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Requirement for *ESP1* in the Nuclear Division of *Saccharomyces cerevisiae*

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Mutations in the *ESP1* gene of *Saccharomyces cerevisiae* disrupt normal cell-cycle control and cause many cells in a mutant population to accumulate extra spindle pole bodies. To determine the stage at which the *esp1* gene product becomes essential for normal cell-cycle progression, synchronous cultures of *ESP1* mutant cells were exposed to the nonpermissive temperature for various periods of time. The mutant cells retained viability until the onset of mitosis, when their viability dropped markedly. Examination of these cells by fluorescence and electron microscopy showed the first detectable defect to be a structural failure in the spindle. Additionally, flow cytometric analysis of DNA content demonstrated that massive chromosome missegregation accompanied this failure of spindle function. Cytokinesis occurred despite the aberrant nuclear division, which often resulted in segregation of both spindle poles to the same cell. At later times, the missegregated spindle pole bodies entered a new cycle of duplication, thereby leading to the accumulation of extra spindle pole bodies within a single nucleus. The DNA sequence predicts a protein product similar to those of two other genes that are also required for nuclear division: the *cut1* gene of *Schizosaccharomyces pombe* and the *bimB* gene of *Aspergillus nidulans.*

INTRODUCTION

Progression of cells through the cell cycle requires coordination of many processes. Mutational analysis of *Saccharomyces cerevisiae* has revealed one mode of coordination to be the existence of dependent pathways, wherein the execution of one event is an essential precondition for entry into later processes (Pringle and Hartwell, 1981). A dependent pathway of this sort serves to explain the faithful execution of cell-cycle functions needed for mitotic spindle formation and elongation in coordination with other cell-cycle events (Byers and Goetsch, 1974). In wild-type cells, duplication of the spindle pole body (the yeast microtubule organizing center) occurs early in the cycle as cells initiate bud formation, and the mitotic spindle then forms while the bud is still small. Later in the cycle, when the bud has become fully enlarged, the spindle elongates and segregation of the genome takes place. The elongated spindle then persists until the time of nuclear division and cytokinesis (Byers, 1981). The terminal phenotypes of many *cdc* mutants demonstrate a variety of dependent relationships, such as the dependency of anaphase spindle elongation on DNA replication and that of cytokinesis on the occurrence of anaphase.

One important basis for dependency relationships in the yeast cell cycle has recently been defined in the concept of checkpoints (Hartwell and Weinert, 1989). This paradigm derives from work of Weinert and Hartwell (1988) who showed that various agents (including some *cdc* mutational defects) causing DNA damage lead to cell-cycle arrest because cell-cycle progression is blocked by a *RAD9*-dependent function. Another checkpoint function was found to impose the requirement that DNA replication be completed before entry into anaphase (Hartwell and Weinert, 1989). Similarly, a number of genes that are involved in a checkpoint that monitors the assembly of microtubule structures have also been identified. Disruption of microtubules with microtubule depolymerizing drugs normally blocks the cell cycle at mitosis (Jacobs *et al.*, 1988). Mutation in the *MAD* (Li and Murray, 1991) or *BUB* (Hoyt *et al.*,

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1991) loci allow cell-cycle progression even in the absence of a mitotic spindle. Clearly, many of the dependent relationships revealed by the *cdc* mutants in *S. cerevisiae* might reflect the activity of such checkpoint functions.

We had previously characterized a temperature-sensitive lethal mutation, *esp1*, that fails to display the type of dependent interactions that typify the *cdc* mutants. Cells mutant for *esp1* can accumulate extra spindle pole bodies (SPBs)[1] during incubation at the nonpermissive temperature, indicating a loss of coordination between SPB duplication and other aspects of the cell division cycle (Baum *et al.*, 1988). One way to explain this phenomenon might be to posit that failure of normal *ESP1* gene function directly causes deregulation of the SPB duplication cycle, such that the initiation of duplication becomes independent of functions that ordinarily would limit it. Alternatively, *esp1* might disrupt the mitotic mechanism, initially leading to missegregation of both SPBs to the same progeny cell and later permitting even larger numbers of SPBs to accumulate in one cell. To distinguish between these possibilities, we have assessed the behavior of synchronized cellular populations to establish when mutant cells first display a structural defect and have obtained evidence that missegregation of the spindle poles is primarily responsible for the observed phenotype. Here, we present an analysis of the effects of *esp1* failure on spindle formation, genome segregation, and cell-cycle control. We also present data showing similarity of the *ESP1* product to related proteins in other eukaryotes.

MATERIALS AND METHODS

Strains

The *S. cerevisiae* strains used in this work are T25A (*MATa/MATa esp1-1/esp1-1 leu2-3,112/leu2-3,112 trp1-289/TRP1 ura3-52/URA3 lys2/LYS2 can1/CAN1 cyh2/CYH2 ADE1/ade1*); SN127-2B (*MATa esp1-1 leu2-3,112 trp1-289 ura3-52 lys2 can1*), SN127h (*MATa/MATa esp1-1/ESP1-1 leu2-3,112/leu2-3,112 trp1-289/TRP1 ura3-52/URA3 lys2/LYS2 can1/CAN1 cyh2/CYH2 ADE1/ade1*); 923-1a (*MATa esp1-1 leu2-3,112 ura3-52 lys2 can1*); 34SN-6JD6C (*MATa/MATa his3Δ1/his3Δ1 leu2-3,112/leu2-3,112 trp1-289/trp1-289 ura3-52/URA3 lys2/LYS2.*

Media

Standard yeast culture media used were yeast extract, peptone, dextrose growth medium (YEPD) (Sherman *et al.*, 1986), YM-1 +glucose (Hartwell, 1967), YM-1 +glucose, pH 3.0 (Moore, 1984), and complete minus leucine + 1 M sorbitol (Hinnen *et al.*, 1978).

Microscopy

Cells were prepared for electron microscopy as previously described (Byers and Goetsch, 1974) and were viewed in a Philips EM300 electron microscope (Mahwah, NJ). Fluorescence light microscopy was performed with a Nikon Microphot FX microscope (Garden City, NY).

[1] Abbreviation used: SPB, spindle pole body.

Indirect immunofluorescent microscopy employed YOL1/34 rat anti-yeast tubulin (Accurate, Westbury, NY) and fluorescein isothiocyanate (FITC)-conjugated goat anti-rat IgG (Accurate) to visualize microtubules and 4,6-diamidino-2-phenylindole (DAPI) staining to visualize DNA as previously described (Baum *et al.*, 1988). Visualization of bud scars was achieved by staining with 0.1% Calcofluor 100 M (Cyanamid, Bound Brook, NJ) (Pringle *et al.*, 1991). Differential interference contrast microscopy was performed with a Nikon Microphot FX microscope by using ethanol-fixed cells (Hutter and Eipel, 1979) mounted in water.

