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Direct experimental evidence for the existence, structural basis and function of astral forces during anaphase B *in vivo*

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Summary

The existence, structural basis and function of astral forces that are active during anaphase B in the fungus, *Nectria haematococca*, were revealed by experiments performed on living cells. When one of the two asters of a mitotic apparatus was damaged, the entire mitotic apparatus migrated rapidly in the direction of the opposing astral forces, showing that the force that accelerated spindle pole body separation in earlier experiments is located in the asters. When a strong solution of the antimicrotubule drug, MBC, was applied at anaphase A, tubulin immunocytochemistry showed that both astral and spindle microtubules were destroyed completely in less than a minute. As a result, separation of the spindle pole bodies during anaphase B almost stopped. By contrast, disrupting only the spindle microtubules with a laser microbeam increased the rate of spindle pole body separation more than fourfold. Taken together, these two experiments show that the astral forces are microtubule-dependent. When only one of the two or three bundles of spindle microtubules was broken at very early anaphase B, most such diminished spindles elongated at a normal rate, whereas others elongated at an increased rate. This result suggests that only a critical mass or number of spindle microtubules needs be present for the rate of spindle elongation to be fully governed, and that astral forces can accelerate the elongation of a weakened or diminished spindle.

Key words: aster, laser microbeam, microtubule, mitosis, spindle.

Introduction

Anaphase of mitosis is the stage during which the two sets of sister chromatids are separated from each other. In most organisms, anaphase is further subdivided into anaphase A in which the chromatids are moved closer to the spindle poles and anaphase B in which the poles are further separated from each other (Inoué and Ritter, 1975).

The mechanisms by which anaphase B is achieved is a matter of current interest and controversy. According to one view, the spindle generates a force that causes its own elongation and thereby pushes the poles apart (Cande *et al.* 1989; McIntosh and McDonald, 1989). In this scenario, the asters would have no role in spindle elongation (Mazia, 1961; McIntosh and McDonald, 1989). This mechanism almost certainly occurs in the many organisms that undergo mitosis in the absence of asters. So far, the majority of the evidence to support such a spindle function in astral mitoses is either indirect or derived from permeabilized cell models and isolated spindles or mitotic apparatuses (MAs) that have been artificially manipulated *in vitro* (Cande *et al.* 1989; Masuda *et al.* 1990; McIntosh and McDonald, 1989). Thus, its relevance to mitotic mechanisms *in vivo* remains to be demonstrated for astral mitoses. However, considerable direct evidence for a pushing force operating during astral mitosis *in vivo* has come from the observations of natural spindle bending (Aist and Bayles, 1991*c*; Bělař, 1929; Cleveland, 1966; von Stosch and Drebes, 1966) and from spindle bending induced by blockage of one spindle pole by a microneedle (Carlson, 1952) or a vacuole (Aist and Bayles, 1991*c*).

Another view – one that has received increasing support over the past several years – is that in astral mitosis the asters pull on the spindle poles during anaphase B and that the spindle has, as a major function, the task of limiting the rate at which the astral forces are allowed to pull the poles apart (Aist and Berns, 1981; Bajer *et al.* 1980; Daub and Hauser, 1988; Girbardt, 1968; Heath *et al.* 1984; King, 1983; Kronebusch and Borisy, 1982). The evidence for this view is largely direct and is based primarily on observations and experiments performed on intact living cells. Thus, it has direct relevance to mechanisms of astral mitosis *in vivo*.

A third possibility is that both astral pulling and spindle pushing contribute to spindle elongation. Recently, Aist and Bayles (1991*c*) demonstrated spindle pushing *in vivo* in *Nectria haematococca*, thus completing (in conjunction with the present report) the necessary evidence that both forces operate during anaphase B in the same organism.

Aist and Berns (1981) conducted the initial laser

microbeam studies that examined the mechanisms of anaphase B in living cells of the fungus, *Nectria haematococca* (anamorph: *Fusarium solani*). They found that when the central spindle was broken by the laser at early anaphase B, the spindle pole bodies (SPBs) separated at three times the rate of SPBs in controls in which the spindle was left intact. This surprising result led them to conclude that cytoplasmic forces, presumably located in the asters, pull on the SPBs and that the spindle governs the rate of SPB separation. However, direct evidence was lacking to show that the newly discovered mitotic force was (i) located in the asters, (ii) mediated by astral microtubules (MTs), and (iii) capable of affecting the rate of spindle elongation. The present sets of experiments were designed and conducted to clarify these points. An abstract of some of this work has been published (Bayles *et al.* 1988).

Materials and methods

General

Cultures of isolate T213 of *Nectria haematococca* Berk. and Br., mating population VI, whose anamorph is *Fusarium solani* f. sp. *pisi* (Mart.) Sacc., were maintained and prepared for microscopic study as before (Aist and Bayles, 1988).

Video microscopy and image processing were performed as described by Hays and Salmon (1990). We used real-time background subtraction, halo suppression and digital contrast enhancement for the original recordings. Entire experiments were recorded on a Sony model VO-5600 U-Matic VCR. The video recordings were then reprocessed using real-time frame averaging (2–3 frames). Motion analysis, data analysis, and photography from the video monitor were done using the reprocessed videotapes as described previously (Aist and Bayles, 1988), except that instead of using a cross-line generator to make measurements, we used a mouse-driven cursor (see Fig. 7), Imagemeasure 1200 software (Microscience Division, Phoenix Technology, Inc., Seattle, WA), and several custom-made programs for data manipulation. Plots were made using Sigma Plot (Jandel Scientific, Corte Madera, CA) and drawn using an IBM Color Plotter (type 7372).

Aster irradiations

To damage one of the two asters in an MA, we used a frequency doubler to generate UV light at a wave-length of 266 nm from the 532 nm output of the pulsed Nd-YAG nanosecond laser (Berns *et al.* 1981; Tao *et al.* 1988). This wavelength was used because it is especially effective for depolymerizing MTs *in vivo* (Tao *et al.* 1988) and because other wavelengths employed in preliminary trials were ineffectual. To avoid excessive absorption of the UV light by glass, we mounted the cells under quartz coverslips and viewed and irradiated them through quartz optics. Although the use of quartz greatly reduced the image quality, we partially compensated for this loss by image processing. The energy of the laser was adjusted by attenuating it to the point that three rapidly fired pulses would cause the gradual disappearance of an interphase nucleolus when it was the test target. This energy level was ~0.12–0.14 μJ/pulse at the plane of the specimen.

Asters were damaged by firing two pulses into them in rapid succession. A successful experiment was defined as one in which the video replay showed that the laser had been fired directly in front of the SPB and within 1–2 μm of it. Any experiment in which targeting did not meet these criteria was discarded. Irradiated control cells were targeted in the cytoplasm beside one of the SPBs and received two similar laser pulses. Such controls were intermingled each day with aster irradiations to randomize any day-to-day variation that may have occurred.

Spindle irradiations

To irradiate either all or part of a spindle, we used the 532 nm wavelength of a pulsed Nd-YAG nanosecond laser (Berns *et al.* 1981; Hays and Salmon, 1990). This laser produces a train of laser pulses at a nearly constant power level. We set the pulse frequency at 10 pulses s^{-1} and routinely irradiated the target with a train of 5–10 pulses. Sometimes, two or three such pulse trains were required to achieve the desired effect. To adjust the laser to a minimum, yet effective, energy level before irradiating the spindle, we targeted nucleoli in interphase nuclei and attenuated the laser beam until one or two pulse trains would cause a visible change in the appearance of the nucleolus. This energy level was 0.6–4.7 μJ/pulse at the specimen plane.

