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Spindle Pole Body Separation in *Saccharomyces cerevisiae* Requires Dephosphorylation of the Tyrosine 19 Residue of Cdc28

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In eukaryotes, mitosis requires the activation of cdc2 kinase via association with cyclin B and dephosphorylation of the threonine 14 and tyrosine 15 residues. It is known that in the budding yeast *Saccharomyces cerevisiae*, a homologous kinase, Cdc28, mediates the progression through M phase, but it is not clear what specific mitotic function its activation by the dephosphorylation of an equivalent tyrosine (Tyr-19) serves. We report here that cells expressing *cdc28-E19* (in which Tyr-19 is replaced by glutamic acid) perform Start-related functions, complete DNA synthesis, and exhibit high levels of Clb2-associated kinase activity but are unable to form bipolar spindles. The failure of these cells to form mitotic spindles is due to their inability to segregate duplicated spindle pole bodies (SPBs), a phenotype strikingly similar to that exhibited by a previously reported mutant defective in both kinesin-like motor proteins Cin8 and Kip1. We also find that the overexpression of *SWE1*, the budding-yeast homolog of *wee1*, also leads to a failure to segregate SPBs. These results imply that dephosphorylation of Tyr-19 is required for the segregation of SPBs. The requirement of Tyr-19 dephosphorylation for spindle assembly is also observed under conditions in which spindle formation is independent of mitosis, suggesting that the involvement of Cdc28/Clb kinase in SPB separation is direct. On the basis of these results, we propose that one of the roles of Tyr-19 dephosphorylation is to promote SPB separation.

In budding (*Saccharomyces cerevisiae*) and fission (*Schizosaccharomyces pombe*) yeasts, passage through various cell cycle transitions requires Cdc28/cdc2 kinase (also called CDK1) (37). The manner in which the mitotic form of the cdc2 kinase is regulated is perhaps one of the most conserved and elaborately studied aspects of the eukaryotic cell cycle. Monomeric cdc2 is inactive as a kinase; its activation requires not only association with cyclins but also a combination of phosphorylation and dephosphorylation of conserved residues such as threonine in the T loop and tyrosine in the glycine-rich ATP binding domain (Thr-167 and Tyr-15, respectively, in cdc2 of *S. pombe*) (13, 36). On the basis of the structural analysis of Cdk2 and the Cdk2-cyclin A complex (12, 42), it is predicted that while phosphorylation of T-loop threonine by Cdk-activating kinase (6, 10, 19, 51) stabilizes cyclin binding, the phosphorylation of Thr-14 and Tyr-15 residues in the ATP binding domain exerts an inhibitory effect by hindering the phosphate transfer from ATP to the substrates. Hence, the dephosphorylation of Thr-14 and Tyr-15 constitutes the final step in the activation of Cdk (29, 43).

The structural rationales provide explanations for many of the genetic and biochemical observations concerning the mitotic activation of cdc2 kinase (3, 13, 14, 21). In *S. pombe*, as in many other organisms, dephosphorylation of the Tyr-15 residue of cdc2 plays an important role in the initiation of mitosis. Phosphorylation of Tyr-15 by the tyrosine kinases wee1 and mik1 impedes cdc2 activity and thus prevents entry into mitosis until this phosphorylation is reversed at the G_2-M transition by a tyrosine phosphatase encoded by the *cdc25* gene (14, 21, 34). These findings suggest that it is the balance among the wee1, mik1, and cdc25 activities that determines the timing of mitosis. As expected, the *wee1 mik1* double mutant attempts mitosis prematurely and as a result undergoes mitotic catastrophe (34). Similarly, substituting Tyr-15 with phenylalanine, a residue which can mimic constitutively dephosphorylated tyrosine, results in the premature onset of mitosis (24). Thus, the state of Tyr-15 phosphorylation is central in the decision to enter mitosis. In addition, Tyr-15 phosphorylation also plays an important role in checkpoint controls which impose cell cycle arrest in response to incomplete DNA synthesis (17).

The role of Cdc28 tyrosine 19 (equivalent to cdc2 Tyr-15) phosphorylation in budding yeast cells is somewhat puzzling. Like cdc2, Cdc28 also shows a marked loss of Tyr-19 phosphorylation in cells arrested in mitosis (2). Surprisingly, however, substitution of Tyr-19 by phenylalanine leads neither to precocious mitosis nor to the abolition of checkpoint controls induced by incomplete DNA replication (2, 52). This raises the possibility that in budding yeast cells, Tyr-19 dephosphorylation is not the rate-limiting step for M-phase initiation. Other pathways whose activation in conjunction with Cdc28 kinase is necessary for triggering mitosis could exist. However, the regulation of Tyr-19 phosphorylation does seem to play a role in a control mechanism that delays nuclear division in response to the inhibition of bud formation (33).

Which mitotic events does cdc2 activation by Tyr-15 dephosphorylation initiate? In fission yeast cells, spindle pole body (SPB) duplication, formation of a bipolar spindle, and chromosome condensation occur after S phase but prior to nuclear division (26). These cellular events are considered to be distinctive features of cells that have entered mitosis. *cdc2* mutants, which arrest in G_2, fail to initiate these processes, suggesting that cdc2 activity is required for the execution of these events. Similarly, in higher eukaryotes cdc2 kinase is thought to trigger spindle assembly, chromosome condensation, and nuclear-membrane breakdown (39). The list of cellular proteins known to be efficiently phosphorylated by cdc2/Cdc28 kinase is long and includes members as diverse as the budding yeast transcription factor Swi5 and the nuclear lamins of mammalian cells (38). However, there is little clue as to how cdc2 causes

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the initiation of mitosis. It also remains unresolved whether cdc2 kinase directly triggers the mitotic events.

In budding yeast cells, progression through M phase requires Cdc28 and four mitotic cyclins encoded by *CLB1*, *CLB2*, *CLB3*, and *CLB4* genes (20, 22, 54). The identities of the proteins that Cdc28/Clb kinase phosphorylates in order to initiate mitosis remain unknown. The terms entry into mitosis and initiation of mitosis, although widely in use, are ill-defined for organisms such as budding yeasts (37). One of the hallmarks of mitosis, chromosome condensation, is difficult to discern in *S. cerevisiae*, though this problem is now partly alleviated by the use of the fluorescent in situ hybridization technique (25). SPB duplication also does not serve as a useful landmark for M-phase initiation, since it occurs early in the cell cycle (8, 45). The SPBs remain attached in a side-by-side configuration for a substantial part of the cell cycle. When the bud grows to about one-third of the size of the mother cell (which coincides with the completion of the S phase), SPBs migrate away from one another, eventually locating themselves on the opposite sides of the nucleus separated by an interdigitated, antiparallel microtubule array constituting a bipolar spindle (reference 8 and this study). Since spindle formation occurs well before nuclear division but after the completion of DNA synthesis, it could be considered a landmark event for the initiation of M phase in budding yeast cells. However, this criterion is weakened by the fact that under certain circumstances budding yeast cells can assemble a bipolar spindle independently of S-phase completion and mitosis. For example, mutants which arrest at the onset of S phase and do not progress into mitosis nevertheless go on to form a short mitotic spindle (7).

The movement of SPBs away from each other requires the activity of kinesin-like motor proteins (48). Cells deficient in the plus-end motors Cin8 and Kip1 fail to assemble a bipolar spindle and arrest with a pair of unseparated SPBs (28, 46, 49). The deletion of a gene encoding a minus-end motor, *KAR3* (16, 35), partially restores the capacity of the *cin8 kip1* double mutant to form a mitotic spindle, suggesting that antagonistic forces are operative in the formation of a bipolar spindle (49). Similar kinesin-related proteins such as bimC in *Aspergillus nidulans* (18) and *cut7* in *S. pombe* (27) are required for spindle assembly. The formation of a bipolar spindle also appears to require Cdc28/Clb kinase. This notion is based on the observation that a quadruple mutant deficient in *CLB1*, *CLB2*, *CLB3*, and *CLB4* undergoes DNA replication but fails to assemble a mitotic spindle and arrests after S phase with an undivided nucleus (20). Electron microscopic examination revealed a pair of SPBs in a side-by-side configuration, suggesting that this mutant, like the *cin8 kip1* double mutant, is unable to segregate the SPBs. However, it is not clear if the incapacity to form a spindle is a direct consequence of the lack of mitotic kinase or an indirect effect of this mutant's failure to initiate mitosis. It is interesting that both the deficiency of motor proteins and that of Cdc28/Clb kinase lead to an inability to assemble a mitotic spindle.

