

Effects of Mitotic and Tubulin Mutations on Microtubule Architecture in Actively Growing Protoplasts of *Aspergillus nidulans*

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ABSTRACT We used immunofluorescent microscopy to characterize microtubule (MT) architecture in wild-type and mutant protoplasts of *Aspergillus nidulans* at interphase and at mitosis. Because the visualization of MTs by immunofluorescence is technically difficult in intact hyphae of *A. nidulans*, we developed a method for removing the cell wall under conditions that do not perturb cell physiology, as evidenced by the fact that the resulting protoplasts undergo nuclear division at a normal rate and that cell cycle mutant phenotypes are expressed at restrictive temperature. Interphase cells exhibited an extensive network of cytoplasmic MTs. During mitosis the cytoplasmic MTs mostly disappeared and an intranuclear mitotic spindle appeared. We have previously shown that the *benA* 33 β -tubulin mutation causes hyperstabilization of the mitotic spindle, and we have presented additional indirect evidence that suggested that the *tubA1* and *tubA4* α -tubulin mutations destabilize spindle MTs. In this paper, we show that the *benA33* mutation increases the stability of cytoplasmic MTs as well as spindle MTs and that the *tubA1* and *tubA4* mutations destabilize both spindle and cytoplasmic MTs.

A central question in cell biology concerns the function of microtubules (MTs)¹ in chromosomal and organellar movement. To explore this question, our laboratory has developed methods for studying the molecular genetics of tubulin in the simple eucaryote *Aspergillus nidulans*. Using two-dimensional gel electrophoresis of the proteins of mutants selected for resistance to the antimicrotubule fungicides benomyl and thiabendazole (25), we have shown that mutations in the *benA* locus define the structural gene for a pair of β -tubulins designated $\beta 1$ and $\beta 2$ (23). Similarly, by studying two-dimensional gel electropherograms of a pseudorevertant (*benA11R7*) of a temperature sensitive *benA* mutant (*benA11*), we were able to identify a second gene, *tubA*, as the structural gene for a pair of α -tubulins designated $\alpha 1$ and $\alpha 3$ (14). These proteins and two other minor tubulin peptides, $\alpha 2$ and $\beta 3$, which are not products of the *benA* or *tubA* genes, have been

characterized by staining Western blots of two-dimensional gel electropherograms with antitubulin antibodies (J. A. Weatherbee and N. R. Morris, manuscript in preparation). No mutations affecting the electrophoretic mobility of the minor tubulin species have yet been found.

We have used certain phenotypic abnormalities caused by mutations in the *benA* and *tubA* genes to define the functions of their corresponding tubulins in the living cell. We know, for example, that one or both *benA* gene products are involved in mitosis, chromosome movement, and nuclear movement. The evidence is that these processes are inhibited by benomyl in wild-type cells, but the inhibition is prevented by *benA* mutations that confer resistance to benomyl and is intensified by *benA* mutations (originally selected against thiabendazole) that cause hypersensitivity to benomyl (16). A second demonstration that one or both *benA* gene products are involved in both mitosis and nuclear movement was made possible by the isolation of *benA33*, a temperature-sensitive mutation that causes a mitotic block at restrictive temperature (17). In *benA33* mutants, although the mitotic spindle forms normally, it has been demonstrated by electron microscopy that

¹ Abbreviations used in this paper: BSA, bovine serum albumin; CMI, chromosome mitotic index; DAPI, 4,6-diamidino-2-phenylindole; MT, microtubule; PBS, phosphate-buffered saline; SMI, spindle mitotic index.

spindle MTs do not disassemble at restrictive temperature. This is direct evidence that spindle MT stability is increased by the *benA33* mutation (17). In addition, the chromosomes fail to move to the poles and nuclear migration is inhibited at restrictive temperature in strains carrying this mutation.

The phenotype of the *benA33* mutation suggested that mitosis and chromosome movement might be inhibited as a consequence of the observed block in spindle MT disassembly. Because nuclear movement is also inhibited in *benA33* mutants and MTs are known to be involved in nuclear movement in *Aspergillus* (7, 16), it seemed plausible that the inhibition of nuclear movement might similarly be explained by hyperstability of cytoplasmic MTs. The suggestion that increased MT stability is responsible for the block in mitosis and the failure of chromosome and nuclear movement appears to be generally correct, since growth inhibition of *benA33* mutants at restrictive temperature can be reversed by chemical agents and *tubA* mutations (see below) that destabilize MTs (17). For a discussion of how MT disassembly may be related to chromosomal and organellar movement, see Inoue (8).

TubA gene products are also involved in both spindle and cytoplasmic MT function. The evidence for this comes from an analysis of the effect of certain *tubA* mutations on the phenotype of *benA33*. Both *tubA1* (isolated as a revertant of *benA11*) and *tubA4* (isolated as a revertant of *benA33*) suppress the temperature sensitivity of *benA33*. Since the effect of the *benA33* mutation is to cause hyperstability of spindle MTs, it seemed likely that the mechanism by which *tubA1* and *tubA4* mutations suppressed *benA33* was by destabilizing MTs. The fact that strains carrying *tubA1* and *tubA4* in the absence of a *benA* mutation are hypersensitive to several unrelated antimicrotubule drugs suggests that these mutations make microtubules less stable to depolymerization by antimicrotubule drugs in general and therefore supports this hypothesis (17).

Although we have shown directly that *benA* gene product is part of the mitotic spindle (17), we have not heretofore presented any direct evidence that the stability of cytoplasmic MTs is altered by the *benA33* mutation. Nor until now have we presented any direct evidence that the *tubA* mutations decrease the stability of either spindle or cytoplasmic MTs. In this paper, we report, on the basis of direct visualization, using antitubulin fluorescence immunocytochemistry, that the *benA33* mutation causes both spindle and cytoplasmic MTs to be hyperstable and that the *tubA1* and *tubA4* mutations destabilize spindle and cytoplasmic MTs.

The visualization of cytoplasmic MTs by immunofluorescence is technically difficult in intact hyphae of *A. nidulans* because of the cell wall. To facilitate the visualization of MTs by immunofluorescence, we developed a method for removing the cell wall under conditions that appear not to cause any major perturbation of cell physiology. That the resulting protoplasts are physiologically normal is demonstrated by the fact that they undergo nuclear division at the same rate as walled germlings and that cell-cycle and tubulin mutants have similar phenotypes in protoplasts and in germlings. Using these protoplasts, we examined the cytoplasmic and spindle MTs of wild-type and mutant tubulin-containing strains of *A. nidulans*, showed that cytoplasmic MTs of the temperature-sensitive, *benA33* β -tubulin mutant are hyperstable, and showed that the microtubules of the *tubA1* and *tubA4* α -tubulin mutants are less stable than those of the wild type.

