Astral Microtubules Are Not Required for Anaphase B in *Saccharomyces cerevisiae*

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Abstract. *tub2-401* is a cold-sensitive allele of *TUB2*, the sole gene encoding β-tubulin in the yeast, *Saccharomyces cerevisiae.* At 18°C, *tub2-401* cells are able to assemble spindle microtubules but lack astral microtubules. Under these conditions, movement of the spindle to the bud neck is blocked. However, spindle elongation and chromosome separation are unimpeded and occur entirely within the mother cell. Subsequent cytokinesis produces one cell with two nuclei and one cell without a nucleus. The anucleate daughter can not bud. The binucleate daughter proceeds through another cell cycle to produce a cell with four nuclei and another anucleate cell. With additional time in the cold, the number of nuclei in the nucleated cells continues to increase and the percentage of anucleate cells in the population rises. The results indicate that astral microtubules are needed to position the spindle in the bud neck but are not required for spindle elongation at anaphase B. In addition, cell cycle progression does not depend on the location or orientation of the spindle.

THE mitotic spindle is composed of two sets of microtubules that emanate from the spindle poles. Kinetochore microtubules attach chromosomes to the spindle poles. Polar microtubules extend from one spindle pole towards the other and interdigitate with polar microtubules from the opposite pole. A third set of microtubules, the astral microtubules, radiate outward from the spindle poles away from the spindle. Chromosome separation occurs at anaphase (Mitchison, 1989; McIntosh and Koonce, 1989; McIntosh and Hering, 1991). In most organisms, this stage is subdivided into anaphase A and B. At anaphase A, daughter chromosomes migrate to opposite spindle poles. At anaphase B, the spindle elongates and the poles move apart. Experimental evidence on anaphase B movement is split between results suggesting that the poles are pushed apart by antiparallel sliding of the interdigitated polar microtubules and that the poles are pulled apart by forces generated by the astral microtubules. Pushing is indicated by the observations that diatom central spindles can elongate in vitro (Cande and MacDonald, 1985; Hogan and Cande, 1990) and that elongating spindles can actually push outwards on the cell cortex in *C. elegans* embryos (Hyman and White, 1987). Evidence for a pulling force is the independent motility of newt cell half spindles (Bajer and Mole-Bajer, 1981). Also, removing the asters of the sea urchin mitotic apparatus blocks anaphase B (Hiramoto et al., 1986) and breaking the central spindle in the fungus *Fusarium*, speeds up anaphase B movement (Aist and Berms, 1991; Aist et al., 1991).

Mitosis in the yeast, *S. cerevisiae*, is largely similar to mitosis in higher eukaryotes (Peterson and Ris, 1976; Byers, 1981). However, the yeast nuclear envelope remains intact throughout mitosis. Yeast spindle poles, termed spindle pole bodies, are embedded in the nuclear envelope. Microtubules extend from the spindle poles into both the nucleus and the cytoplasm. The kinetochore and polar microtubules reside within the nucleus and the astral microtubules reside in the cytoplasm. For this reason, yeast spindle microtubules have been referred to as intranuclear microtubules and yeast astral microtubules have been called cytoplasmic microtubules. In this paper, we use the terms astral and spindle microtubules rather than cytoplasmic and intranuclear microtubules to agree with the terminology used for most other eukaryotes.

S. cerevisiae grows by forming a bud at a predetermined site on the mother cell. Before anaphase, the spindle must be positioned through the bud neck to ensure that spindle elongation delivers one set of chromosomes to both the mother and bud. Cytokinesis then occurs at the bud neck to produce two unbudded daughter cells. Studies using tubulin mutants or microtubule destabilizing drugs demonstrate the principal role of yeast microtubules in mitosis (Huffaker et al., 1988; Jacobs et al., 1988). They are required for establishing the position of the spindle in the bud neck and for the subsequent separation of chromosomes. In this paper we describe a cold-sensitive allele of *TUB2*, the sole gene encoding β-tubulin in *S. cerevisiae.* At a restrictive temperature, cells containing this allele lack astral microtubules but are able to assemble a mitotic spindle. Under these conditions, movement of the spindle too the bud neck is blocked. However, spindle elongation and chromosome separation are unimpeded and occur entirely within the mother cell. The resulting nuclear division is apparently normal and sufficient to allow cells to progress through the cell cycle.

Table I. Yeast Strains

Strain	Genotype
CUY17	*MATα ura3-52 leu2-3,112*
CUY66	*MATα ura3-52 ade2-101 tub2-401*
CUY67	*MATa ura3-52 lys2-801 tub2-401*
CUY554	*MATa ura3-52 leu2-3,112* *MATα URA3 LEU2*
CUY555	*MATa tub2-401 ura3-52 ade2-101 lys2-801 his4-539* *MATα tub2-401 ura3-52 ADE2 LYS2 HIS4*

Materials and Methods

Strains and Medium

Yeast strains used in this study are listed in Table I. All are essentially isogenic to S288C. Cells were grown in YPD medium which contains 1% yeast extract, 2% bacto-peptone, and 2% glucose. Solid medium contained 2% agar.

DAPI Staining

Cells were washed with water and fixed in methanol/acetic acid (3:1) for 30 min at room temperature. Fixed cells were washed twice with water and sonicated briefly to disrupt clumps unless otherwise indicated. They were then stained with 1 μg/ml 4',6'-diamidino-2-phenylindole (DAPI) for 30 min at room temperature.

Immunofluorescence

For microtubule staining, cells were fixed at their last growth temperature by adding formaldehyde directly to the culture to a final concentration of 4% and incubating at this temperature for 2 h. Fixed cells were permeabilized by incubation with 25 μg/ml zymolyase 100,000 in 1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5, and 25 mM β-mercaptoethanol at 30°C for 30 min. Permeabilized cells were stained for immunofluorescence essentially as described by Kilmartin and Adams (1984). Rat monoclonal anti-yeast-α-tubulin antibody, YOL1/34, was a gift from J. Kilmartin (Medical Research Council Laboratory of Molecular Biology, Cambridge, United Kingdom). Fluorescein-conjugated goat anti-rat IgG antiserum was obtained from Cappel Research Products (Durham, NC). DNA was stained with 1 μg/ml DAPI for 5 min at room temperature before mounting.

