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In Vitro Reactivation of Spindle Elongation in Fission Yeast *nuc2* Mutant Cells

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Abstract. To investigate the mechanisms of spindle elongation and chromosome separation in the fission yeast *Schizosaccharomyces pombe*, we have developed an in vitro assay using a temperature-sensitive mutant strain, *nuc2*. At the restrictive temperature, *nuc2* cells are arrested at a metaphase-like stage with short spindles and condensed chromosomes. After permeabilization of spheroplasts of the arrested cells, spindle elongation was reactivated by addition of ATP and neurotubulin both at the restrictive and the permissive temperatures, but chromosome separation was not. This suggests that the *nuc2* cells are impaired in function at a stage before sister chromatid disjunction. Spindle elongation required both ATP and exogenous tubulin and was inhibited by adenylyl imidodiphosphate (AMPPNP) or vanadate. The ends of yeast half-spindle microtubules pulse-labeled with biotinylated tubulin moved past each other during spindle elongation and a gap formed between the original half-spindles. These results suggest that the primary mechanochemical event responsible for spindle elongation is the sliding apart of antiparallel microtubules of the two half-spindles.

THE molecular mechanisms that are required for the orderly segregation of chromosomes during mitosis have been studied extensively, but still remain poorly understood. One promising approach for analyzing this problem is the genetic analysis of mutants that are defective in mitotic events. Many cell division cycle (*cdc*) and cell-size mutants have been isolated from a variety of yeasts and other fungi (Pringle and Hartwell, 1981; Nurse, 1985; Yanagida et al., 1985). The fission yeast *Schizosaccharomyces pombe* is an attractive model system for analyzing mitosis. Unlike the budding yeast *Saccharomyces cerevisiae*, *S. pombe* cells divide in the center of the cell and have a series of mitotic events that is more characteristic of higher eukaryotes. These events include the disappearance of cytoplasmic microtubule arrays, chromosome condensation, spindle formation, and chromosome separation accompanied by spindle elongation. In *S. pombe*, several mitotic mutants that are arrested at specific mitotic phases or are defective in chromosome separation have been isolated and characterized, and genes responsible for some of these mutations, including alpha- and beta-tubulin and DNA topoisomerase II have been identified (Toda et al., 1984; Hiraoka et al., 1984; Uemura and Yanagida, 1984, 1986; Uemura et al., 1987; Hirano et al., 1986, 1988, 1989; Ohkura et al., 1989; Adachi and Yanagida, 1989).

Another approach for analyzing mitosis is the development of systems in which mitotic events are reproduced in vitro. Using amphibian egg extracts and isolated nuclei, several laboratories have developed in vitro systems for studying nuclear envelope breakdown, chromosome condensation, and spindle formation (Lohka and Maller, 1985; Miake-Lye and Kirschner, 1985; Suprynowicz and Gerace, 1986; Newport and Spann, 1987). The ability of chromosomes to capture and translocate on microtubules toward the plus end (Mitchison and Kirschner, 1985*a*,*b*) and to move toward the minus end by depolymerization of microtubules (Koshland et al., 1988) has been demonstrated using isolated chromosomes. In our laboratory, the mechanisms of anaphase chromosome movement have been studied in lysed mammalian cells (Cande, 1982) and with spindles isolated from the diatom *Stephanopyxis turris* (Cande and McDonald, 1985, 1986; Masuda and Cande, 1987; Wordeman and Cande, 1987; Baskin and Cande, 1988; Masuda et al., 1988; Wordeman et al., 1989). Recently, we have shown using isolated diatom spindles that the forces responsible for spindle elongation are produced by mechanochemical enzymes that mediate the sliding apart of antiparallel microtubules of the two half-spindles, and that the role of tubulin polymerization is to increase the extent of spindle elongation (Masuda and Cande, 1987; Masuda et al., 1988).

We sought to develop a system in which genetic analysis could be combined with in vitro analysis of function. Such a system would be a powerful tool for understanding the roles of specific gene products in the orderly segregation of chromosomes. Genetic analysis of the mutants can be used to define the gene products responsible for the mutant phenotypes, and their functions can be studied in vitro.

To develop an in vitro system for studying the mechanisms of chromosome segregation in *S. pombe*, we have chosen a temperature-sensitive mutant strain *nuc2* (Hirano et al.,

1988) as starting material. The *nuc2* cells allow us to obtain a large number of mitotic cells, because they are arrested during mitosis with a short spindle and condensed chromosomes when incubated at the restrictive temperature. The gene product of *nuc2* is a 67-kD protein that is found in a fraction containing nuclear scaffold-like proteins (Hirano et al., 1988).

Using the *nuc2* cells arrested at the restrictive temperature, we developed a method to permeabilize cells and stabilize spindles with retention of some spindle functions. Because the *nuc2* cells are arrested at a metaphase-like stage, we examined whether we could induce anaphase in vitro in the permeabilized cells. Normally at anaphase in *S. pombe*, spindles elongate from 2–3 to 10–13 μm (Hiraoka et al., 1984). Although chromosome-to-pole movement has not been demonstrated in *S. pombe*, chromosome separation definitely occurs as the spindle elongates during anaphase. In the permeabilized *nuc2* cells, spindle elongation was reactivated, but chromosome separation was not. This suggests that *nuc2* cells are impaired in function at a stage before sister chromatid disjunction.

We used these permeabilized cells to study the mechanism of spindle elongation in the fission yeast, and found that the two half-spindles slide apart during spindle elongation. The physiology of spindle elongation and the rearrangements of microtubules that occur in fission yeast in vitro are similar to those of diatom spindle elongation in vitro (Cande and McDonald, 1985, 1986; Masuda and Cande, 1987; Masuda et al., 1988). This suggests that the sliding apart of two half-spindles may be a general mechanism for spindle elongation in many organisms.

Materials and Methods

Preparation of Neurotubulin, Glu-Tubulin, and Biot-Tubulin

Neurotubulin was isolated from twice-cycled bovine brain microtubule protein by ion exchange chromatography on DEAE-Sephadex, and biotinylated as described previously (Masuda and Cande, 1987). Glu-tubulin was prepared by incubation of twice-cycled bovine brain microtubule protein with pancreatic carboxypeptidase A (Worthington Biochemicals, Malvern, PA), followed by ion exchange chromatography on DEAE-Sephadex according to the method of Webster et al. (1987). Unmodified neurotubulin, biot-tubulin, and glu-tubulin were frozen in liquid nitrogen and stored in small aliquots at −80°C. A molecular mass of 110 kD was used to calculate tubulin concentration.