MATERIALS AND METHODS

Strains: FGSC4 (Glasgow wild type) is available from the Fungal Genetic Stock Collection (Arcadia, CA). The other strains used were LB2-5 (*bimE7*, *yA2*, *cuxE16*), BR02 (*benA33*, *pabaA1*, *yA2*), L014 (*tub1*, *proA1*, *yA2*, *suA1*, *ade20*), and BEN9R7 (*tubA1*, *benA11*, *biA2*). L0197 (*tubA4*, *yA2*, *pabaA1*) and L0199 (*tubA4*, *benA33*, *yA2*, *pabaA1*) were generous gifts from Dr. B. R. Oakley (Ohio State University).

Growth Conditions: Conidia of *Aspergillus nidulans* were harvested weekly from cultures grown on plates containing 2% agar, 2% dextrose, and 0.5% yeast extract. All plates were incubated at 32°C, except for the extremely heat-sensitive *benA33* strains, which grew best at 25°C. Young germlings to be used for protoplasting were prepared by inoculating a 250-ml Erlenmeyer flask containing 50 ml of YGT (yeast extract 5 g/liter, glucose 20 g/liter with 0.2% Tween-80 to improve germination synchrony [1]) with an inoculum of 2×10^6 spores/ml. Spores were germinated at 32°C for 10 h with agitation on a New Brunswick Gyrotory Shaker (New Brunswick Scientific Co., Edison, NJ). Before protoplasting, germlings were washed three times with medium lacking Tween-80.

Protoplast Preparation and Growth: 2 ml of packed germlings are resuspended in 10 ml of medium followed by the addition of an equal volume of 1 M MgSO₄ containing 20 mg/ml Novozyme 234 (Novo Laboratories, Wilton, CT). Digestion of germ tubes was complete within an hour at 32°C with gentle agitation. After 75 min, undigested conidial bulbs were removed by centrifugation at 500 g for 2 min. The protoplast suspension was then diluted to 250 mM MgSO₄ with YG and the protoplasts harvested by centrifugation. Alternatively, protoplasts were generated in 250 mM MgSO₄, and the undigested material removed by filtration through miracloth.

In growth experiments, time 0 was 15 min after the removal of undigested material from protoplasts. Protoplasts were fixed for 30 min in 2.5% glutaraldehyde, 0.05 M phosphate buffer, pH 7.3, pelleted and then resuspended in distilled water. 1 μ g/ml of 4,6-diamidino-2-phenylindole (DAPI) was added and the samples were scored for the average number of nuclei per protoplast and chromosome mitotic index (CMI).

Antitubulin Indirect Immunofluorescence of Protoplasts: Protoplasts were fixed for 20 min in either 4% ultrapure electron microscopy grade formaldehyde, 8 mM phosphate buffer, pH 7.3, or in 4% formaldehyde, 100 mM PIPES, 30 mM EGTA, pH 7.3. Fixation in 100 mM PIPES, pH 7.3 (without EGTA), gave the same immunofluorescence patterns as those obtained with phosphate buffer. The presence of EGTA was required for optimal preservation of cytoplasmic MTs in strains not containing the *benA33* mutation.

Fixed protoplasts were washed twice with phosphate-buffered saline (PBS) and once with PBS containing 1% BSA. 10- μ l aliquots were then air-dried onto coverslips. The coverslips were washed in methanol for 8 min and acetone for 6 min at -20°C. After rehydration in PBS-BSA, samples were stained for 45 min at 37°C with a rat monoclonal antibody against yeast α -tubulin (generously contributed by Dr. J. Kilmartin, Cold Spring Harbor Laboratory, YOL 1/34) at a 1:100 dilution or a mouse monoclonal antibody against chicken β -tubulin (generously contributed by Dr. S. Blöse, DM1B) at a dilution of 1:500. Samples were washed three times with PBS-BSA and then stained with fluorescein isothiocyanate-conjugated anti-rat or anti-mouse IgG respectively (Cappel Laboratories, West Chester, PA) diluted 1:20. Coverslips were mounted in nine parts glycerol and one part PBS, pH 8.5, containing 0.5 μ g/ml DAPI and 0.5% *n*-propyl gallate. Fluorescence microscopy was conducted using Zeiss epifluorescence optics.

Electron Microscopy: Samples for electron microscopy were fixed in 3% glutaraldehyde, 100 mM PIPES, 30 mM EGTA, pH 7.3 and postfixed in 1% buffered osmium tetroxide before dehydration through alcohol and embedding in Spurr Resin. Gold and silver thin sections stained with uranyl acetate and lead citrate were observed in a Philips EM 300.

RESULTS

Protoplast Preparation and Characterization

We examined *Aspergillus* protoplasts by phase-contrast microscopy. The protoplasts were spherical and contained an internal vesicle that increased in size as a function of incubation time. Nuclei were easily visualized by staining with the DNA-specific, fluorescent dye, DAPI (Fig. 1, *A* and *B*) and, as in yeast, mitochondrial DNA was seen as pinpoint dots of fluorescence (27). When we examined the protoplasts by electron microscopy, we saw that the cell wall was absent and