Nuclear pores were stained as described by Davis and Fink (1990). Mouse monoclonal anti-yeast-nuclear pore antibody, mAb 942, was a gift from L. Davis (Duke University, Durham, NC). Affinity-purified fluorescein-conjugated rabbit anti-mouse IgG was obtained from Jackson Labs (West Grove, PA).

Isolation of Small Unbudded Cells

Small unbudded cells were isolated by the protocol of Sloat and Pringle (1978). Cells were grown in 50 ml of medium at 30°C to early stationary phase, washed with sterile water, and resuspended in 10 ml 2% Ficoll. They were then layered on top of a Ficoll step gradient (10 ml each of 10, 8, 6, and 4% Ficoll) and spun for 18 min at 600 rpm in a Beckman GP centrifuge. 1-ml fractions were removed from the top of the gradient and observed under a light microscope. Top fractions that contained >98% unbudded cells were pooled. The unbudded cells were washed three times with sterile water and resuspended in medium or spread on plates.

Immunoblot Analysis

Cells were harvested, washed, and spheroplasted by incubation with 25 μg/ml zymolyase 100,000 in 1.2 M sorbitol, 0.1 M potassium phosphate, pH 7.5, and 25 mM β-mercaptoethanol at 37° for 20 min. Spheroplasts were collected by centrifugation, resuspended in 0.5 ml cracking buffer (10 mM sodium phosphate pH 7.2, 1% β-mercaptoethanol, 1% SDS, 8 M urea) containing protease inhibitors (1 μg/ml phenanthroline, 1 mM PMSF, 1.5 μg/ml benzamidine hydrochloride), and incubated at 37°C for 30 min. Samples were then boiled for 5 min and run on a 10% acrylamide gel. Immunoblotting was performed as described by Burnette (1981). Antiserum 206 (a gift from F. Solomon, Massachusetts Institute of Technology, Cambridge, MA) was used to detected yeast β-tubulin and antiserum B28 (a gift from R. Ye and A. Bretscher, Cornell University, Ithaca, NY) was used to detect yeast actin. Blots were exposed to Kodak X-OMAT AR film and quantitated by densitometry.

Results

Nuclear and Cell Division in Asynchronous Cultures

tub2-401 cells are cold-sensitive and fail to grow at temperatures below 20°C. To determine the arrest phenotype of these cells, asynchronous cultures were grown at 30°C and shifted to either 14° or 18°C. The results of these experiments are tabulated in Fig. 1 and representative cells are shown in Fig. 2. The generation time for wild type cells is ~8 h at 14°C and 4 h at 18°C. *tub2-401* cells shifted to 14°C for two generation times (16 h) displayed a typical cell cycle arrest. Greater than 90% of the cells possessed a single large bud. The nuclear DNA in these cells was visualized by DAPI staining. The large-budded cells contained a single nucleus randomly located within one of the cell bodies. Thus, incubation at 14°C blocks both migration of the nucleus to the bud neck and nuclear division. This is similar to the arrest phenotype of *tub2-401* cells grown at 11°C (Huffaker et al., 1988).

tub2-401 cells shifted to 18°C for two generation times (8 h) did not display a uniform cell cycle arrest. However, the percentage of unbudded cells increased with additional time at the restrictive temperature. After four generation times (16 h), nearly 70% of the cells were unbudded. DNA staining revealed two unusual classes of cells. Most of the unbudded cells that accumulated contained no detectable nuclear DNA although they contained mitochondrial DNA that stained normally. The number of "anucleate" cells increased with time at 18°C. After 8 h, 31% of the cells in the population were anucleate. After 20 h, 54% of all cells and 80% of the unbudded cells were anucleate.

			A			B						
Strain	Temp.	Time										
TUB2	30°		35	41	24	0	35	46	19	0	0	0
TUB2	18°	8 h	42	29	29	0	42	32	26	0	0	0
TUB2	14°	16 h	41	39	20	0	41	44	15	0	0	0
tub2-401	30°		31	33	36	0	31	46	21	0	2	0
tub2-401	18°	4 h	44	23	33	21	18	43	2	5	11	0
tub2-401	18°	8 h	43	15	42	31	9	35	2	3	19	1
tub2-401	18°	12 h	59	14	27	41	12	22	1	5	15	3
tub2-401	18°	16 h	69	10	21	55	10	14	0	4	12	5
tub2-401	18°	20 h	67	12	21	54	11	9	0	3	14	10
tub2-401	14°	16 h	1	1	98	0	1	98	0	0	1	0

Figure 1. Cell and nuclear morphologies. Haploid cells were grown at 30°C and shifted to 14° or 18°C for the times indicated and stained with DAPI. (*A*) The percentages of cells that were unbudded, small-budded, or large-budded are indicated. A large-budded cell possesses a bud whose diameter is at least three-fourths the diameter of the mother cell. (*B*) Percentages of unbudded and budded cells with various nuclear morphologies. Budded cells include both small and large-budded cells. The categories include from left to right: unbudded cells with no nucleus, unbudded cells with one nucleus, budded cells with one nucleus, budded cells with two nuclei properly distributed between mother and bud, unbudded cells with two nuclei, budded cells with two nuclei in mother, and budded cells with more than two nuclei in mother.