Criteria for a successful experiment were established in preliminary trials and were verified by video replay immediately after each experiment was done; experiments not meeting these criteria were discarded. For whole-spindle irradiations, we confirmed that the targeting had been accurate, that the irradiation had produced a clear zone in the spindle, and that the severed ends of the spindle moved independently of each other. Typically, one or both of the spindle segments rotated shortly after irradiation, as reported previously (Aist and Berns, 1981). At the beginning of anaphase B in *N. haematococca* the central spindle is often composed momentarily of two or three separate bundles of MTs before these bundles coalesce into a single, large bundle comprising the entire central spindle (Aist and Berns, 1981). For irradiations of only one such bundle of spindle MTs, we confirmed that the targeting had been accurate, that the bundle had been broken, and that the remainder of the spindle was left intact.

Standard MBC treatment

The fungus was grown on slides coated with a thin layer of yeast extract–glucose–Gelrite (YEG/G) medium but with 2% Gelrite (Scott Laboratories, Inc., Fiskville, RI) and 0.1% $MgCl_2$ added. This formulation provided a firmer substratum for the hyphae during the subsequent tubulin immunofluorescence staining procedure. The fungus was treated by applying 150 μl of either 0.5 μg ml^{-1} methyl benzimidazole-2-ylcarbamate (MBC) in 0.25% dimethyl sulfoxide (DMSO) or 0.25% DMSO alone to the slide for 60 s then draining, adding fixative immediately, and processing for tubulin immunofluorescence as described below. This 'weak' MBC solution was the lowest concentration that would give partial MT breakdown in 60 s and was used to demonstrate the potency of MBC as an antimicrotubule drug in *N. haematococca*.

Monitoring MBC effects in vivo

To monitor the effects of MBC on live cells, hyphae were mounted in a Dvorak chamber (Nicholson Precision Instruments, Gaithersburg, MD), and the entire experiment was videotaped. The hyphae were first grown on round Dvorak coverslips onto which 300 μl of YEG/G medium had been pipetted. Before mounting, excess medium was cut away, a cut was made across the remaining block of medium, and the two pieces of medium with hyphae were separated to form a channel 1–2 mm wide. When a selected cell reached mid-anaphase A, either 200 μl of MBC at 10 μg ml^{-1} in 0.25% DMSO or 0.25% DMSO alone was injected into the chamber, parallel to the channel, such that the solution flowed freely through the channel. Only hyphae within 260 μm (2 microscope fields) of the channel were used, to ensure rapid contact of sufficient solution with the hyphae. This 'strong' solution of MBC was used in these experiments to produce rapid and complete MT breakdown and to compensate for dilution of the MBC by its diffusion into the medium. Rates of separation of SPBs were determined as described previously (Aist and Bayles, 1991*c*).

Tubulin immunocytochemistry

After much trial and error, we settled on the following tubulin immunofluorescence staining procedure, which is partially based on that of H. C. Hoch (unpublished) and That *et al.* (1988). The fixative solution contained 3% paraformaldehyde (Electron Microscopy Sciences) in PHEMO buffer (That *et al.* 1988) (60 mM Pipes, 25 mM Hepes, 10 mM EGTA, 2 mM $MgCl_2$, pH 6.9, containing 10% DMSO) and was applied directly to the slide. After a 1 h fixation the slide was drained and PHEMO buffer added. Then

excess medium was cut away leaving a 0.25-inch square containing hyphal tips. A well was made by gluing a small glass ring to the slide with rubber cement. All subsequent solutions were applied and removed with a pipette. The hyphae were rinsed twice, 5 min each, with PHEMO buffer.

Partially degrading the cell wall with enzymes, without destroying the hyphal structure, was the key to the success of the procedure. We used a two-step process. First, 50 mM phosphate buffer, pH 6.0, was applied for 5 min. Novozyme 234 (Novo Nordisk BioLabs, Inc., Danbury, CT) at 5 mg ml^{-1} in 50 mM phosphate buffer, pH 6.0, was applied for 1 min and rinsed immediately with 50 mM phosphate buffer, pH 7.0, to stop the reaction. This was followed by a 5 min rinse with the same buffer at pH 6.0, to prepare for the next enzyme. Chitinase (from *Streptomyces griseus*, Sigma Chemical Co. lot no. 39F-4012, 14.8 units mg^{-1}) at 5 units ml^{-1} in phosphate buffer, pH 6.0, containing 2 mM phenylmethylsulfonyl fluoride (PMSF, a protease inhibitor) was applied for 5 min at 37 °C and for 25 min at 24 °C. This was followed by 3 rinses, 5 min each, with phosphate buffer at pH 7.0. To permeabilize the plasma membrane, the hyphae were treated with Triton X-100 at 0.1 % in the phosphate buffer, pH 7.0 for 10 min, followed by five 3-min rinses with the same buffer.

Primary antibody, YOL 1/34, raised against yeast alpha tubulin (generously provided by Dr J. Kilmartin, Medical Research Council, Cambridge, UK, and later purchased from Accurate Chemical and Scientific Corp., Westbury, NY), was diluted 1:100 in 50 mM phosphate buffer, pH 7.0, applied, and the hyphae were incubated for 2 h at 37 °C. This was followed by 5 rinses, 5 min each, in the same buffer. The specimens were left overnight at 4 °C in the final rinse. The next day the secondary antibody, FITC-conjugated goat anti-rat IgG (Sigma Chemical Co.), diluted 1:100 in phosphate buffer, pH 7.0, was applied and the hyphae were incubated for 1 h at 37 °C, then rinsed 5 times, 5 min each, with the same buffer.

The mounting medium contained 70 % glycerol in 30 mM phosphate buffer, pH 8.3, with 0.1 % *n*-propyl gallate (an antibleach agent) and 1 μg ml^{-1} 4′,6-diamidino-2-phenylindole (DAPI, a DNA-binding fluorescent probe). The fluorescence was viewed with a Zeiss IM35 microscope equipped with an HBO 50 W mercury lamp and the Zeiss filter sets for FITC and DAPI, a 100× Phase-Neofluar objective (NA 1.3), a 16× projection lens and a Hamamatsu C2400–08 SIT video camera. The rest of the image processing system was as described by Aist and Bayles (1988). Images were frame averaged using a setting of 8 frames before videotaping. Photographs were taken either of the microscope image with Kodak T-MAX 400 film or of the video image with Kodak Technical Pan film.

In some cases, after MBC treatment in the Dvorak chamber, the hyphae were fixed and processed for tubulin immunofluorescence. The same fixative solution as described above, but with 6 % paraformaldehyde (to allow for dilution), was injected into the Dvorak chamber ~60 s after MBC injection. The specimens were then removed from the chamber and further processed for immunofluorescence as described above.

Results

General effects of laser irradiations

Although irradiations with a 532 nm wavelength Nd-YAG millisecond laser (Aist and Berns, 1981) induced wall appositions and caused mitochondria in the vicinity to swell, no side-effects were observed in the present study employing the nanosecond laser at the 532 nm wavelength. Furthermore, no dark globule of laser-damaged material was produced as it was before, and instead of the cut ends of the spindle depolymerizing rapidly toward the SPBs (Aist and Berns, 1981), the spindle segments often were retained more-or-less in their entirety through most or all of the SPB separation phase. Therefore, we consider the nanosecond laser to be far superior to the millisecond laser because of the lack of visible side-effects with the former.

With UV laser irradiations, an affected zone 4–6 μm across developed, usually within 10–20 s. In this zone, the mitochondria lost their predominantly longitudinal orientation, began to exhibit rapid Brownian motion, and appeared to become segmented into smaller units that sometimes became swollen. All of these changes, except swelling, may very well have resulted from depolymerization of the MTs in the target area (Howard and Aist, 1977; Howard and Aist, 1980). The use of two laser pulses instead of one allowed us to reduce the laser power to a level below that which would cause the cells to form wall appositions (cf. Aist and Berns, 1981).