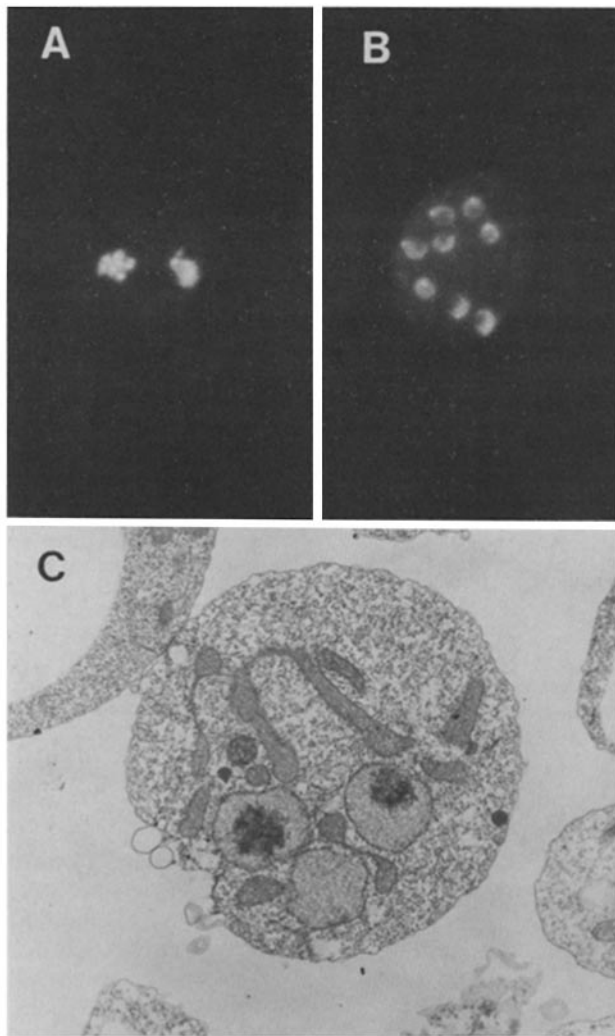


FIGURE 1 Fluorescence and electron microscopy of *Aspergillus* protoplasts. Protoplasts were grown at 32°C for 3.5 h and fixed by the addition of an equal volume of buffered 5% glutaraldehyde. Fluorescence microscopy of DAPI-stained (A) mitotic and (B) interphase nuclei of protoplasts. (C) Thin section electron micrograph of a mitotic protoplast. $\times 8,200$.

the cytoarchitecture well preserved (Fig. 1C). (For a comparison with germling ultrastructure, see reference 18.) At least 75% of the young protoplasts were nucleated, of which 75% were uninucleate and 25% binucleate. Most of the protoplasts contained interphase nuclei that were ovoid with a spherical staining defect caused by the nucleolus (Fig. 1B); but mitotic nuclei were also seen (Fig. 1A). These were irregular, lacked a nucleolus and were more strongly fluorescent than interphase nuclei.

The physiological state of the protoplasts vis-a-vis that of the germlings was evaluated by comparing their respective rates of nuclear division (Fig. 2). We determined the nuclear doubling time of wild-type protoplasts at 32°C, 37°C, and 42°C, the permissive, optimal, and restrictive temperatures for *Aspergillus* mitotic mutants. At 32°C the nuclear doubling time was 102 min and at 42°C 110 min for protoplasts, compared to 98 and 115 min, respectively, for germlings (1). The mitotic indices of the protoplasts and germlings were similar (data not shown).

Germlings of *A. nidulans* may contain multiple nuclei in

the same hyphal compartment; and, as in other coenocytic cells, nuclear division is synchronous at high growth rates (21). Protoplasts similarly may be multinucleate, and, as expected, division of all the nuclei in a protoplast was also synchronous (for example, see Figs. 3, B and D, and 4).

Immunofluorescent Visualization of Microtubules in Wild-type Protoplasts

Protoplast MTs were studied by indirect immunofluorescence using two monoclonal antibodies, YOL 1/34, raised against yeast α -tubulin, and DM1B, raised against avian β -tubulin. Both antibodies stained cytoplasmic and spindle MTs equally well, confirming that both cytoplasmic and spindle MTs contain α - and β -tubulins. Optimal preservation of cytoplasmic MTs required the presence of EGTA in the fixation buffer. In the absence of EGTA the cytoplasmic MTs had a granular appearance (Fig. 3A); whereas in the presence of EGTA they were filamentous (Fig. 3C). EGTA was not required for optimal fixation of the mitotic spindle (Fig. 3, B and D), indicating that spindle MTs may be more stable to fixation than cytoplasmic MTs in *A. nidulans*.

Interphase protoplasts exhibited a conspicuous network of cytoplasmic MTs with larger protoplasts having more extensive networks than smaller protoplasts. No obvious MT organizing center(s) were apparent for these MT networks. At mitosis most cytoplasmic MTs disappeared. If cells were fixed without EGTA, cytoplasmic MT networks were never observed during mitosis (Figs. 3B and 4). In samples fixed in the presence of EGTA a few cytoplasmic MTs were seen during mitosis (Fig. 3D), often radiating from the spindle poles in the position expected of astral MTs, but these were sparse compared to the cytoplasmic MTs of interphase cells (Fig. 3C). Thus except for the astral MTs, most wild-type cytoplasmic MTs apparently either depolymerize or become unstable to fixation during mitosis.

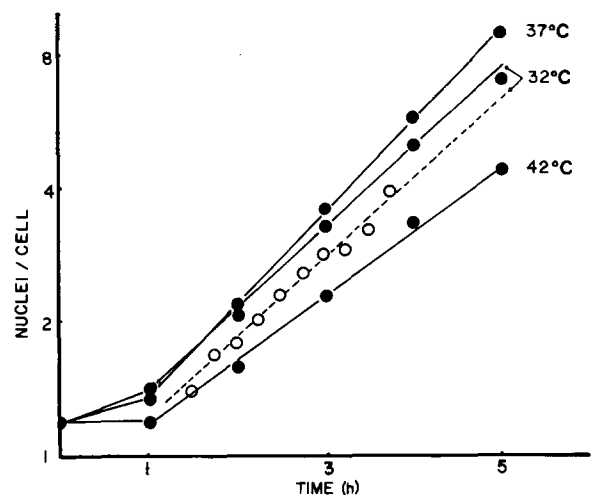


FIGURE 2 The rate of nuclear division in *Aspergillus* protoplasts. Wild-type germlings were protoplasted at 32°C and, 1 h after the addition of Novozyme, aliquots were shifted to the indicated temperatures. The increase in the average nuclear number per protoplast (●) at 32°C, 37°C, and 42°C appears to be linear after an initial lag period. For comparison, the rate of nuclear increase in germlings (○) is also presented. A minimum of 170 cells were counted for each determination. Nuclear doubling times were derived from the slopes of these lines.