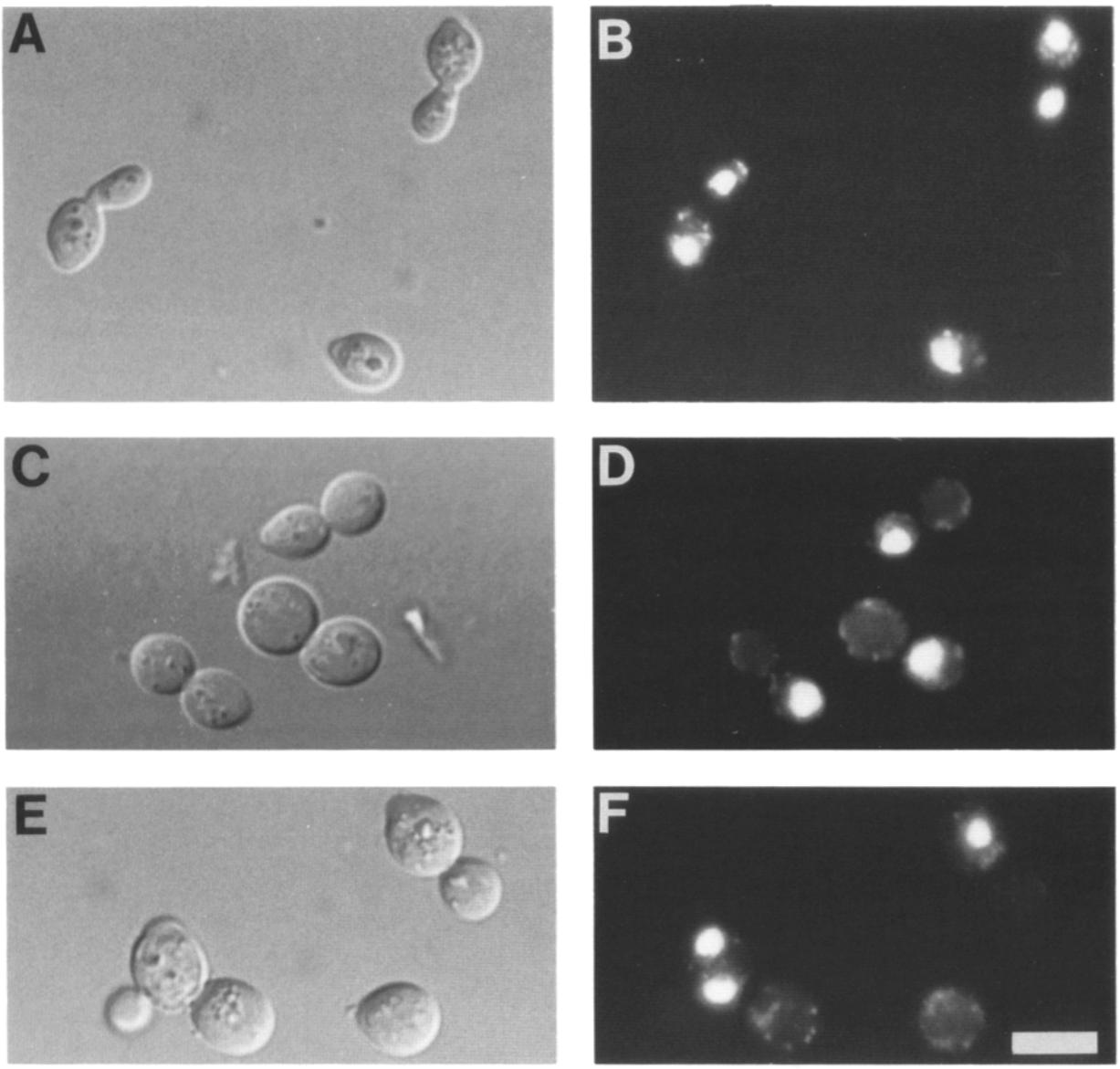

Figure 2. DAPI staining of DNA in *tub2-401* cells. Haploid *tub2-401* cells were grown at 30°C (*A* and *B*) and shifted to 14°C for 16 h (*C* and *D*) or 18°C for 8 h (*E* and *F*). Nomarski optics reveals cell morphology (*A*, *C*, and *E*) and DAPI fluorescence reveals cellular DNA (*B*, *D*, and *E*). Bar, 10 μm.

The nucleated cells were also unusual. Many of the budded cells contained two nuclei in one cell body, presumably the mother cell (see data on cell clusters below), and none in the bud. Some of the unbudded cells also contained two nuclei. The size and intensity of staining indicated that each region represented a complete nucleus. After 8 h, 33% of the cells with nuclei displayed this "binucleate" phenotype. After 20 h, nearly 60% of the nucleated cells were binucleate. The average number of nuclei also increased as a function of time. By 20 h, three or more nuclei could be detected in >20% of the nucleated cells. Rare cells with as many as eight nuclei were observed, but usually it was difficult to distinguish more than three or four nuclei in one cell.

We believe that these numbers underestimate the percentage of binucleate and multinucleate cells. Cells were scored as binucleate only if two distinctly separate DAPI staining regions could be observed. Given the relative diameters of the DAPI staining region and the cell body, we estimate that two nuclei lying on a line >45° out of the plane of view would be scored as one nucleus. Consequently, nearly half of the binucleate cells would be scored as mononucleate. This could account for most of the mononucleate cells observed at later times.

The simplest hypothesis to explain the generation of anucleate and binucleate cells at 18°C is that nuclear division takes place entirely within the mother cell. The completion of nuclear division is apparently sufficient to allow a cell to continue through the cell cycle and produce two unbudded cells, one anucleate and one with two nuclei. The anucleate cell can not bud again but the binucleate cell can go through another round of the cell cycle to produce a second anucleate daughter and a multinucleate mother cell.

This hypothesis was supported by examining cells which were not dispersed by sonication or digestion with enzymes

