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Journal

EXPERIMENTAL MYCOLOGY, 17(3)

ISSN

0147-5975

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Publication Date

1993-09-01

DOI

10.1006/emyc.1993.1018

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The Mechanics of Anaphase B in a Basidiomycete as Revealed by Laser Microbeam Microsurgery

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Accepted for publication April 12, 1993

BAYLES, C. J., AIST, J. R., AND BERNS, M. W. 1993. The mechanics of anaphase B in a basidiomycete as revealed by laser microbeam microsurgery. *Experimental Mycology* 17, 191-199. Cytoplasmic forces were found to be actively pulling on the spindle pole bodies during anaphase B in the dikaryotic, basidiomycete fungus, *Helicobasidium mompa*. When the spindle of one nucleus was severed with a laser microbeam at mid anaphase B, its two spindle pole bodies separated at a much faster rate than did those of the intact spindle in the other nucleus of the same cell. Since astral microtubule populations apparently reach their maximum during anaphase B in this fungus, we suggest that these microtubules may be involved in the cytoplasmic pulling forces. The spindle appears to act primarily as a governor, regulating the rate at which the spindle pole bodies are separated. © 1993 Academic Press, Inc.

INDEX DESCRIPTORS: Mitosis; anaphase B; basidiomycete; aster; microtubule.

Helicobasidium mompa is a dikaryotic, clampless basidiomycete that causes root rot of mulberry and other plants in Asia (Ito, 1949). Light microscopic and ultrastructural studies of mitosis in this fungus were first conducted by Bourett and McLaughlin (1986). Anaphase in *H. mompa*, as in most eukaryotic organisms, is subdivided into anaphase A, during which the chromatids move toward the spindle poles, and anaphase B, which begins when the chromatids reach the poles and during which the poles continue to separate from each other (Inoué and Ritter, 1975). Spindle elongation occurs during anaphase A as well as anaphase B in many organisms, including *H. mompa*. Bourett and McLaughlin (1986) noted that at the anaphase B–telophase transition, the separation rate of the daughter nuclei increased rapidly, and they suggested that the spindle had broken and that forces in the cytoplasm were pull-

ing the spindle pole bodies (SPBs)¹ apart. Their ultrastructural studies showed that astral microtubules (MTs) were present by late metaphase and that their number had increased by late anaphase B, suggesting a role for astral MTs in this pulling force during telophase (Bourett and McLaughlin, 1986).

Cytoplasmic forces were shown earlier to pull on the SPBs during anaphase B in the ascomycete, *Nectria haematococca*, in laser microbeam experiments performed on living cells (Aist and Berns, 1981). Further studies showed that these forces were localized in the asters, were mediated by astral MTs, and were capable of affecting the rate of spindle elongation (Aist *et al.*, 1991). Three-dimensional reconstructions from serial sections displayed an extensive network of astral MTs in this fungus (Aist and Bayles, 1991a). Recent genetic experiments with mutants of the yeast fungus, *Saccharomyces cerevisiae*, have suggested a role for the asters during anaphase B: a mutant without asters produced only a short, thick, unelongated spindle (Berlin *et al.*, 1990), and another mutant, this one without one of

¹ Abbreviations used: SPB, spindle pole body; MT, microtubule; YEG, yeast extract–glucose medium; spn1, the first of the two spindles to break naturally; spn2, the other spindle in the same cell.

the half-spindles but with two full sets of astral MTs, managed to fully separate the two SPBs without a central spindle (Winey *et al.*, 1991). Experimental evidence for astral pulling in several animal species (Bergan, 1960; Daub and Hauser, 1988; Hiramoto *et al.*, 1986; Lutz *et al.*, 1988) suggests that astral forces are common and widespread among species with astral mitosis.

Breaking the central spindle during anaphase B with a laser microbeam is an effective way of demonstrating that cytoplasmic (presumably astral) forces are active during this mitotic stage. To date, this approach has been used to study astral forces in only one organism, *N. haematococca* (Aist and Berns, 1981; Aist *et al.*, 1991). To show that this result is not limited to one organism, we describe here the similar use of the laser microbeam to determine whether cytoplasmic forces pull on the SPBs of a basidiomycete, *H. mompa*, during anaphase B.