Preparation of Spheroplasts of nuc2 Cells

The *nuc2* cells (Hirano et al., 1988) were grown in YPD[1] medium (0.1% yeast extract, 0.2% polypeptone, and 0.2% glucose) at 26°C, the permissive temperature. After the cell density reached 3–5 × 10^6/ml, cells were incubated at 36°C, the restrictive temperature, for 4–5 h. Cells were collected by low-speed centrifugation, washed once with 0.1 M Tris-H_2SO_4 (pH 9.4) and incubated in the same solution containing 10 mM DTT for 5 min at 36°C. They were then washed twice with YPD medium containing 1 M sorbitol (YPDS medium), resuspended at 10^8/ml in YPDS medium containing 0.6–1.0 mg/ml Zymolyase 100T (Seikagaku Kogyo Co., Tokyo, Japan), and incubated at 36°C for 30–60 min until more than half of the cells became spheroplasts.

Permeabilization of Spheroplasts

Spheroplasts were washed twice with MESS solution (0.1 M MES, pH 6.5, 5 mM EDTA, 1 mM spermidine, 0.5 mM spermine, 1 M sorbitol, 10% DMSO, 0.1 mM rac-6-hydroxy-2,5,7,8-tetramethyl-chromane-2-carboxylic acid [trolox; Fluka Chemical Corp., Ronkonkoma, NY], 5 mM DTT, 0.2 mM PMSF, and proteolytic inhibitors [40 μg/ml benzyl arginyl methyl ester, 40 μg/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone, 40 μg/ml *p*-tosyl-L-arginine methyl ester, 4 μg/ml leupeptin, and 4 μg/ml pepstatin A]), then resuspended in the same solution containing 0.5% Triton X-100 on ice, and incubated for 10 min on ice. Permeabilized cells were washed twice in MESS solution without sorbitol, and sedimented onto poly-L-lysine-coated coverslips at 4°C. Coverslips were stored in MESS solution containing 2 mg/ml BSA without sorbitol on ice. The cells that had not been converted into spheroplasts were not susceptible to permeabilization by this method.

Reactivation of Spindle Functions

Permeabilized cells were washed with PMEG solution (75 mM Pipes, 10 mM $MgSO_4$, 5 mM EGTA, 40 mM beta-glycerophosphate, 0.1 mM ATP-gammaS, 0.1 mM trolox, 5 mM DTT, and proteolytic inhibitors [pH 6.8]) containing 10% DMSO, and incubated at 36°C or 22°C in PMEG without DMSO in the presence of 6 mM ATP, 3 mM GTP, 20 μM taxol, and 20 μM neurotubulin by pipetting 40 μl of the solution directly onto coverslips. Reactivation was terminated by fixation in PMEG solution containing 10% DMSO, 2.6% paraformaldehyde and 0.2% glutaraldehyde.

Indirect Immunofluorescence and Light Microscopy

After fixation for 15 min and reduction in 1 mg/ml sodium borohydride in PBS/methanol (1:1) for 15 min, the coverslips were incubated with selected antibodies. Rabbit polyclonal anti-glu-tubulin antiserum (Gundersen et al., 1984) and a mouse MPM-2 mAb (Davis et al., 1983) were kindly provided by Dr. Jeannette Bulinski (University of California, Los Angeles), and Dr. Potu Rao (University of Texas, M. D. Anderson Cancer Center), respectively. Other antibodies were purchased as follows: a mouse mAb against chicken alpha-tubulin (clone DM1A) from Sigma Chemical Co., St. Louis, MO; a rat anti-tyr-tubulin mAb (clone YL1/2) (Kilmartin et al., 1982; Wehland et al., 1983) from Accurate Chemical & Scientific Corp., (Westbury, NY); and a rabbit anti-biotin antibody from Enzo Biochem Inc. (New York, NY). For visualization of total microtubules in the permeabilized cells (Fig. 1), cells were incubated with mouse anti-chicken alpha-tubulin (DM1A) (1:500) for 1 h or overnight, and with fluorescein-conjugated goat anti-mouse IgG (1:50) for 30 min. For double-staining with anti-glu tubulin and anti-tyr tubulin antibodies (Fig. 4), cells were stained with anti-glu tubulin (1:50) for 30 min and then with fluorescein-conjugated sheep anti-rabbit IgG (1:80) for 30 min, followed by incubation with anti-tyr tubulin (1:50) for 1 h and with rhodamine-conjugated goat anti-rat IgG (1:40) for 30 min. For double-staining with MPM-2 antibody and with anti-tyr tubulin (Fig. 5), cells were incubated with MPM-2 antibody (1:50) for 2 h and with fluorescein-conjugated anti-mouse IgG for 1 h, followed by incubation with anti-tyr tubulin and with rhodamine-conjugated goat anti-rat IgG. For double-staining with anti-biotin and anti-tyr tubulin (Fig. 7), cells were stained with anti-biotin (1:100) for 1 h and with fluorescein-conjugated sheep anti-rabbit IgG (1:80), followed by incubation with anti-tyr tubulin and with rhodamine-conjugated anti-rat IgG. After cells were incubated in 0.1 μg/ml diamioinophenyl indole (DAPI) for 5 min, the coverslips were mounted in PBS containing 90% glycerol and 100 mg/ml 1,4-diazabicyclo(2.2.2)octane. Cells were observed using a Zeiss photomicroscope III equipped with epifluorescence optics and 100× neofluar lens. Lengths of spindles were measured from immunofluorescence images and spacings between zones of biot-tubulin incorporation on a TV monitor using a DAGE-MTI low-light level TV camera.

Results

Permeabilization of nuc2 Cells

A temperature-sensitive mutant strain, *nuc2*, forms short, uniform spindles (~2–3 μm long) when arrested at the restrictive temperature (36°C) (Hirano et al., 1988). The spin-

1. *Abbreviations used in this paper*: DAPI, 4′-6-diamidino-2-phenylindole; MESS, 0.1 M MES, pH 6.5, 5 mM EDTA, 1 mM spermidine, 0.5 mM spermine, 1 M sorbitol, 10% DMSO, 0.1 mM trolox, 5 mM DTT, 0.2 mM PMSF, and proteolytic inhibitors; PMEG, 75 mM Pipes, 10 mM $MgSO_4$, 5 mM EGTA, 40 mM beta-glycerophosphate, 0.1 mM ATP-gammaS, 0.1 mM trolox, 5 mM DTT, and proteolytic inhibitors; YPD, 0.1% yeast extract, 0.2% polypeptide, 0.2% D-glucose.