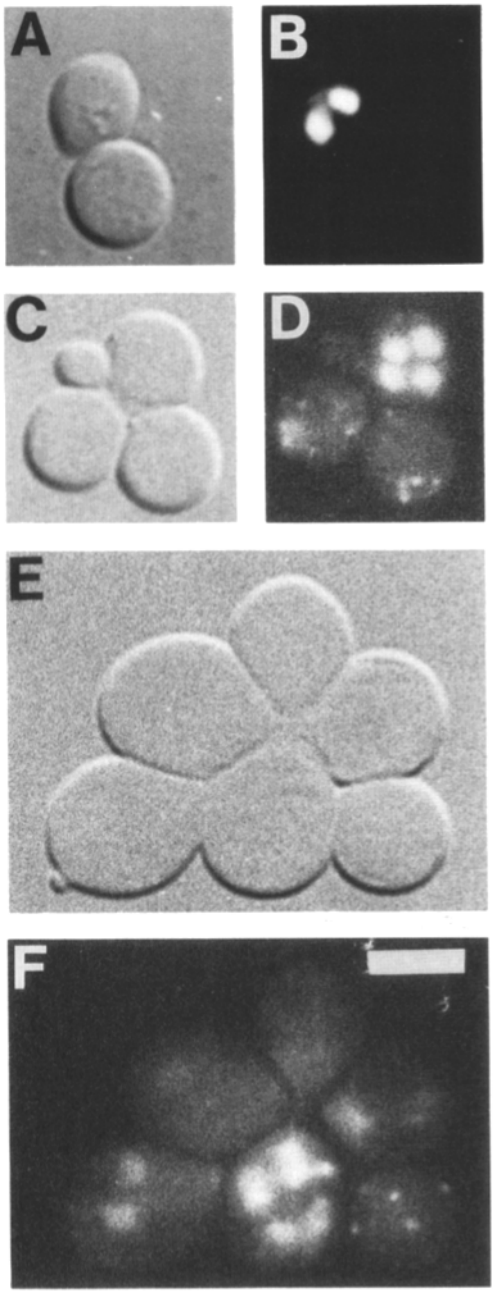

Figure 3. DAPI staining of DNA in *tub2-401* cell clusters. Haploid *tub2-401* cells were grown at 30°C and shifted to 18°C for 4 h (*A* and *B*), 8 h (*C* and *D*), and 20 h (*E* and *F*). Nomarski optics reveals cell morphology (*A*, *C*, and *E*) and DAPI fluorescence reveals cellular DNA (*B*, *D*, and *E*). Cell clusters were not dispersed by sonication before viewing. Bar, 10 μm.

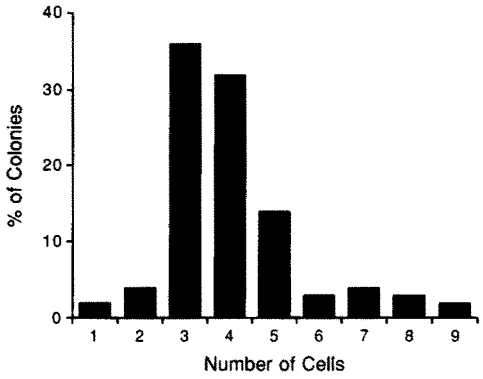

Figure 4. Cell division of *tub2-401* cells at 18°C. Haploid *tub2-401* cells were grown to early stationary phase at 30°C. Unbudded cells were then isolated on ficol gradients, spread on plates, and incubated at 18°C. After 4 d, the number of cells in each microcolony was counted under a microscope.

that remove the cell wall. In the absence of these treatments, the daughter cells produced at 18°C tended to remain associated with the mother cell producing small cell clusters. Most clusters consisted of one mother cell that contained multiple nuclei surrounded by anucleate daughter cells. Fig. 3 (*A* and *B*) shows a large-budded cell produced after 4 h at 18°C. It is binucleate indicating that nuclear division has occurred within the mother cell. Fig. 3 (*C* and *D*) shows a small cell cluster produced after 8 h at 18°C. The mother cell contains four nuclei indicating that nuclear division has occurred twice at the restrictive temperature. Two anucleate cells, presumably daughters from the two mitotic divisions, are attached to the mother cell. In addition, the mother cell has extended a small bud indicating that it is beginning the next cell cycle. Fig. 3 (*E* and *F*) shows a larger cell cluster reduced after 20 h at 18°C. The mother cell is surrounded by five anucleate daughters indicating that it has gone through five cell divisions. The mother cell contains at least six nuclei and probably contains more that are difficult to distinguish. By this time, the DNA staining is often more diffuse which also makes it difficult to recognize individual nuclei. In this cluster, one of the daughters contains two nuclei and is putting out a bud of its own (lower left cell in Fig. 3, *E* and *F*). This is unusual; nuclei were observed in daughter cells in <10% of the clusters. It is apparent from examining cell clusters that *tub2-401* cells can undergo several rounds of budding and nuclear division at 18°C. In general, nuclei are not distributed to the daughter cells but remain within the mother cell.

Not all cells will continue to divide for five generations at 18°C. When unbudded cells were plated at 18°C for several days, 80% of them produced microcolonies containing only three, four, or five cells (Fig. 4). Assuming that each mother cell produced only anucleate daughters that can not bud again, these microcolonies represent two, three, and four generations of growth, respectively.

Nuclear Division in Synchronous Cultures

Our data from asynchronous cultures indicated that at 18°C nuclear division occurred predominately within the mother cell; large-budded cells with two nuclei in the mother and none in the bud were much more prevalent than those with one nucleus in the mother and one in the bud. However, the steady state levels of these two classes of cells reflect not only the relative rates at which they are produced but also the amount of time that they exist. For example, cytokinesis might be delayed in binucleate cells resulting in a higher steady state level of this class of cells.

To measure more accurately the fraction of nuclear divisions that occur within the mother cell, we examined a population of cells as it first entered mitosis. A uniform population of unbudded *tub2-401* cells was isolated from cells

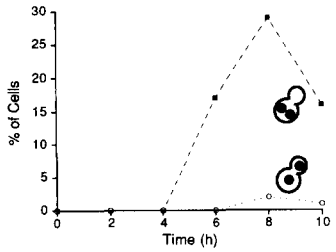

Figure 5. Nuclear division in synchronized *tub2-401* cells. Unbudded haploid *tub2-401* cells were isolated on Ficoll gradients, added to fresh medium at 0 h, and incubated at 18°C. At the times indicated, cells were fixed and stained with DAPI. (○) Percentages of cells that contain one DAPI staining region in the mother cell and one in the bud. (■) Percentages of cells that contain two DAPI staining regions in the mother cell and none in the bud.

grown at 30°C and diluted into medium at 18°C. Because the unbudded cells were isolated from saturated cultures, they did not begin the cell cycle immediately or in a highly synchronous manner. Cells that completed nuclear division first appeared after 6 h at 18°C (Fig. 5). Their numbers peaked at 8 h when 30% of the population contained two nuclei. At each time point, >95% of these cells contained both nuclei in the mother cell; <5% contained a nucleus in the mother and the bud. These results demonstrate that nuclear division occurs predominately within the mother cell.

Nuclear Envelope Staining

In the experiments described above, DAPI staining was used to determine the number of nuclei in each cell. Because DAPI stains the chromosomes, two separate regions of DAPI staining indicate that chromosome separation has occurred. This does not prove that the nucleus has actually divided. The separated sets of chromosomes could be present within a single nuclear envelope. Antibody that specifically recognizes yeast nuclear pores was used to visualize the nuclear envelope by immunofluorescence. In *tub2-401* cells grown at 18°C for two generations, the nuclear pore staining mirrored the DAPI staining (Fig. 6). Binucleate cells that contained two separated regions of DAPI staining also contained two distinct nuclear envelopes. These results indicate that nuclear division is completed within the mother cell. Cells that failed to stain with DAPI also failed to stain with the nuclear pore antibody. Thus, the anucleate cells contain neither chromosomes nor nuclear envelope.