Cell-Cycle Synchronization and Temperature Shift Experiments

T25A or 923-1a cells were grown in YM-1+glucose, pH 3.0 at 23°C to a density of 0.5-2 × 10^6/ml and then arrested in the G1 phase of the cell cycle by the addition of 800 nM synthetic α-factor (Sigma, St. Louis, MO) and further incubation for 2.5 h. Maintenance of cell-cycle arrest in G1 was routinely monitored by phase microscopy to confirm the absence of buds. The characteristics of G1 arrest were also checked in some experiments by examining the arrested cells for the expected unipolar array of microtubules by indirect immunofluorescence with anti-tubulin antibodies (Adams *et al.*, 1984) and by analyzing the content of DNA by flow cytometry. α-factor was removed by sedimenting the cells for 2 min at ~1500 g, rinsing in growth media preincubated at 36 or 23°C as appropriate, resedimentation, and resuspension in growth media.

Yeast cells were prepared for flow cytometry by fixation in ethanol and staining with propidium iodide (Sigma) (Hutter and Eipel, 1979). Samples were analyzed with the use of a FACSCAN flow cytometer by using the CELLFIT and LYSYS software (Becton Dickinson, San Jose, CA). Sorted fractions were obtained by use of a ORTHO System Model 50h fluorescence-activated cell sorter.

Cloning and Sequencing of the ESP1 Locus

Plasmids carrying the *ESP1* locus were isolated by transforming SN127-2B spheroplasts (Hinnen *et al.*, 1978) with a yeast genomic plasmid library in YeP13 (Nasmyth and Tatchell, 1980) and plating on −leu medium at 37°C. Plasmids which complemented the temperature-sensitive lethality of *esp1-1* were isolated from ts^+ yeast colonies and transformed into *Escherichia coli* strain RR1 (Maniatis *et al.*, 1982). A clone conferring complementing activity (CP1) was subjected to transposon mutagenesis as described by Seifert *et al.* (1986) by using a Tn3 element carrying the yeast *HIS3* gene (a gift of Lee Hartwell and Dan Burke). Several plasmids containing single insertions at different sites were introduced into yeast strain 34SN-6D6C by lithium acetate transformation (Ito *et al.*, 1983) and after selection on −leu −his medium were assessed for their ability to complement the ts lethality of *esp1-1*. This and similar analysis of subcloned fragments revealed that most of the insert in CP1 was required for complementation. The entire ~7.0-kb insert in CP1 was sequenced by using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977) modified for the use of ^{35}S-dATP (Biggin *et al.*, 1983) and T7 polymerase (Sequenase; USB, Cleveland, OH). A series of unidirectionally deleted templates for sequencing was generated by transferring restriction fragments isolated from the insert in CP1 into the vector pVZ1 and employing the exonuclease III method (Henikoff, 1987). Both strands were sequenced in their entirety.

Northern Blot Analysis

RNA was isolated from strain AB320 (Nasmyth and Tatchell, 1980) undergoing exponential growth in YEPD media by cell breakage with glass beads in the presence of phenol sodium dodecyl sulfate (SDS) as previously described (Maccechini *et al.*, 1979). Poly A^+ RNA was prepared by oligo(dT) selection as previously described (Aviv and Leder, 1972). Approximately 5 μg of the poly A^+ RNA was electrophoresed on 1% agarose. The RNA was transferred to nitrocellulose

(Thomas, 1980) and was hybridized with a ^{32}P-labeled probe derived from a *Bam*HI/*Eco*RI digest of CP1 (see Figure 7) that was prepared by nick translation as described (Maniatis *et al.*, 1982).

RESULTS

ESP1 Function is Essential During the Mitotic Phase of the Cell Cycle

To determine when the *ESP1* gene function first becomes essential in the cell cycle, we analyzed the effect of exposing synchronized cultures of temperature-sensitive *esp1* mutant cells to restrictive conditions at various times after entry into the cell cycle. Mutant cells grown at the permissive temperature (23°C) were arrested in G1 by treatment with the mating pheromone α-factor as described in MATERIALS AND METHODS and then transferred to fresh medium to permit synchronous entry into the cell cycle at the nonpermissive temperature (36°C). Aliquots removed from the 36°C culture at various times were restored to permissive conditions by plating on solid medium at 23°C, thereby permitting resumption of *ESP1* function. Aliquots plated within 2 h after removal of α-factor retained good viability, but plating at successively later times revealed that many cells had become inviable. Monitoring cell-cycle progression by light microscopy demonstrated that the period crucial for induction of inviability at 36°C coincided with the stage of transition from a medium sized bud to a large one (Figure 1A, □, ■). In contrast, wild-type cells subjected to the same regimen retained full viability (Figure 1A, ○, ●).

The absence of lethality until the mutant population had progressed through a late stage of bud growth suggested that the *ESP1* function was unimportant during earlier stages of the cell cycle. It remained possible, on the other hand, that the observed lag instead reflected the functional half-life of the thermolabile *esp1* product. Other experiments were performed to address this possibility. We reasoned that if there actually were an essential role for the *ESP1* gene product early in the cell cycle, then mutant cells exposed to the nonpermissive temperature during the arrest with α-factor should already have lost *ESP1* function before removal of the pheromone and would die immediately upon release. Accordingly, *esp1* cells were arrested in G1 with α-factor while at the permissive temperature of 23°C. Without removal of the pheromone, the culture was split in two, and one-half was transferred to 36°C while the other half was kept at the permissive temperature. After an additional 3 h of incubation in pheromone, both portions were washed free of pheromone, releasing them from G1 at 36°C. As in the preceding experiment, successive transfer from 36 to 23°C served to establish when restoration of permissive conditions prevented inviability. Both the portion held at 23°C and the one shifted to 36°C remained fully viable while idling at G1 arrest in the presence of α-factor (Baum *et al.*, 1988; Figure 1B). Upon transfer to fresh medium and passage through the cell cycle, both cultures underwent a progressive loss of viability as before. Significantly, the rate of loss in viability was indistinguishable for the two portions, occurring ~2 h after release from α-factor as both cultures progressed to a large budded state (Figure 1B). We interpret these results to mean that the lag in loss of viability does not depend on a gradual rate of thermal denaturation of the *ESP1* gene product but in-