All irradiated cells included in the data sets remained alive, and their daughter nuclei appeared normal, as before (Aist and Berns, 1981).

Damaging one of the two asters of an MA

Because breaking the central spindle in the earlier study (Aist and Berns, 1981) resulted in a threefold increase in the rate of separation of the SPBs, it was inferred that a mitotic motor that pulls on the SPBs resides somewhere in the cytoplasm, presumably in the astral regions. To test directly the validity of the inference that this motor is located in the aster, we used a UV laser to damage just one of the two asters in the MA and then monitored the subsequent migration of the entire MA. The rationale was that if such pulling forces are localized to the asters, then by selectively damaging one aster we should be able to weaken it sufficiently to cause the other aster to predominate long enough to pull the MA for a considerable distance in the direction of its forces.

When one of the two asters was damaged by the UV laser in early anaphase B, the entire MA migrated rapidly away from the targeted region (Figs 1 and 2; Table 1). In control cells irradiated in the cytoplasm beside the SPB, there was little or no migration. Both the distance and the rate of migration were much greater (by two- to threefold) when the aster was irradiated than when the cytoplasm was irradiated (Table 1). After an initial 30–60 s period, several of the targeted asters had apparently recovered, because the direction of MA migration then reversed.

Effects of MBC on the integrity of the MA

MBC is a potent inhibitor of MT polymerization in some fungi, and it has been shown to be highly effective against MTs in another *Fusarium* species (Howard and Aist, 1980). Because we wanted to use MBC to detect a role for astral MTs in the astral pulling forces, we have studied the

Table 1. *Effects of irradiating one aster with a 266 nm wavelength laser microbeam on migration of the mitotic apparatus in* Nectria haematococca

Target	Direction[a] A/T/N (no. of cells)	Distance[b] (μm±1 S.D.)	Rate[c] (μm min^{-1}±1 S.D.)
Aster	9/0/0	4.5±2.0 a	20.5±6.9 a
Cytoplasm	2/1/7	1.9±0.9 b	7.0±3.1 b

Values within a column not followed by the same letter are significantly different ($P \leq 0.01$) by the two-sample *t*-test.

[a] A, away from the target site; T, toward the target site; N, none (migration was <2.0 μm), determined during the first 30 s after irradiation.

[b] Measured during the first 30 s after irradiation.

[c] Measured during the first 30 s after irradiation, but including only the time during which the mitotic apparatus was in motion.

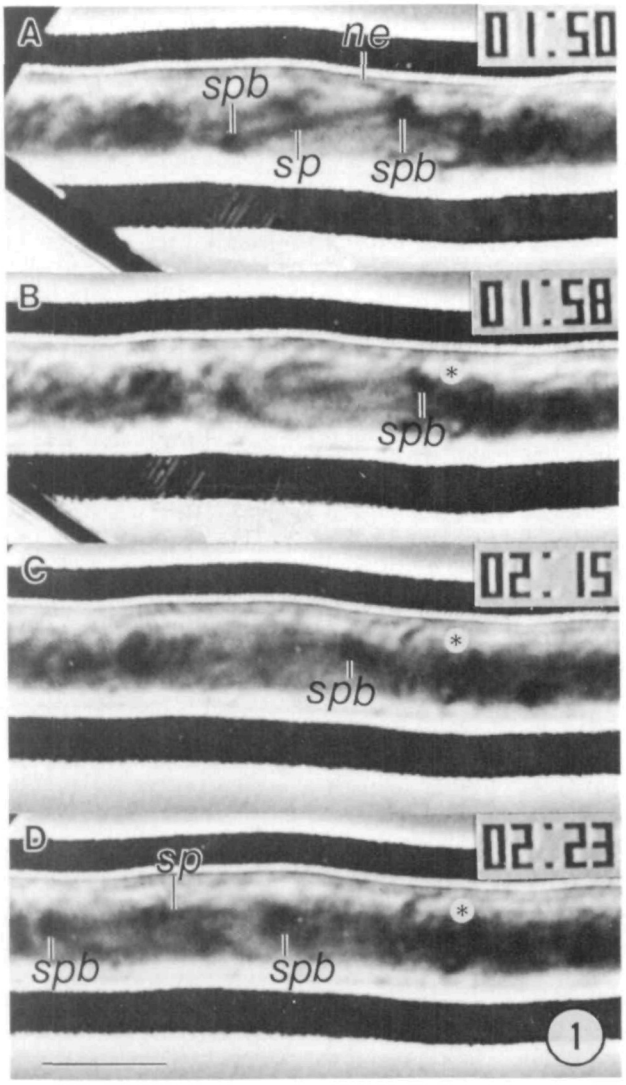

Fig. 1. Phase-contrast video micrographs of an experiment in which one aster in *Nectria haematococca* was irradiated with two rapid-fire pulses from a 266 nm laser beam. (A) The nucleus at early anaphase B, immediately before the laser was fired. The nuclear envelope (ne), spindle (sp) and spindle pole bodies (spb) are visible. (B) Taken during firing of the invisible, 266 nm UV laser shots (asterisk). In (C) and (D), the mitotic apparatus moves farther and farther away from the target site. Elapsed time (in min:s) is shown in the upper right corner of each frame. Bar, 5 μm.

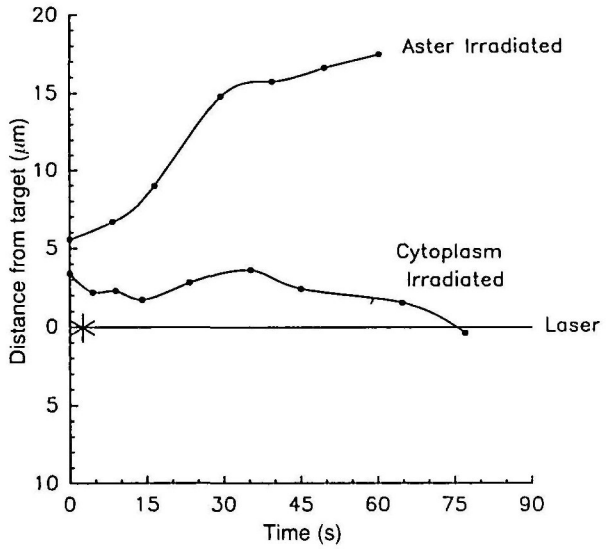

Fig. 2. Plots of the migration (relative to the site of laser irradiation at the asterisk) of aster-irradiated and cytoplasm-irradiated mitotic apparatuses in *Nectria haematococca*. In either case, the target received two rapid-fire pulses from a 266 nm UV laser at time 0. Note that when the aster was irradiated, the mitotic apparatus moved rapidly away and far from the target site; whereas, when the cytoplasm beside the SPB was irradiated, the mitotic apparatus moved slowly and relatively little.

ability of MBC to destroy the MA in *N. haematococca*. Fig. 3 shows the MTs at various stages of mitosis. In untreated control cells, the metaphase spindle is relatively short and thick with some MTs splaying out to the sides, and there are no astral MTs (Fig. 3A). Bundles of spindle MTs are often prominent during anaphase A, and astral MTs are evident (Fig. 3C). Throughout anaphase B (Fig. 3E,G), the astral MTs remain prominent. A weak solution of MBC is enough to cause considerable disruption of the spindle in only 1 min (Fig. 3B, D, F, H). At late anaphase B, just the length of spindle that is enclosed in the envelope of the incipient daughter nucleus may sometimes survive this treatment (Fig. 3H).