DNA Replication

We have shown previously that incubation of *tub2-401* cells at 11°C blocks nuclear division and causes cells to accumulate with a 2C DNA content (Huffaker et al., 1988). At 18°C, nuclear division is not blocked. Because DNA replication is normally a prerequisite for chromosome segregation and nuclear division, we expected that the DNA content of *tub2-401* cells would increase with time of incubation at 18°C.

Flow cytometry was used to measure the DNA content of individual *tub2-401* cells at various times after a shift to 18°C (Fig. 7). Before the shift, cells with both a 1C and 2C DNA content were observed. These are cells in the G1 and G2/M phases of the cell cycle, respectively. After 4 h at 18°C, <10% of the nucleated cells contained a 1C DNA content. Most (85%) contained a 2C DNA content and some (15%) with 4C DNA content could be observed. (Anucleate cells can not be detected by this technique.) By 8 h, most nucleated cells contained a 4C DNA content. Only 20% still

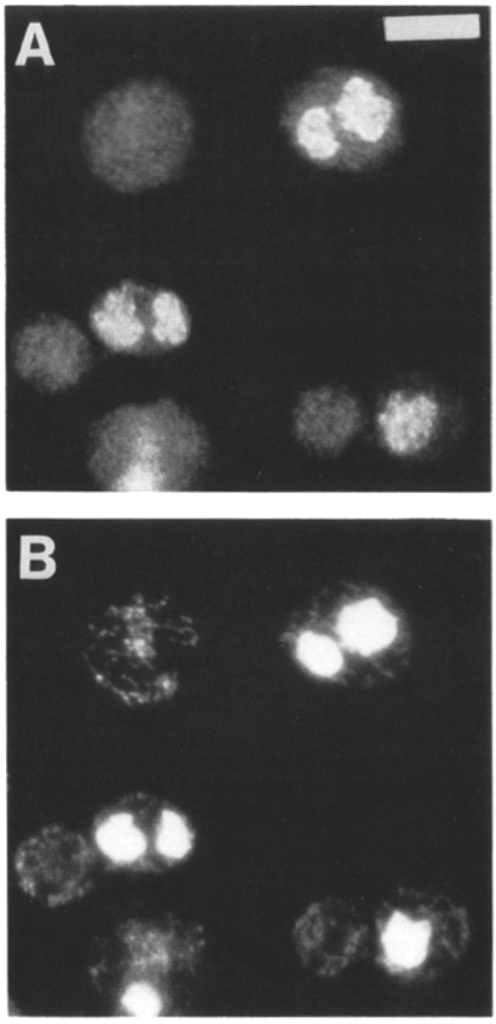

Figure 6. Nuclear envelope staining of *tub2-401* cells. Diploid cells homozygous for the *tub2-401* allele were grown at 30°C and shifted to 18°C for 8 h. (*A*) Immunofluorescence using a nuclear pore-specific antibody reveals nuclear envelope. (*B*) DAPI fluorescence reveals cellular DNA. Bar, 10 μm.

contained a 2C DNA content after 8 h at 18°C. We conclude that DNA replication continues in most nucleated cells at 18°C; the DNA content doubles every 4 h, the generation time at this temperature.

Microtubule Assembly In Vivo

Immunofluorescence was used to visualize cellular microtubules. Our results with wild type cells are similar to those obtained by Kilmartin and Adams (1984) which are in good agreement with previous electron microscopic observations of yeast microtubules (Byers, 1981; Peterson and Ris, 1979). Cells were placed into one of three classes based on their spindle structure. The results of these experiments are tabulated in Table II and representative cells are shown in Fig. 8 (*A* and *B*).

Cells without spindles made up the first class. This class included cells early in the cell cycle that had not yet formed a spindle. As expected, most of these cells were unbudded. Microtubule staining consisted of a bright dot plus one or more bundles of microtubules that extended from this dot. We could not determine whether these bundles extended into

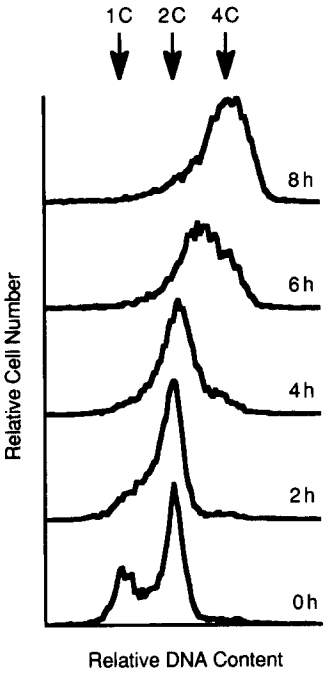

Figure 7. DNA content of *tub2-401* cells. Diploid cells homozygous for the *tub2-401* allele were grown at 30°C and shifted to 18°C for the times indicated. The DNA content of individual cells was determined by flow cytometry using the protocol of Hutter and Eipel (1978).

the nucleus or cytoplasm. Also included in this class were large-budded cells that had completed chromosome separation and spindle breakdown. These cells contained two sets of microtubule arrays, one in the mother and one in the bud, similar to the one seen in unbudded cells.

Cells with spindles were divided into two additional classes, those with short spindles and those with long spindles. Cells that have completed pole separation but not yet entered anaphase contain short spindles. These spindles were usually ~1 μm in length and stretched across the nucleus. Cells with spindles that appeared <1 μm were also included in this class; we assumed that these contained normal length short spindles that were not parallel to the field of view. Cells that have entered anaphase contain long spindles. These were easily distinguished from short spindles because spindle elongation is relatively rapid and produces spindles several times the length of short spindles. In addition, chromosome segregation was apparent in these cells, either as an elongation or a separation of the DAPI staining. Astral microtubules extended outward from both poles of most short and long spindles. Spindle and astral microtubules were readily distinguished by their geometry; astral microtubules generally extended at an angle from the spindle creating a sharp bend at the spindle pole. Also, the spindle stained brighter than the astral microtubules. Astral microtubules were observed in 85% of the wild type cells. (We sometimes had to focus through a cell to observe astral microtubules because they did not always lie in the plane of view. They are not apparent in every cell in a given photograph.)