MATERIALS AND METHODS

H. mompa Tanaka, isolate CB S 278.51 from cherry (kindly provided by D. J. McLaughlin, Department of Botany, University of Minnesota, St. Paul, MN), was maintained on yeast extract–glucose (YEG) medium (Aist and Bayles, 1988). For light microscopy, the fungus was grown on slides dipped in a modified YEG and maintained in a moist chamber. The modified medium (2% yeast extract, 0.1% glucose, 12% gelatin, and 1% Gelrite) was developed to improve the optical clarity of the fungus. The gelatin increased the refractive index of the medium to match that of the cytoplasm (Girbardt, 1968), thereby decreasing the halo effect of the cell walls and increasing the contrast of phase-dense organelles such as the SPBs. This modified medium had no effect on the actual growth rate; however, hyphae at the colony margin were more sparse.

The clearly visible mitotic apparatus, a

rarity in fungi, and the presence of two synchronously dividing nuclei, which provides a built-in control for each irradiation, were the main reasons for choosing *H. mompa* over many other basidiomycetes that were examined for this study.

Nuclei in the penultimate cells of *H. mompa* generally migrated toward the base of the new branch before dividing; thus, the proximity of these nuclei to the branch could be used as an indicator of their impending mitosis (Bourett and McLaughlin, 1986). However, the movement of the nuclei into the branch during mitosis often precluded a clear view of both mitotic apparatuses. Fortunately, mitosis in the apical cells occurred soon before or after that in the penultimate cells, providing a fairly reliable marker for the onset of mitosis in the apical cells. Only apical cells were used for these experiments.

Phase-contrast optics and a Planapo 100X (N.A. 1.30) objective were used. Video microscopy and image processing were performed at the Beckman Laser Institute and Medical Clinic (Irvine, CA), as described previously (Aist *et al.*, 1991); however, background subtraction was not available for some sequences. Experiments were recorded on a Sony Model VO-5800H U-Matic high-resolution VCR. The video recordings were later reprocessed using real-time frame averaging (two frames), halo suppression, and digital contrast enhancement. Motion analysis, data analysis, and photography were done using the reprocessed videotapes, and final plots were made as described previously (Aist *et al.*, 1991). The apparent accuracy that is achievable with these measurement procedures is approximately $\pm 0.05 \mu\text{m}$ (Aist and Bayles, 1991b).

The spindle of one nucleus was irradiated during mid anaphase B while the other nucleus served as a simultaneous unirradiated control within the same cell. To irradiate the spindle, we used the 532-nm wavelength of a pulsed Nd-YAG nanosecond la-

ser (Berns *et al.*, 1981) as described previously (Aist *et al.*, 1991). The spindle was irradiated with pulse trains of 5 pulses/0.5 s. Usually two or three pulse trains, applied within a few seconds of each other, were necessary to break the spindle. To adjust the laser to an effective energy level before irradiating the spindle, we targeted mitochondria in other tip cells and attenuated the laser beam until one or two pulse trains would cause a slight change in the appearance of the mitochondria.

Criteria for a successful experiment were the same as those that were established previously (Aist *et al.*, 1991), and they were verified in each case by video replay. We confirmed visually that the irradiation had produced a definite clear zone in the spindle and/or that the spindle segments moved independently of each other (Fig. 3), indicating that the spindle was actually broken. Typically, one or both of the spindle segments rotated shortly after the irradiations broke the spindle, as reported previously (Aist and Berns, 1981; Aist *et al.*, 1991). Experiments not meeting at least one of these criteria were discarded. These same criteria were applied to the unirradiated, control nuclei to determine the time at which the spindle broke naturally (Fig. 2). Twelve unirradiated cells and seven irradiated cells were used for the final analyses.