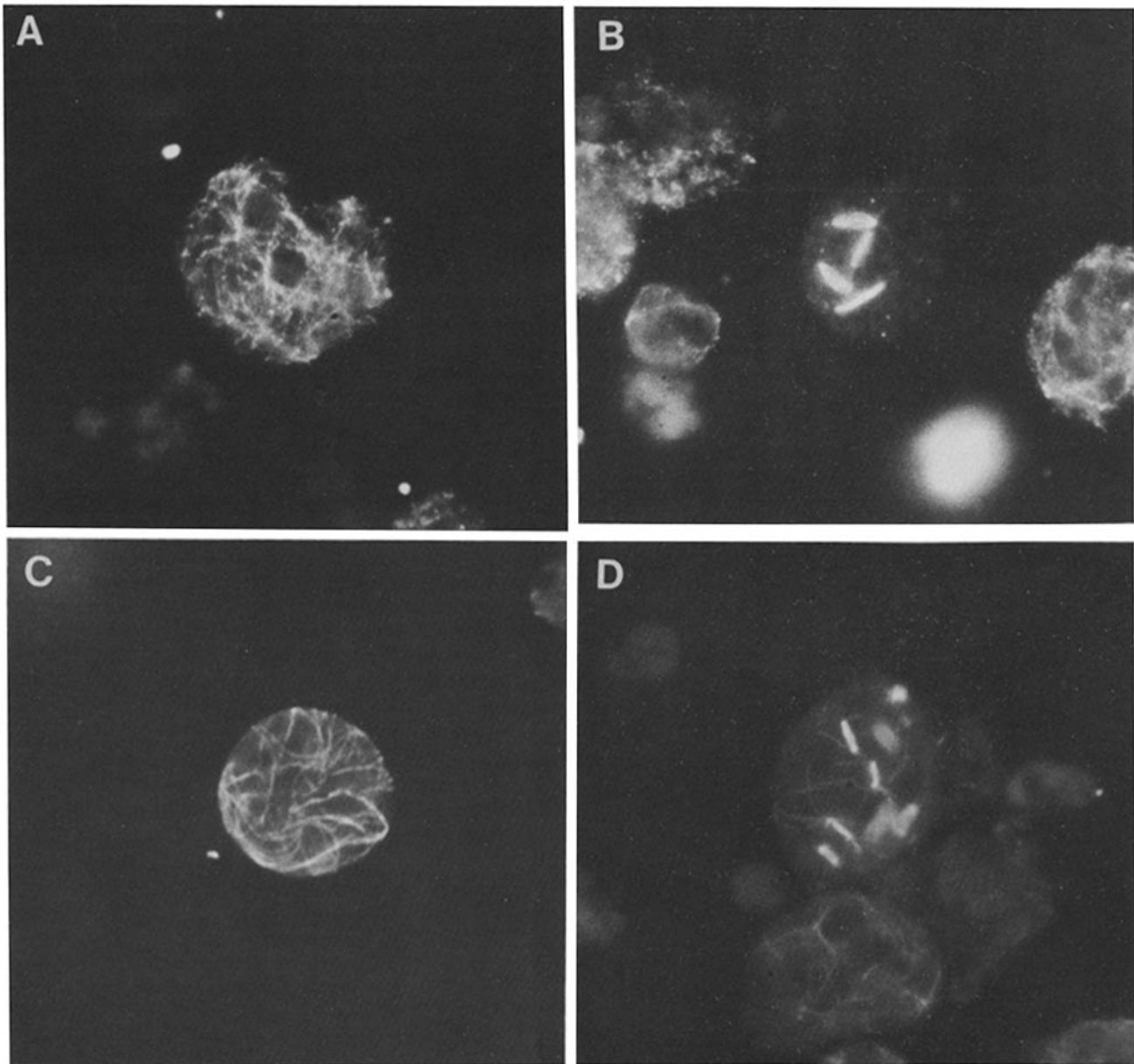


FIGURE 3 Antitubulin immunofluorescence micrographs of FGSC4 protoplasts illustrate the differential stability of spindle and cytoplasmic microtubules in strains not containing the *benA33* β -tubulin mutation. Cells were stained with the β -tubulin antibody DMIB (from S. Blose [Cold Spring Harbor Laboratory]). Wild-type protoplasts were fixed with 4% formaldehyde in 100 mM PIPES, pH 7.3, in the absence (A and B) or presence (C and D) of 30 mM EGTA. Both interphase (A and C) and mitotic (B and D) cells are shown. $\times 3,600$.

Protoplasts in mitosis were easily identified by the presence of mitotic spindles and condensed chromatin (Fig. 4). As noted above, mitosis was synchronous among the nuclei of any one protoplast. If one nucleus in a protoplast was in mitosis all of the nuclei in that protoplast were in mitosis. The stages of mitosis could be ascertained from the length of the mitotic spindle and the degree of separation of the chromatin. In logarithmically growing wild-type protoplasts, $\sim 5\%$ of the cells were in mitosis. Of these, 25% exhibited spindles $< 1 \mu\text{m}$ in length completely within the chromatin mass (Fig. 4, A and B), 65% had intermediate spindles between 2–3 μm in length (Fig. 4, C and D), and 10% were either in anaphase (Fig. 4, E and F) or in telophase (Fig. 4, G and H). The percentage of cells in mitosis and the frequency distribution of the stages of mitosis gives the approximate duration of each

stage. If the overall duration of mitosis is 5 min (5% of 102 min at 32°C), then the short spindle stage lasts ~ 1.25 min, the intermediate spindle stage ~ 3.25 min and anaphase plus telophase ~ 0.5 min.

Expression of the Cell Cycle Mutation *bimE7* in Protoplasts

BimE7 is a temperature-sensitive, conditional lethal mutation that arrests the cell cycle in mitosis at restrictive temperature, and is non-allelic with the *benA* or *tubA* loci (12). This strain contains tubulin that is indistinguishable from wild-type tubulin in its electrophoretic characteristics (N. R. Morris, unpublished data) and in the requirement of EGTA for optimal preservation of cytoplasmic MTs. When rapidly