In this work, we explore the physiological consequences of a mutant allele of *CDC28* (*cdc28-E19*) in which Tyr-19 has been replaced by glutamic acid, a residue that can mimic an irreversibly phosphorylated tyrosine. We find that, although able to traverse the Start and S phases, cells expressing *cdc28-E19* arrest with an undivided nucleus, indicating that Tyr-19 dephosphorylation is required for mitosis in *S. cerevisiae*. These cells are unable to form bipolar spindles because of their inability to segregate SPBs. The failure of the mutant allele to promote SPB separation is also observed under conditions under which spindle assembly is no longer dependent on the initiation of mitosis, indicating a direct dependence of SPB segregation on the dephosphorylation of Tyr-19. The phenotype caused by the overexpression of a wee1 homolog in wild-type cells is consistent with this notion. On the basis of these results, we suggest that SPB segregation in *S. cerevisiae* requires dephosphorylation of the Tyr-19 residue of Cdc28.

MATERIALS AND METHODS

Strains and growth media. All strains used in this study were derived from the wild-type strain W303. The *cdc28-4* mutant had been made isogenic by backcrossing at least three times to the wild-type strain. Cells were routinely grown in yeast extract-peptone (YEP) medium containing adenine (50 mg/liter) supplemented with either glucose or galactose. Raffinose was also added to the galactose medium.

Synchronization procedures. In many experiments involving the *cdc28-4* mutant, cells were synchronized by nutrition starvation since this mutant does not release efficiently from pheromone-induced G_1 arrest. To obtain stationary-phase cultures, cells were plated on YEP-raffinose and incubated at 24°C for 60 to 72 h. The cells were scraped from the plates and inoculated directly into YEP-glucose or YEP-raffinose and YEP-raffinose-galactose media at a starting optical density at 600 nm (OD_{600}) of 0.2 to 0.3. Samples collected at various times were used for the preparation of total RNA and cell extracts. The synchrony was determined by immunofluorescence staining of the spindle, and the DNA content was measured by flow cytometry.

For pheromone-induced G_1 arrest, *bar1*Δ cells from overnight cultures were diluted to a starting OD_{600} of 0.1. They were allowed to grow at 24°C to an OD_{600} of 0.3, at which point α-factor was added to a final concentration of 0.8 μg/ml. When >95% cells had arrested in G_1, the cultures were filtered and washed extensively to remove α-factor and inoculated either into fresh YEP-glucose or YEP-raffinose or YEP-raffinose-galactose medium. Samples were collected at various times and analyzed.

Cell extracts, kinase assays, and Western blot (immunoblot) analysis. For the determination of Cdc28/Clb2 kinase, cells were harvested by centrifugation at 4°C and washed with ice-cold stop mix (54). The pellet was resuspended in appropriate amounts of lysis buffer and mixed with an approximately equal amount of acid-washed glass beads (0.5-mm diameter; Biospec). Cells were broken by two bursts of vigorous vortexing (IKA-Vibrax-VXR), each lasting 3 min. The glass beads and cell debris were removed by centrifugation for 5 min in a microcentrifuge. The cell extracts were further cleared by being centrifuged twice (15 min each) at maximum speed in a microcentrifuge. The resulting supernatant was used to assay mitotic kinase activity. Immunoprecipitation using polyclonal antibodies against Clb2 and kinase assays were performed as described by Surana et al. (53). The kinase activity was quantitated with a PhosphorImager (Molecular Dynamics).

For the detection of Cdc28 tyrosine phosphorylation, Cdc28 was first immunoprecipitated according to Tyers et al. (55), using anti-Cdc28 antibodies (1:20 dilution) from cell extract equivalent to 8 mg of total protein. The immunocomplexes were washed six times with buffer 3 (0.1% Nonidet P-40, 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 50 mM Tris-HCl [pH 7.5]), resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membrane. The membrane was first probed with anti-Cdc28 antibodies (1:1,000 dilution) and then was stripped and reprobed with antiphosphotyrosine antibodies [5 mg/ml; monoclonal immunoglobulin G2b(κ); Upstate Biotechnology, Inc.] All Western blot analyses were performed with an enhanced chemiluminescence kit (Amersham) according to the manufacturer's instructions.

Electron microscopy. For electron microscopy, yeast cells were prepared according to the method of Byers and Goetsch (9), with the exception of the infiltration and embedding steps. Cells in 100% ethanol were transferred to 100% acetone, and an equal volume of Spurr resin (Polysciences Inc.) was added. The samples were mixed well and left overnight at room temperature. After another 24 h or more of infiltration in 100% resin with two to three changes, cells were transferred into 0.5-ml Eppendorf tubes, pelleted by centrifugation at a low speed, and incubated overnight at 80°C to allow the resin to harden.

To determine the structure of spindles in cells, an SPB was first located, and the particular cell in which the SPB was located was followed through serial sections on either side of that section until the second SPB was located.

Genetic manipulations. All DNA manipulations were performed according to the method of Sambrook et al. (47). Tyr-19→Glu mutation in the *CDC28* sequence was generated by means of PCR amplification. The nucleotide sequences (5′→3′) of the PCR primers containing the mutation are as follows: bottom strand, TTGGCCAGGT**CTTAAG**TCTAACGCTTTATAAACAACACCTTCT GTACCTT (the *Afl*II site is in boldface type, and the mutated site is underlined; position, ~478 bp); top strand, ATCGTT**CTCGAG**ATAGTTT (the *Xho*I site is in boldface type; position, ~60 bp). The 400-bp *Afl*II-*Xho*I PCR fragment thus obtained was subcloned into the corresponding sites in the *CDC28* sequence. The resulting mutated *CDC28* allele was isolated as a 1.2-kb *Asu*II-*Dra*II sequence and was blunt-end ligated to the *GAL1* promoter in a *URA3*-selectable *CEN* vector. The clone was sequenced in both orientations over the PCR-amplified region to ensure the absence of other mutations.

The plasmid for *SWE1* gene disruption was constructed by first cloning a

3.2-kb *Ssp*I-*Bam*HI fragment containing the entire *SWE1* gene into a BlueScript vector. An ~1.15-kb *Bgl*II-*Cla*I fragment within the open reading frame was then replaced by a 1.2-kb *Bam*HI fragment containing the *HIS3* gene. A wild-type yeast strain was transformed with a 3.2-kb *Xho*I-*Sac*I fragment from the disruption plasmid to obtain HIS^+ transformants. The disruption of the *SWE1* gene in these clones was confirmed by Southern blot analysis. The *GAL-SWE1* strain was constructed with an integrative vector carrying *GAL-SWE1* (a gift from R. N. Booher).

Other techniques. Yeast transformation was carried out by the lithium acetate method. Total RNA was isolated according to the method of Cross and Tinkelenberg (11), and Northern (RNA) blot analyses were performed as described by Price et al. (44). The method of Kilmartin and Adams (31) was used for immunofluorescence and photomicroscopy. To analyze DNA distribution by flow cytometry, cells were fixed in 70% ethanol overnight at 4°C, washed once with 0.2 M Tris-HCl (pH 7.5) containing 20 mM EDTA, and resuspended in the same buffer. Cells fixed in this way were treated with RNase (1 mg/ml) for 4 h at 37°C, washed once with phosphate-buffered saline (PBS), resuspended in 0.1 ml of propidium iodide solution (50 μg/ml in PBS), and incubated overnight at 4°C. The cell suspension was diluted 10 times with PBS and sonicated before analysis by flow cytometry.

RESULTS

***cdc28-E19* allele and cell cycle progression.** A marked reduction in the Cdc28 phosphorylation on Tyr-19 in mitotic cells is consistent with the role of tyrosine dephosphorylation in M-phase progression (2). However, unlike in fission yeast cells, replacing Tyr-19 with phenylalanine (*cdc28-F19*) does not result in precocious mitosis (2, 52), raising the possibility that the state of Tyr-19 phosphorylation might not be critical for mitosis in budding yeast cells. To examine this, we constructed a *CDC28* allele in which Tyr-19 was replaced by glutamic acid (*cdc28-E19*) and tested its ability to complement the Start-defective *cdc28-4* mutant. This substitution could approximate the Cdc28 protein constitutively phosphorylated on tyrosine. Centromere-based plasmids carrying either *cdc28-F19* or *cdc28-E19* alleles driven by their native promoters were introduced into *cdc28-4* mutant cells, and the resulting transformants were incubated at 37°C for 36 h. While *cdc28-F19* allowed the *cdc28-4* mutant to grow well, the *cdc28-E19* allele failed to complement the temperature-sensitive mutant, suggesting that Cdc28 constitutively phosphorylated on Tyr-19 cannot support progression through the cell cycle (Fig. 1A). The *cdc28-E19* allele driven by the *CDC28* native promoter was also introduced into a wild-type strain to test whether this mutant allele was recessive or dominant. The fact that the wild-type cells expressing *cdc28-E19* grow as well as the cells lacking it (Fig. 1B) suggests that it is a recessive allele.