RESULTS

Unirradiated Cells

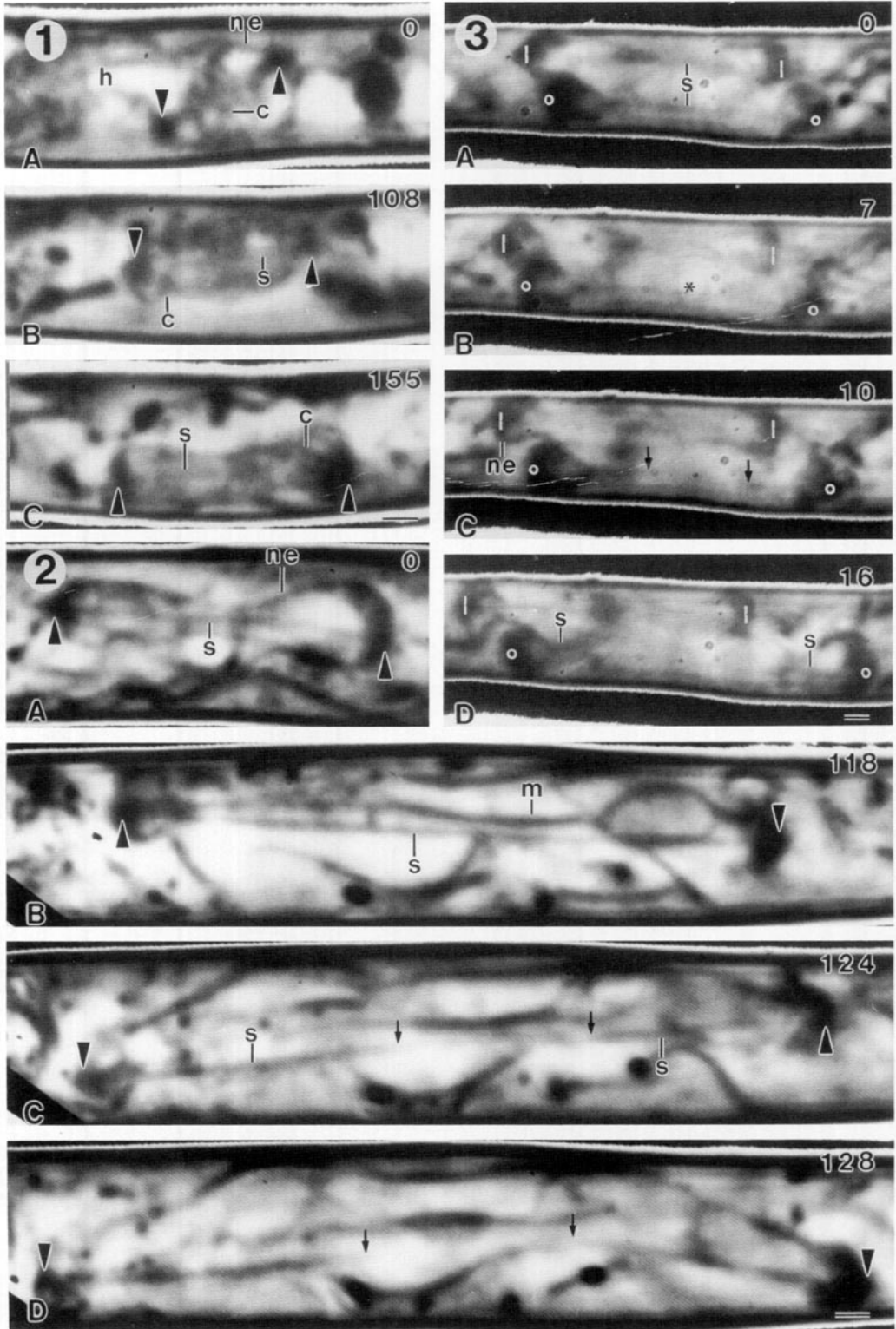
The two nuclei of the tip cells divided synchronously, with one spindle (spn1) often slightly ahead of the other (spn2). During metaphase, the two spindles were often positioned perpendicularly or obliquely within the cell (Fig. 1A). They typically rotated during anaphase A and came to lie side-by-side for anaphase B (Fig. 3A). Anaphase A was characterized by the chromatids appearing less distinct as they moved to the poles (Figs. 1B–1C). Spindle elongation began during anaphase A, and

the two spindles elongated at the same rate (3.7 $\mu\text{m}/\text{min}$) (Table 1) during this phase. Anaphase A lasted for an average of 31 s during which time the spindles increased in length from 6.6 to 8.1 μm .