Microtubules were examined in *tub2-401* cells after growth at 18°C for two generations (8 h). Most of the cells with no spindles contained only a dot of staining near the perimeter of the nucleus that presumably represented scant microtubules at the spindle pole body. In only 15% of the cells of this class did microtubules extend from the dot of staining. As was the case with wild type cells, we could not determine whether these extended into the nucleus or cytoplasm. Cells with short and long spindles were observed in about the same percentages in *tub2-401* cells as in wild type cells. However, astral microtubules were conspicuously absent in *tub2-401* cells with both short and long spindles (Table II and Fig. 8, *E–J*). Only 15% of the cells with spindles contained detectable astral microtubules at either pole. When observed, these were faint, short fibers and not nearly as prominent as astral microtubules in wild type cells.

Several interesting features were noticed about the spindles that formed at 18°C. First, the location of the spindles was aberrant. In wild type cells an elongated spindle always extended through the bud neck and had one pole in the mother cell and one in the bud (Fig. 8, *A* and *B*). The spindles formed in *tub2-401* cells were contained entirely within the mother cell (Fig. 8, *E–J*). Second, the orientation of the spindle relative to the cell axis was usually incorrect. In wild type cells, the spindle always aligned with the long axis of the cell (Fig. 8, *A* and *B*). The spindles formed in *tub2-401* cells not only failed to extend through the bud neck but seldom aligned with the long cell axis. Instead, they appeared to orient randomly in the mother cell, sometimes perpendicular to the normal direction (Fig. 8, *E* and *F*). Third, 10–15% of the spindles were bent (Fig. 8, *G* and *H*). In most of these cases, the length of the spindles exceeded the diameter of the mother cell. This suggests that the spindles attempt to achieve their normal full length and must bend to fit within the confines of the mother cell. Fourth, a number of cells with two spindles were observed (Fig. 8, *I* and *J*). These were more numerous after longer shifts to 18°C and most likely represent cells undergoing a second nuclear division at this restrictive temperature. Nuclear division in these cells appeared to be synchronized. Tetranucleate cells with only one spindle were not observed. Sometimes the spindles were parallel but often they crossed over one another. This indicates that the direction of spindle elongation within a single cell is also random.

Table II. Percentages of Microtubule Structures Observed in Wild Type and tub2-401 Cells

		No spindle		Short spindle		Long spindle	
Strain	Temperature	Dot plus MTs	Dot only	Plus astral MTs	Minus astral MTs	Plus astral MTs	Minus astral MTs
	°C						
TUB2	18	60	1	22	5	11	1
tub2-401	18	9	52	4	23	2	10
tub2-401	14	1	99	0	0	0	0

Diploid cells, homozygous for the indicated allele, were grown at 30°C and shifted to 18°C for 8 h or 14°C for 16 h. Immunofluorescence with α-tubulin-specific antibody was used to visualize microtubules. Microtubule structures were classified as described in the text.