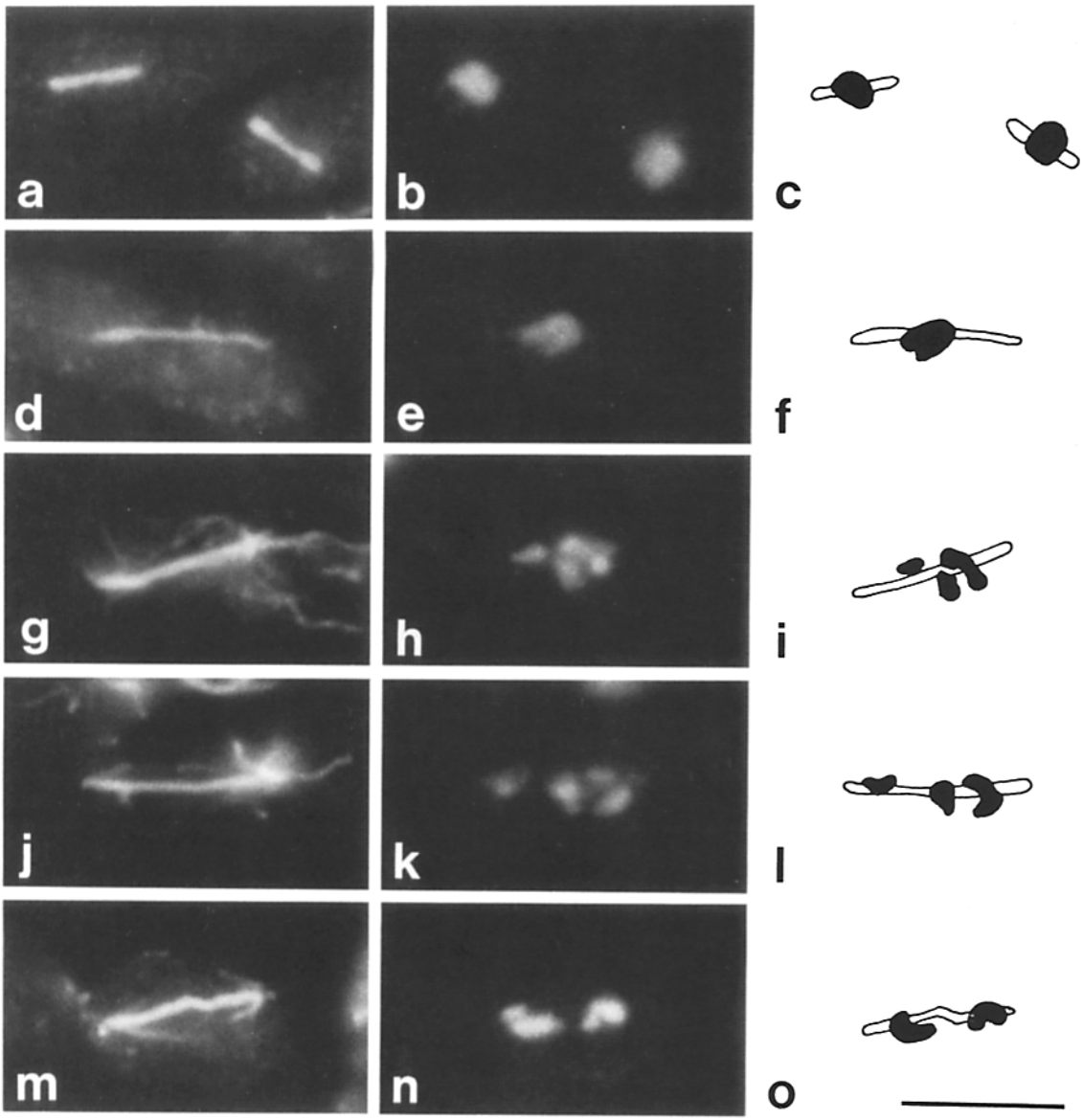

Figure 1. Reactivation of spindle elongation and chromosome movement in the permeabilized *nuc2* cells. The permeabilized *nuc2* cells were incubated in PMEG solution in the presence of 6 mM ATP, 3 mM GTP, 20 μM neurotubulin, and 20 μM taxol for 15 min at 36 or 22°C. Spindles and chromosomes in the permeabilized cells were shown by immunofluorescence with anti-chicken alpha-tubulin (*left*) and DAPI (*middle*). The relative positions of spindle and chromosomes were shown by schematic drawings (*right*). (*a–c*) Before reactivation; (*d–l*) after reactivation at 36°C; (*m–o*) after reactivation at 22°C. Bar, 5 μm.

dle passes through a metaphase-plate like configuration of three condensed chromosomes. Permeabilized cells were prepared from spheroplasts of arrested cells. Immunofluorescence microscopy showed that 50–80% of the spheroplasts had short spindles and condensed chromosomes with a metaphase-like configuration. Other spheroplasts showed cytoplasmic arrays of microtubules and decondensed chromosomes that were typical of interphase cells (Hagan and Hyams, 1988; Toda et al., 1981) (data not shown). Spheroplasts were permeabilized with 0.5% Triton X-100 in the presence of 10% DMSO. In the absence of DMSO spindle microtubules immediately disassembled. The permeabilized cells retained the short spindle and condensed chromosomes (Fig. 1, *a* and *b*). Cytoplasmic microtubules of interphase cells also remained in the permeabilized cells.