Table 2 summarizes data from a larger sample of mitotic cells treated in this way. In the vast majority of cells not treated with MBC, both the aster and the spindle were complete, whereas in none of the MBC-treated cells were both asters and spindles complete. Asters were more sensitive to the treatment, being absent in 70% of the cells, while spindles were absent in 35%. Moreover, spindles were complete in 25% of the MBC-treated cells, but none of these cells had complete asters.

Effects of MBC versus *breaking the spindle on the rate of SPB separation*

To determine whether or not the astral MTs are a necessary component of the astral pulling mechanism, we used MBC to destroy the astral MTs and determined the rate of SPB separation during the ensuing anaphase B. Because MBC treatment affects both the astral and spindle MTs, we included a set of experiments in which the central spindle was broken at early anaphase B by laser microbeam irradiation. The rationale was as follows: breaking the spindle will inactivate only the spindle, leaving the astral MTs intact, whereas MBC will destroy MTs in both the spindle and the asters; the difference in SPB separation rates in the two sets of experiments will therefore be attributable to the presence of intact astral MTs in the laser-irradiated cells.

A typical experiment is illustrated in Fig. 4. A strong solution of MBC was injected into the Dvorak chamber at mid-anaphase A (Fig. 4B), and the central spindle was still visible a few seconds later, at early anaphase B (Fig. 4C). Within the next few seconds the central portion of the spindle disappeared, and only short segments of it persisted in the incipient daughter nuclei during the remainder of anaphase B (Fig. 4D, E). Meanwhile, there was only a very slight further separation of the SPBs (Fig. 4C–F). Oscillation of the mitotic apparatus (Aist and Bayles, 1988) and lateral movements of the incipient

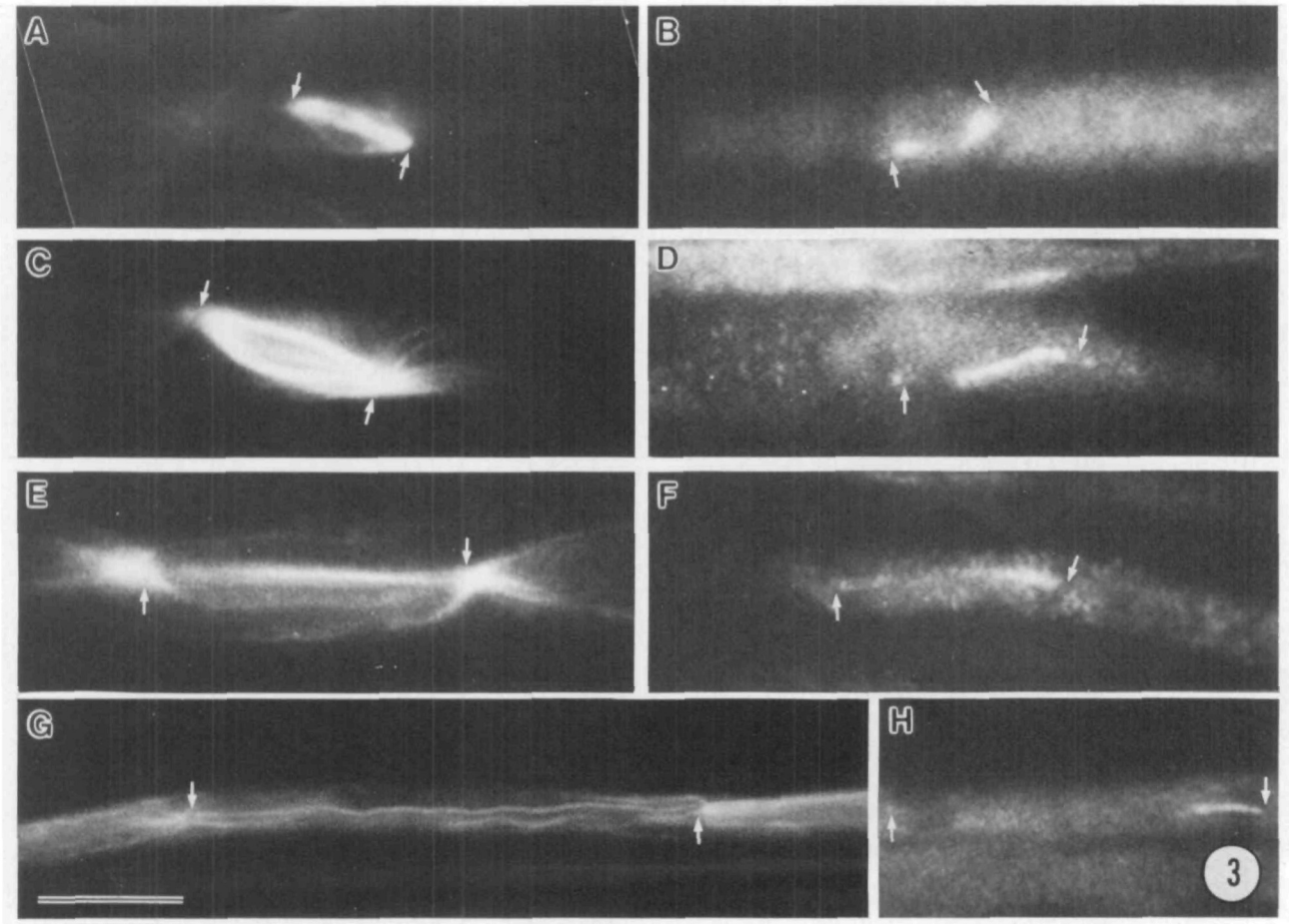

Fig. 3. Microtubules in the mitotic apparatus of *Nectria haematococca* with (B,D,F,H) or without (A,C,E,G) a weak (0.5 $\mu g\,ml^{-1}$) MBC treatment before fixation and tubulin immunocytochemistry. The approximate locations of the spindle pole bodies are shown by the arrows. At metaphase (A), most of the microtubules form a relatively short, thick central spindle between the spindle pole bodies, whereas others are more divergent. Astral microtubules are absent. During anaphase A (C), several bundles of spindle microtubules are apparent, as are astral microtubules. The asters remain prominent throughout anaphase B (E,G) as the central spindle elongates. After a 60 s treatment, the asters in the MBC-treated cells have been severely degraded or destroyed and the spindles are discontinuous and distorted, signs of advanced degradation. Metaphase (B), anaphase A (D), mid-anaphase B (F) and late anaphase B (H) are shown. The late anaphase B figure (H) has a spindle remnant within the incipient daughter nucleus at the right. Note that there is a diffuse, punctate localization of tubulin in the MBC-treated cells.

Table 2. *Effects of a weak solution of MBC on microtubules of the mitotic apparatus in* Nectria haematococca *at anaphase B, as visualized by tubulin immunofluorescence*

Condition of aster/spindle	Control[a] (%)	DMSO[b] (%)	MBC[c] (%)
Complete/complete	98	86	0
Partial[d]/complete	2	8	25
Partial/partial[e]	0	4	4
Absent/complete	0	0	24
Absent/partial	0	0	12
Absent/absent	0	2	35
	(n=65)[f]	(n=50)[f]	(n=72)[f]

[a] Specimens were fixed without any treatment.
[b] Specimens were fixed after 60 s in 0.25 % DMSO.
[c] Specimens were fixed after 60 s in 0.5 $\mu g\,ml^{-1}$ MBC in 0.25 % DMSO.
[d] An aster was judged to be partial when it was clearly reduced in size relative to asters in control cells.
[e] A spindle was judged to be partial when segments of it were missing.
[f] Results are from at least three separate experiments conducted on different days.

daughter nuclei (Aist and Bayles, 1991*c*) were both severely inhibited by this strong MBC solution.

