and therefore we could not have detected this possible contamination with the ATP assay that we used. Such bound ATP would serve as energy for only one round of hydrolysis, and therefore would not be likely to support the movements that we observe which occur over distances of several micrometers. Finally, objects moved in the presence of 100 μM vanadate, a concentration that inhibits both of the known minus end–directed, MT-dependent motor enzymes. We conclude that the movements of particles and chromosomes described here are unlikely to be the result of ATP hydrolysis by a mechanoenzyme; the most likely alternative source of fuel for movement is the energy derived from the depolymerization of MTs.

Our evidence suggests strongly that MT depolymerization in our system resulted from subunit loss at the plus ends of the polymers. Structural work has shown that the MTs emanating from Tetrahymena basal bodies are oriented with their plus ends distal to the pellicle (Heidemann and McIntosh, 1980). Although MTs could, in principle, grow from the minus ends of the basal bodies on the far side of the pellicle and extend through the near side of the pellicle to introduce polymers of the opposite polarity, direct observation has shown that such arrangements occurred infrequently, if at all (Heidemann and McIntosh, 1980). Since the minus ends of the pellicle-initiated MTs were always bound to basal bodies, they would be unlikely to serve as sites for tubulin dissociation. Furthermore, the mean velocities of particle and chromosome movements seen (26 μm/min and 16 μm/min, respectively) were similar to the rates of rapid shortening for MT plus ends in vitro (27 μm/min in Walker et al., 1988). Finally, the observation that labile MTs grown from the plus ends of EGS-fixed MT stubs can move particles or chromosomes as they depolymerize is difficult to reconcile with the hypothesis of minus end–dependent depolymerization.

Several factors may have contributed to the observed variability in particle velocity. Interactions between multiple MTs bundled into a single, phase-dense fiber could have modulated the overall rate of MT depolymerization. The release of tubulin from depolymerizing MTs might have caused local fluctuations in the concentration of soluble tubulin, even in the presence of a continuous flow of (tubulin-free) buffer, and thereby affected the instantaneous rate of MT depolymerization. The differences in perfusion flow velocity from one experiment to another also may have provided different amounts of viscous drag, causing variation in the velocities of pellicle-directed movements. Also, the connections between chromosomes and multiple MT fibers

seen in Fig. 4 could have influenced the apparent rate of movement, as several fibers may have been pulling the chromosomes in different directions. These factors may also explain, in part, the pauses we observed during movement. Because the pauses seen in our assay occurred relatively frequently, we think that they are probably of a different nature from the infrequent pauses described by Walker et al. (1988) during the rapid shortening of MTs in vitro.

Our observations suggest that the free energy released by MT depolymerization after subunit dilution is sufficient to do work on objects that remain attached to depolymerizing MTs. The transduction of depolymerization energy into force can be understood with a model in which subunit loss from the MT end biases the diffusion of an object bound to the polymer. The observation that dynein plus ADP-vanadate can couple MTs to glass, yet permit them to move in the presence of vanadate ions by what looks like one-dimensional diffusion (Vale et al., 1989), suggests that a dynein-like molecule may contribute to the dynamic MT binding that we see. In this model, dynein would serve as a coupling factor, not a motor in the conventional sense, since the concentration of ATP that is present at the time of movement seems too low for motor action. Note, however, that a coupling factor used in this way is actually one component of the motor since it interacts with MTs in a way that transduces the chemical energy of MT depolymerization into a biased diffusion that results in mechanical work. The localization of cytoplasmic dynein to the kinetochores of mammalian chromosomes (Pfarr et al., 1990; Steuer et al., 1990) is consistent with this enzyme playing a coupling role for chromosomes in vivo, but we do not yet know whether the vesicles and particles that move in our system are also associated with dynein. Furthermore, some of the chromosome–MT interactions in our system seem to involve not only the kinetochores, but also the chromosome arms, which show no evidence of dynein binding. At present, we have no information to assess the relative strengths of MT–arm vs MT–kinetochore interactions in vitro. In vivo, the interactions between kinetochores and spindle fibers dominate the behavior of chromosomes but other kinds of MT–chromosome interactions can be important (e.g., Reider et al., 1986). Experiments with our system to compare the relative strength of these different types of MT–chromosome interactions are planned.