Reactivation of Spindle Elongation

To reactivate spindle function in permeabilized cells, we used conditions similar to those used for the reactivation of isolated diatom spindles (Masuda et al., 1988). The permeabilized cells were incubated in PMEG solution containing 6 mM ATP, 3 mM GTP, 20 μM neurotubulin, and 20 μM taxol at the restrictive temperature (36°C) or at the permissive temperature (22°C) (see Materials and Methods). We found that spindle elongation occurred in the permeabilized cells both at 36 and 22°C (Figs. 1 and 2). Although the isolated diatom spindles can elongate in ATP in the absence of exogenous tubulin (Cande and McDonald, 1985), spindle elongation in the permeabilized *nuc2* cells required the addition of both ATP and exogenous tubulin. Without tubulin,

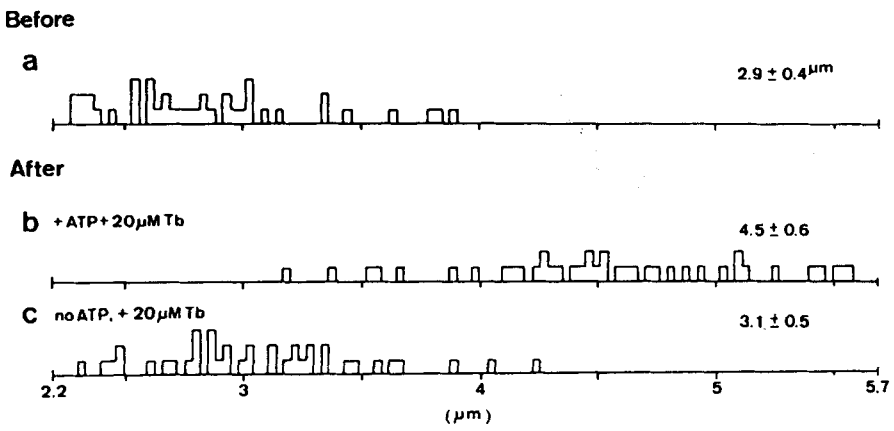

Figure 2. Histograms of spindle lengths before (*a*) and after (*b* and *c*) reactivation. The permeabilized cells (*a*) were incubated at 36°C for 15 min in PMEG solution containing 3 mM GTP, 20 μM neurotubulin, and 20 μM taxol in the presence (*b*) or absence (*c*) of 6 mM ATP. The average length of the spindles (n = 40) for each experimental condition is given.

spindles in the permeabilized cells disassembled during the incubation period. Taxol had no effect on spindle disassembly. Neurotubulin was incorporated into the spindle, around the spindle poles as new microtubules in the nucleus, and as cytoplasmic microtubules both in the presence (Fig. 1) and absence (data not shown) of ATP. Without ATP, however, spindles did not elongate in the presence of neurotubulin (Fig. 2). Neither ATP-gammaS, GTP, ITP, or ADP induced spindle elongation.

The rate and extent of spindle elongation were dependent on the tubulin concentration and incubation temperature (Fig. 3). Spindles elongated faster in the higher concentration of tubulin (0.1–0.2 μm/min on average in 20 μM tubulin), but still slower than in vivo (~1 μm/min) (Hiraoka et al., 1984). Spindles elongated faster at 36°C, the restrictive temperature, than at 22°C, the permissive temperature.

Chromosome separation or disjunction was not required for spindle elongation. When spindles elongated at 36 or 22°C, chromosomes tended to remain at the center of the spindle (Fig. 1, *d–f*). However, in a small number of spindles (range, 1 to 10%), movement of chromosomes away from the spindle midzone occurred as the spindles elongated (Fig. 1, *g–o*). In some cases, we observed three chromosome masses occupying different positions along the elongated spindle (Fig. 1, *g–l*); in other cases, we observed two chromosome masses, in which one was usually larger than the other, at different positions along the spindle (Fig. 1, *m–o*). In *S. pombe*, there are three linkage groups (Kohli et al., 1977), and three sets of chromosomes can be seen by DAPI staining in mitotically arrested cells (Umesono et al., 1983). The pattern of chromosome movement we observed in vitro is consistent with a mechanism of chromosome translocation without separation of sister chromatids. Even when chromosome movement occurred, most of the chromosomes did not move to the ends of the spindle; and in those spindles where elon-

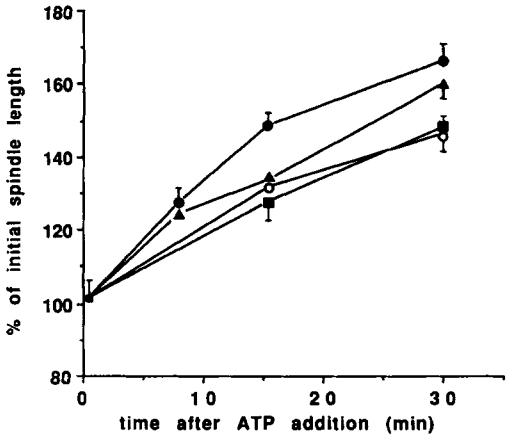

Figure 3. Dependence of spindle elongation on tubulin concentration and incubation temperature. Spindle elongation in the permeabilized cells was reactivated in PMEG solution containing 6 mM ATP, 3 mM GTP, 20 μM taxol, and (●) 20 μM, (▲) 10 μM, or (■) 5 μM neurotubulin at 36°C, or (○) 20 μM neurotubulin at 22°C. Lengths of 30 spindles were measured and averaged for each point. SEM for some points are given.

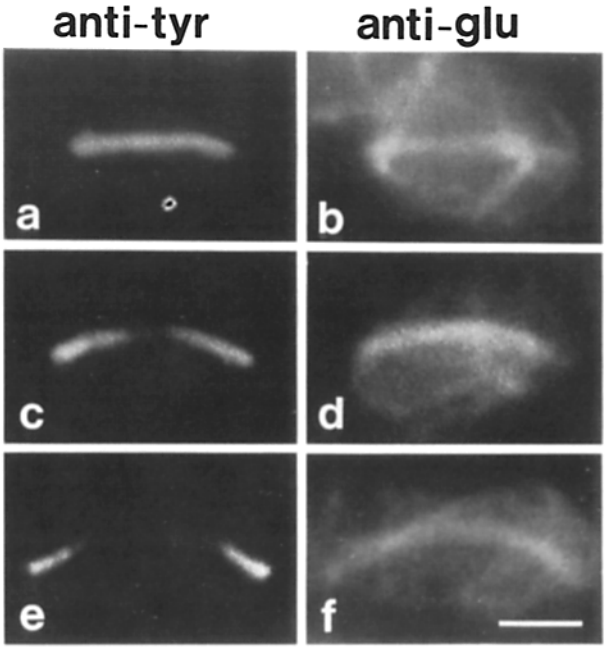

Figure 4. Behavior of yeast spindle microtubules during spindle elongation in glu-tubulin. Spindle elongation in permeabilized cells was reactivated in PMEG solution containing 6 mM ATP, 3 mM GTP, 20 μM taxol, and 20 μM glu-tubulin for 15 min at 36°C. Three representative elongated spindles are shown that were double-stained with anti-tyr-tubulin (*a*, *c*, and *e*) and anti-glu-tubulin (*b*, *d*, and *f*) antibodies. Bar, 2 μm.

gation did not occur, chromosomes always remained in the spindle midzone. Those results suggest that anaphase A-like movement, that is, chromosome-to-pole movement, did not occur in the permeabilized cells.