Fig. 5 presents graphically a typical set of experiments. Injection of distilled water or DMSO (controls) had no effect, and the SPB separation rates were normal. Injection of a strong MBC solution almost stopped SPB separation within 20 s and it remained almost nil thereafter.

We repeated these experiments ten times and compared the results with those from breaking the spindle with a laser microbeam (Table 3). The spindle remained intact and the SPB separation rate was normal in the untreated, distilled water-treated and DMSO-treated controls. In the MBC-treated cells, five of the ten spindles vanished rapidly – before mid-anaphase B – following injection. Two others vanished gradually, disappearing completely by late anaphase B. And three remained throughout anaphase B, but only as an unusually thin thread of low contrast. One of these residual spindles became greatly bent (not shown) and remained so for more than 30 s of anaphase B. These diminished, persistent spindles had no apparent effect on the SPB separation rate, which was

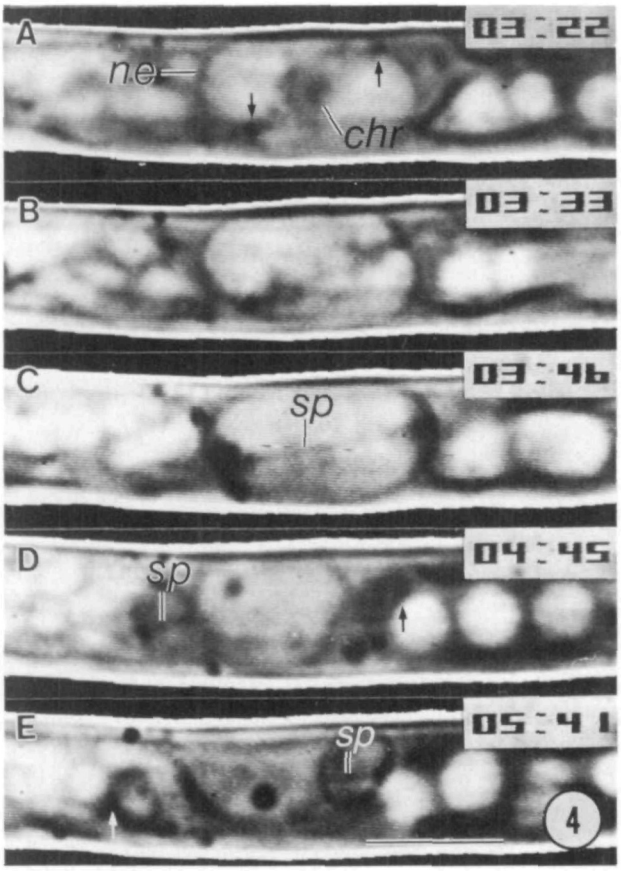

Fig. 4. Phase-contrast video micrographs from an experiment in which a strong solution (10 $\mu g\,ml^{-1}$) of MBC was injected into the Dvorak chamber at anaphase A. Spindle pole bodies are visible at the arrows and elapsed time (in min:s) is shown at the upper right corner of each frame. The nucleus progressed from metaphase (A) into anaphase A (B), when injection was performed. (C) The fading central spindle (sp) at early anaphase B, just a few seconds after injection. The entire central two-thirds of the spindle was gone by mid-anaphase B (D), and only short segments of it survived within the incipient daughter nuclei (D, E). Although the incipient daughter nuclei did not separate fully, nucleoli developed within them at the usual time. chr, chromosomes; ne, nuclear envelope. Bar, 5 μm.

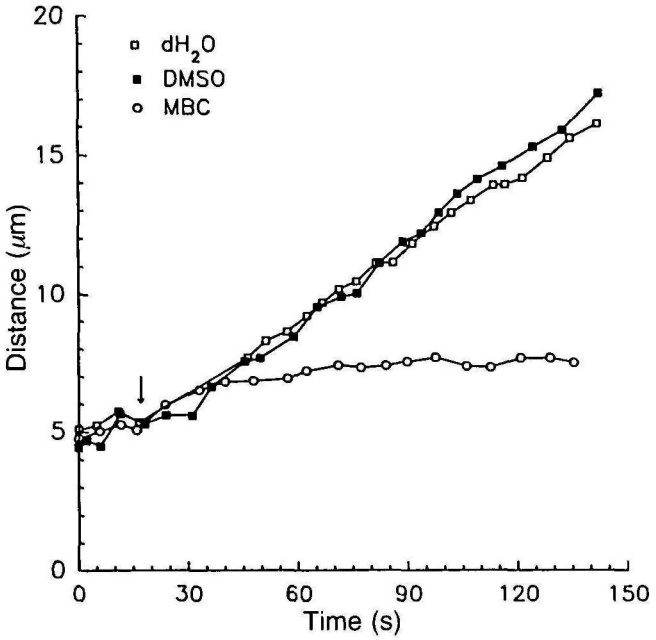

Fig. 5. Plots of spindle pole body separation in representative experiments in which either a strong (10 $\mu g\,ml^{-1}$) MBC solution or a control solution was injected into the Dvorak chamber at anaphase A (arrow). The control injections (distilled water or DMSO) permitted normal rates of separation, whereas MBC almost stopped separation within 15–20 s.

Table 3. *Effects of a strong solution of MBC and of breaking the central spindle with a laser microbeam, on the condition of the spindle and separation of spindle pole bodies during anaphase B in* Nectria haematococca

Treatment (n)	Spindle	Rate ($\mu m\,min^{-1}$)±1 S.D.[a]
Control[b] (7)	Intact	5.4±1.7 (a)
dH_2O[c] (10)	Intact	6.0±1.6 (a)
DMSO[d] (11)	Intact	5.2±1.3 (a)
MBC[e] (10)	Degraded	0.6±0.3 (b)
Laser[f] (9)	Broken	27.4±11.4 (c)

Specimens were treated and videotaped while growing in a Dvorak chamber.

[a] Values not followed by the same letter (a, b or c) were significantly different ($P \leq 0.001$) by the two-sample t-test.

[b] No treatment was applied.

[c] Distilled water was injected into the chamber at anaphase A.

[d] 0.25 % DMSO was injected into the chamber at anaphase A.

[e] 10 $\mu g\,ml^{-1}$ MBC in 0.25 % DMSO was injected into the chamber at anaphase A.

[f] The central spindle was broken by laser irradiation at early anaphase B.

reduced by an order of magnitude by the MBC treatment (Table 3). In contrast, breaking the central spindle with the laser microbeam while leaving the astral MTs intact increased the rate of SPB separation more than fourfold. We confirmed that injection of a strong MBC solution does rapidly destroy the MTs of the mitotic apparatus, using tubulin immunocytochemistry (Fig. 6).

Breaking only one of the two or three bundles of spindle MTs

During anaphase A and very early anaphase B there are often two to three discrete bundles of MTs comprising the central spindle (Aist and Berns, 1981). In the present set of experiments, we purposely broke only one bundle to see if we could upset the balance of forces between the spindle and the asters, and show an effect of astral forces on the spindle elongation rate. The rationale was that if astral pulling forces can contribute to elongation of the central spindle, then we should see an increase in the spindle elongation rate when we weaken the spindle by breaking part of it. When one of the bundles of spindle MTs was broken by the 532 nm laser at very early anaphase B (Fig. 7), the subsequent rate of SPB separation was usually unaffected (Fig. 8). However, in three of nine such experiments there was an increase in the subsequent SPB separation rate (Figs 8 and 9). This increase was beyond the range of rate increases that occurred either naturally in unirradiated cells or in nucleoplasm-irradiated controls during very early anaphase B. By comparison, in only one of the ten whole spindle-irradiated nuclei did the rate of SPB separation remain within the normal range, and the range of separation rates was much greater than when only one bundle was broken (Fig. 8).