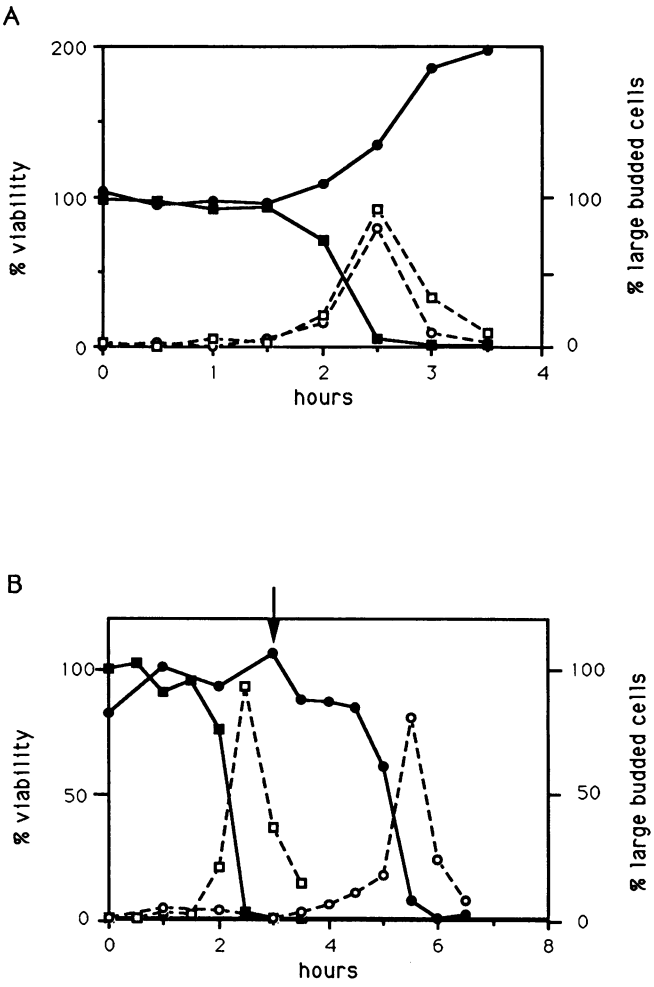

Figure 1. Time course of changes in viability and cell morphology of *esp1* diploid strain T25A after transfer to the restrictive temperature (36°C) at T = 0 h. (A) Mutant (□, ■) or wild-type cells (○, ●) were synchronized by treatment with α-factor for ~3 h at 23°C followed at 0 h by transfer to fresh medium at 36°C. At the indicated times aliquots were plated to assess viability (●, ■) and fixed to determine the distribution of cell morphologies. Open symbols indicate the fraction of large budded cells. (B) Mutant cells were synchronized as in (A) except that the culture was split into two portions. One portion was transferred directly from α-factor arrest to 36°C treatment in fresh medium (□, ■), whereas the other portion remained in α-factor containing medium at 36°C for another 3 h before transfer (at time indicated by arrow) to fresh medium at 36°C (○, ●). Closed symbols represent percent viability and open symbols, percent large budded cells.

stead reflects a stage-specific requirement for *ESP1* late in the cell cycle. Consistent with this interpretation, transfer of synchronous cultures from permissive to nonpermissive conditions at various times during cell-cycle progression caused lethality only when transfer took place before the large budded stage, when spindle elongation occurs.

Mitotic Spindle Defects Coincide with the Induction of Lethality by esp1

Previous experiments performed on asynchronously dividing cells had established that loss of *ESP1* function eventually results in a multipolar phenotype, but this striking defect becomes evident long after the induction of lethality (Baum *et al.*, 1988). Having now found that synchronously dividing mutant cells become committed to massive lethality at a specific stage of the cell cycle, we next sought to identify any primary morphological defect that might later lead to multipolarity. Accordingly, *esp1* cells undergoing a synchronous cell cycle at the nonpermissive temperature were monitored for indices of cell-cycle progression and plated to assess their viability. In addition, we fixed and processed aliquots for indirect immunofluorescence with anti-tubulin antibody to determine spindle morphology and for DAPI staining to characterize chromosomal DNA distribution. Microtubule arrays were found to remain normal in mutant cells during all stages leading up to and including G2, when a short spindle typically is found to lie entirely within the mother cell (Figure 2C1). Then, when wild-type cells had entered mitosis and the mutant cells similarly bore a large bud, several distinct spindle abnormalities become evident in the mutants incubated at 36°C (shown in Figure 2, C3 and E). Most of the mutant cells (85%) lacked normal mitotic spindles and instead contained microtubule arrays characterized by discontinuous and/or unusually faint staining, whereas the microtubules in other cells were found to lie in the type of curvilinear arrays shown in Figure 2E. Indeed, these aberrant microtubule arrays were so distorted that it was generally impossible to photograph them in a single plane of focus. By way of contrast, the discrete spindles of wild-type cells at this stage were regularly arrayed with their centers situated within the neck of the bud and their poles extending into either half of the cell (Figure 2A). In the remaining 15% of mutant cells, a spindle of short length similar to that normally seen early in G2 was aberrantly positioned entirely within the bud (Figure 2C2). The bud was easily distinguished from the mother cell, which had retained the "shmoo" form acquired during mating pheromone arrest (Cross *et al.*, 1988). A further indication of mitotic abnormality was signaled by the position of chromosomal DNA, as seen by DAPI staining. Whereas the chromosomal DNA in a wild-type cell usually spans the neck or has segregated equally to each pole by this stage of the cycle (Byers, 1981; Figure 2B), the DNA in many mutant cells lay largely within one half (usually the bud) of the large budded cell (Figure 2D3). Therefore, both spindle staining and DNA localization established the lethal phase to be associated with a structural defect in the mitotic spindle as diagrammed in Figure 2F.

Examination of the aberrant spindles by electron microscopy clarified the nature of these abnormal mitotic arrays (Figure 3). Normally, bundles of intranuclear microtubules emanating from either pole of the spindle interdigitate with each other to form a discrete mitotic spindle (Byers and Goetsch, 1974). In *esp1* cells undergoing mitotic failure, the microtubules radiating from the two poles failed to retain this interdigitation, leading to the generation of two independent half spindles with few pole-to-pole microtubules. Furthermore, the spindle pole bodies failed to achieve their normal positions within the mother cell and bud, both often coming to lie within the same half of the cell at the time cytokinesis begins (note cleavage furrow formation in Figure 3). By serial-section electron microscopy, we analyzed 17 *esp1* mutant cells entering into cytokinesis as indicated by the presence of a cleavage furrow, and found that in 16 of these cells both SPBs were near one another in the same end of the cell. These defects then lead to an aberrant cell division in which both spindle pole bodies remain in a single progeny cell. Presumably, passage of the cleavage furrow through the bud neck while it still is occupied by a lobe of the nucleus leads to a misdivision of the nuclear contents (Figure 3, inset) similar to the "cut" phenotype of *S. pombe* (Hirano *et al.*, 1986). Significantly, we found no evidence for premature duplication of spindle poles at the time of this initial misdivision, including 16 cells that were analyzed in complete sets of serial sections. Therefore the earliest morphological defect evident upon failure of *ESP1* function is the loss of proper spindle behavior.

Aberrant Chromosome Segregation upon Failure of ESP1 Function

Because *esp1* permitted cell division despite an obvious aberration in spindle behavior, it was of interest to determine the consequences of this division on the segregation of chromosomal DNA. Haploid *esp1* cells were arrested in G1 of the cell cycle with α-factor and then released synchronously at the nonpermissive temperature. Aliquots removed from the culture at various times after release were analyzed by flow cytometry and light microscopy (Figure 4A). At the time of release from α-factor, most of the cells in the population (>95%) were arrested in an unbudded state with a 1C content of DNA. By 1 h after release, most cells had completed S-phase and had gained a 2C content of DNA. By 2 h, most cells had undergone cytokinesis to yield two distinct populations of progeny. Most cells in one population had a DNA content of less than 1C (the hypoploid