***Cdc28-E19* is defective in SPB separation.** To determine the cell cycle stage at which the *cdc28-E19* allele is particularly defective, stationary-phase cells of the *cdc28-4* mutant harboring a *CEN* plasmid containing either *cdc28-E19* or *CDC28* (driven by the native promoter) were allowed to resume the cell cycle in glucose medium at 37°C. The parental *cdc28-4* strain was used as a control. Samples were withdrawn at regular intervals and analyzed for bud emergence, DNA replication, and spindle formation. As expected, while the strain expressing the wild-type *CDC28* continued to progress through the cell cycle and formed spindles normally, the *cdc28-4* strain arrested as unbudded cells (Fig. 2). In the case of the *cdc28-E19* strain, however, ~67% of the cells had arrested with large buds, a single nucleus, and 2 N DNA content at 4 h after the release (Fig. 2); the remaining cells failed to recover from the stationary-phase arrest. Immunofluorescence staining by anti-tubulin antibodies showed that 54% of the budded cells were unable to assemble spindles and each contained a single brightly stained spindle pole; in the remainder, each had a short spindle. This phenotype suggests that although unable to induce nuclear division, Cdc28-E19 is Start active and is capable of promoting DNA replication.

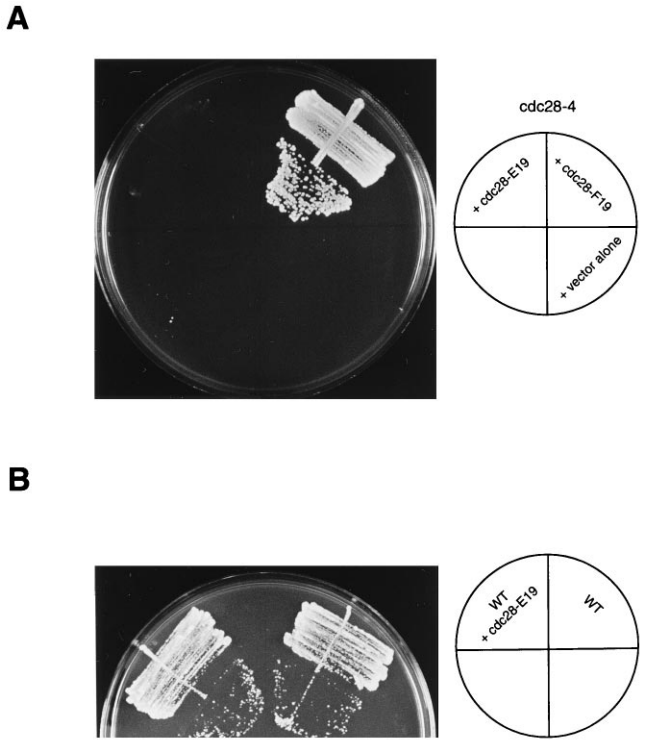


FIG. 1. *cdc28-4* mutation is suppressed by the *cdc28-F19* but not by the *cdc28-E19* allele. (A) The Start-defective *cdc28-4* mutant was transformed with the mutant alleles driven by the native *CDC28* promoter or an empty plasmid which served as a negative control (vector alone). The transformants were streaked on glucose plates prewarmed to 37°C. The plates were photographed after 2 days at 37°C. (B) Wild-type (WT) cells with or without the *cdc28-E19* allele driven by the native promoter were plated on a glucose plate at 24°C and photographed after 2 days.

However, from these results it is difficult to ascertain whether Cdc28-E19 is defective in spindle assembly. The spindle formation seen in some of the *cdc28-E19* cells could be due to the fact either that Cdc28-E19 is partially active with regard to spindle assembly or that Cdc28-4 protein, though defective in Start functions, could be capable of mediating spindle formation to some extent. If Cdc28-E19 is indeed capable of partially promoting the spindle assembly, then increased concentration of the mutant protein would be expected to cause spindle formation in a greater proportion of cells. To test this, stationary-phase cells of the *cdc28-4* mutant carrying *GAL1* promoter-driven *cdc28-E19* were allowed to resume cell cycle progression in either glucose (in which *GAL1* is inactive) or galactose medium at 37°C. Samples withdrawn at regular intervals were first analyzed for bud emergence and the expression of the *RNR1* gene (15), both of which are Start-dependent events. As expected, cells growing in glucose medium remained unbudded and neither transcribed the *RNR1* gene (the slight accumulation of RNR1 transcript in these cultures could be due to the uneven loading of RNA) nor replicated their DNA throughout the course of the experiment (Fig. 3A and B). Cells expressing *cdc28-E19* because of induction by galactose, on the other hand, budded (>80% by 3.5 h) and showed accumulation of *RNR1* transcript, indicating that the overexpression of the *cdc28-E19* allele too can promote Start-related events. These cells had also completed S phase as indicated by flow cytometry (Fig. 3B, lowermost panel) but eventually arrested with 2 N DNA content and an undivided nucleus (Fig. 3B).

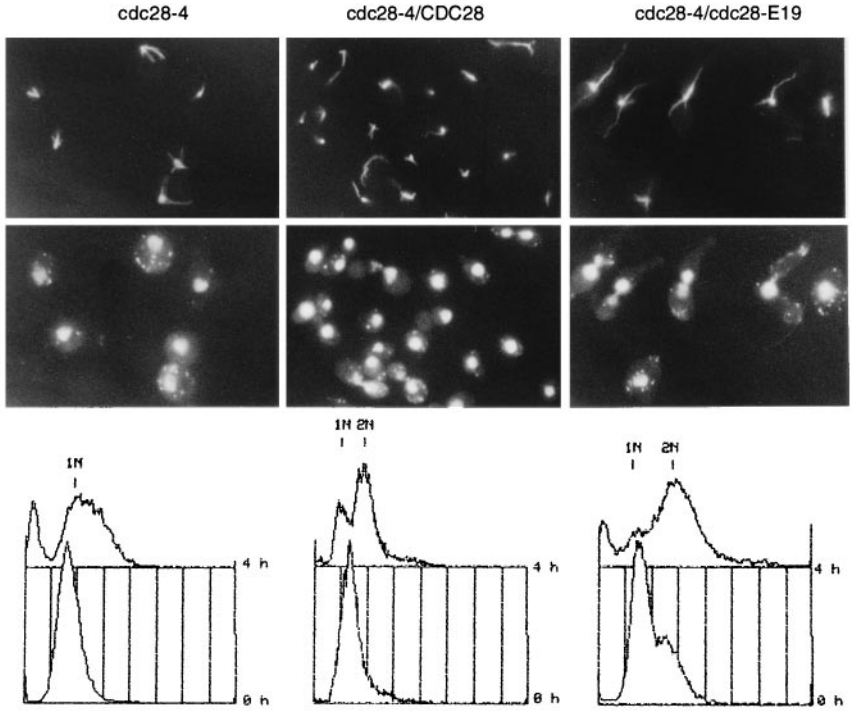
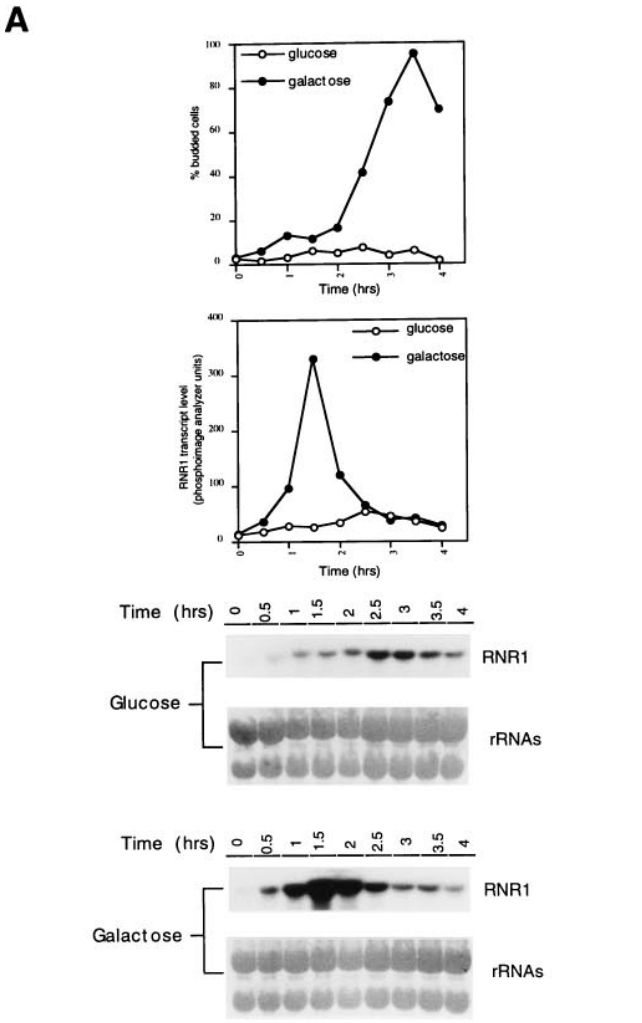