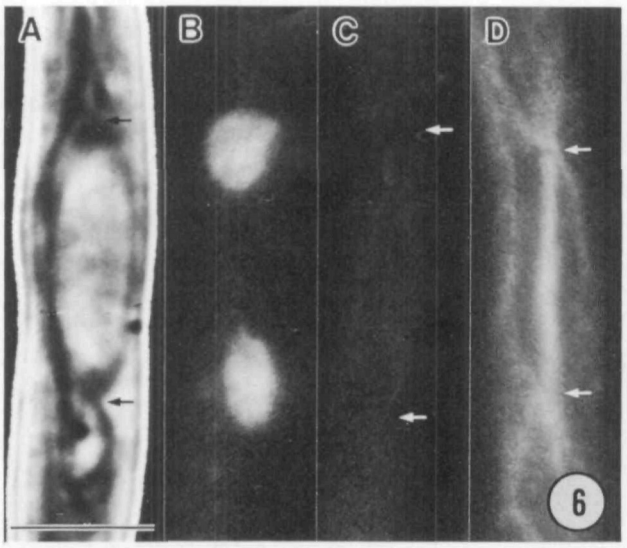

Fig. 6. Light micrographs illustrating the rapid destruction of microtubules of the mitotic apparatus of *Nectria haematococca* by 10 $\mu g\,ml^{-1}$ MBC injected into the Dvorak chamber at anaphase A. The approximate locations of the spindle pole bodies are indicated by the arrows. (A) The mitotic nucleus at mid-anaphase B, ~60 s after injection and ~5–10 s before fixation. Note that the central spindle is not visible. The incipient daughter nuclei of the same nucleus are shown with DAPI fluorescence in (B) and tubulin immunofluorescence in (C). Note that microtubules are absent and that a diffuse, punctate fluorescence is present instead. (D) The microtubules in an untreated control at the same stage, for comparison. Bar, 5 μm.

Discussion

The results of purposely targeting and damaging one of the two asters of an MA using a UV laser have provided the first direct, conclusive evidence that these cytoplasmic forces are localized in the astral region. Laser damage to one aster apparently reduced its pulling force on the associated SPB, thereby allowing the undamaged aster to predominate for a considerable period of time and to pull the entire MA in the direction of its forces. Failure of the cytoplasm-irradiated controls to respond in a similar way confirms the localization of the cytoplasmic forces to the astral region and eliminates the possibility that the results of irradiating one aster are due to nontarget effects.

Because the 266 nm wavelength of laser light can be expected to damage a wide range of proteins in the target

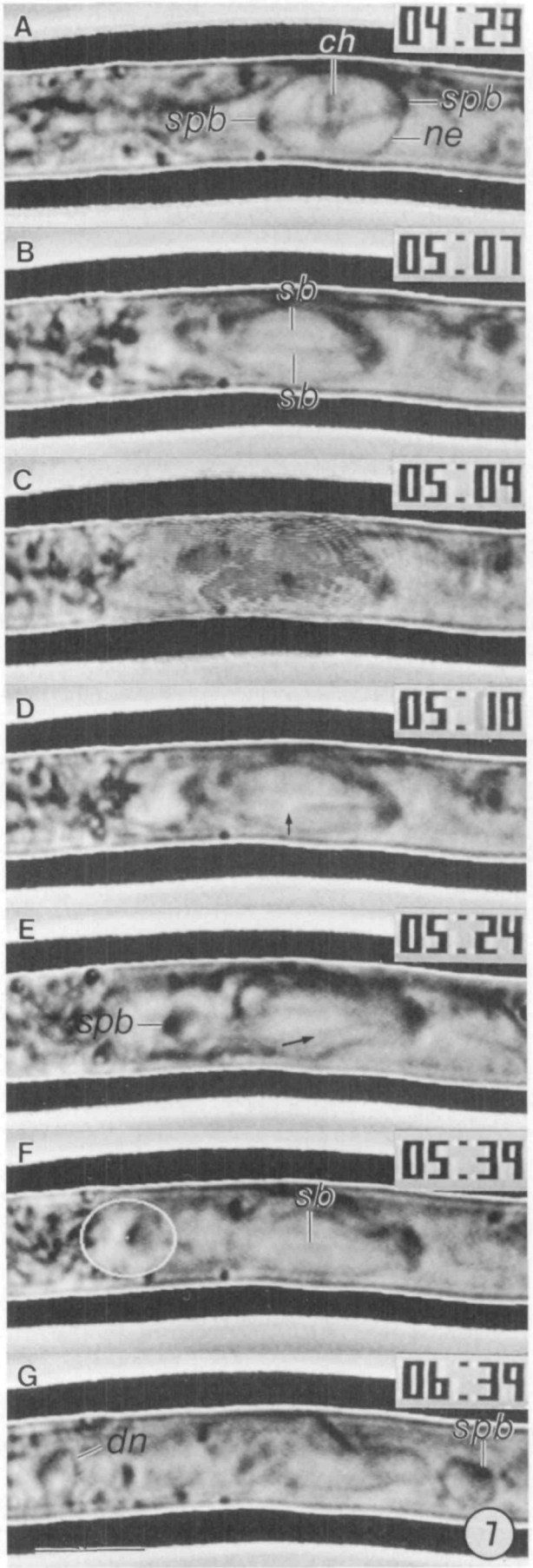

Fig. 7. A time-lapse series of phase-contrast video micrographs showing the laser microbeam irradiation and breaking of a bundle of spindle microtubules (sb) and illustrating how measurements were made. (A) Metaphase, showing the chromosomes (ch), nuclear envelope (ne) and spindle pole bodies (spb). (B) Early anaphase B, showing two bundles of spindle microtubules. (C) Irradiation of the lower bundle with the 532 nm laser microbeam. (D) Immediately after irradiation; the arrow points to the gap in the lower spindle bundle. (E) The broken spindle bundle (arrow) pointing away from the opposite SPB. (F) Mid-anaphase B, showing the cursor used to record the location of the SPBs. The coordinates of the single (white) pixel in the center of the circle were input to a computer file. The unbroken spindle bundle is still visible. (G) Early telophase, showing the young daughter nuclei (dn). Bar, 5 μm.

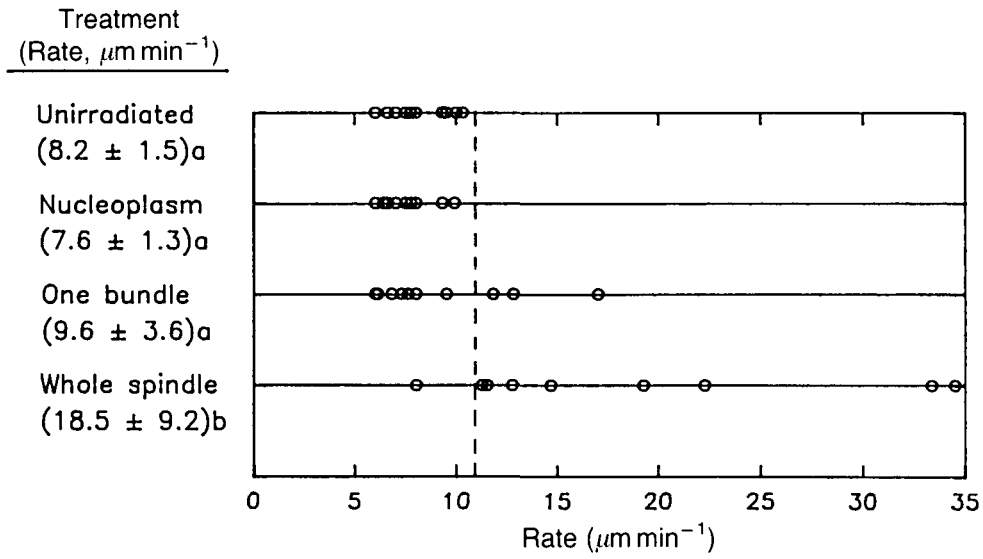

Fig. 8. A graph showing the linear distributions and averages (±1 S.D.) of individual SPB separation rates for four treatments. Values not followed by the same letter (a or b) are significantly different ($P<0.05$) by the nonpaired *t*-test. The broken line represents the upper limit of the normal range for SPB separation rates, as exemplified by both the unirradiated and the nucleoplasm-irradiated controls. Breaking the whole spindle increased the range of rates obtained about sixfold and moved all but one rate to above the normal range, whereas breaking only one bundle of spindle microtubules produced an intermediate range (> a twofold increase) and moved three of the nine rates to above the normal range.

area, it seems likely that several components of the astral motility system (e.g. MTs and motor molecules) were affected. Therefore, this approach cannot be used to demonstrate a role for specific components of the motility system, but it can and did show where it is located.