Effect of AMPPNP and Vanadate on Spindle Elongation

We examined the effects of inhibitors of microtubule-based motility, vanadate and AMPPNP, on spindle elongation. Vanadate is an inhibitor of ciliary, flagellar, and cytoplasmic dynein ATPase activity (Gibbons et al., 1978; Paschal and Vallee, 1987). A nonhydrolyzable ATP analogue, AMPPNP blocks kinesin-induced microtubule motility in the presence of ATP (Vale et al., 1985). Both inhibit diatom spindle elongation in vitro (Cande and McDonald, 1986; Masuda and Cande, 1987). Spindle elongation in *S. pombe* was inhibited by an equimolar mixture of AMPPNP and ATP (3 mM/3 mM) or by one-tenth molar ratio of vanadate to ATP (0.6 mM/6 mM). Although the concentrations of vanadate and AMPPNP required to inhibit spindle elongation in yeast were higher than in diatoms, the molar ratios of vanadate and AMPPNP to ATP required for inhibition were similar.

Behavior of Yeast Spindle Microtubules during Elongation

Spindle elongation required exogenous tubulin, which was incorporated into spindles and around spindle poles. Because we had no antibody that specifically recognizes *S. pombe* tubulin, we used the following strategy to monitor the changes in distribution of *S. pombe* microtubules and exogenous neurotubulin during spindle elongation. In many types of cells, alpha-tubulin exists in two different forms, tyrosinated and detyrosinated. In *S. pombe*, alpha and beta-tubulins have a carboxy-terminal tyrosine (Toda et al., 1984; Hiraoka et al., 1984) and are recognized by anti-*S. cerevisiae* tubulin antibody (clone YL1/2; Kilmartin et al., 1982) (Adachi et al., 1986), which specifically recognizes tyrosinated tubulin (Wehland et al., 1983). In contrast, anti-detyrosinated tubulin antibody, which specifically recognizes detyrosinated (glu) tubulin (anti-glu-tubulin antibody; Gundersen et al., 1984) does not cross-react with any microtubules in *nuc2* cells (Masuda, H., unpublished observations). To distinguish endogenous tubulin from exogenous tubulin by immunofluorescence, we added detyrosinated neurotubulin (glu-tubulin) to spindles for reactivation. The spindles were then double-stained with anti-tyrosinated (tyr) tubulin and anti-glu-tubulin antibodies. Yeast spindle microtubules were recognized by the anti-tyr-tubulin antibody, but not by anti-glu-tubulin antibody. Exogenous tubulin was recognized by anti-glu-tubulin antibody, but not by anti-tyr-tubulin antibody.

When spindle elongation was induced by addition of ATP and glu-tubulin, exogenous microtubules were found in whole spindles and around the poles (Fig. 4). In longer spindles, a gap was observed at the center of the original yeast spindle when visualized with anti-tyr-tubulin antibody (Fig. 4, *c* and *e*). This gap was filled with exogenous microtubules, as seen with anti-glu-tubulin antibody. The spindles with a noticeable gap had elongated, on average, to >160–170% of the initial spindle length. The percentage of the spindles with a gap to total spindle number varied among experiments (~0–60% in 20 μM tubulin for 15 min). These results suggest that two yeast half-spindles increase in the distance between them during spindle elongation.

Site of Spindle Pole Bodies

We used a marker for the spindle pole bodies to confirm that the pole-to-pole distance increased during spindle elongation. We found that the MPM-2 antibody, which recognizes mitosis-specific phosphorylated epitopes (Davis et al., 1983), stained both ends of spindle, which correspond to the site of spindle pole bodies, and chromosomes in the permeabilized *nuc2* cells (Fig. 5, *a*, *c*, and *e*). In addition, the spindles themselves sometimes were weakly stained with the antibody. The antibody stained decondensed interphase chromosomes, but not the spindle pole bodies in interphase cells. Similar staining patterns with MPM-2 antibody have been shown using mammalian tissue cultured cells (Vandre et al., 1986). After the permeabilized cells were incubated in ATP and glu-tubulin, the antibody stained both ends of the elongated yeast spindle that was stained with anti-tyr-tubulin antibody (Fig. 5, *b* and *d*) and sometimes weakly stained other parts of spindle. Chromosomes in both mitotic and interphase cells were no longer stained with the antibody (Fig. 5, *d* and *f*), presumably due to the extraction of the proteins recognized by the antibody during reactivation.

Behavior of Microtubule Ends of Yeast Half-Spindles during Elongation

To obtain further evidence that the two yeast half-spindles slid apart, we examined the behavior of microtubule ends during spindle elongation. The experimental design is diagrammed in Fig. 6. Spindles were incubated for 1 min at 22°C with 10 μM biotinylated neurotubulin (biot-tubulin) in the absence of ATP to label the microtubule ends within each half-spindle with biot-tubulin. Using these conditions, we limited the amount of tubulin incorporation into the spindle so that discrete zones of labeling within the half-spindles were observed. If incubation occurred for longer periods or at higher concentrations, neurotubulin was incorporated throughout the spindle (Fig. 4).

In roughly half of the pulse-labeled spindles, two regions inside the spindle (*arrows* in Fig. 7, *A–b*; Table I) and regions around the spindle pole bodies were labeled with biot-tubulin (Fig. 7 *A–a* and *b*). In addition, sites near the spindle pole bodies, presumably at the cytoplasmic face of the pole body, were labeled. This incorporation may be at the sites from which astral microtubules emanate when spindles elongate in vivo (Tanaka and Kanbe, 1986; Hagan and Hyams, 1988). In many of these spindles, the zones of incorporation within each half-spindle and around the spindle pole bodies merged together and it was not possible to distinguish between the various sites of incorporation (Fig. 7, *A–c* and *d*; Table I).