The accumulation of all the chromatids at the poles signified the transition to anaphase B. The nuclei began to change in shape from oval to dumbbell (Fig. 2A), and by late anaphase B the nuclear envelope had collapsed to the center of the spindle while flaring out and encircling the incipient daughter nuclei at each end. Eventually, the daughter nuclei were pinched off (Fig. 2B), and the intercalary portion of the nuclear envelope disappeared. The rate of spindle elongation increased as anaphase B progressed, averaging 4.2 $\mu\text{m}/\text{min}$ during the first 30 s, a rate not significantly different from that of anaphase A, and increasing to 7.0 $\mu\text{m}/\text{min}$ during the last 30 s, significantly faster than the initial rate (data not shown). The average rate of spindle elongation throughout all of anaphase B was 5.4 $\mu\text{m}/\text{min}$, which was significantly faster than that of anaphase A. Both nuclei followed this pattern of elongation (Fig. 4a).

The spindles increased in length to an average of 16.7 μm during anaphase B (Table 1), and this phase lasted an average of 93 s. There were no significant differences between the two nuclei for these parameters. When one spindle (spn1) reached a length of ca. 17 μm , it was observed to break, and then the rate of separation of its SPBs increased almost immediately to an average of 44 $\mu\text{m}/\text{min}$ (Table 1). We define the beginning of telophase as the time when the spindle was observed to break naturally (Fig. 2C). After the spindle broke, one of the SPBs often moved laterally so that its attached spindle segment no longer pointed toward the other SPB (Fig. 2D). This independent movement of the spindle segments confirmed that the spindle was no longer intact.

The two spindles either broke at nearly the same time, or a lag time of up to 34 s



(average 14 s) occurred between them. During the time of rapid SPB separation of spn1, the intact spindle (spn2) continued to elongate at the significantly slower, average rate of 11 $\mu\text{m}/\text{min}$ (Fig. 4a). When spn2 reached 17 μm in length, it was observed to break, and its SPBs then sped apart at an average rate of 25 $\mu\text{m}/\text{min}$, a rate that was not significantly different from that of spn1.

Irradiated Cells

In the seven irradiated cells, the two spindles elongated at an average rate of 3.7 $\mu\text{m}/\text{min}$ during anaphase A, and the length of the spindles increased from 6.1 to 8.4 μm . There was no difference between the two nuclei in their rates of spindle elongation during anaphase A, and these rates did not differ from those in the unirradiated

cells (Table 1). The nontarget spindle elongated at an average rate of 5.8 $\mu\text{m}/\text{min}$ during anaphase B, similar to that in the unirradiated cells. When the spindle of one nucleus (target) was seen to be broken experimentally with the laser microbeam during mid anaphase B (Figs. 3A–3C), then its SPBs immediately moved apart at an increased rate, 30 $\mu\text{m}/\text{min}$, that was similar to the rate after a naturally occurring break. Importantly, this increased rate of SPB separation never began until the spindle was seen to be broken, even though one or two pulse trains of laser light had already been applied to the spindle. One spindle segment often rotated such that it was no longer pointing toward the other SPB, confirming that the spindle had been severed (Fig. 3d). The irradiated spindles were an average of

FIGS. 1–3. Phase-contrast, time-lapse videomicrographs of mitosis in living hyphae of *Helicobasidium mampa*. Elapsed time (in s) is shown in the upper-right corner of each frame.

FIG. 1. Early stages of mitosis in an unirradiated cell. Arrowheads point to the apparent positions of the spindle pole bodies. A, metaphase. Chromosomes (c) are associated with the middle two-thirds of the spindle, which extends on either side of the chromosome cluster to each spindle pole body. The hole (h) in the nuclear envelope (ne) is characteristic of basidiomycete mitosis. B, early anaphase A. Chromosomes (c) can be seen as they migrate poleward along the spindle (s), and they are especially numerous, in this example, above the spindle. C, later anaphase A. Arms of some chromosomes (c) can be seen extending back from the spindle pole region, while a few chromosomes are still moving poleward, along the spindle (s). Bar, 1 μm .