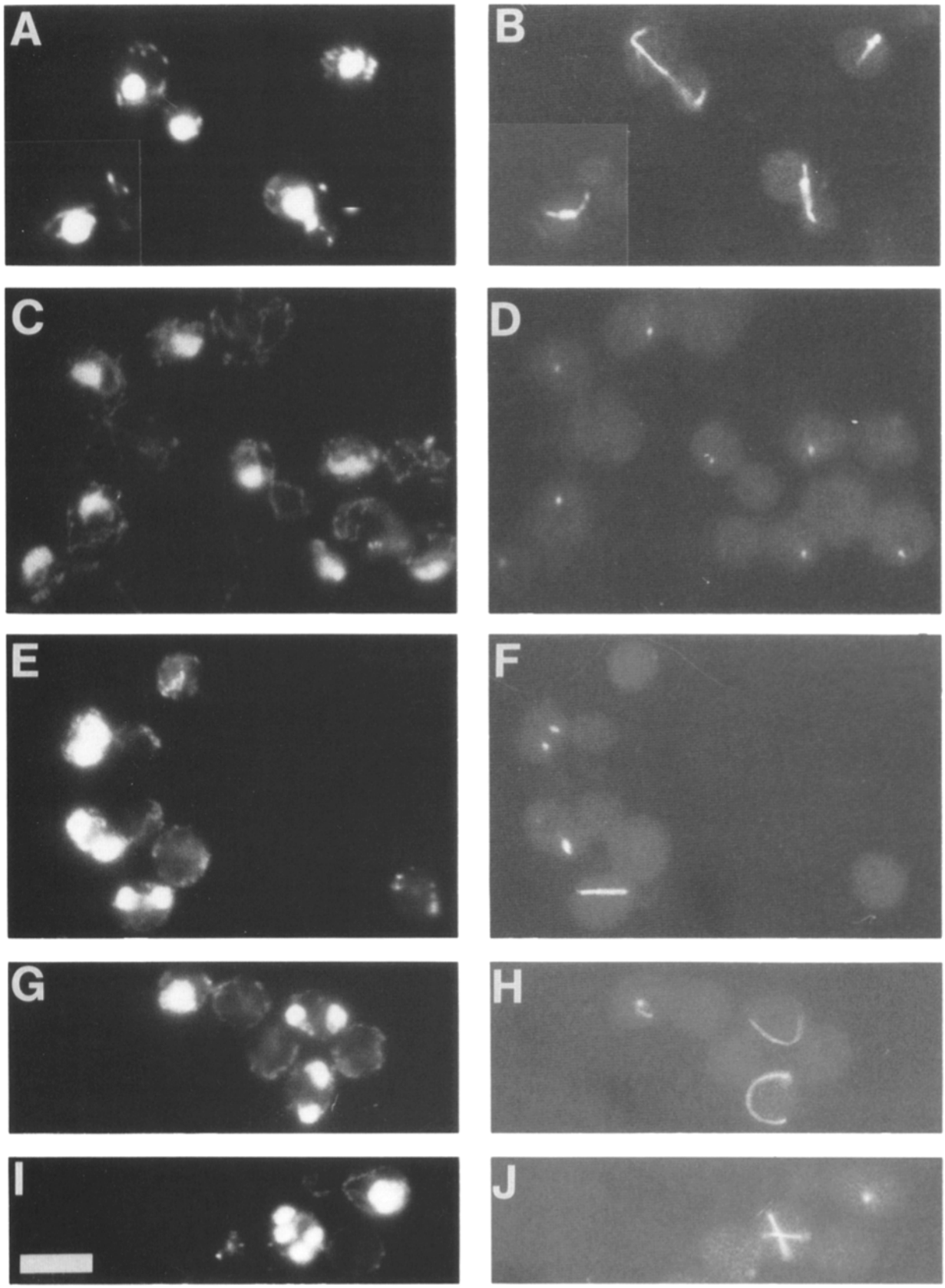

Figure 8. Microtubules in wild type and *tub2-401* cells. (*A* and *B*) Wild type diploid cells grown at 30°C. (*C* and *D*) *tub2-401* homozygous diploid cells grown at 30°C and shifted to 14°C for 16 h. (*E–J*) *tub2-401* homozygous diploid cells grown at 30°C and shifted to 18°C for 8 h. (*A*, *C*, *E*, *G*, and *I*) DAPI fluorescence reveals cellular DNA. (*B*, *D*, *F*, *H*, and *J*) Immunofluorescence using α-tubulin-specific antibody reveals microtubules. Bar, 10 μm.

After a shift to 14°C for two generation times (16 h), most *tub2-401* cells contained only a single dot or more rarely two dots of staining (Table II and Fig. 8, *C* an *D*). Less than 1% of the cells contained visible microtubules extending from the dots. Thus, at 14°C, *tub2-401* cells did not contain any significant spindle or astral microtubules. This pattern of staining differed from that seen after incubation at 11°C. Cells shifted to 11°C for two generations contained no visible microtubule staining, not even dots (Huffaker et al., 1988).

β-Tubulin Levels

The microtubule assembly defect in *tub2-401* cells becomes progressively more severe as the temperature is lowered. One explanation for this effect would be that the stability of the mutant protein decreases with temperature. The following experiment demonstrated that this is not the case. Immunoblotting was used to determine the levels of β-tubulin in wild type and *tub2-401* cells grown at 30°C and shifted to 18°C for 8 h or 14°C for 16 h. The levels of actin were determined as an internal control. As shown in Fig. 9, there was no significant variation in the levels of β-tubulin in any of the samples.

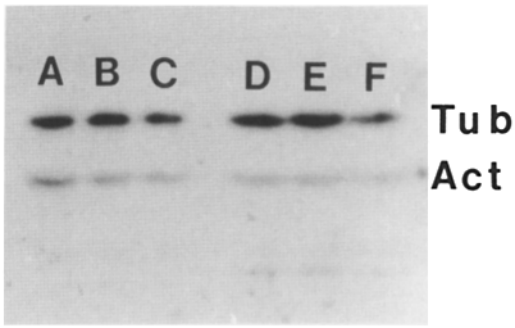

Figure 9. Immunoblot analysis of β-tubulin and actin levels. Haploid wild type and *tub2-401* cells were grown at 30°C and shifted to 18°C for 8 h or 14°C for 16 h. (lane *A*) wild type, 30°C; (lane *B*) wild type, 18°C; (lane *C*) wild type, 14°C; (lane *D*) *tub2-401*, 30°C; (lane *E*) *tub2-401*, 18°C; (lane *F*) *tub2-401*, 14°C. Tub, β-tubulin; Act, actin. When normalized to the levels of actin, the relative levels of β-tubulin were as follows: wild type, 30°C = 1.00; wild type, 18°C = 0.98; wild type, 14°C = 0.88; *tub2-401*, 30°C = 1.10; tub2-401, 18°C = 1.05; tub2-401, 14°C = 0.88.

Discussion

Astral Microtubules Are Not Required for Anaphase B

At 18°C the *tub2-401* mutation causes a defect in astral microtubule assembly in vivo; 85% of the cells lacked detectable astral microtubules. In contrast, both short and long spindles were observed in about the same ratios as in wild type cells, indicating that the mutation does not have a significant effect on spindle formation or elongation. However, spindle elongation occurred entirely within the mother cell producing a binucleate and anucleate cell. From these results we conclude that astral microtubules are required for establishing the correct position of the spindle in the bud neck but are not needed for spindle elongation. Previous results have also indicated that astral microtubules are needed to move the spindle into the bud neck (Huffaker et al., 1988; Jacobs et al., 1988). The accompanying paper (Palmer et al., 1992) demonstrates that astral microtubules are required as well to maintain the orientation of the spindle once it has reached the bud neck.

Other than its location, anaphase in these cells appeared to be normal by every criteria that we measured. Elongated spindles were observed in mutant cells at about the same frequency as in wild type cells. They often appeared to obtain the length of fully elongated spindles in wild type cells even though they had to bend to fit into one cell body. The intensity of DAPI staining was similar between separated sets of chromosomes indicating that chromosome segregation was not grossly affected. Finally, nuclear division appeared to be completed producing a cell with two distinct nuclear envelopes. Because all of our analyses were done at the level of the light microscope, we can not exclude the possibility that the mutation affects the ultrastructure of the spindle or results in increased levels of chromosome missegregation. This mutation has a severe effect on spindle microtubules at 14°C so it is not unreasonable to expect that some defect in spindle function might be detected at 18°C by more sensitive assays.

Three lines of evidence support the conclusion that astral microtubules are not needed for anaphase B movement. First, astral microtubules of any type were observed in only 15% of the cells. Therefore, most of the cells that completed nuclear division lacked detectable astral microtubules. The conclusion that these cells in fact contained no astral microtubules depends on the sensitivity of the technique used to detect them. Previous studies have demonstrated that immunofluorescence is capable of detecting single microtubules in vitro (Mitchison and Kirschner, 1984) and in vivo in animal cells (Rieder and Alexander, 1990). We feel it is reasonable to conclude that immunofluorescence could detect single microtubules in yeast as well. We cannot exclude the possibility that astral microtubules were present in *tub2-401* cells at 18°C but not preserved by formaldehyde fixation. Astral microtubules were observed in almost all wild type cells and spindle microtubules were observed in *tub2-401* as frequently as in wild type cells. Thus, we would still have to invoke a specific defect in astral microtubules in *tub2-401* cells to explain an increased sensitivity to fixation.