Spindles that were pulse-labeled with biot-tubulin were then incubated in the presence of ATP and glu-tubulin at 36°C to induce elongation. After elongation, spindles were double-stained with anti-biotin and anti-tyr-tubulin antibodies to examine the redistribution of biot-tubulin (Fig. 7 *B*; Table I). The glu-tubulin is not stained under these conditions. As shown above, spindles that have elongated to >160–170% of the initial length had a gap at the center. In the elongated

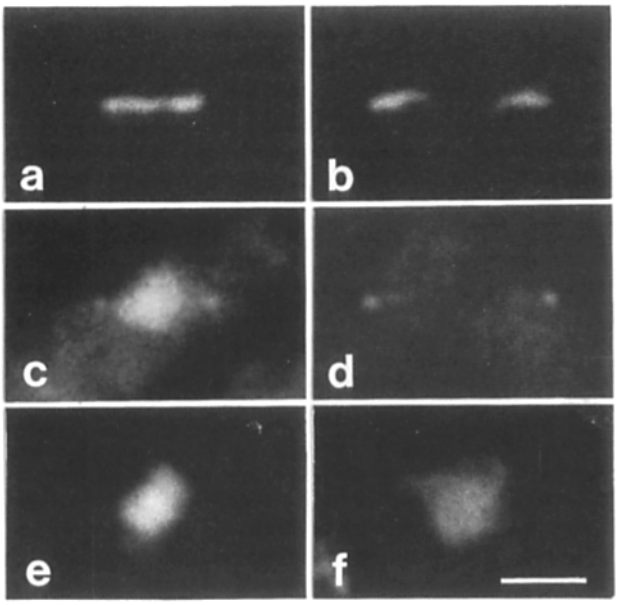

Figure 5. Sites of spindle pole bodies before and after spindle elongation. Spindle elongation in the permeabilized cells was reactivated in PMEG solution containing 6 mM ATP, 3 mM GTP, 20 μM taxol, and 20 μM neurotubulin for 15 min at 36°C. Two different cells before (*a*, *c*, and *e*) and after (*b*, *d*, and *f*) reactivation were triple-stained with anti-tyr-tubulin (*a* and *b*), MPM-2 antibody (*c* and *d*), and DAPI (*e* and *f*). Bar, 2 μm.

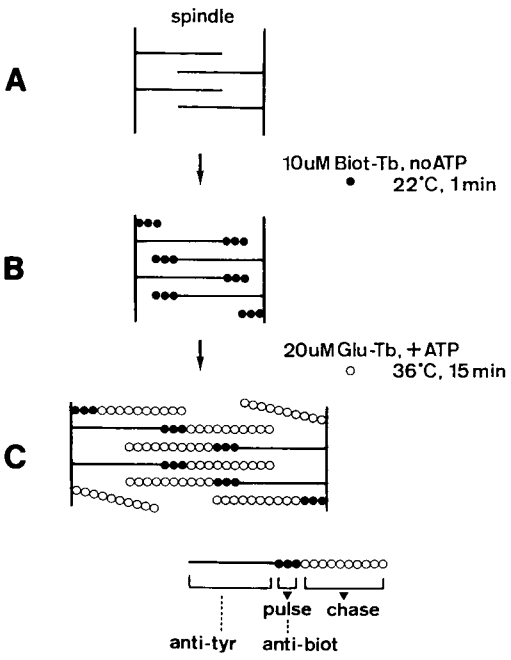

Figure 6. Schematic diagram of experiments for monitoring the behavior of microtubule ends of yeast half-spindles during spindle elongation. The ends of microtubules in yeast spindles (*A*) were pulse-labeled with biot-tubulin (biot-tb) in the absence of ATP (*B*). After free biot-tb was washed off, spindle elongation was induced by addition of glu-tb and ATP (*C*). Spindle microtubules and biot-tb on the ends were observed by immunofluorescence with anti-tyr tubulin and anti-biotin antibodies, respectively.