FIG. 2. Late stages of mitosis in an unirradiated cell. Arrowheads point to the apparent positions of the spindle pole bodies. A, early anaphase B. The spindle (s) is continuous from pole to pole, and the nuclear envelope (ne) is constricted to a narrow isthmus near the middle. B, late anaphase B. Although greatly elongated, the spindle (s) is still intact. A mitochondrion (m) lies close to the spindle, in this example. C, early telophase, just as the continuity of the spindle (s) has been lost near the center, between the arrows. D, early telophase. During just the first 4 s after the spindle broke naturally, the spindle pole bodies separated an additional 2.5 μm . Meanwhile, the spindle pole body to the right migrated laterally (downward, in the figure), leaving its attached spindle segment at an angle, relative to the other spindle segment. This independent movement of the two spindle segments confirms that the spindle had broken naturally. The arrows point to the apparent ends of the spindle segments. Bar, 1 μm .

FIG. 3. Part of anaphase B in an irradiated cell. The two nuclei are dividing in parallel; white bars and circles mark the apparent positions of the spindle pole bodies in the two nuclei, respectively. A, 7 s before irradiation, the two spindles (s) can be seen between their respective spindle pole bodies, lying parallel to each other. B, the lower spindle is being irradiated by the laser microbeam, at the asterisk. C, 3 s after irradiation, the irradiated spindle is discontinuous between the arrows, in the vicinity of the targeted portion. Parts of the nuclear envelope (ne) are visible. D, after the spindle was broken, the spindle pole bodies separated an additional 2.4 μm in just 6 s, whereas those of the unbroken spindle, lying just above, separated only 0.35 μm during the same time period. The two, shortened, remaining segments of the irradiated spindle (s) can be seen; the one on the left has rotated out of parallel with the one on the right, confirming that the spindle was broken by the laser microbeam. Bar, 1 μm .

TABLE 1
Comparisons of Various Parameters of the Two Nuclei in Unirradiated Cells and in Laser
Microbeam-Irradiated Cells of *Helicobasidium mompa*

| Treatment ^a | Rate ($\mu\text{m}/\text{min} \pm 1 \text{ SD}$) of spindle pole body separation during | | | Spindle length ($\mu\text{m} \pm 1 \text{ SD}$) at the time it was broken |
|------------------------|--|---------------------------|--------------------------|--|
| | Anaphase A | Anaphase B | Telophase | |
| Unirradiated | | | | |
| spn1 | $3.4 \pm 1.8 \text{ a}^b$ | $5.4 \pm 0.7 \text{ a}$ | $33 \pm 14 \text{ a}$ | $16.6 \pm 3.4 \text{ a}$ |
| spn2 | $3.9 \pm 2.4 \text{ a}$ | $5.5 \pm 1.5 \text{ a}$ | $25 \pm 6.9 \text{ a}$ | $16.9 \pm 2.3 \text{ a}$ |
| Irradiated | | | | |
| Target | $3.4 \pm 0.3 \text{ a}$ | $5.2 \pm 0.9 \text{ a}^c$ | $30 \pm 8.9 \text{ a}^d$ | $13.3 \pm 1.7 \text{ b}$ |
| Nontarget | $3.4 \pm 0.9 \text{ a}$ | $5.8 \pm 0.7 \text{ a}$ | $35 \pm 13 \text{ a}$ | $17.2 \pm 1.6 \text{ a}$ |

^a Twelve unirradiated cells and seven irradiated cells were used in the analyses.

^b Numbers within a column not followed by the same letter are significantly different ($P < 0.05$) by the two-sample *t* test. All possible pairwise comparisons were made.

^c Rate up until the spindle was broken by the laser microbeam in mid anaphase B.

^d Rate after the spindle was broken by the laser microbeam in mid anaphase B.

13 μm long when they were severed with the laser microbeam, which was significantly shorter than the length (17 μm) of the unirradiated spindles when they broke naturally (Table 1).