The effects of irradiating asters of *Nectria*, a fungus, were similar to those of irradiating asters in animal cells. Hyman (1989) used a laser microbeam to destroy astral MTs in the nematode, *Caenorhabditis elegans*, and doing so interfered with astral migration. Hiramoto *et al.* (1986) were able to control the direction of aster migration in sand dollar eggs by irradiating the aster asymmetrically; the aster always moved in the direction associated with the larger array of astral MTs. Similarly, Bajer *et al.* (1980) found that when asters were naturally asymmetric, the centrosome moved in the direction of the most extensive astral rays.

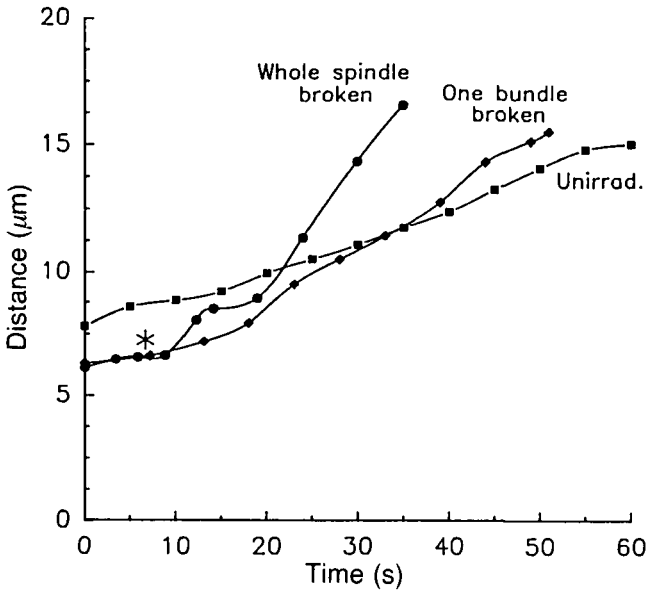

Fig. 9. Plots of SPB separation comparing the effects of breaking the whole spindle, one bundle of spindle microtubules and none of the spindle (unirradiated) with a 532 nm laser (asterisk) at early anaphase B in *Nectria haematococca*. In three of nine experiments in which one bundle was broken, the rate of SPB separation increased to a rate intermediate between the other two, as shown.

The finding that one aster can move an entire MA and that it can do so at rates averaging 20 μm min^{-1} suggests that the aster can build up a very considerable pulling force in *Nectria*. This is true also for the marine worm, *Chaetopterus*, in which the MA in an oocyte can be pulled by one aster through the cytoplasm at an average rate of 45 μm min^{-1} (Lutz *et al.* 1988). One could be led to speculate that if such large forces are tugging at the spindle poles and are capable of moving them at such high velocities, any pushing force that the spindle is capable of generating may be largely superfluous *in vivo*. This speculation is further supported by the finding that, in living cultured newt cells, the spindle poles separate faster and farther when interzonal MTs are absent than when they are present (Bajer *et al.* 1980).

Our tubulin immunofluorescence protocol is apparently very faithful in preserving and visualizing the mitotic apparatus in *N. haematococca*, since our images of them in control cells agreed closely with our reconstructions from freeze-substituted cells (Aist and Bayles, 1991*a*,*b*; Jensen *et al.* 1991). The results following the weak MBC treatments confirm that MBC is a potent inhibitor of MT polymerization in *N. haematococca*. The partial-to-complete destruction of most mitotic apparatuses in only 1 min at 0.5 μg ml^{-1} MBC illustrates the utility of MBC in studying the role of astral and spindle MTs in mitosis in this fungus. Thus, the injection of a 20-fold greater concentration of MBC into the Dvorak chamber would be expected to destroy the MTs of the mitotic apparatus within seconds, and this result was confirmed by both video microscopy of living cells and tubulin immunofluorescence. Injection of 10 μg ml^{-1} MBC during anaphase A effectively disrupted the structural integrity of both the asters and the spindle early in anaphase B.

Taken together, the results of disrupting both spindle and asters using MBC and of disrupting only the spindle using the laser microbeam, show that the astral forces are dependent specifically on astral MTs. In both experiments the central spindle is inactivated by disruption of its component MTs. But only with MBC treatment were the astral MTs disrupted. Thus, the huge reduction in the SPB

separation rate (from 27.4 to 0.6 $\mu m\,min^{-1}$) can be attributed to the lack of astral MTs in the MBC experiment, thereby revealing the role of the intact MTs in transmitting astral forces to the SPBs in the laser microbeam experiment. That MBC neutralized the astral forces was confirmed also by the severe inhibition of both oscillations of the mitotic apparatus and lateral movements of the incipient daughter nuclei that occurred in living, control cells.

The results of breaking only one of the bundles of spindle MTs were unexpected in that the SPB separation rate was usually unaffected (Fig. 8), even though in each case it was confirmed by video replay that a bundle had been broken and that the other bundle(s) remained intact. We infer that the undamaged, complete spindle has more than enough MTs to generate sufficient counterforce to maintain the rate of SPB separation within the normal range, because destroying the pole-to-pole continuity of one-third to one-half of them usually had no effect on the rate. However, in three of the nine experiments the rate was clearly increased. Taken together, these results suggest that only a critical mass or number of spindle MTs – not the whole spindle – need be present and active in order for the SPB separation rate to be fully regulated. Perhaps in the three exceptional cases this critical number was no longer present after one bundle had been broken, and the residual spindle allowed the SPBs to separate at an accelerated, yet still-regulated, rate. This result and interpretation are in agreement with those of King (1983), who found that as the number of spindle MTs at anaphase B in yeast was reduced naturally to two, the rate of spindle elongation held constant at 0.36 $\mu m\,min^{-1}$, and that a marked increase (to 4.16 $\mu m\,min^{-1}$) occurred when the number of spindle MTs was further reduced from two to one. Similarly, Hays and Salmon (1990) found that the poleward force at the kinetochore in metaphase of grasshopper spermatocytes depends on the number of MTs in the kinetochore fiber. Alternatively, since breaking the whole spindle produced a very wide range of SPB separation rates (Fig. 8), there was apparently also a wide range in the magnitude of force generated by asters in different cells. Thus, breaking only one bundle may have allowed an increase in the SPB separation rate only in those cells where the astral force was larger than average.

Our result (Figs 8 and 9) shows that the astral pulling force in *Nectria* can increase the rate of spindle elongation when the spindle counterforces are experimentally weakened at early anaphase B. In the later stages of anaphase B the number of MTs in the central spindle becomes substantially diminished by natural causes (Aist and Bayles, 1991*b*), and the asters may help to maintain the constant spindle elongation rate that occurs to the end of this stage (Aist and Bayles, 1988).