The second line of evidence is that many of the spindles in the binucleate cells were much longer than the diameter of the mother cell and bent around the cell periphery. Pulling by astral microtubules could not produce a spindle length greater than the diameter of the cell. Therefore, the poles must have been pushed by forces generated within the spindle. Bent spindles often appeared to attain a normal full length. This suggests that spindle length is not determined by the confines of the cell but is an intrinsic property of the spindle.

The third line of evidence is that the orientation of spindles in the binucleate cells was random. Spindles were observed in every direction across the mother cell, sometimes perpendicular to the normal direction. In cells with two spindles, the spindles were usually oriented in different directions. Thus, even within one cell, spindle orientation appeared random. Astral microtubules must be anchored at some cellular site if they are going to exert a force on the spindle poles.

Because they are required for establishing the position of the spindle in the bud neck, it is likely that such a site is located in the mother cell and bud. In order for astral microtubules to play a role in the randomly oriented spindle elongations that occur in *tub2-401* cells, they would need anchoring sites randomly located around the mother cell. This we view as unlikely. The fact that spindle elongation occurs regardless of the orientation of the spindle indicates that the components needed for this process reside within the nucleus.

The results presented here demonstrate that forces generated within the spindle are sufficient for spindle elongation and chromosome separation. This does not rule out the possibility that astral microtubules normally do exert a pulling force on the spindle poles at anaphase B. Studies on another yeast mutant suggest that poles may separate in the absence of a bipolar spindle. The *mps2* (Winey et al., 1991) mutant fails to assemble a normal spindle pole at its restrictive temperature. Instead the new pole can only nucleate astral microtubules; the old pole assembled before the temperature shift is normal and assembles both spindle and astral microtubules. This creates a monopolar spindle but both spindle poles nucleate astral microtubules. In this situation the poles do separate, one moves into the bud and other remains in the mother cell. Because the monopolar spindle cannot push the poles apart, the astral microtubules may be pulling the poles. Thus, both pushing and pulling forces could be acting at anaphase B. Our results show that pushing forces are sufficient for chromosome segregation. It is not known whether the pulling forces are sufficient to separate chromosomes. In the *mps2* mutant, chromosomes are not separated but remain attached to one pole.

Cell Cycle Progression

Successful completion of the cell cycle requires an ordered sequence of events. In most eukaryotic cells this sequence is enforced by checkpoints, points in the cell cycle that cannot be passed unless certain prior events have been completed (Hartwell and Weinert, 1989). Eukaryotic cells that have been treated with agents that disrupt microtubules arrest in M phase of the cell cycle. In yeast, disruption of all cellular microtubules can be achieved by drugs or mutations (for example, *tub2-401* cells at 14°C). Cells arrest with a large bud and a single undivided nucleus containing a replicated set of chromosomes. Cytokinesis and progression to the next cell cycle are blocked. Thus, microtubules perform some function that is required to pass a mitotic checkpoint. Hoyt et al. (1991) and Li and Murray (1991) have identified several genes that are needed for this checkpoint to function.

tub2-401 cells at 18°C are able to progress past this mitotic checkpoint. Although nuclear division takes place entirely within the mother cell, the cell cycle is not blocked. Two unbudded cells are produced, one with two nuclei and one lacking a nucleus. The binucleate cell can continue through another round of the cell cycle, budding, replicating its DNA, and dividing each nucleus to produce a tetranucleate and another anucleate cell. This process continues on average for three to four generations until, presumably, the ploidy becomes too great for cell viability. Because these cells lack astral microtubules, we conclude that astral microtubules do not perform any function that is required to pass the mitotic checkpoint. The obvious interpretation of these results is that the checkpoint monitors some aspect of spindle assembly. It apparently does not depend on the location or orientation of the spindle.

Microtubule Assembly in tub2-401 Cells

tub2-401 is a cold-sensitive allele of the gene encoding β-tubulin that causes a defect in microtubule assembly at restrictive temperatures. This defect becomes progressively more severe as the temperature is lowered. At 30°C, the permissive temperature, *tub2-401* cells contain all the microtubule structures observed in wild type cells. At 18°C, spindle microtubules are observed but astral microtubules are primarily lacking. At 14°C, neither spindle nor astral microtubules are present. These results demonstrate that both spindle and astral microtubules are affected by this alteration in β-tubulin, but the astral microtubules are more sensitive to cold temperatures than spindle microtubules.

One interpretation of the *tub2-401* phenotype is that this mutation makes the polymerization of tubulin subunits intrinsically more cold sensitive. Because yeast contain only one β-tubulin, both spindle and astral microtubules must be affected. Spindle microtubules may be more stable than astral microtubules by virtue of their association with other components of the spindle. For example, spindle microtubules may be stabilized by interactions with chromosomes or microtubule-associated proteins that cross-link interdigitated polar microtubules. We assume that astral microtubules also interact with other proteins, but the stability afforded by these associations may not be as great.

A second explanation depends on the fact that the yeast nuclear envelope remains intact throughout the cell cycle. Because the spindle and astral microtubules lie in two separate cellular compartments, yeast cells must have some mechanism to ensure the proper distribution of tubulin subunits. Yeast may preferentially import tubulin into the nucleus to provide sufficient subunits for spindle formation and elongation. If the concentration of tubulin subunits is higher in the nucleus than in the cytoplasm, spindle microtubules might be able to assemble under conditions that inhibit astral microtubule assembly. The *tub2-401* mutation may produce such a condition at 18°C.

It is also possible that the *tub2-401* mutation does not affect the intrinsic polymerization properties of tubulin subunits. The mutation may instead alter the interaction of microtubules with their associated proteins. If this is the case, the greater cold sensitivity of astral microtubules could be explained in a number of ways. For example, spindle and astral microtubules probably interact with different sets of proteins. The *tub2-401* mutation may block a subset of these interactions. The interactions that are lost as a result of the mutation may be more critical for astral microtubule stability; hence, they become more cold-sensitive.

It is not likely that the mutation interferes with the formation of the α-tubulin-β-tubulin dimer. The monomer proteins are probably not stable (Kemphues et al., 1982; Katz et al., 1990), so such a defect would be expected to reduce the levels of tubulin. In contrast, the levels of β-tubulin in the mutant remain at wild type levels at all temperatures.

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