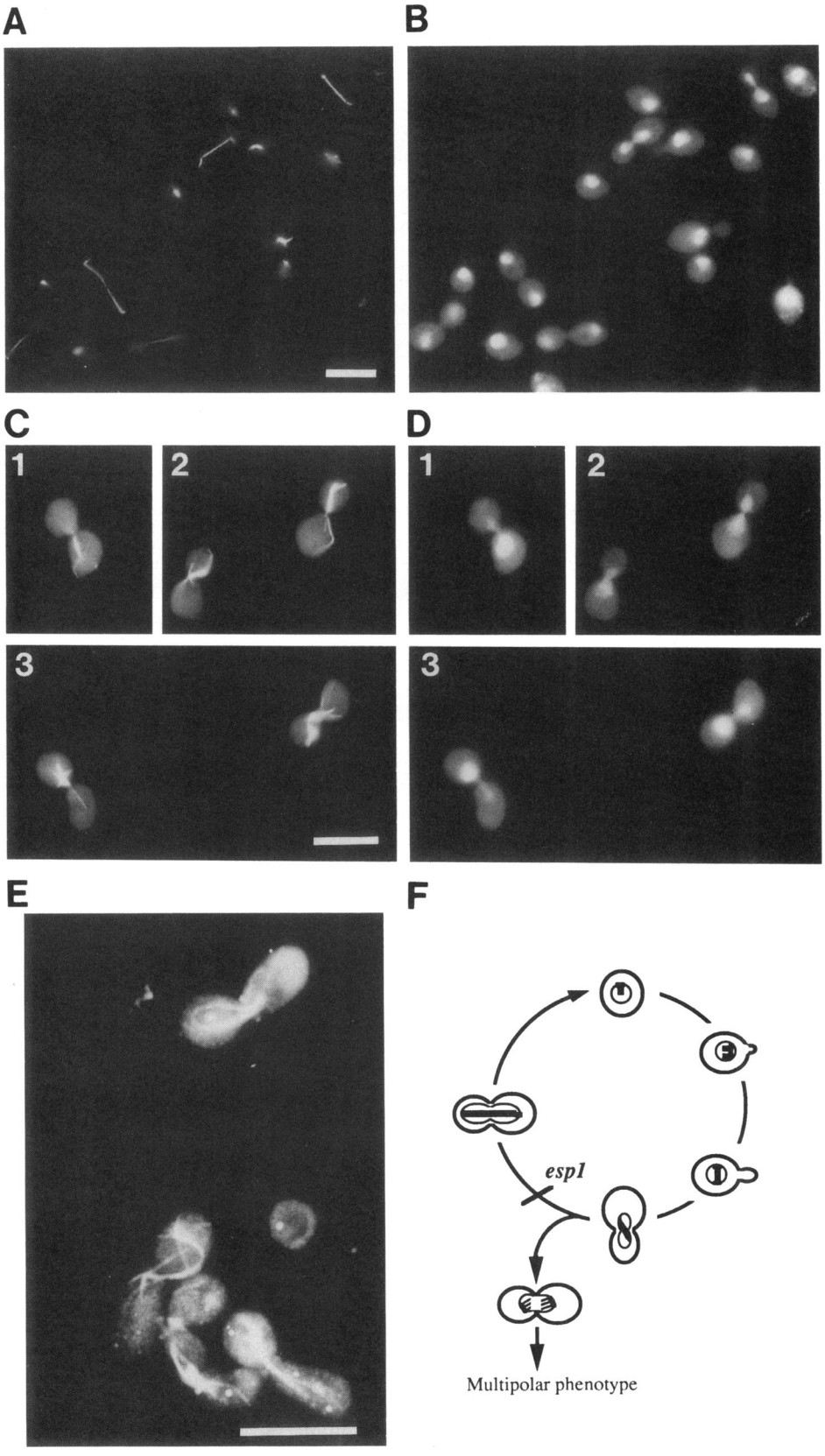

Figure 2. Immunofluorescent staining of *esp1* mutants in the lethal phase of the cell cycle. Yeast cells were stained to display microtubules (A, C, E) or DNA (B, D) as described in MATERIALS AND METHODS: (A, B) control heterozygote strain, SN127h (*esp1/ESP1*) incubated for 2 h 15 min at 36°C after release from α-factor, (C, D, and E) mutant strain T25A (*esp1/esp1*) treated identically. Schematic drawing (F) indicates normal cell cycle and the point of departure by *esp1* cells; scale bar, 10 μm.

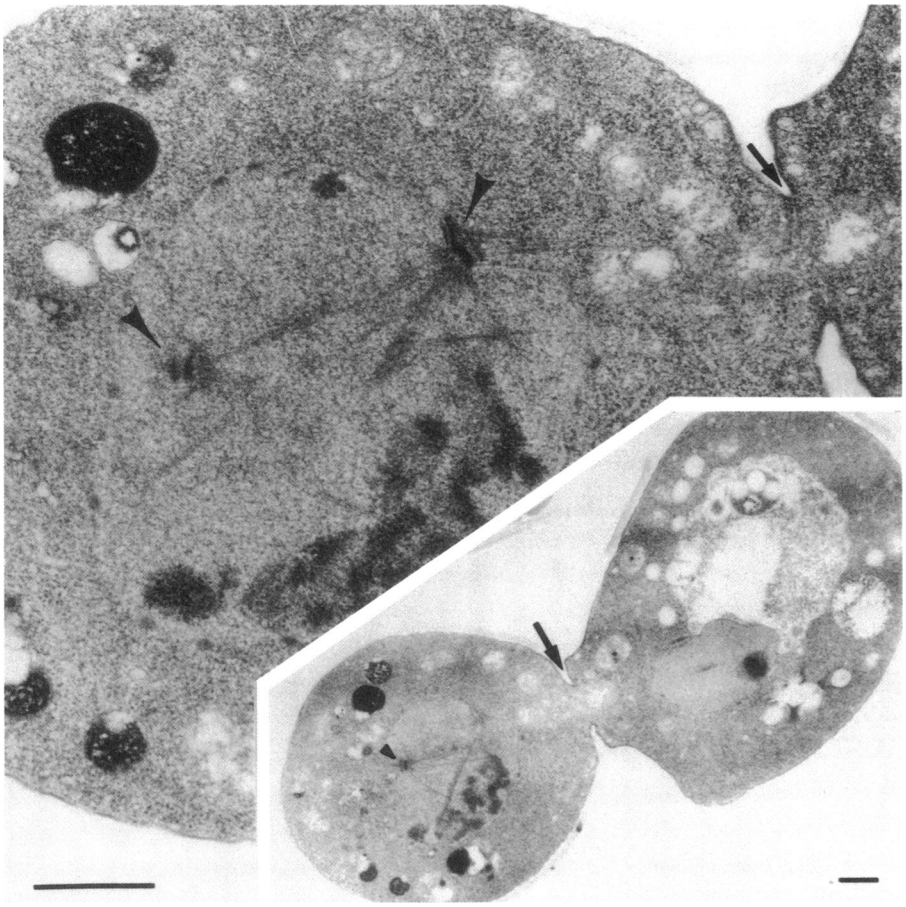

Figure 3. Electron microscopy of an *esp1* mutant in the lethal phase. T25A (*esp1/esp1*) cells from the 2 h 15 min time point in Figure 1A were fixed and sectioned for electron microscopy. The inset shows an adjacent section at lower magnification. Arrows indicate the position of the cleavage furrow; arrowheads indicate positions of SPBs; scale bar, 0.5 μm.