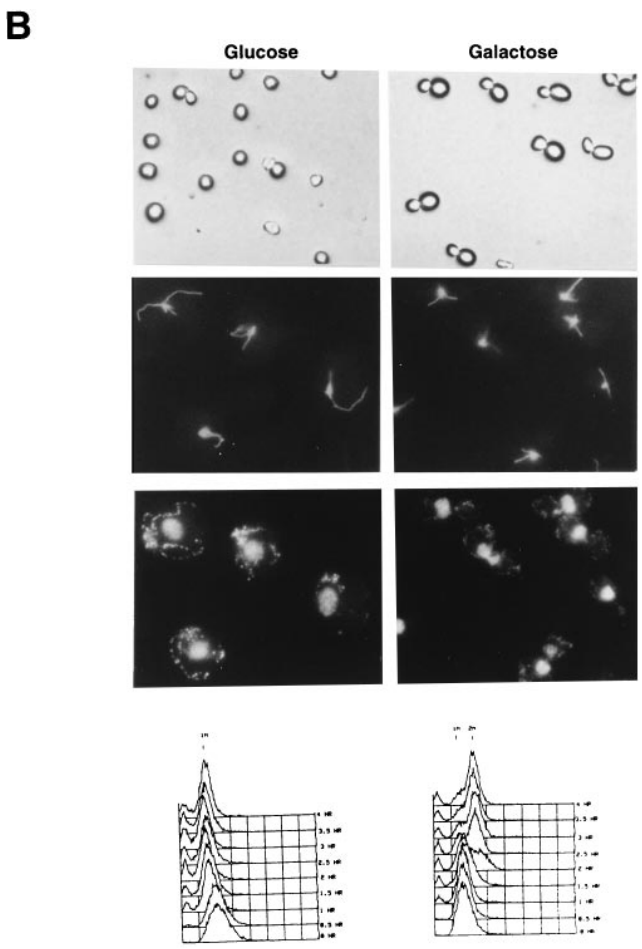
FIG. 2. Phenotype of *cdc28-4* cells expressing native promoter-driven *cdc28-E19*. Stationary-phase cells of the *cdc28-4* strain either carrying no plasmid (left column) or carrying a centromere-based plasmid containing native promoter-driven *CDC28* (middle column) or *cdc28-E19* (right column) were released into glucose medium at 37°C. The 4-h samples stained with antitubulin antibodies (top row) and diamidinophenyl indole (DAPI) (middle row) are shown. The lowermost panels show fluorescence-activated cell sorter profiles of the 0- and 4-h samples.

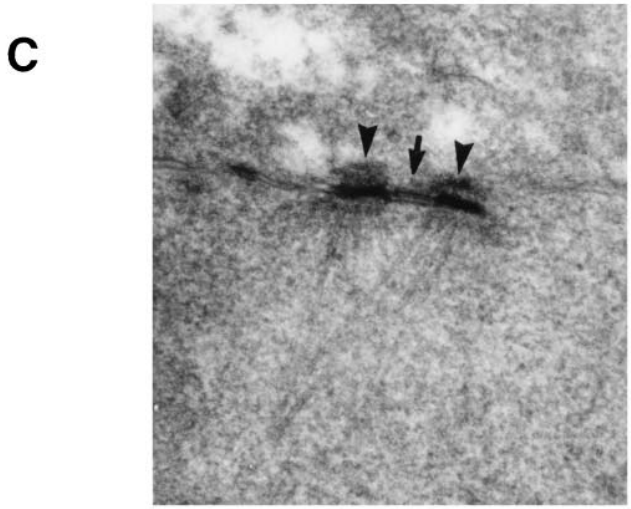
Staining with antitubulin antibodies showed that ~85% of the budded cells lacked a bipolar spindle; instead, these cells contained microtubules emanating from a single brightly stained spot (Fig. 3B). The lack of a mitotic spindle in these cells is not a transient but a terminal feature, in that this phenotype persists even after prolonged incubation. Electron microscopic examination revealed that in >95% of the cells examined, the spindle poles had duplicated but remained in a side-by-side configuration still attached by a bridge (Fig. 3C). Thus, in the culture overexpressing Cdc28-E19, the proportion of cells devoid of mitotic spindles is greater than that in the culture in which the mutant allele is expressed from its native promoter. These results imply that spindle formation observed in some of the cells carrying native promoter-driven Cdc28-E19 might have been due to the Cdc28-4 protein. When overexpressed, Cdc28-E19 perhaps outcompeted the Cdc28-4 protein for the mitotic cyclins (see below), precluding any spindle assembly. We conclude therefore that Cdc28-E19, though active with respect to Start and S-phase functions, is defective in promoting SPB segregation.

Timing of bipolar spindle formation in wild-type cells. If SPB separation is triggered only when cells have initiated mitosis and not by activation of Cdc28/Clb kinase per se, then the failure of *cdc28-E19* to cause SPB segregation would not be surprising since cells expressing this allele do not progress into mitosis. It has been previously shown that spindle poles migrate away from one another when the bud attains one-third of the size of the mother cell, a stage at which cells are expected to have already completed DNA synthesis (8). Since the timing of SPB migration is critical to the interpretation of our data, we sought to determine the kinetics of spindle formation directly in relation to S phase. To do this, wild-type cells were first synchronized in G_1 by pheromone treatment and then allowed to resume cell cycle progression at 24°C in pheromone-free medium. The DNA contents of cells withdrawn at 10-min intervals were analyzed by flow cytometry, and the presence of bipolar spindles was determined by both electron microscopy and indirect immunofluorescence staining. As shown in Fig. 4, ~80% of the cells duplicated their spindle poles within 50 min of release from pheromone arrest. By 70 min, the majority of the cells shows a 2 N DNA content and >95% of them contain separated SPBs or spindles. Staining with antitubulin antibodies confirmed that >75% of these cells possessed clearly discernible short mitotic spindles. Therefore, in the wild-type cells mitotic spindle formation is concomitant with the completion of S phase.

Dependence of bipolar spindle formation on dephosphorylation of Tyr-19. Given that wild-type cells assemble bipolar spindles after S phase, it is possible that the absence of mitotic spindles in *cdc28-E19* cells is not due directly to a functional defect of the *cdc28-E19* allele but is rather a reflection of the inability of these cells to initiate mitosis. To address this, we examined the effect of the *cdc28-E19* allele on spindle formation under conditions under which spindle assembly occurs independently of mitosis. In *S. cerevisiae*, cells treated with hydroxyurea (HU) arrest early in S phase and do not undergo mitosis (i.e., nuclear division) but form short bipolar spindles. Stationary-phase cells of the *cdc28-4* mutant carrying either *GAL-CDC28* or *GAL-cdc28-E19* on a *CEN* plasmid were allowed to resume cell cycle progression at 37°C in galactose medium containing HU. The cultures were monitored for their ability to transcribe the *RNR1* gene, duplicate their DNA, and assemble bipolar spindles. As expected, chromosome duplication does not occur in these cells, although the peak corresponding to 1 N DNA content continues to broaden with time (Fig. 5B). In cells expressing the wild-type *CDC28* gene, *RNR1*