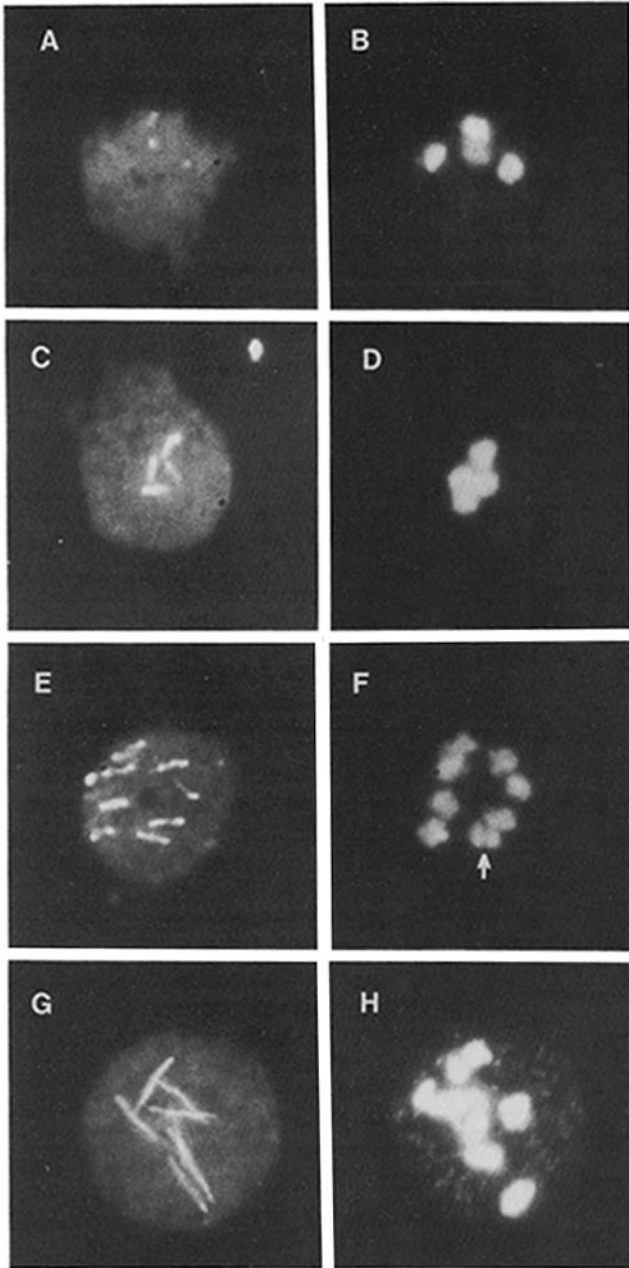


FIGURE 4 Simultaneous antitubulin (left panels) and DAPI (right panels) staining of mitotic nuclei. The progression of mitosis is depicted in this series of micrographs of protoplasts that have wild-type tubulin. (A and B) small spindles $<1 \mu\text{m}$ in length, (C and D) larger spindles that are still contained within the chromatin mass, (E and F) anaphase spindles with separated chromosomes (see arrow), and (G and H), telophase spindles connecting two nuclei. All spindle types are preserved even when fixed in the absence of EGTA. CMI and SMI did not vary by >1 or 2% in a given experiment. $\times 3,600$.

growing germlings of *bimE7* are switched from permissive to restrictive temperature, mitotic nuclei accumulate as a function of time (13). Protoplasts prepared from a strain carrying *bimE7* also expressed this phenotype (see Fig. 5). At permissive temperature, the chromosome and spindle mitotic indices (CMI and SMI) of *bimE7* germlings and protoplasts were higher than wild-type strains. The CMI and SMI for the wild-type strain FGSC4 at 32°C were $5\text{--}6\%$ and $4\text{--}5\%$, respectively.

For *bimE7* strains at 32°C the CMI ranged from 8 to 11% and the SMI ranged from 7 to 10% . When *bimE7* protoplasts were shifted from 32°C to 40°C , the CMI and SMI reached peaks of 90% and 50% , respectively. Presumably this difference between the CMI and SMI can be attributed to the lability of the mitotic spindle relative to chromatin condensation.

At 40°C there was no increase in nuclear number per protoplast as a function of time. As in mitotic wild-type protoplasts, *bimE7* protoplasts blocked in mitosis had few or no cytoplasmic MTs when fixed in the presence of EGTA. The decline in the SMI of *bimE7* protoplasts after 3 h at restrictive temperature (Fig. 5B) is similar to that previously reported for mycelia (12). This decline may reflect the deleterious effects of keeping *bimE7* protoplasts at restrictive temperature for several hours, since protoplasts maintained at restrictive temperature for >3 h have decreased viability upon shifting to permissive temperature (data not shown).

Analysis of *bimE7* protoplasts at half hour intervals (between 2 to 4 h) after shifting to restrictive temperature showed a complete absence of anaphase and telophase spindles. This may be contrasted to control protoplasts growing at permissive temperature in which 8% of 200 spindles counted were in anaphase or telophase. Thus, the mitotic block in *bimE7* may be due to a defective gene product normally required for anaphase and telophase. Alternatively, the mitotic process as a whole may be so lengthened that spindles degenerate before reaching anaphase.

Effect of the *benA33* β -Tubulin Mutation on Cytoplasmic MT Stability

Antitubulin immunofluorescence was used to analyze the distribution and stability of cytoplasmic MTs in strains carrying the *benA33* mutation. As noted above, proper fixation of cytoplasmic MTs in wild-type protoplasts of *A. nidulans* required the presence of EGTA in the fixation buffer (Fig. 3). In contrast, the cytoplasmic MTs of *benA33* protoplasts were well preserved even without EGTA (Fig. 6A). More dramatic evidence for increased stability of the cytoplasmic MTs of *benA33* protoplasts was seen during mitosis. Cytoplasmic MTs mostly disappear during mitosis in wild-type protoplasts (as described above). However, in the vast majority ($>85\%$, $n = 200$) of mitotic protoplasts carrying the *benA33* mutation the cytoplasmic MT networks were strikingly well preserved

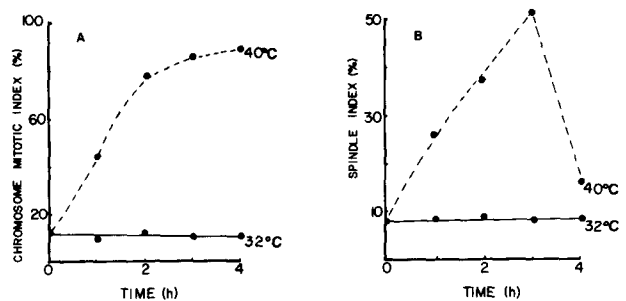


FIGURE 5 The effect of restrictive temperature on *bimE7* protoplasts. Protoplasts of *bimE7* were shifted from permissive temperature (32°C) to restrictive temperature (40°C) at time 0. The effect of this shift on the (A) chromosome mitotic index, and (B) the spindle mitotic index is shown. More than 175 cells were counted for each time point.