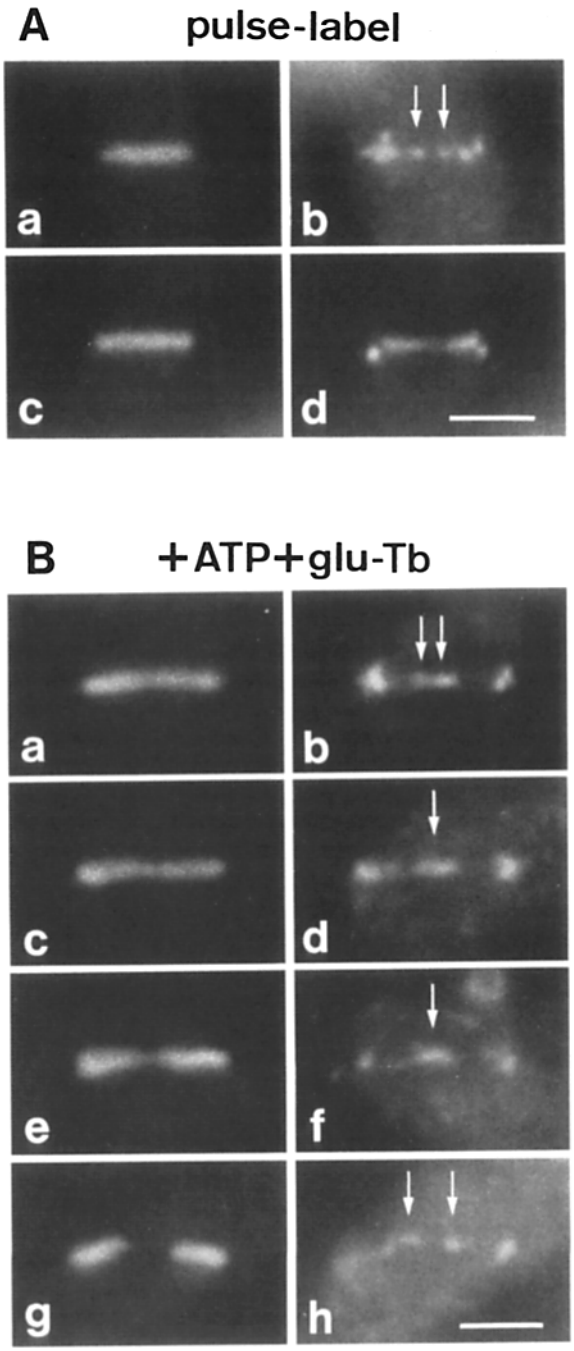

Figure 7. (*A*) Pulse-labeling of spindles with biot-tubulin. Permeabilized cells were incubated in PMEG solution containing 3 mM GTP, 20 μM taxol, and 10 μM biot-tubulin in the absence of ATP for 1 min at 22°C. They were then washed with the solution in the absence of biot-tubulin. Two spindles are shown which were double-stained with anti-tyr-tubulin (*a* and *c*), and anti-biotin (*b* and *d*). Note that two regions inside the spindle (*arrows*), regions around spindle pole bodies, and regions near the spindle pole bodies outside the spindle were labeled with biot-tubulin. (*B*) Reactivation of spindles pulse-labeled with biot-tubulin. Spindles pulse-labeled with biot-tubulin were incubated in PMEG solution containing 6 mM ATP, 3 mM GTP, 20 μM taxol, and 20 μM glu-tubulin for 15 min at 36°C. Spindles were double-stained with anti-tyr-tubulin (*a*, *c*, *e*, and *g*) and with anti-biotin (*b*, *d*, *f*, and *h*). Typical examples of the elongated spindles are shown which were divided into four groups based on the behavior of yeast spindle microtubules and of biot-tubulin inside the spindle (*arrows*). Bar, 2 μm.

Table I. Distribution of Pulse-labeled Biotinylated Tubulin Incorporation in the Spindle

Classes		%	Spindle length		Spacing*
		n = 40	μm	%	μm
Before reactivation					
Two discrete zones	(Fig. 7 *A–a* and *b*)	57	2.26 ± 0.30	105	0.90 ± 0.22
Two broad zones	(Fig. 7 *A–c* and *d*)	43	2.02 ± 0.30	94	1.34 ± 0.24
After reactivation					
Two discrete zones	(Fig. 7 *B–a* and *b*)	40	2.95 ± 0.40	137	0.68 ± 0.18
One discrete zone	(Fig. 7 *B–c* and *d*)	15	3.05 ± 0.24	141	–
One zone spanning gap	(Fig. 7 *B–e* and *f*)	32	3.59 ± 0.33	166	–
Two zones flanking gap	(Fig. 7 *B–g* and *h*)	13	3.92 ± 0.54	181	0.92 ± 0.30

Spindles before and after reactivation were divided into two and four classes, respectively, according to the images from immunofluorescence with anti-biotin and anti-tyr-tubulin antibodies (Fig. 7). Spindle lengths were determined in microns and as percentage of initial spindle length (average 2.16 μm).
* Center-to-center distance between two labeled zones inside the spindle.

spindles with no gap, we observed two labeled regions, which were closer to each other than those before reactivation (Fig. 7 *B–a* and *b*; Table I), or just one labeled region at the center of the spindle (Fig. 7 *B–c* and *d*). In the elongated spindles with a small gap, we observed one biotin-labeled region that spanned the gap (Fig. 7 *B–e* and *f*). A small number of the elongated spindles with a gap showed two labeled regions at the ends of the large gap (Fig. 7 *B–g* and *h*). These results suggest that yeast spindles elongate in vitro by a mechanism involving microtubule sliding; that is, as spindles elongate in the presence of glu-tubulin and ATP, the half-spindle microtubules with their zones of incorporated biot-tubulin slide past each other (see Fig. 6 and Discussion).

Discussion

Function of the Mitotic Apparatus in Permeabilized *nuc2* Cells

We have developed a permeabilized cell model for studying the functions of the mitotic apparatus in *nuc2* cells arrested at a restrictive temperature. Because the *nuc2* cells are apparently arrested at a metaphase-like stage with a short spindle and condensed chromosomes, we examined whether we could reactivate spindle elongation and chromosome separation in vitro. Spindle elongation occurred both at restrictive and permissive temperatures, and was faster at 36°C, the restrictive temperature, than at 22°C, the permissive temperature. This suggests that the machinery for spindle elongation is functional in *nuc2* cells. In contrast, chromosome disjunction or separation did not occur at either temperature. This is consistent with the observation that *nuc2* cells are irreversibly arrested at a metaphase-like stage in vivo (Hirano, T., unpublished data). The machinery for sister chromatid disjunction may be nonfunctional or have never been turned on. These results suggest that the *nuc2* cells are impaired in function at a stage before sister chromatid disjunction.

The observation that *nuc2* cells are functional in vitro for spindle elongation is consistent with a hypothesis that the *nuc2* gene product may be a nuclear scaffold protein, or a chromosomal protein perhaps associated with specific sites such as the centromere (Hirano et al., 1988), because mutations of these proteins are not likely to affect spindle elongation.

Spindles in permeabilized *nuc2* cells were labile in structure and function, and addition of exogenous tubulin was required for reactivation of spindle elongation. The rate and extent of spindle elongation reactivated in the presence of neurotubulin and ATP were less than in vivo. Spindles in permeabilized *nuc2* cells elongated from 2–3 to 3–6 μm at 0.1–0.2 μm/min on average, whereas in vivo spindles at anaphase elongate from 2–3 up to 13 μm at 1 μm/min (Hiraoka et al., 1984). The in vivo observations show that tubulin incorporation into spindles is required during anaphase to support extensive spindle elongation. We observed that a small number of yeast spindles elongated in ATP and neurotubulin to form a large gap between the two half-spindles (Figs. 4 *e* and 7 *B–g*; Table I). In some experiments, these spindles elongated to more than the double of the initial spindle length (as shown in Fig. 4 *e*) at a rate of 0.4 μm/min on average (data not shown). These observations suggest that neurotubulin incorporated into spindle can participate in yeast spindle elongation by interacting with yeast spindle microtubules. Because the percentage of the spindles with a large gap varied among experiments (~0–30%), our method for in vitro reactivation of spindle elongation may not be optimal for obtaining in vitro rates and extent of elongation similar to those in vivo.