peak), whereas the other cell population (the hyperploid peak) consisted of cells with a DNA content of 2C or greater. The proportion of cells in the hypoploid peak increased slightly during the course of the experiment, indicating that cells of this type continued to be produced at later times. Over the same period of time, the hyperploid peak progressively broadened and shifted to higher DNA contents. Some cells continued to gain in DNA content even 5 h after release from α-factor, reaching contents in excess of 4C. Arrest of asynchronous *esp1* cultures cells gave similar results except that DNA synthesis was more restricted at the later times (unpublished data). In overview, these findings indicate that gross chromosome missegregation resulting from *esp1* failure leads to the production of two distinct populations of cells, one containing little or no DNA while the other receives the bulk of the DNA. The latter cells presumably then enter at least one additional new cell cycle, further increasing their DNA content on their passage through S-phase. When the two populations were sorted for DNA content with the use of a fluorescence-activated cell sorter and then examined by light microscopy, cellular morphology of the two populations was found to differ strikingly. Ninety-nine percent of the cells from the hypoploid peak were unbudded (Figure 4B) while 98% of those in the hyperploid peak were budded (Figure 4C). It is therefore clear that only those cells that received the bulk of the genome were able to undergo further bud emergence and DNA replication.

A potential explanation for the accumulation of extra SPBs after *esp1*-induced mitotic failure is that SPB duplication becomes deregulated, losing any coupling with other aspects of the cell cycle. Alternatively, the reason for the accumulation of extra SPBs might be that *esp1*-induced mitotic failure causes missegregation of SPBs along with missegregation of the chromosomes. If those cells acquiring two SPBs in the aberrant cell division initiate a new cell cycle and duplicate those SPBs, the resulting cell would have four SPBs in a single nucleus. To assess these alternatives, we arrested *esp1* mutant cells with α-factor and then released them at the nonpermissive temperature. Once the new cell cycle was initiated, α-factor was added again to arrest cells after the initial *esp1*-mediated misdivision. Four hours later, the cells were fixed and processed for immunofluorescence staining of tubulin. If the *esp1* defect caused unrestrained SPB duplication, then extra SPBs might accumulate even on re-exposure to α-factor. Instead, 97%

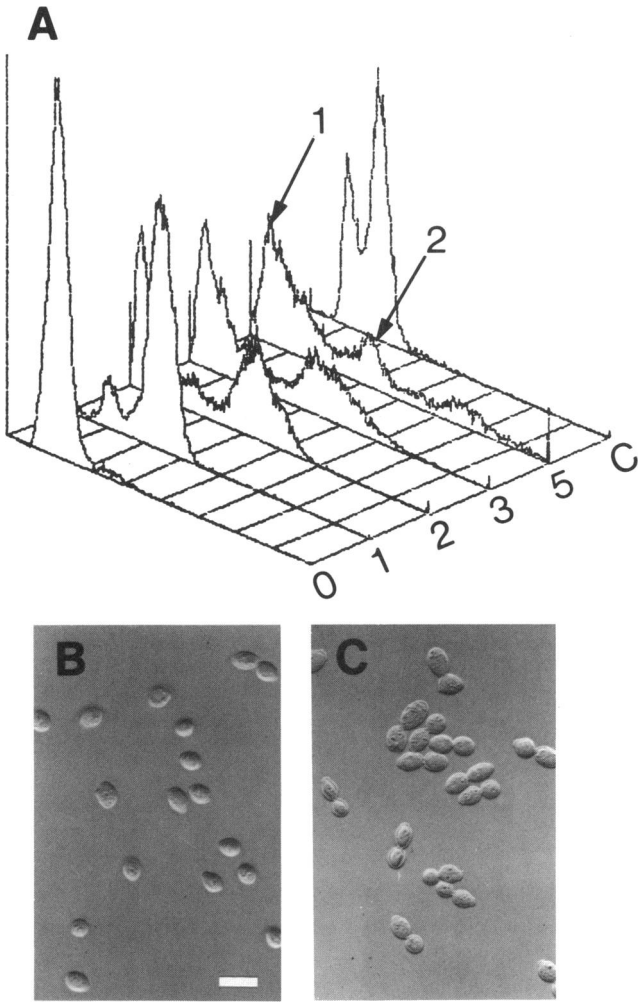

Figure 4. Flow cytometric analysis of *esp1* mutants released from α-factor at the restrictive temperature. (A) Strain 923-1a was arrested with α-factor at 23°C and then washed free of it and incubated at 36°C. Aliquots were taken at the arrest (0 h) and at 1, 2, 3, and 5 h after release. An aliquot (profile C) of the asynchronous culture taken before α-factor treatment serves as a control to display DNA contents expected for G1 (left) and G2 (right) cells. (B) and (C) An asynchronous culture of T25A cells were grown to log phase and then shifted to the restrictive temperature. Cells were fixed and sorted for a hypoploid population whose relative position is indicated by arrow 1 and a hyperploid peak whose relative position is indicated by arrow 2 in A. Light microscopy shows that cells of the sorted hypoploid peak (B) are unbudded, whereas those of the hyperploid peak (C) are budded; scale bar, 10 μm.

of the cells re-exposed to α-factor had no more than two SPBs (Table 1). To confirm that α-factor readdition had effectively blocked accumulation of multiple SPBs, these cells were also viewed by electron microscopy. None of the 20 unbudded cells observed by electron microscopy had more than two SPBs, confirming the immunofluorescence results (unpublished data). Those few cells (3% of the total; Table 1) that accumulated extra SPBs probably resulted from leakage past the α-factor block because most were budded cells. In the control culture, without α-factor readdition, 33% of the cells accumulated multiple SPBs (Table 1). We also noted in this experiment that a large number of cells in both cultures failed to stain with anti-tubulin antibodies (Table 1). Although we cannot prove that these cells actually had no SPBs (rather than simply failing to be permeabilized sufficiently for antibody staining of their microtubules), provisionally finding cells of this type is consistent with a missegregation of the spindle pole bodies. More importantly, the fact that virtually none of the cells that could be scored unambiguously contained more than two SPBs established that further potential cycles of SPB duplication had been inhibited by readdition of α-factor. It must be the case that any further SPB duplication requires renewed passage through the "start" phase of the cell cycle after the *esp1*-induced mitotic failure, and that *esp1* does not uncouple SPB duplication from the cell-cycle control point at "start".

Asymmetric Segregation of Nuclear Material at Cell Division

A marked heterogeneity among growing yeast cells arises from the fact that cell division is asymmetric, yielding a mother cell and daughter cell (the progeny cell with a newly formed wall). To inquire whether the two distinct cellular populations found after *esp1* misdivision represented mother and daughter cell populations, we analyzed the origins of their walls by visualizing the bud scar. This structure is deposited at the neck of the bud during each cell cycle and then remains with the mother cell when cell division is completed. The two products of cell division can easily be distinguished by staining the bud scars for their high content of chitin by use of the fluorescent compound Calcofluor

Table 1. Effect of α-factor readdition after *esp1*-mediated misdivision

	Unstained[a] (%)	1 or 2 SPBs[b] (%)	>2 SPBs[b] (%)
TA25 + α-factor	56	40	3
TA25	48	16	33

TA25 mutant cells were synchronized in the G1 phase of the cell cycle by treatment with α-factor. The α-factor was removed by washing, and the cells were shifted to 36°C. After 45 min, when >90% of the cells had formed buds, the culture was divided, and α-factor was added to one portion while the incubation at 36°C was continued. After 4 more hours at 36°C, cells were fixed and stained to visualize microtubules as described in MATERIALS AND METHODS.