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References

Aist, J. R. and Bayles, C. J. (1988). Video motion analysis of mitotic events in living cells of the fungus *Fusarium solani*. *Cell Motil. Cytoskel.* **9**, 325–336.

Aist, J. R. and Bayles, C. J. (1991*a*). Ultrastructural basis of mitosis in the fungus *Nectria haematococca* (sexual stage of *Fusraium solani*). I. Asters. *Protoplasma* **161**, 111–122.

Aist, J. R. and Bayles, C. J. (1991*b*). Ultrastructural basis of mitosis in the fungus *Nectria haematococca* (sexual stage of *Fusarium solani*). II. Spindles. *Protoplasma* **161**, 123–136.

Aist, J. R. and Bayles, C. J. (1991c). Detection of spindle pushing forces *in vivo* during anaphase B in the fungus *Nectria haematococca*. *Cell Motil. Cytoskel.* **19**, 18–24.

Aist, J. R. and Berns, M. W. (1981). Mechanics of chromosome separation during mitosis in *Fusarium* (Fungi Imperfecti): New evidence from ultrastructural and laser microbeam experiments. *J. Cell Biol.* **91**, 446–458.

Bajer, A. S., De Brabander, M., Molè-Bajer, J., De Mey, J., Paulaitis, S. and Geuens, G. (1980). Mitosis: the mitotic aster, interzone and functional autonomy of monopolar half-spindle. In *Microtubles and Microtubule Inhibitors* (ed. M. De Brabander and J. De May), pp. 399–425. Elsevier/North-Holland Biomedical Press, Amsterdam.

Bayles, C. J., Aist, J. R., Tao, W. and Berns, M. W. (1988). Astral forces in fungal mitosis. *J. Cell Biol.* **107**, 455a.

Bělař, K. (1929). Beiträge zur Kausalanalyse der Mitose. III. Untersuchungen an den Staubfadenhaarzellen und Blatmeristemzellen von *Tradescantia virginica*. *Z. Zellforsch. mikrosk. Anat.* **10**, 73–134.

Berns, M. W., Aist, J., Edwards, J., Strahs, K., Girton, J., McNeill, P., Rattner, J. B., Kitzes, M., Hammer-Wilson, M., Liaw, L.-H., Siemens, A., Koonce, M., Peterson, S., Brenner, S., Burt, J., Walter, R., Bryant, P. J., van Dyk, D., Coulombe, J., Cahill, T. and Berns, G. S. (1981). Laser microsurgery in cell and developmental biology. *Science* **213**, 505–513.

Cande, W. Z., Baskin, T., Hogan, C., McDonald, K. L., Masuda, H. and Wordeman, L. (1989). *In vitro* analysis of anaphase spindle elongation. In *Cell Movement*, vol. 2, *Kinesin, Dynein, and Microtubule Dynamics* (ed. F. D. Warner and J. R. McIntosh), pp. 441–452. Alan R. Liss, New York.

Carlson, J. F. (1952). Microdissection studies of the dividing neuroblast of the grasshopper, *Chortophaga viridifasciata* (De Greer). *Chromosoma* **5**, 200–220.

Cleveland, L. R. (1966). Reproduction by binary and multiple fission in *Gigantomonas*. *J. Protozool.* **13**, 573–585.

Daub, A.-M. and Hauser, M. (1988). Taxol affects meiotic spindle function in locust spermatocytes. *Protoplasma* **142**, 147–155.

Girbardt, M. (1968). Ultrastructure and dynamics of the moving nucleus. In *Aspects of Cell Motility. XXIInd Symp. Soc. exp. Biol.* (ed. P. L. Miller), pp. 249–259. Cambridge University Press.

Hays, T. S. and Salmon, E. D. (1990). Poleward force at the kinetochore in metaphase depends on the number of kinetochore microtubules. *J. Cell Biol.* **110**, 391–404.

Heath, I. B., Rethoret, K. and Moens, P. B. (1984). The ultrastructure of mitotic spindles from conventionally fixed and freeze-substituted nuclei of the fungus Saprolegnia. *Eur. J. Cell Biol.* **35**, 284–295.

Hiramoto, Y., Hamaguchi, Y., Hamaguchi, M. S. and Nakano, Y. (1986). Roles of microtubules in pronuclear migration and spindle elongation in sand dollar eggs. In *Cell Motility: Mechanism and Regulation* (ed. H. Ishikawa, S. Hatano and H. Sato), pp. 349–356. Alan R. Liss, Inc., New York.

Howard, R. J. and Aist, J. R. (1977). Effects of MBC on hyphal tip organization, growth, and mitosis of *Fusarium acuminatum*, and their antagonism by D_20. *Protoplasma* **92**, 195–210.

Howard, R. J. and Aist, J. R. (1980). Cytoplasmic microtubules and fungal morphogenesis: ultrastructural effects of methyl benzimidazole-2-ylcarbamate determined by freeze-substitution of hyphal tip cells. *J. Cell Biol.* **87**, 55–64.

Hyman, A. A. (1989). Centrosome movement in the early division of *Caenorhabditis elegans*: a cortical site determining centrosome position. *J. Cell Biol.* **109**, 1185–1193.

Inoué, S. and Ritter, Jr, H. (1975). Dynamics of mitotic spindle organization and function. In *Molecules and Cell Movement* (ed. S. Inoué and R. E. Stephens), pp. 3–30. Raven Press, New York.

Jensen, C. G., Aist, J. R., Bayles, C. J., Ballard, S. M. and Jensen, L. C. W. (1991). Ultrastructural basis of mitosis in the fungus *Nectria haematococca* (sexual stage of *Fusarium solani*). III. Intermicrotubule bridges. *Protoplasma* **161**, 137–149.

King, S. M. (1983). A regulatory model for spindle function during mitosis. *J. theor. Biol.* **102**, 501–510.

Kronebusch, P. J. and Borisy, G. G. (1982). Mechanics of anaphase B movement. In *Biological Functions of Microtubules and Related Structures* (ed. H. Sakai, H. Mohri and G. G. Borisy), pp. 233–245. Academic Press, Tokyo.

Lutz, D. A., Hamaguchi, Y. and Inoué, S. (1988). Micromanipulation studies of the asymmetric positioning of the maturation spindle in *Chaetopterus* sp. oocytes: I. Anchorage of the spindle to the cortex and migration of a displaced spindle. *Cell Motil. Cytoskel.* **11**, 83–96.

Masuda, H., Hirano, T., Yanagida, M. and Cande, W. Z. (1990). *In*

vitro reactivation of spindle elongation in fission yeast *nuc2* mutant cells. *J. Cell Biol.* **110**, 417–425.
MAZIA, D. (1961). Mitosis and the physiology of cell division. In *The Cell*, vol. 3 (ed. J. Brachet and A. E. Mirsky), pp. 77–412. Academic Press, New York.
MCINTOSH, J. R. AND MCDONALD, K. L. (1989). The mitotic spindle. *Sci. Am.* **261**, 48–56.
TAO, W., WALTER, R. J. AND BERNS, M. W. (1988). Laser-transected microtubules exhibit individuality of regrowth, however most free new ends of the microtubules are stable. *J. Cell Biol.* **107**, 1025–1036.
THAT, T. C. C. T., ROSSIER, C., BARJA, F., TURIAN, G. AND ROOS, U.-P. (1988). Induction of multiple germ tubes in *Neurospora crassa* by antitubulin agents. *Eur. J. Cell Biol.* **46**, 68–79.
VON STOSCH, H. A. AND DREBES, G. (1965). Der Stemmkörper und andere Besonderheiten der Diatomeenzytoologie. *Naturwissenschaften* **52**, 311–312.

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