After irradiation, while the daughter nuclei of the target nucleus were separating rapidly, the nontarget spindle continued to elongate at an average rate of 6.7 $\mu\text{m}/\text{min}$ for 18–60 s (average 41 s), until it reached a length of ca. 17 μm (Fig. 4b). At this point the nontarget spindle was observed to break naturally, and then its SPBs separated rapidly, averaging 34 $\mu\text{m}/\text{min}$. These rates, which are comparable to those in the unirradiated cells, show that the irradiation did not affect spindle elongation in the nontarget nucleus. The fact that the SPBs of the target nucleus separated at a rate similar to that of a natural break shows that the irradiation affected only the spindle of that nucleus and did not affect extranuclear forces acting on the SPBs. The irradiated cells apparently remained healthy, and the new septum was formed in a timely fashion. Due to the slow growth rate of this fungus, it was not practical to observe the subsequent mitosis in the resultant tip cell.

DISCUSSION

Reliable interpretation of our results depends on accurate identification of the central spindle in the video images and adequate verification that the spindle was broken. Bourett and McLaughlin (1986) established that, during anaphase B, the central spindle of *H. mompa* consists of a long, thin bundle of MTs that appears as a line connecting the two poles in living cells. We verified this *in vivo* appearance of the central spindle and improved upon its visual clarity by video-enhanced, phase-contrast microscopy. Although we did not demonstrate ultrastructurally that we had severed MTs with the laser microbeam in this study, we observed and applied the same *in vivo* criteria for spindle breaking that were confirmed ultrastructurally to be valid for breaking of the central spindle of *N. haematococca* (Aist and Berns, 1981). There seems to be no reasonable interpretation of the video images in the present study, other than that we did, in fact, break the central spindle.

Our data show that breaking the central spindle with the laser microbeam during

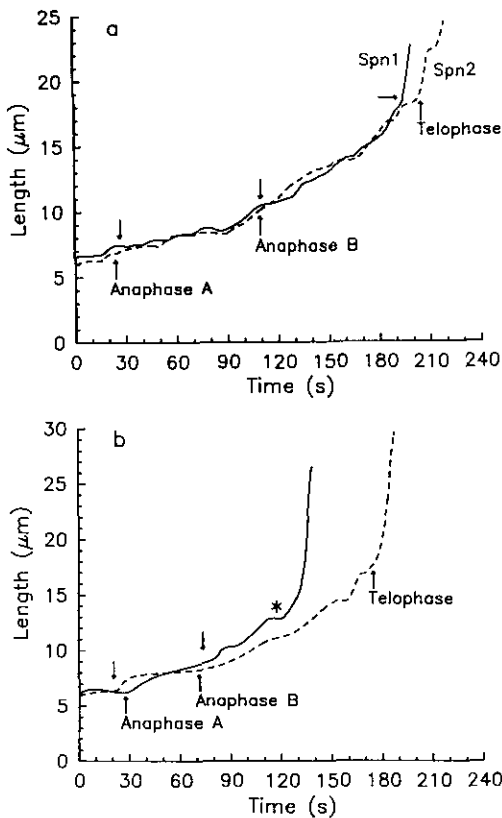


FIG. 4. Plots of spindle elongation and spindle pole body separation of the two nuclei in a representative (a) unirradiated cell and (b) irradiated cell. Data were taken every 3–5 s, and the curves pass directly through all the data points. Arrows indicate the boundaries between the phases of mitosis as identified (independently from the generation of the curves) by direct observation of mitotic events recorded on the videotapes. The asterisk indicates the time of laser irradiation. Rates of spindle elongation often fluctuated; however, some of the undulations in the curves may be due to one or both poles being slightly out of focus momentarily.

mid-anaphase B in *H. mompa* produced the same effect as that of a spindle breaking naturally at late anaphase B; a markedly increased rate of separation of the SPBs. There were no adverse effects on either the target or the nontarget nucleus, and the spindle in the latter continued to elongate and broke naturally in the same manner as did those in the unirradiated cells. These

results show that the forces acting on the SPBs after natural spindle breaking were present already and fully active at mid-anaphase B. These forces may be at least partly responsible for spindle elongation in *H. mompa*, as was shown to be the case for *N. haematococca* (Aist and Berns, 1981; Aist *et al.*, 1991). In *N. haematococca*, the rates of spindle elongation for anaphases A and B were 3.0 and 6.0 $\mu\text{m}/\text{min}$, respectively (Aist and Bayles, 1988), similar to those found here for *H. mompa*. After the spindle was broken with a laser microbeam during early anaphase B, the daughter nuclei of *N. haematococca* separated at an increased rate of ca. 27 $\mu\text{m}/\text{min}$ (Aist *et al.*, 1991), a rate that is comparable to that reported here for *H. mompa*.