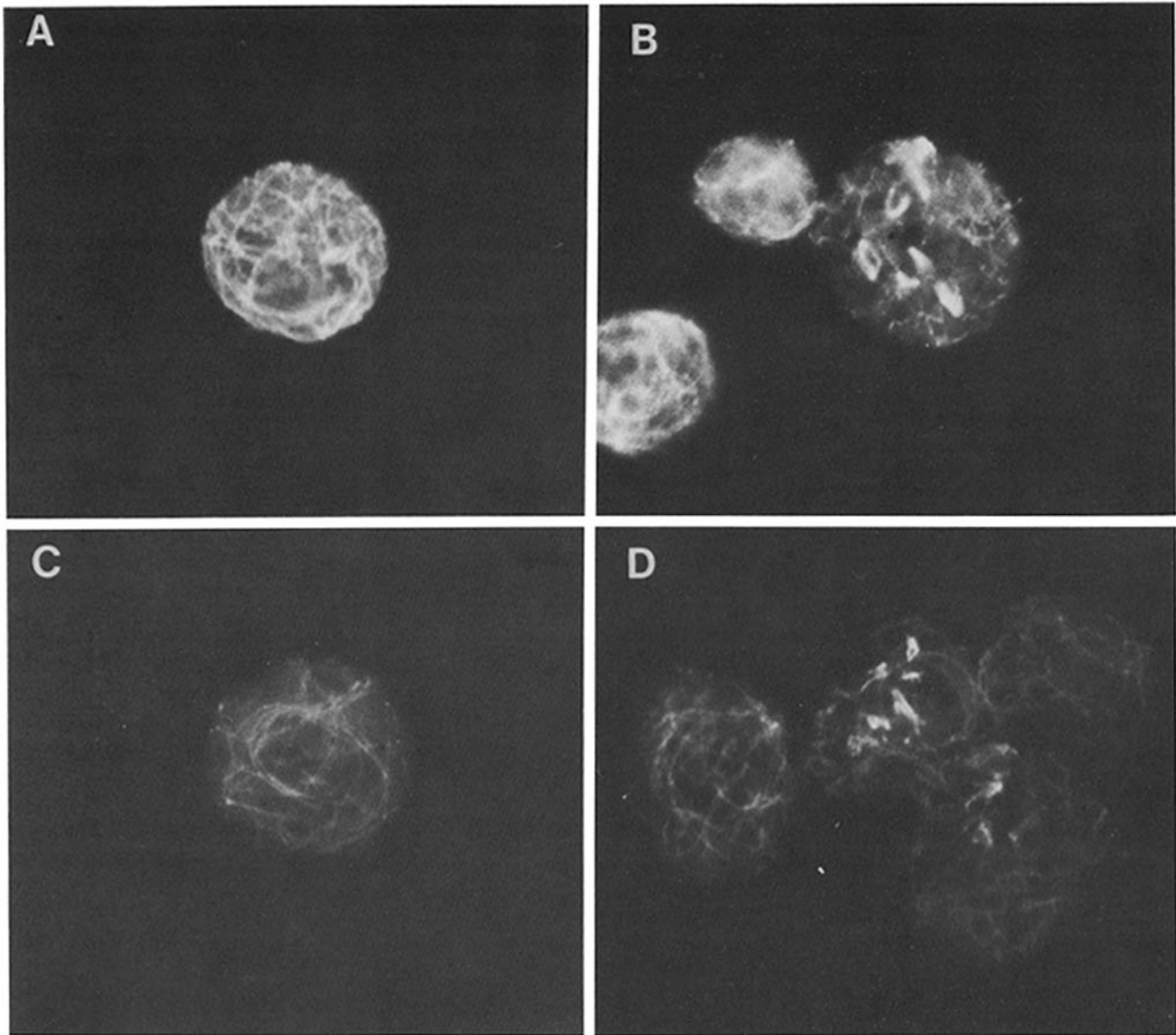


FIGURE 6 Antitubulin immunofluorescence of *benA33* protoplasts. Enhanced microtubule stability in protoplasts carrying the *benA33* tubulin mutation is shown in these immunofluorescent micrographs of (A and C) interphase and (B and D) mitotic cells. Note the persistence of cytoplasmic MTs during mitosis in the absence (A and B) or presence (C and D) of 30 mM EGTA. For comparison with protoplasts containing wild-type tubulin see Figs. 4 and 5. $\times 3,600$.

(Fig. 6). Whatever the mechanism that causes cytoplasmic MTs to depolymerize during mitosis in wild-type protoplasts, whether they are depolymerized *in vivo* or are simply unstable to fixation, the *benA33* mutation appears to have an opposing effect. It either makes them resistant to depolymerization or stable to fixation. Thus, although we do not understand precisely the nature of the effect, the *benA33* mutation “hyperstabilizes” both spindle and cytoplasmic MTs.

Effect of *tubA* α -Tubulin Mutations on the Stability of Spindle and Cytoplasmic MTs

There are two known α -tubulin mutations in *Aspergillus*, *tubA1* and *tubA4*. Each of these mutations suppresses the temperature sensitivity of *benA33* and both cause *Aspergillus* to be supersensitive to a variety of unrelated antimitotic chemicals (17; Oakley, B. R. personal communication). This supersensitivity to antimitotic agents can in turn be sup-

pressed by *benA33*. These data have been interpreted to mean that the effect of the *tubA* mutations is to decrease MT stability (see reference 17 for the detailed argument). Immunofluorescent staining with antitubulin antibodies of strains carrying *tubA* mutations has now provided direct evidence in support of this hypothesis. Although staining of wild-type spindle MTs has been highly reproducible, we have only rarely been able to visualize mitotic spindles in strains containing either the *tubA1* or *tubA4* mutations, even though these strains were demonstrably undergoing nuclear division (Table I). Mitotic activity was assayed by measuring chromosome and spindle mitotic indices and counting the number of nuclei at the beginning and end of the experiment (Table I). A strain containing the *bimE7* mutation was used as one of the controls in this experiment because its twofold elevated mitotic index compared to wild type at permissive temperature made it easy to score spindles and mitotic nuclei. The stability to fixation of *bimE7* and wild-type MTs was indistinguishable.