[a] This is the number of cells shown by DAPI staining to contain mitochondrial DNA but failing to stain with the antibody directed against tubulin. More than 95% of TA25 cells grown under permissive conditions stained with the antibodies against tubulin.

[b] Scored as focal points of microtubule arrays.

White 200R (Pringle *et al.*, 1991). A clear bias in the fate of mother versus daughter cells was found when arrested *esp1* cells were examined after such staining. The unbudded cells were found largely to be mother cells, 87% of them bearing bud scars; by contrast, 86% of the budded cells were daughter cells, lacking a bud scar and displaying only the more lightly staining birth scar that is characteristic of daughter cells (Figure 5A and Table 2). This pattern was confirmed by viewing unsonicated cells, where the two unabscised products of the original misdivision often remained together (Figure 5B). In these cases, the mother cell failed to bud again, whereas the daughter cell had then budded once more. We therefore conclude that the prevalent pattern of arrest after *esp1*-mediated misdivision is that the daughter cell receives the bulk of the DNA and initiates a new cell cycle, budding once more. The mother cell receives little or no DNA and remains unbudded.

Confirmation that the bulk of the nucleus generally resides in the daughter cell upon *esp1* misdivision was found in observations on cells arrested with α-factor and released at the nonpermissive temperature. Cells arrested with α-factor acquire a projection, which is retained upon budding, thereby permitting one to distinguish this cell (the mother cell) from the more evenly rounded daughter cell. Figure 3 (inset) shows a cell fixed just before cytokinesis; much of the nucleus, including both spindle pole bodies, lies within the bud. The presumptive products of this division would be (1) a daughter cell with two spindle pole bodies and most of the nucleus and (2) a mother cell with no spindle pole bodies and little or no nucleus. Many other cells similar to that shown in Figure 3 have been observed by electron microscopy (unpublished data).

Table 2. Bud scar number and cell morphology

Morphology	Number of bud scars					% With scar
	0	1	2	>2	Total	
Unbudded	65	245	116	74	500	87
Small budded	33	1	2	0	36	8
Large budded	254	26	10	6	296	14

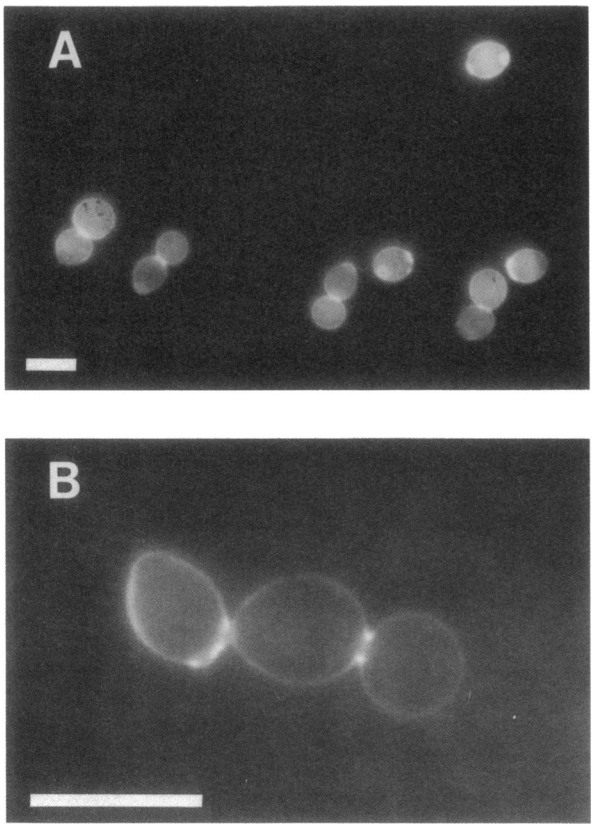

Figure 5. Bud scar staining of T25A (*esp1/esp1*) cells after 4.5 h at 36°C. Cells were grown to log phase at 23°C and then shifted to 36°C for 4.5 h. Aliquots of cells were fixed and stained with calcofluor to reveal bud scars. Cells in A were sonicated to separate cells that had completed cytokinesis, while viewing an unsonicated sample B consistently revealed that only the daughter cells (lacking the bud scar) budded again; scale bar, 10 μm.

Molecular Analysis of the ESP1 Gene Reveals Similarity to the cut1 Locus of S. pombe and the bimB Locus of Aspergillus nidulans

The *ESP1* gene was cloned by transforming *esp1* cells with a cloned library of genomic yeast DNA and demanding complementation of the temperature-sensitive lethality caused by the mutation. Among plasmids found to confer complementation, plasmid pCP1 bore the smallest insertion (7 kb) and was analyzed in detail (Figure 6A). First, to determine which portion of the cloned insert contained the *ESP1* gene, we performed insertional mutagenesis in *E. coli*, by using a bacterial transposon carrying the *HIS3* gene. Insertional alleles distributed over various representative segments of the insert were chosen for transformation into *esp1* cells to test their ability to complement the lethal phenotype. We found that insertions 5 and 15 failed in the complementation test and therefore presumably disrupted the *ESP1* gene, whereas others (insertions 6, 9, and 34) permitted complementation and therefore probably lay outside the gene.

To test more stringently for the effects of these insertions on *ESP1* function, restriction fragments bearing the insertions were used to replace homologous portions of either allele in an *ESP1/esp1* diploid. To accomplish this, we used the one step gene replacement method (Rothstein, 1983), selecting for the *HIS3* yeast gene contained within the transposon to isolate transformants. As expected, one-half of the His^+ transformants (226/480) for insertion 5, which lay in the complementing