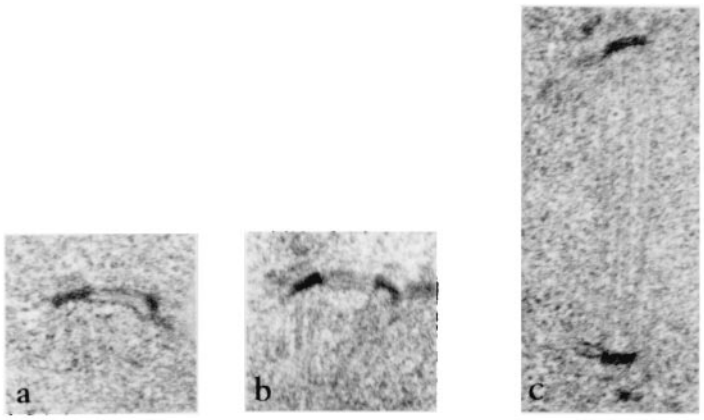




	No. of paired SPBs	No. of unpaired SPBs
cdc28-4/pGAL-cdc28-E19	23/24 (95.8%)	1/24 (4.2%)
cdc28-4/pGAL-CDC28	9/24 (37.5%)	15/24 (62.5%)

FIG. 3. Phenotype of *cdc28-4* cells expressing *GAL-cdc28-E19*. (A) Stationary-phase *cdc28-4* mutant cells carrying *GAL-cdc28-E19* were released into either glucose or galactose medium prewarmed at 37°C. Samples were collected at 30-min intervals and analyzed. The upper and lower graphs show the budding index and a quantitative estimation of the *RNR1* transcript level, respectively. *RNR1* transcript (bottom panels) was analyzed by Northern blotting (~30 μg of RNA per well) and quantitated with a PhosphorImager (Molecular Dynamics). (B) Cell cycle arrest phenotype from the 4-h sample. From top to bottom are shown phase-contrast photomicroscopy, microtubule staining with antitubulin antibody, nuclear staining with DAPI, and DNA content, as measured by flow cytometry, showing a 2 N peak in the galactose sample. (C) Electron micrograph of a cell from the 4-h galactose sample at 37°C, showing two SPBs (arrowheads) still attached to each other by a bridge structure (arrow). Scale, 5 mm = 0.1 mm. The proportions of cells with paired or unpaired SPBs in the 4-h galactose sample are also tabulated.

transcript appears at 1.5 h after the release and short spindles can be seen in ~80% of the cells after 4.5 h (Fig. 5). The *cdc28-E19* cells, although slightly delayed, also activate *RNR1* transcription (Fig. 5A). Bud emergence in these cells shows a similar delay (data not shown), suggesting that they are sluggish in traversing Start. Remarkably, however, *cdc28-E19* cells fail to assemble bipolar spindles even 6 h after the release from stationary phase. Electron microscopy revealed that at 4.5 h, the spindle poles had duplicated in all of the 20 cells examined but remained paired in a side-by-side configuration as in Fig. 3C (data not shown). In cells expressing CDC28, on the other hand, 90% of the cells had formed spindles (20 cells examined). These observations strongly argue that the failure of *cdc28-E19* cells to assemble spindles is not a consequence of



Time (min)	Single SPB with or without satellite	Paired SPBs attached by a bridge	Separated SPBs or spindles	No. spindles seen by immunofluorescence	Facs profile
30	23/30 (76.7%)	7/30 (23.37%)	0/30 (0%)	0/200 (0%)	
40	12/29 (41.1%)	7/29 (58.6%)	0/29 (0%)	0/200 (0%)	
50	0/30 (0%)	24/30 (80.0%)	6/30 (20.0%)	4/204 (2.0%)	
60	0/28 (0%)	6/28 (21.4%)	22/28 (78.6%)	80/242 (33.1%)	
70	0/30 (0%)	1/30 (3.3%)	29/30 (96.7%)	216/282 (76.6%)	

FIG. 4. The separation of spindle poles is correlated with the completion of DNA replication. Wild-type cells synchronized in G_1 by α-factor treatment were released into glucose medium at 24°C. The state of the spindle poles in samples withdrawn at 10-min intervals (first column) was analyzed by electron microscopy. SPBs in 28 to 30 different cells from each time point were followed through serial electron microscopic sections and classified as single SPB without or with satellite attached (a), duplicated SPBs attached by a bridge structure (b), and separated SPBs or spindles (c). The fifth column tabulates the percentages of cells with short mitotic spindles visualized by immunofluorescence microscopy, and the last column shows the distribution of DNA content as determined by flow cytometry. Scale, 4.7 mm = 0.1 mm. Facs, fluorescence-activated cell sorter.

their inability to initiate mitosis but is specifically due to the inability of the *cdc28-E19* allele to promote SPB separation.

Overexpression of *SWE1* abolishes bipolar spindle formation. In *S. pombe*, cdc2 is inactivated by the phosphorylation of Tyr-15 by wee1 kinase (14). Similarly, *SWE1*, a budding-yeast homolog of *wee1*$^+$, has been shown to phosphorylate Tyr-19 of Cdc28, causing a drastic reduction in the activity of Cdc28/Clb2 kinase complex in vitro (5). If dephosphorylation of Tyr-19 is indeed required for spindle formation, as our results suggest, then overexpression of *SWE1* will be expected to render cells incapable of assembling bipolar spindles. To test this hypothesis, wild-type cells carrying the *GAL-SWE1* construct (integrated at the *SWE1* locus) were first synchronized in G_1 by α-factor treatment, induced by the addition of galactose for 2 h while the cells were still arrested, and then allowed to resume cell cycle progression at 24°C in galactose medium containing HU. The control culture was grown in raffinose medium and was not induced. Samples were withdrawn at various times and

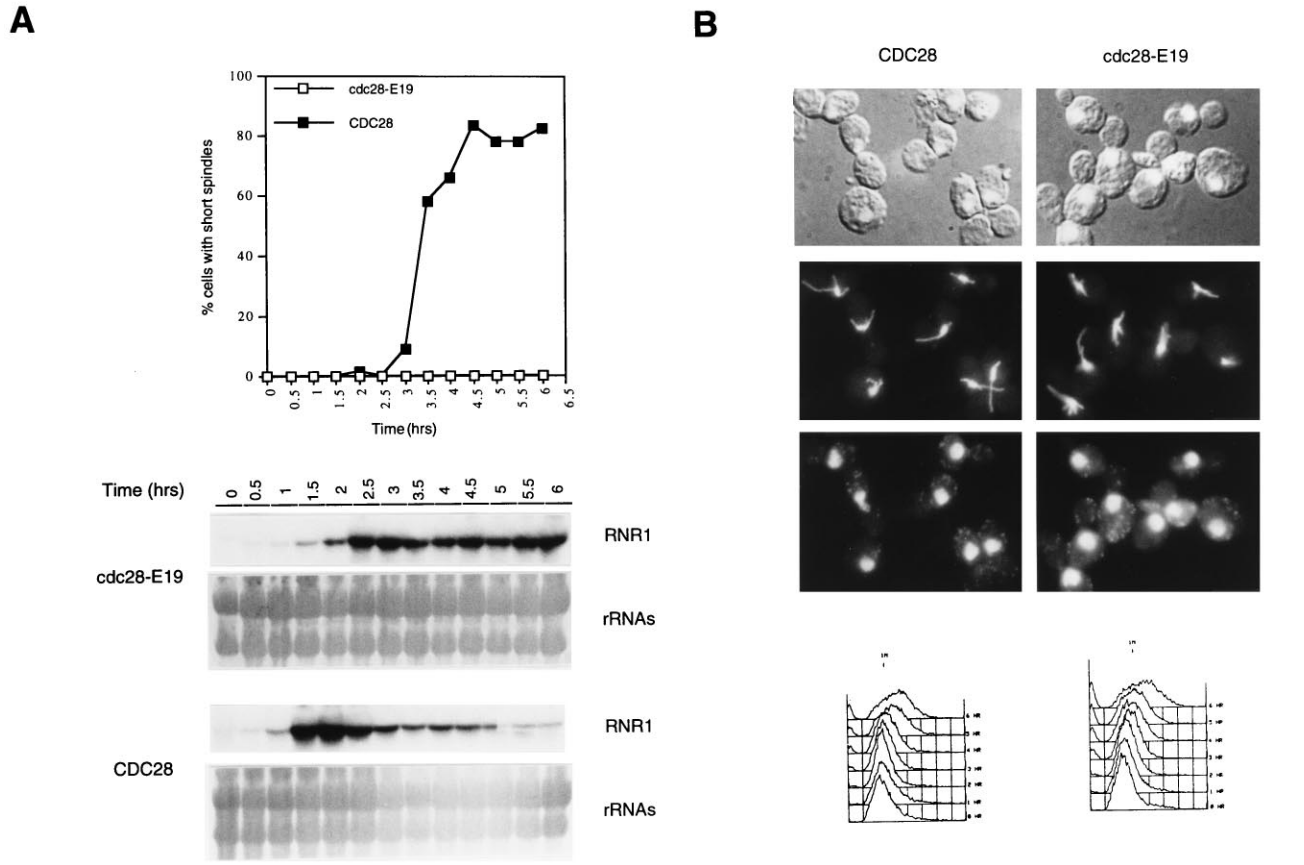
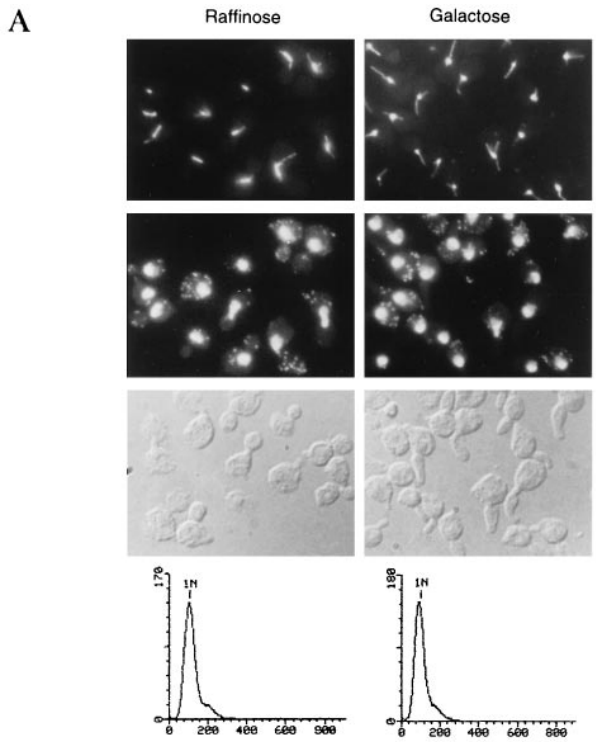