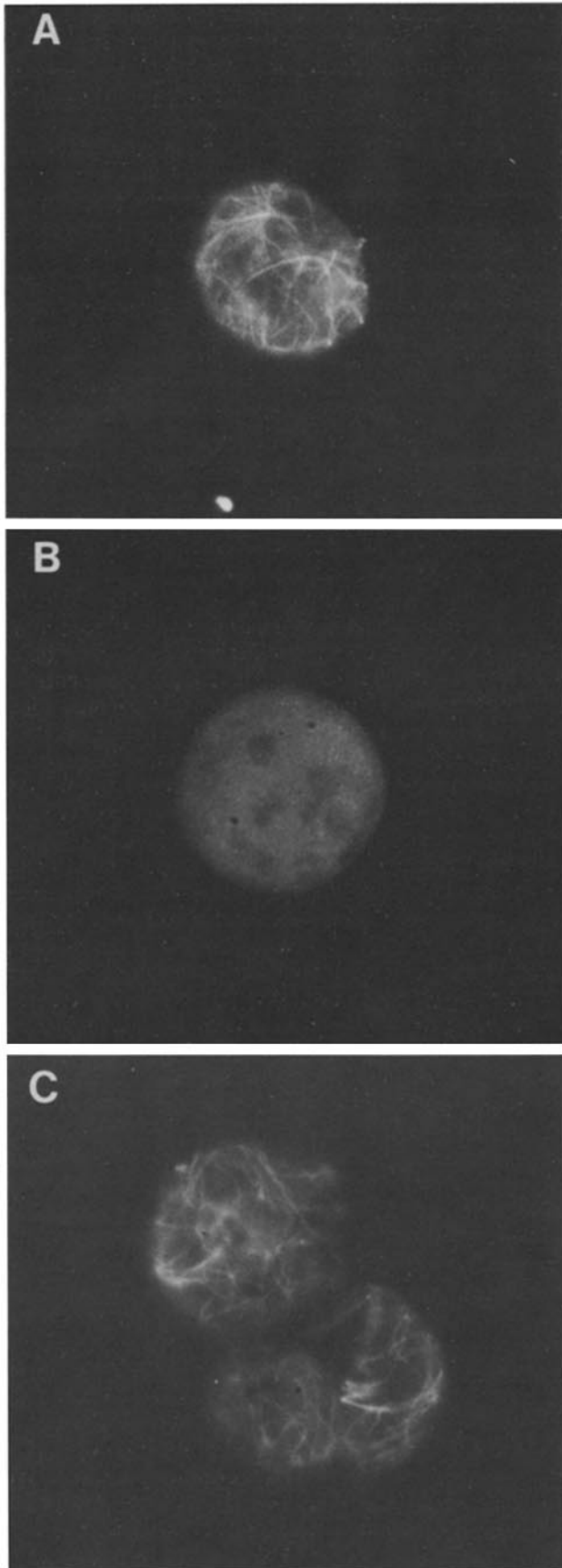


FIGURE 7 Hypostability of microtubules in protoplasts containing *tubA4* mutations. Antitubulin immunofluorescence of protoplasts fixed in the presence of 30 mM EGTA. Interphase protoplasts of wild-type strains (A) display complex networks of cytoplasmic MTs

TABLE I
Spindle Hypostability in Protoplasts Carrying *tubA1* and *tubA4* Mutations

	CMI	SMI	Nuclear in- crease
	%	%	×
FGSC4	5.2	4.3	*
<i>bimE7</i>	10.6	10.0	1.9
<i>tubA1</i>	6.0	0.5	2.2
<i>tubA4</i>	7.0	0.0	1.7

Protoplasts were grown at 32°C for 2 h, then fixed in the presence of EGTA. The CMI was determined by DAPI fluorescence while the SMI was determined by immunofluorescence. Nuclear increase represents the increase in the number of nuclei at the time of fixation. Note that although the nuclear increase was approximately twofold for all mutant strains, few *tubA1* and no *tubA4* spindles were found. This indicates that the mutant α -tubulins were hypostable during fixation. A minimum of 400 cells were scored for each determination.

* Not determined in this experiment; see Fig. 1.

The number of nuclei per protoplast increased in the *tubA* strains at about the same rate as in the *bimE7* strain. The chromosome mitotic indices of the *tubA* strains were similar, but slightly higher than those for the wild-type strain. The increases in nuclear number, as well as the evidence of mitotic activity, indicates that the *tubA* nuclei were actively dividing. However, the spindle mitotic indices, as compared to the chromosome mitotic indices, of these strains were substantially lower than in the *bimE7* or wild-type controls. The SMI/CMI ratios for the control strains were close to unity, whereas in the *tubA* cultures the SMI/CMI ratio was essentially zero.

If a mitotic spindle is required for mitosis in *Aspergillus*, then our failure to observe spindles in *tubA1* and *tubA4* protoplasts undergoing active nuclear division probably indicates that the spindle MTs of these strains are unstable to fixation under the same conditions that give good fixation of wild-type and *bimE7* spindle MTs. A similar observation was made with respect to cytoplasmic MTs. We did not observe cytoplasmic MTs in either *tubA1* or *tubA4* protoplasts fixed under conditions (with EGTA) that gave excellent preservation of wild-type cytoplasmic MTs (Fig. 7). The two most probable explanations of this observation are either that *tubA1* and *tubA4* containing strains have no cytoplasmic MTs, which we think unlikely, or that the cytoplasmic MTs of *tubA1* and *tubA4* are less stable to fixation than wild-type MTs. A third possibility, which also applies to the spindle MT observations, is that these *tubA* mutations alter the epitope recognized by the YOL 1/34 α -tubulin antibody. To control for this, we examined the immunocytochemistry of strains carrying *tubA1* and *tubA4* in the presence of *benA33*, which is known to suppress the hypersensitivity of these strains to benomyl and therefore might be expected to suppress MT instability. In the two *benA*, *tubA* double mutants that we examined (*benA33*, *tubA1*, and *benA33*, *tubA4*), staining of cytoplasmic MTs was indistinguishable from wild type (Fig. 7C). Because the staining of cytoplasmic MTs in strains carrying *tubA1* and *tubA4* is restored by mutations in the *benA* gene, it is apparent that the YOL 1/34 epitope on α -tubulin is not immunologically altered by these *tubA4* mutations. The *tubA* mutants, therefore, either do not have cyto-

while no MTs were seen in protoplasts of (B) *tubA4*. MT stability was restored by the suppressing β -tubulin mutation *benA33* (C: *tubA4*, *benA33*, double mutant). $\times 3,600$.

plasmic MTs or, as is more likely the case, contain cytoplasmic MTs that are unstable to fixation. This experiment also confirms our hypothesis that *benA33* suppresses the multiple drug sensitivity of *tubA1* and *tubA4* by increasing MT stability, thereby counteracting the decreased MT stability caused by these mutations.