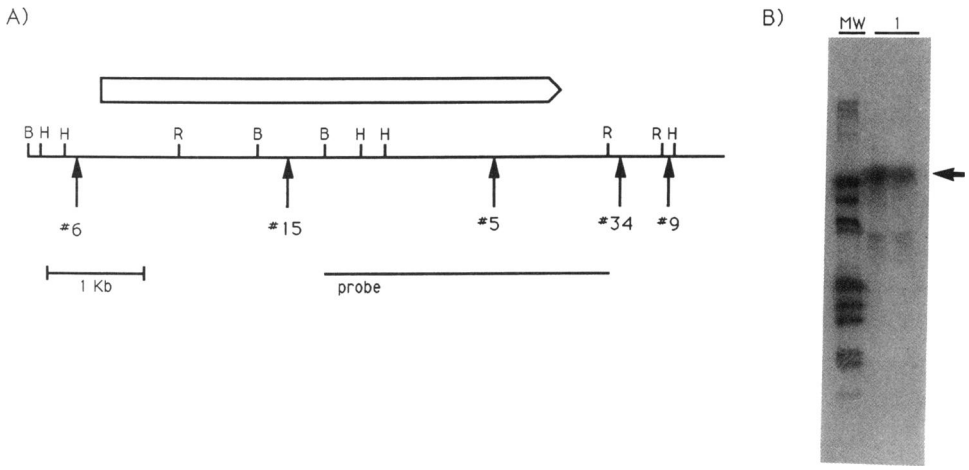

Figure 6. Restriction map of *ESP1*-complementing region and RNA blot analysis of the *ESP1* gene. Restriction sites are indicated: B, *Bam*HI; H, *Hind*III; R, *Eco*RI. The sites of Tn3 transposon inserts that were analyzed are indicated. Inserts 15 and 5 failed to complement the *esp1* defect. The potential open reading frame is indicated by the large arrow above the sequence. (B) The RNA blot at right was probed with the 3 kb *Bam*HI-*Eco*RI segment from the 3′ end of the complementing region, revealing a message of 5.3 kb. The molecular weight standards are λ DNA digested with *Eco*RI and *Hind*III. The arrow indicates the *ESP1* message that runs slightly above the 5.15 kb standard.

region, were temperature-sensitive, indicating that the wild-type *ESP1* gene had been disrupted to uncover the recessive temperature-sensitive allele. When transformants heterozygous for the insertion were sporulated and their meiotic products analyzed, only two spores per tetrad were viable and these lacked the insertion at the *ESP1* locus, as indicated by their invariably being His^- (unpublished data). Examination of germinated spores containing the inactive *esp1* insertion allele almost always gave rise to a phenotype similar to that found for the temperature-sensitive allele at mitotic arrest, namely one unbudded and one budded cell (unpublished data). We conclude that the segment cloned corresponds to the *ESP1* gene, which must be essential for cell viability, and that the phenotype we described for the temperature-sensitive allele probably represents the null state. Consistent with these conclusions, the terminal phenotypes of four other independent *esp1* alleles have been characterized by fluorescence-activated cell sorter (FACS) analysis and immunofluorescent staining for microtubules; all were indistinguishable from *esp1-1* for the phenotypes described (unpublished data).

DNA sequence analysis of the *ESP1*-complementing sequences revealed a potential open reading frame of 4719 bases encoding a protein of 181 502 Da. Total RNA was isolated from cells and analyzed by northern analysis by using the cloned *ESP1* gene as a probe. A 5.3 kb RNA species was detected whose size is consistent with the size of the open reading frame (Figure 6B). A computer-assisted search of similarities between the deduced *ESP1* gene product and the GENbank database revealed substantial similarities to the *cut1* gene of *S. pombe* (Uzawa *et al.*, 1990) and the *bimB* gene of *A. nidulans* (May *et al.*, 1992). The segment of sequence similarity covers 371 amino acids at the carboxy terminus with 37.7% identity to the *S. pombe* sequence and 36.4% identity to the *bimB* gene product (Figure 7). Uzawa *et al.* (1990) identified a putative calcium binding pocket that is conserved in all three sequences. This sequence is underlined in Figure 7. The *ESP1* gene contains a putative *CDC28* phosphorylation site (TPSK) starting at amino acid position 955. Mutagenesis of this site had no apparent effect on *ESP1* gene function (J. McGrew and B. Byers, unpublished data).

DISCUSSION

Mutational defects in *esp1* have previously been shown to lead to a lethal state characterized by the accumu-

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ESP1 (S. cerevisiae)      (1-1202)QLLNNIENSWFNGVQGFFSPEV
cut1 (S. pombe)           (1-1465)QLLENIEISWLGGFKGIFNPHK
bimB (A. nidulans)        (1-1691)NLLQNIENVWFGGFRGIFSPIP

VDNSLFEKFKDRFYEILHQNLPSRKL-YGNPAMFIKVEDWVIELFLKL-NPARKS
IDTSLFAKFSSQFQNIIAKNFNMDKK-----TPVPTLSPEILELFITL-GKPGYE
LCEKSLARFASAFENILENHLPSRRKGSRAQGPKLTLHPNVLELFVGVKGLDDQE

IFLSKMEDLIYFVLDILLFHGEENAYDEIDFSMLHVQLEEQIKKYRATMT---TN
GYEQLLEDLIYFILDIFQFRGLHFAYDEIDTDQLSMDLQDALNAYFNNYVS--EE
DPEDTLMDLLYFVVDILQFQGERNAYDEVDFDMMVVETLDAVRAYHEAAKDQATQ

SIFHTFLVVSSSCHLFPWECLSFLKDLSITRVPSYVCLNKLLSRFHVQLPLQVTI
NRSHTVLVLDKSVHQFPWESLPCLNRQSVSRVPSL---SILRDILSNHLSLMESM
RPNNTVLVLDKSLHLFPWESLPCLQGLPVCRVPSL---ECLRDRVLHLRSGKQSA

QDNISMILNPNGDLSRTESKFKGMFQKIIDAKPSSQLVMNEKPEEETLLKMLQNS
LKCVKKQVHIYINPSLDLKHTQEMFEHKLV-EGGWKGLIASQPSNRDFIKMLSGN
LSIDRRNGTYILNPTGDLKTTQETFEKDLSSLKGWTGMVNRQPTEDEFKDSLQSK

NLFVYIGHGGGEQYVRSKEIKKCTKIAPSFLLGCSSAAMKYYGKLEPTGTIYTYL
DFFLYFGHGGGEQYTTSYDLATLKRCAVTILMGCSSGALYECGSFEPWGTPLDYL
SLFLYFGHGSGAQYIRGRTVKRLDRCAVAFLMGCSSGTLTEAGEYEPYGTPMNYL

LGGCPMVLGNLWDVTDKDIDKFSEELFEKMGFRCNTDDLN----GNSLSVSYAVS
SAGCPTLVANLWDVTDKDIDRFSLKMLESWGLFENKAPF-----VNSTSICTAVS
QAGSPALVATLWDVTDKDIDRFAKATFEHWGLIGNGHRGNEGIGEAGVALDAAVS

KSRGVCHLRYLNGAAPVIYGLPIKFVS* (1573)
ESRSCCHLRYLNGAAPVIYGIPAYIIP* (1827)
QSRGACVLKYLNGAAPVVYGVPGVFLH* (2067)
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Figure 7. Similarities among the predicted amino acid sequences at the carboxy termini of the *ESP1* gene, the *cut1* gene of *S. pombe*, and the *bimB* gene of *A. nidulans*, shown in single letter amino acid code. Sequence identities between any two or all the members are indicated in boldface and the dashes indicate gaps. The underlined sequence indicates a potential calcium binding site (Uzawa *et al.*, 1990). The Genbank accession number for the entire DNA sequence is LO7289.

lation of extra SPBs (Baum *et al.*, 1988). We report here that the initial induction of lethality coincides with aberrant behavior of the mitotic spindle. This leads to a failure to complete nuclear division in the first cycle of nuclear division after transfer to the restrictive temperature. Concomitantly, there is massive chromosome nondisjunction, the daughter cell generally acquiring most of the DNA and both spindle poles. This cell then often proceeds to initiate a new cell cycle, leading to increases in both DNA content and the number of SPBs. Because *esp1*-mediated misdivision occurs well before any extra spindle poles have begun to appear, it seems likely that the accumulation of extra SPBs is a secondary consequence of the initial defect in mitosis.