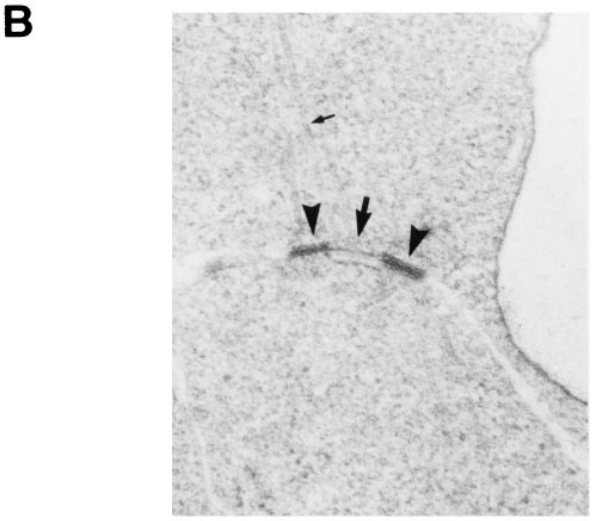
FIG. 5. Spindle formation in HU-arrested *cdc28-E19* cells. *cdc28-4* mutant cells carrying either *GAL-cdc28-E19* or *GAL-CDC28* were released from stationary phase into raffinose or galactose medium prewarmed at 37°C. At 15 min after the release, HU was added at a concentration of 15 mg/ml. Samples were collected at half-hourly intervals and analyzed. (A) The graph shows the percentages of cells with short spindles for cells released into galactose medium. The gels show the *RNR1* transcript in the galactose samples, measured by Northern blotting (~30 μg of RNA per well). The data for raffinose samples are not shown. (B) Arrest phenotype of cells from the 6-h galactose sample. From top to bottom are shown a differential interference contrast (Nomarski) micrograph, microtubule staining with antitubulin antibody, nuclear staining with DAPI, and distribution of DNA content, as determined by flow cytometry.

analyzed for the presence of mitotic spindles. As expected, cells bud normally in raffinose medium, form clearly discernible short spindles, and eventually arrest with 1 N DNA content (Fig. 6A, left panels). In galactose medium, where Swe1 is overexpressed, buds begin to emerge at about the same time as in raffinose but they are noticeably elongated. Cells eventually arrest with 1 N DNA content and monopolar spindles (Fig. 6A, right panels). When analyzed by electron microscopy, SPBs in all of the 20 cells examined were found in a side-by-side configuration (Fig. 6B), as seen in *cdc28-E19* cells, suggesting that overexpression of Swe1 renders cells unable to segregate SPBs.

It is possible that the prevention of spindle assembly due to an excess of Swe1 is independent of its capacity to phosphorylate Tyr-19. To test this notion, we introduced a *CEN* plasmid carrying *GAL-cdc28-F19* into a wild-type strain containing an integrated copy of *GAL-SWE1*. Cdc28-F19 cannot be phosphorylated by Swe1 because of the replacement of Tyr-19 by phenylalanine. Cells of this strain were synchronized by pheromone treatment, released at 24°C into galactose medium containing HU, and then stained with antitubulin antibodies at various times. The cells, coexpressing *SWE1* and *cdc28-F19* because of galactose induction, eventually arrested with large buds and 1 N DNA content; approximately 65% of these cells contain short mitotic spindles (Fig. 6C). This phenotype is identical to that of wild-type cells growing in raffinose medium containing HU (Fig. 6A, left panels). Thus, the phenotype caused by Swe1 overexpression can be partially alleviated by the presence of *cdc28-F19*. This suggests that the abolition of bipolar spindle formation by an excess of Swe1 is largely due to its ability to phosphorylate Tyr-19.

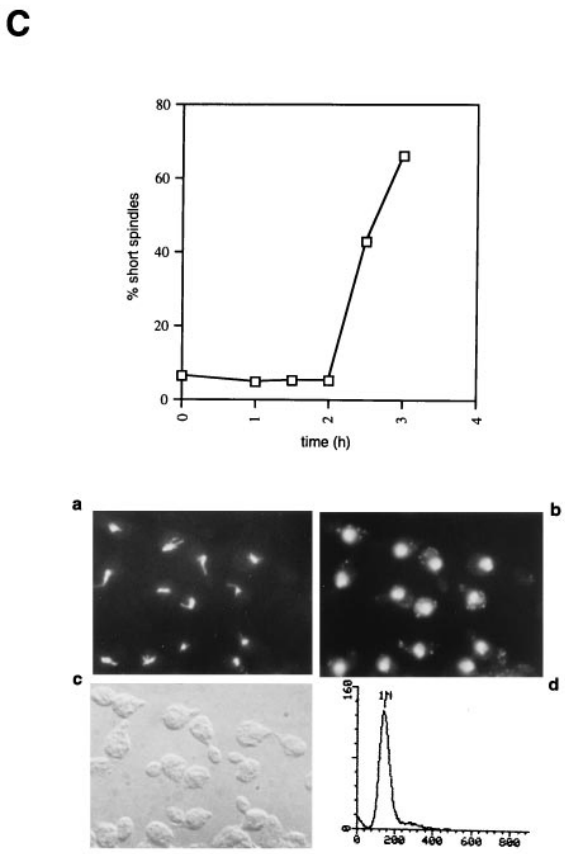
Clb2-associated kinase activity in cells overexpressing either *cdc28-E19* or *SWE1*. Since Swe1 diminishes Cdc28/Clb2 kinase activity in vitro by phosphorylating Tyr-19 (5), it is conceivable that the absence of a bipolar spindle in cells expressing either *cdc28-E19* or *SWE1* from the *GAL1* promoter is due to a complete lack of the mitotic cyclin-associated H1 kinase activity. To examine this possibility, stationary-phase cells of the *cdc28-4* strain carrying either *GAL-cdc28-E19* or *GAL-CDC28* on a single-copy vector were released into either glucose or galactose medium at 37°C. Cell extracts were made from samples withdrawn at various times, Clb2-associated kinase was immunoprecipitated with anti-Clb2 antibodies, and the kinase activity was measured with histone H1 as a substrate. As expected, cells released in glucose remain arrested in G_1 and show barely detectable H1 kinase activity (Fig. 7A, upper panel). In galactose, however, Clb2-associated H1 kinase activity begins to rise 2 h after the release and continues to increase throughout the course of the experiment. Since





GAL-SWE1 strain	No. of paired SPBs	No. of unpaired SPBs
galactose	20/20 (100%)	0/20 (0%)
raffinose	0/20 (0%)	20/20 (100%)