We predict that chromosome separation would occur during spindle elongation if sister chromatids have undergone disjunction, and chromosomes remain attached to the spindle. However, chromosome separation did not occur during spindle elongation in *nuc2* cells. In most cases, chromosomes remained in the center of the elongating spindle. In some cases, chromosomes without sister chromatid disjunction were dislocated from the cell center distally along the elongating spindle. This suggests that at least in some of permeabilized cells chromosomes remained attached to the spindle and were pulled away from the spindle midzone by the elongating spindle without disjoining sister chromatids. It has been shown recently that isolated sea urchin spindles increase in pole-to-pole distance when incubated in sea urchin tubulin and ATP or GTP (Rebhun and Palazzo, 1988), and that there is a random distribution of chromosomes away from metaphase plate in metaphase starting material. Although these observations suggest that *nuc2* cells at the restrictive temperature are blocked in function before sister chromatid disjunction, it remains to be determined whether the *nuc2* gene product plays a direct role in this event.

The Mechanism of Spindle Elongation in Fission Yeast

We have shown here that spindle elongation in permeabilized *nuc2* cells can be reactivated in the presence of ATP and exogenous tubulin. We predicted that similar molecular mechanisms were required for spindle elongation in the diatom *S. turris* and the fission yeast *S. pombe*, because chromosome separation in both organisms is caused mainly by spindle elongation accompanied by tubulin polymerization (Masuda et al., 1988). Diatom spindles are favorable structures for electron microscopy and are well documented ultrastructurally (reviewed by Pickett-Heaps and Tippit, 1978), whereas *S. pombe* spindles are difficult to study by conventional microscopy and have not been extensively analyzed. Nevertheless, the overall principles of spindle architecture in both organisms are similar. The *S. pombe* spindle is composed of long parallel arrays of microtubules that are nucleated by spindle pole bodies embedded in the nuclear envelope (Tanaka and Kanbe, 1986). Although a zone of microtubule overlap is not apparent in spindles at early stages, there is a pronounced zone of microtubule overlap in telophase spindles. Short discontinuous microtubules are found on the periphery of the spindle pole which may correspond to kinetochore microtubules (Tanaka and Kanbe, 1986). The diatom spindle is composed of a central spindle and a peripheral array of kinetochore microtubules (Pickett-Heaps and Tippit, 1978; McDonald et al., 1977, 1979). The central spindle comprises two sets of microtubule bundles that interdigitate in the middle of the spindle. The interdigitated microtubules are fairly uniform in length, and the zone of microtubule overlap is distinct and visible by light microscopy (Pickett-Heaps et al., 1980; Cande and McDonald, 1985, Masuda et al., 1988). We have previously shown using isolated diatom spindles that the forces required for spindle elongation are generated by enzymes in the overlap zone that mediate the sliding apart of antiparallel microtubules of the two half-spindles (Cande and McDonald, 1985, 1986; Masuda and Cande, 1987; Masuda et al., 1988). We suggest that similar mechanochemical enzymes are involved in spindle elongation in the fission yeast and the diatom, based on the physiological and morphological similarity of the process in vitro in both organisms.

Spindle elongation in the permeabilized *nuc2* cells was highly specific for ATP, and was inhibited by AMPPNP or vanadate. The rate and extent of spindle elongation were dependent on the concentration of exogenous tubulin, although we do not know the mechanism. Similar results have been obtained using the isolated diatom spindles, except that diatom spindles can elongate in ATP in the absence of exogenous tubulin (Cande and McDonald, 1986; Masuda and Cande, 1987).

Immunofluorescence microscopy with anti-tyr-tubulin antibody that recognizes yeast spindle microtubules showed that during reactivation in glu-tubulin and ATP, the original yeast spindles increased in length, and formed a gap at the spindle midzone after elongation >60–70% of the original spindle length (Fig. 4). This suggests that the overlap zone of yeast half-spindles is broad and is ~60–70% of the original spindle length. Previously, we have shown that diatom spindles can be reactivated both in the presence and absence of exogenous tubulin and form a gap between the original half-spindles after they elongate by more than the size of the original zone of microtubule overlap (~20% of the original spindle length) (Cande and McDonald, 1985, 1986; Masuda and Cande, 1987; Masuda et al., 1988).

The pulse-label experiments showed that two regions inside the yeast spindle were labeled with biot-tubulin as discrete bands (Fig. 7 *A*). When the pulse-labeled spindles elongated in exogenous tubulin and ATP, the two bands were closer together, or combined to form one band. When the yeast spindle microtubules formed a gap at the midzone, the labeled tubulin filled the gap, or was found at the edges of the gap (Fig. 7 *B*, Table I). These results can be interpreted as follows: (*a*) isolated yeast spindles are composed of two half-spindles with microtubules of relatively uniform length and with a relatively large zone of microtubule overlap; (*b*) pulse-labeled biot-tubulin remains at the ends of yeast half-spindle microtubules during spindle elongation; and (*c*) the distance between the microtubules of the original half-spindles increases during spindle elongation (Fig. 6). Similar results have been obtained using isolated diatom spindles. When the diatom spindles are preincubated in biot-tubulin in the absence of ATP, biot-tubulin is incorporated into the ends of microtubules in the overlap zone as two discrete bands. When these spindles are then reactivated in ATP in the absence of exogenous tubulin, they elongate more than the size of the original overlap zone and form a large gap between the original half-spindles filled with biot-tubulin (Masuda et al., 1988).

The apparent disorder of tubulin incorporation into yeast spindles, when spindles were continuously incubated in glu-tubulin, is not due to a random distribution of microtubule ends throughout the spindle, but may reflect the small size of the yeast spindle (2–3 μm) when compared with the diatom spindle (8–10 μm), and the high nucleation activity of the spindle pole bodies of these metaphase-like spindles.

It is unlikely that pulling forces contribute to the yeast spindle elongation we observed in vitro. Few or no cytoplasmic microtubules are observed in the permeabilized *nuc2* cells that have spindles. During spindle reactivation, new cytoplasmic microtubules are nucleated off the spindle poles, but the arrangement and number varies from cell to cell and does not correlate with the extent of spindle elongation.

Implications

In this study we have demonstrated that we can study mitotic mechanisms in vitro in an organism, *S. pombe*, that is amenable to analysis by genetic and molecular techniques. We have shown that *nuc2* cells are competent to undergo spindle elongation in vitro. The mechanism of spindle elongation in *S. pombe*, i.e., the sliding apart of the half-spindles is similar to that observed in diatoms in vitro, suggesting that this may be a common component of spindle mechanics in many eukaryotic cells.

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