The fact that both spindle and cytoplasmic MTs were rendered unstable to fixation by the *tub4A* mutations, constitutes direct evidence that the *tubA* α -tubulin is a constituent of both classes of MTs. Additional evidence came from analysis of the *benA*, *tubA* double mutants, which gave MT immunofluorescent staining patterns and stability characteristics similar to those of wild-type protoplasts. These findings confirm our original conclusion (17) that because *benA33* causes both spindle and cytoplasmic MTs to be hyperstable, and *tubA1* reverts the *benA33* phenotype, one or both *tubA* gene products, α 1- and α 3-tubulins must also be a component of both spindle and cytoplasmic MTs.

DISCUSSION

The development of a simple method for generating protoplasts that are normal with respect to their ability to transverse the cell cycle provides a new tool for studying cell cycle events in the genetically manipulable lower eucaryote *A. nidulans*. The protoplasting method we have described is not in itself new. It is well known that fungal wall material can be removed by enzymatic digestion to generate viable protoplasts (19, 26). Moreover, the method we have described closely resembles that of DeVries and Wessels (5), who used *Trichoderma harzianum* lytic enzyme (the principle constituent of Novozyme 234) with $MgSO_4$ as the osmotic buffer to make fungal protoplasts. DeVries and Wessels (5) noted that the protoplasts produced by this method grew slowly in buffered enzyme solution, but the growth rate of the protoplasts was not quantitated. The slow growth in buffer that they observed suggested to us that protoplasts might grow more rapidly if they were generated in a nutrient medium. When we tested this *Aspergillus*, it turned out to be correct. In this paper, we present the first quantitative account of nuclear division in fungal protoplasts proceeding at a growth rate nearly identical to that of young, rapidly growing germlings.

Because protoplasts are more readily fixed and are more permeable to antibody than walled germlings, their use has greatly facilitated our immunochemical, light, and electron microscopic investigations of the cytoarchitecture of *A. nidulans*. A special virtue of these protoplasts is that, since their nuclei divide at a rapid rate, there is no question that they are alive and physiologically normal. Using mitotic mutants (12, 13, 18) and tubulin mutants (14, 17), we have blocked these protoplasts at particular, well defined points in the cell cycle and have examined the effects of the mutations on cytoarchitecture.

Wild-type *Aspergillus* protoplasts exhibit a cytoplasmic microtubular network during interphase and a mitotic spindle at mitosis. During mitosis, as in higher eucaryotes, the cytoplasmic microtubular network largely disappears. This was noted in wild-type protoplasts and was confirmed using the mutant *bimE7*, in which large numbers of mitotic nuclei accumulate at restrictive temperature, making it much easier to score mitotic phenotypes than in the wild type. Because cytoplasmic MTs generally disappear or reorganize as the mitotic spindle forms in most eucaryotes, there is considerable interest in the composition of spindle versus cytoplasmic

MTs. A particularly interesting question is whether the tubulin of the cytoplasmic MTs is used to build the spindle MTs and if so how polymerization at different sites is controlled.

The observation that mutations in *benA* and *tubA* affect both spindle and cytoplasmic MTs does not necessarily mean that the same *benA* proteins are found in both cytoplasmic and spindle MTs. There are two electrophoretically differentiable *benA* gene products β 1- and β 2-tubulin (23). Some of these may be located in the spindle and others in cytoplasmic MTs. Similarly there are two *tubA* gene products, α 1- and α 3-tubulins (14; J. A. Weatherbee and N. R. Morris, manuscript in preparation) which also might be differentially located. To know for certain which proteins are in the spindle and which are in cytoplasmic MTs, it will be necessary to isolate spindle and cytoplasmic MTs and to analyze their proteins. In addition to locating the *benA* and *tubA* gene products, it will be of interest to determine where the other two tubulin species, the α 2-, α 3-, and β 3-tubulins, are located. The fact that mitotic protoplasts that contain only spindle MTs can be accumulated at restrictive temperature using *bimE7* protoplasts should greatly facilitate the isolation and analysis of spindle and cytoplasmic MTs.

Considering the differences in location, function, and stability between spindle and cytoplasmic MTs, we expect that cytoplasmic and spindle MTs will exhibit biochemical differences. Evidence for structural and functional heterogeneity of tubulin has accumulated within recent years. Multiple forms of α -tubulin exist in the alga *Polytomella*, some of which predominate in different classes of MTs (11). This heterogeneity appears to be a consequence of posttranslational modification in *Polytomella* (10). However, diversity of tubulins dependent on the expression of different tubulin genes has also been demonstrated in a variety of organisms including *Aspergillus* (14, 23; J. A. Weatherbee and N. R. Morris, manuscript in preparation), *Chlamydomonas* (24), *Drosophila* (20, 22), chick (2, 3), and human (4). Because of the pattern of expression of β -tubulin genes in various cell types, Lopata et al. (9) have speculated that genetically different tubulins are used for mitotic and interphase microtubules in a modern version of the multiple-tubulin hypothesis originally proposed by Fulton and Simpson (6). Although this suggestion may be generally correct, in *Aspergillus* the products of the same α - and β -tubulin genes, *tubA* and *benA*, are used for both spindle and cytoplasmic MTs, in *Drosophila* the same testes specific β -tubulin, β -2T, is used for both spindle and flagellar MTs. Also the evidence so far suggests that *Saccharomyces cerevisiae* has only one β -tubulin gene (15).

The nuclear membrane of *Aspergillus* does not break down during mitosis, and this has important theoretical and practical consequences. We would like to know if tubulins are partitioned or in equilibrium across the nuclear membrane; and if they are partitioned, do they move from cytoplasm to nucleus during the transition from interphase to mitosis (and vice versa)? The fact that cytoplasmic MTs persist during mitosis in strains carrying the *benA33* mutation suggests, but does not prove, that there may be separate cytoplasmic and nuclear tubulin pools. In other words, cytoplasmic MT breakdown does not appear to be a necessary prerequisite to spindle MT polymerization. One practical consequence of the failure of the nuclear membrane to break down during mitosis in *Aspergillus* is that it should be possible to isolate intact, membrane bound nuclei from mitotic and interphase protoplasts to analyze nuclear tubulins and other nuclear proteins

to determine whether particular tubulins are nucleus specific.

Protoplasts have generally proven to be excellent starting material for the gentle isolation of nuclei and other organelles from fungi (26). The protoplasts described in this paper have the additional advantage that they traverse the cell cycle normally and can be blocked at various points in the cell cycle using other temperature-sensitive mitotic mutants (12; Bergen, L. G., manuscript in preparation). We hope to be able to use the *Aspergillus* protoplast system not only to study the tubulins of *Aspergillus* at interphase and mitosis, but also to examine MT-associated proteins that may be related to the control of mitosis.

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