FIG. 6. Phenotype of cells overexpressing *SWE1*. (A) Wild-type cells carrying *GAL-SWE1* were synchronized by α-factor treatment, induced for 2 h by addition of galactose, and then released into galactose medium containing HU (15 mg/ml). The control culture was similarly treated and was not induced. The arrest phenotypes of cells from the 3-h samples (from top to bottom, microtubule staining with antitubulin antibody, nucleus staining with DAPI, and cell morphology as viewed under Nomarski optics, respectively) and the distribution of DNA content (lowermost panels) are shown. (B) Electron micrograph of a cell from the 3-h galactose sample, showing side-by-side SPBs (arrowheads) attached by a bridge (arrow), similar to cells expressing *cdc28-E19*. The small arrow points to cytoplasmic microtubules. The number of cells showing such a phenotype as determined by serial electron micrographs is shown in the table. Scale, 5.4 mm = 0.1 mm. (C) Arrest phenotype of cells coexpressing *SWE1* and *cdc28-F19* in galactose medium. A culture synchronized in G_1 by α-factor treatment was released into YEP-galactose medium containing 15 mg of HU per ml, and samples were collected at hourly intervals. Cells from the 3-h sample are shown as follows: (a) mitotic spindles stained with antitubulin antibody, (b) nuclear staining with DAPI, (c) cell morphology under Nomarski optics, and (d) DNA distribution profile. The proportion of cells that form short spindles at various times after release from α-factor is shown in the graph at the top.



endogenous *cdc28-4* allele encodes a temperature-sensitive protein with little H1 kinase activity at 37°C, the kinase activity measured in this experiment must be almost entirely due to Cdc28-E19. Although it appears slightly later, the level of the kinase activity in cells expressing Cdc28-E19 is comparable to that in cells expressing the wild-type Cdc28 (Fig. 7A, lower panel). Therefore, we conclude that Cdc28-E19, although unable to promote SPB separation and progression through mitosis, is active as a histone H1 kinase.

To determine the effect of *SWE1* overexpression on Cdc28/Clb2 kinase, wild-type cells harboring *GAL-SWE1* were grown in raffinose medium and synchronized in G_1 by α-factor treatment. One half of the culture was then induced with the addition of galactose, while the other half continued to grow in raffinose. After 2 h, both cultures were released into their respective pheromone-free media. As cells growing in raffinose do not express Swe1, they continue their progression through the cell cycle and show an expected oscillation in the Clb2-associated kinase activity (Fig. 7B, upper panel). The kinase activity in cells expressing Swe1 because of growth in galactose is first detected 60 min after the release but continues to rise through the entire course of the experiment (Fig. 7B, middle panel). These cells are unable to form mitotic spindles and eventually arrest with 2 N DNA content (data not shown). To determine if the overexpression of *SWE1* indeed leads to increased tyrosine phosphorylation of Cdc28 in this experiment,

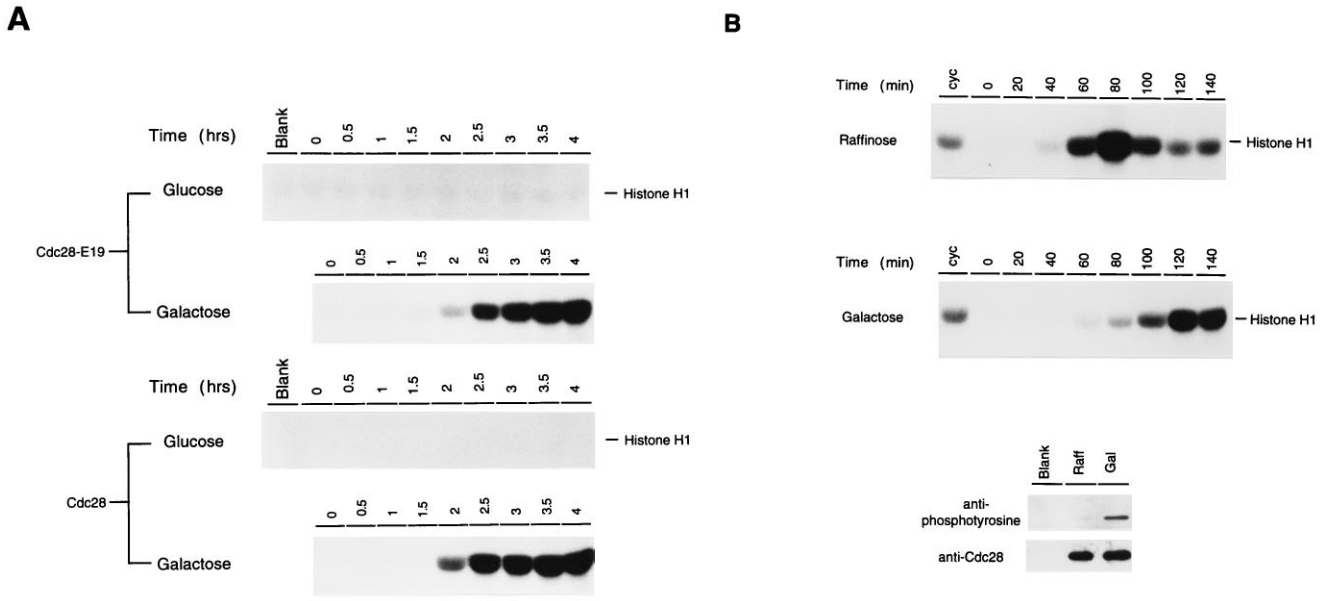


FIG. 7. Clb2-associated histone H1 kinase activity. (A) Stationary-phase *cdc28-4* mutant cells carrying *GAL-cdc28-E19* or *GAL-CDC28* were released into either glucose or galactose medium prewarmed at 37°C. Samples were collected every 30 min, and the cells were harvested for preparation of cell extracts. Immunoprecipitation of Cdc28/Clb2 complex and kinase assays were performed as described in Materials and Methods. The kinase activities at various times after release are shown. (B) Wild-type cells carrying *GAL-SWE1* were grown in raffinose medium and synchronized in G_1 with α-factor treatment. One half of the culture was then induced with galactose for 2 h, while the other half remained in raffinose medium. Thereafter, both cultures were filtered and released into their respective pheromone-free media. Samples were collected every 20 min and assayed for Clb2-associated histone H1 kinase activity (top and middle panels). Cdc28 was immunoprecipitated from the 140-min samples, resolved by SDS-PAGE and probed with either antiphosphotyrosine antibodies or anti-Cdc28 antibodies (bottom panel).

Cdc28 was immunoprecipitated from the 140-min samples and analyzed by Western blotting using either anti-Cdc28 antiserum or antiphosphotyrosine antibodies. While Cdc28 antiserum can detect Cdc28 in both raffinose- and galactose-grown cells, the antiphosphotyrosine antibodies detect Cdc28 only in cells grown in galactose (Fig. 7B, lower panel). These results show that Cdc28, though phosphorylated on tyrosine by Swe1, can still associate with Clb2 and is active as an H1 kinase in vivo.

Transcription of various genes in cells expressing *cdc28-E19*. Since it appears from our data that the *cdc28-E19* allele is capable of performing Start- and S-phase-related functions, we tested whether cells expressing the *cdc28-E19* allele were also normal with regard to the transcription of genes that are turned on in either late G_1 or G_2 phase of the cell cycle. We chose *HCS26*, *CLN1*, and *RNR1* as genes whose transcription in late G_1 is dependent on Start (32). Of these, *HCS26* and *CLN1* transcription is activated in late G_1 via an Swi4/6 cell cycle box element (32). The transcription of *CLB5* (50) also comes on in late G_1 but is driven by a promoter element called the *Mlu*I cell cycle box. *SWI5* and *CLB2*, on the other hand, are transcriptionally activated in S or G_2 phase by a promoter element which requires binding of transcription factor Mcm1 (1). To determine the effect of *cdc28-E19* on the transcription of these genes, stationary-phase cells of the *cdc28-4* strain, carrying either wild-type *CDC28* or the *cdc28-E19* allele driven by the *GAL1* promoter, were allowed to resume cell cycle progression at 37°C in either glucose or galactose medium. Cells were harvested at various times, total RNA was prepared, and the levels of various transcripts were determined by Northern blot analysis. As expected, cells growing in glucose arrested in G_1 as unbudded cells and showed very low transcript levels (Fig. 8). The detectable amount of *RNR1* and *CLN1* RNAs present in the lanes corresponding to the latter time points in *cdc28-E19* samples could have been due either to loading differences or to slight leakiness of the arrest. In galactose medium, transcripts for all six genes are induced in both *CDC28*- and *cdc28-E19*-expressing cells. There are, however, differences in the levels of various transcripts and in the timings of their appearance in the two strains. It is difficult to interpret such differences, because the release of these cultures from stationary phase is not very synchronous. Nevertheless, these results indicate that cells expressing the *cdc28-E19* allele are not defective with regard to the transcriptional activation of the six genes we have tested.