Although *esp1* clearly causes a defect in nuclear division, the specific role of the *ESP1* product in this process remains obscure. The structural aberrations of the spindle revealed by immunofluorescence and electron microscopy suggest that the *ESP1* function is required in some manner to maintain the appropriate interdigitation of antiparallel microtubules extending into the central spindle from either pole. Although spindles of normal appearance are present in early stages after initial exposure to restrictive conditions, spindle structure becomes abnormal when anaphase elongation normally would occur. This might reflect a role for *ESP1* in the behavior of macromolecular components that drive pole-to-pole microtubules in a poleward direction or that mediate the concomitant elongation of individual microtubules during spindle elongation (Hogan and Cande, 1990). On the other hand, spindle disruption could derive from an inability of the spindle to separate the chromosomes, as might be the case if the mechanism permitting sister chromatid separation were defective. Uzawa *et al.* (1990) have pointed out the difficulty in distinguishing these alternatives in their characterization of the "cut" phenotype resulting from mutation in the highly similar protein encoded by the *cut1* gene of *S. pombe*.

If the observed defect in spindle function is sufficient to cause the *esp1* phenotype, then it remains to be explained how this leads to the accumulation of extra SPBs. It seems plausible to posit that, when the primary mitotic dysfunction occurs, this failure alone is responsible for later segregation of both poles to one of the two progeny cells. This cell would then execute the "start" function and enter a new cell cycle with the further duplication of the SPBs, thereby acquiring an even greater number of SPBs within a single nucleus. Consistent with this model, temperature shifts of synchronized cultures have shown that failure of nuclear division leads to segregation of both SPBs and much of the nucleus to a single progeny cell (Figure 3). Further support for the model comes from our observation that the aberrant division produces two populations of cells, one displaying a DNA content less than 1C and the other receiving the bulk of the DNA (Figure 4A). The latter cells are able to bud again, many continuing to undergo DNA synthesis 5 h after transfer to the restrictive temperature (Figure 4, A and C). Some of these latter cells must also undergo further cycles of SPB duplication, since up to eight SPBs have been seen in a single cell (Baum *et al.*, 1988). Whether this more pronounced multipolarity arises from repeated cycles of misdivision in a subset of cells or from an uncoupling of the SPB duplication cycle from budding is obscured by cumulative heterogeneity in the total population. Whatever mechanisms may be responsible for continued SPB duplication at these later stages of arrest, it is now clear that the initial phases of multipolarity result from misdivision. Disruption of the spindle leads to missegregation of both poles to a single nucleus, which thereby becomes the first derivative of *esp1* failure with an abnormal complement of SPBs.

The sequence similarity between *ESP1*, *cut1* (Uzawa, *et al.*, 1990), and *bimB* (May *et al.*, 1992) suggests these proteins confer a common function, and support for this viewpoint is provided by the finding that mutations in all three genes cause related defects in nuclear division. Mutations in *ESP1* and *cut1* both lead to failure of nuclear division without blocking cytokinesis. In *S. cerevisiae*, where the initiation of cellular reproduction is asymmetric, cytokinesis progresses to completion, leaving much of the nucleus in one progeny cell, where further SPB duplication can take place. Perhaps the preferential segregation of the nucleus to the bud in *esp1* cells reflects the behavior of a mechanism for nuclear motility, such as that demonstrated by Palmer *et al.* (1989), but operating aberrantly in *esp1* cells because of the absence of a rigid spindle. Alternatively, the segregation of the nucleus to the daughter cell might result from a defect in the behavior of cytoplasmic microtubules, which have been shown to control positioning of the nucleus in the bud neck (Huffaker *et al.*, 1988). In contrast to the way division proceeds in budding yeast, nuclear division and cytokinesis of the fission yeast *S. pombe* take place in a symmetric manner. Consequently, the undivided nucleus present after *cut1* failure lies squarely in the path of the cleavage furrow and appears to block completion of cytokinesis. Interestingly, when the cytokinesis of *cut1* mutants was prevented by a second mutation in the cytokinetic process, the doubly mutant cells progressed into a multipolar state similar to that exhibited by *esp1* strains of *S. cerevisiae* (Creanor and Mitchison, 1990; Uzawa *et al.*, 1990). Effects on cytokinesis are irrelevant in *A. nidulans*, which grows as a multinucleated mycelium. In this organism, the related defect in *bimB* causes repeated failure of nuclear division, leading to the formation of large polyploid nuclei that appear to contain multiple poles (May *et al.*, 1992). Thus under appropriate conditions, mutations in all three genes can lead to a multipolar phenotype. It will be intriguing in the future to discover whether similar mutations in yet other species lead to multipolarity

or whether these phenomena will be restricted to organisms that exhibit closed mitosis, as do these various Ascomycetes. It is also interesting to note that *esp1*, *cut1*, and *bimB* all contain a potential calcium binding site (Figure 7). Transient alterations in the level of intracellular calcium have been observed during mitosis in a number of cell types (Hepler, 1989), and the activity of the *ESP1* gene product and its homologs could potentially be regulated by such fluctuations.

Conditional mutations in many genes required for specific aspects in the yeast cell division cycle cause cell-cycle arrest when the cells are transferred to nonpermissive conditions (Hartwell and Weinert, 1989). Although the *ESP1* function also appears to be required at a specific point in the cell cycle, mutations in this gene do not immediately cause any obvious cell-cycle arrest or delay. Hartwell and Weinert (1989) proposed that mutations that cause cell-cycle arrest do so either because they inactivate a crucial gene product that is a directly required for the next step in the cell cycle or because the mutational defect activates an extrinsic control mechanism, called a checkpoint, that enforces dependency on the cell cycle. Because *esp1* mutants fail to arrest the cell cycle despite an overt defect in nuclear division, it may be the case that there is no checking function for the relevant process in these cells. On the other hand, it is also tenable that *ESP1* plays an essential role in the checkpoint function, which cannot be executed properly in the mutant cell. The data available at present do not differentiate between these possibilities. These uncertainties are not unique to *esp1* for mutations in *top2* (Holm *et al.*, 1985), *ndc1* (Thomas and Botstein, 1986), and *mps1* (Winey *et al.*, 1991) similarly cause defects in nuclear division without leading to arrest of the cell cycle. Perhaps all of these genes represent a subset of nuclear functions that are not subject to checkpoint control. In the present case, it has been possible to explore the relevance of *ESP1* to one type of checkpoint functions in a straightforward manner. Namely, the *BUB* (Hoyt *et al.*, 1991) and *MAD* (Li and Murray, 1991) checkpoint functions can be assessed by determining whether cell-cycle progression is arrested by microtubule depolymerization. We have found that, unlike the case for *bub* or *mad* mutants, *esp1* strains retain the ability to arrest in response to microtubule depolymerizing drugs (J. McGrew and B. Byers, unpublished data). Therefore *ESP1* appears to play no essential role in the microtubule-related checkpoint functions defined by the *bub* and *mad* mutations.

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