In *N. haematococca*, the forces acting on the SPBs were localized to the asters and were shown to be MT-dependent (Aist *et al.*, 1991). Astral MTs in *H. mompa* are present at late metaphase and increase in number during anaphase (Bourett and McLaughlin, 1986). Our present results showed that the rate of separation of the SPBs increased during anaphase. Thus, these astral MTs may be involved in the force that is pulling on the SPBs during anaphase and telophase and could be at least partially responsible for spindle elongation during anaphase. We were unable to visualize either spindle or astral MTs in *H. mompa* using fluorescent antibody methods, despite exhaustive efforts (unpublished results).

The increase in the rate of spindle elongation during late anaphase B relative to early anaphase B in the control cells generally occurred just before the spindle broke, giving the impression that the spindle was slowly being ripped apart. This is in contrast to *Ceratocystis fagacearum*, in which the spindle breaks suddenly and the loose ends actually recoil slightly (Aist, 1969). Both cases, however, suggest that there are cytoplasmic forces pulling on the SPBs.

Thus, the main role of the spindle in astral mitoses may be to limit the rate at which the astral forces are allowed to pull the poles apart. Direct evidence for this role has come from *in vivo* observations and experiments with several species of fungi and animals (Aist *et al.*, 1991; Aist and Berns, 1981; Bajer *et al.*, 1980; Bergan, 1960; Daub and Hauser, 1988; Girbardt, 1968; Heath *et al.*, 1984; Hiramoto, 1986; King, 1983; Kronebusch and Borisy, 1982).

Others have suggested that the spindle generates a force that causes its own elongation and thereby pushes the poles apart (Cande *et al.*, 1989; McIntosh and McDonald, 1989; McIntosh and Pfarr, 1991). Direct evidence for the capacity of the spindle to generate a pushing force in the presence of asters has come from *in vivo* observations of bending or buckling of the spindle when constraints were present on the poles (Aist and Bayles, 1991b; Belar, 1929; Cleveland, 1966; von Stosch and Drebes, 1966). Spindle bending also occurred in *Schizosaccharomyces pombe*, despite the presence of (presumably) unconstrained asters (Tanaka and Kanbe, 1986). And in a mutant of *S. cerevisiae* that lacks asters, the spindle alone proved capable of separating the SPBs and occasionally bent itself into a C-shape in the mother cell in which it was confined (Sullivan and Huffaker, 1992).

It is most likely that both astral pulling and spindle pushing contribute to spindle elongation in some organisms, and this is indeed the case with *N. haematococca* (Aist *et al.*, 1991; Aist and Bayles, 1991b; Aist and Berns, 1981). With *H. mompa*, spindle bending was not observed, but we now have direct evidence of cytoplasmic pulling forces and spindle counterforces both acting on the SPBs during anaphase B. Taken together with the relevant genetic experiments with yeast (Berlin *et al.*, 1990; Sullivan and Huffaker, 1992; Winey *et al.*, 1991), the experimental evidence suggests that astral pulling forces, spindle counter-

forces, and spindle pushing forces may all be common among fungi.

ACKNOWLEDGMENTS

The authors thank Gexin Du, Cornell University, and Steven Cheng, Beckman Laser Institute and Medical Clinic, for technical assistance. This work was supported by NSF Grant DCB-8916338 and Hatch Project NYC-153-332 (to J.R.A.) and by NIH Grants RR 01192 and CA 32248, DOE Grant DE-FG03-91ER61227, and ONR Grant N00014-91-C-0134 (to M.W.B.).

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