The magnitude of the force acting on objects moving in our system can be approximated, because we have observed movement against a flow of buffer. The Stokes' equation suggests that the minimum force exerted on moving objects was $0.35–1.6 \times 10^{-7}$ dynes, depending on the rate of buffer flow in a particular experiment. This force is 2,000–200 times less than the maximum force a spindle can produce during anaphase A (Nicklas, 1983), but it is 3.5–16-fold greater than the force required to overcome viscous drag in the living spindle (Nicklas, 1988).

We do not know the equilibrium constants for GTP- or GDP-tubulin addition and loss during the rapid shortening of MTs, but we can determine the work done per mole of subunits lost in our system. Each subunit lost will shorten a MT just over 0.6 nm, and if the mean force acting during this time is $10^{-7}$ dynes, there must be an energy change of <0.1 Kcal/mole of subunits lost. Since this is less than the average thermal energy per degree of freedom at 37°C, the "MT depolymerization motor" need not be very efficient to account for our observations.

There are some encouraging parallels between the movements seen in our system and those observed in cells. The organization and density of pellicle-initiated MTs in our system is similar to that of the MTs which grow from a centrosome during mitosis (Heidemann and McIntosh, 1980; Roos, 1973). The velocities and characteristics of particle movements seen during MT depolymerization are similar to, though somewhat slower than, the centripetal movements of particles seen on asters (reviewed in Rebhun, 1972). Also, the in vitro chromosome movements in our system resemble the poleward movements of chromosomes in vivo during anaphase A; both are associated with MT depolymerization and occur at a constant rate. Although the motions we see are faster than those seen in anaphase A of most cells, they both appear to be limited by the rate of MT depolymerization (reviewed in Inoue, 1981). Since we are diluting the soluble tubulin concentration almost to zero, while a cell would more likely modify the tubulin association equilibrium constant, the greater speed of chromosome movement in our system is not surprising. Because the minimum force generated by MT depolymerization is substantial, it is plausible that the work produced by this process could contribute to mechanisms of motion in vivo.

There are, at present, distinct limitations to the current value of our system as a model for cell motility. We need to know more about the associations between MTs and chromosomes in our assay before we can speculate with any confidence about its relevance for the motions in cells (e.g., anaphase A). For example, we need to learn whether the kinetochores in our system are the strongest sites of attachment between a chromosome and the MTs with which it associates, as they are in vivo. It will also be important to determine whether cytoplasmic dynein or other kinetochore proteins are essential for the movements seen. Until we can deepen our understanding of the molecules required for these movements, their relevance for understanding cell motility will remain unknown. Nonetheless, our observations, together with those of Miyamoto and Hotani (1988) demonstrate by simple, real-time assays that MT polymerization–depolymerization may serve as a force generator for cellular morphogenesis and motility.

This paper is dedicated to Shinya Inoue whose penetrating insight into both physical and biological processes predicted the phenomenon described here long before it was a practical subject for study.

We are grateful to Mary Morphew and Eileen O'Toole for their help with specimen preparation for EM and to Paul Furcinitti for assisting us with the computer analysis; to the reviewers of the manuscript for their valuable comments; and to Corey Nislow and Curt Pfarr for their critical reading of the manuscript.

This work was supported by a grant from the National Institutes of Health (GM 33787) to J. R. McIntosh and a grant from La Foundation de la Recherche Medicale et la Foundation Philippe to M. Coue who was on leave from the Centre Nationale de la Recherche Scientifique.

Received for publication 24 August 1990 and in revised form 4 December 1990.

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