Deficiency of Swe1 does not alter timing of spindle assembly. As noted before, wild-type cells begin to assemble bipolar spindles after the completion of S phase, though the SPBs duplicate prior to the initiation of DNA replication. What prevents SPB separation during S phase? The fact that overproduction of Swe1 severely impairs the cells' ability to segregate spindle poles implies that Swe1 could play an important role in determining the timing of spindle assembly. Therefore, we first compared the kinetics of spindle formation with the transcriptional activation of *SWE1*. Wild-type cells synchronized in G_1 by α-factor treatment were released from the arrest at 24°C in pheromone-free medium. Cell samples were withdrawn at 10-min intervals and analyzed for the appearance of *SWE1* transcript and short spindles. The amount of *SWE1* RNA, detectable within 10 min of release from G_1, peaks at 30 min and is lowest from 70 to 90 min. The proportion of cells with short, bipolar spindles is maximum at 70 min (Fig. 9A), a time when the abundance of *SWE1* transcript is at its minimum. This pattern is repeated in the subsequent cycle.

The inverse correlation between spindle formation and the appearance of *SWE1* RNA prompted us to examine whether the deficiency of *SWE1* function causes alteration in the timing of spindle assembly. Cells of the *swe1*Δ strain were synchronized in G_1 by pheromone treatment and then permitted to

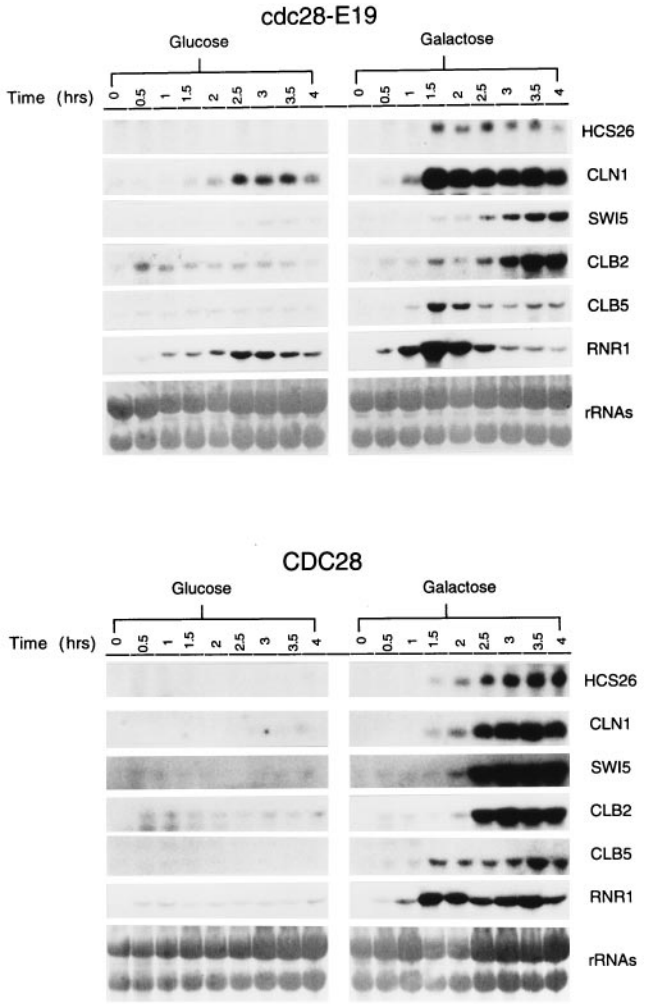


FIG. 8. Profile of various RNA transcripts from *cdc28-4* mutant cells carrying *GAL-cdc28-E19* or *GAL-CDC28*. Stationary-phase cells were released into either glucose or galactose medium prewarmed at 37°C. Samples were collected at half-hourly intervals, and total RNA was isolated and analyzed by Northern blot analysis. The amount of RNA (~30 μg per lane) loaded was visualized by methylene blue staining of the rRNAs.

resume cell cycle progression at 24°C. Spindle formation was monitored by immunofluorescence staining of cell samples withdrawn at various times. As shown in Fig. 9B, the proportion of cells without spindles in the *swe1*Δ strain decreases sharply after 60 min of release and reaches a minimum within the next 30 min during the first cycle. This pattern is almost identical to that for *SWE1*-proficient cells treated in a similar manner. The kinetics of the appearance of bipolar spindles in both strains are also similar during the second cycle. From these data, we conclude that while overexpression of *SWE1* affects SPB segregation, and therefore spindle formation, it is not a limiting factor in determining the timing of spindle assembly.

DISCUSSION

Chromosome separation or nuclear division is a clear indication that cells are well into M phase. However, mitotic events such as chromosome condensation, bipolar spindle formation, and nuclear-membrane breakdown occur before chromosome segregation and are, therefore, considered to mark the initiation of mitosis. In many eukaryotes, these events are easily discernible, while in others, there are only a few cytologically visible signs of M-phase initiation (37). The problem of scarce mitotic landmarks is particularly highlighted in organisms such as the budding yeasts, in which the nuclear membrane remains intact throughout the cell cycle and condensed chromosomes are difficult to observe (25).

S. cerevisiae differs in yet another aspect. Unlike the fission yeasts and some other eukaryotes, replacing the highly conserved tyrosine 19 (equivalent to Tyr-15 in cdc2) with phenylalanine in Cdc28 does not lead to precocious mitosis (2, 52), raising the possibility that the dephosphorylation of Tyr-19 might not be critical for mitosis. However, it has been reported that the delay in nuclear division in response to a transient disruption of cell polarity involves phosphorylation of Cdc28 on Tyr-19 (33). This argues that the regulation of Tyr-19 phosphorylation is important for mitosis (33). Our finding that *cdc28-E19* cells arrest prior to nuclear division (Fig. 2) also shows that Tyr-19 dephosphorylation is clearly necessary for mitotic progression in budding yeast cells. A detailed characterization of the arrest phenotype further reveals that cells expressing *cdc28-E19* undergo Start and DNA replication but are incapable of assembling a bipolar spindle (Fig. 3A and B). The inability of *cdc28-E19* cells to form spindles appears to be due to their failure to separate the spindle poles (Fig. 3C). Since these cells exhibit high levels of Clb2-associated histone H1 kinase activity (Fig. 7A), the defect in spindle assembly is not due to the inability of mutant Cdc28 to interact with mitotic cyclins. This is consistent with our observation that the expression of the *cdc28-E19* allele from the *GAL1* promoter severely retards the growth of wild-type cells (data not shown), presumably because the mutant protein competes with the wild-type Cdc28 for mitotic cyclins. It is noteworthy that the phenotype of *cdc28-E19* cells is similar to that of the *cdc28-1N* mutant (41, 54) which also arrests with a large bud, 2 N DNA content, and high-level Clb2-associated kinase activity. However, whereas *cdc28-E19* cells are unable to promote spindle assembly, the *cdc28-1N* mutant can assemble a short mitotic spindle but fails to extend it at the nonpermissive temperature. The involvement of a serine/threonine kinase in the biogenesis of the mitotic spindle is not unprecedented. In *Drosophila melanogaster* and *S. pombe*, serine/threonine kinases Aurora and plo1, respectively, have been implicated in centrosome segregation (23, 40).

It is known that activation of cdc2 via Tyr-15 dephosphorylation is required for the initiation of mitosis-specific processes. However, it is not clear if the triggering of these events is directly caused by activated cdc2 or is a consequence of the cells' entry into mitosis. Although the absence of a bipolar spindle in *cdc28-E19* cells suggests that tyrosine 19 dephosphorylation is required for SPB separation, this dependence may not be direct. A unique feature of *S. cerevisiae* has allowed us to explore this further. When arrested in S phase, either through certain *cdc* mutations or by treatment with HU, budding yeast cells do not progress into mitosis but nonetheless assemble a bipolar spindle. We find that in the presence of HU, where spindle assembly can be dissociated from the initiation of mitosis, spindle pole separation is still dependent on the cells' ability to dephosphorylate Tyr-19 (Fig. 5). This strongly implies a direct dependence of SPB separation on Tyr-19 dephosphorylation.

The abolition of spindle assembly due to overexpression of *SWE1* in HU-treated cells is consistent with this conclusion (Fig. 6A and B). In a previous report, it was suggested that an excess of Swe1 interferes with the elongation of the mitotic spindle but does not preclude its formation (5). These exper-

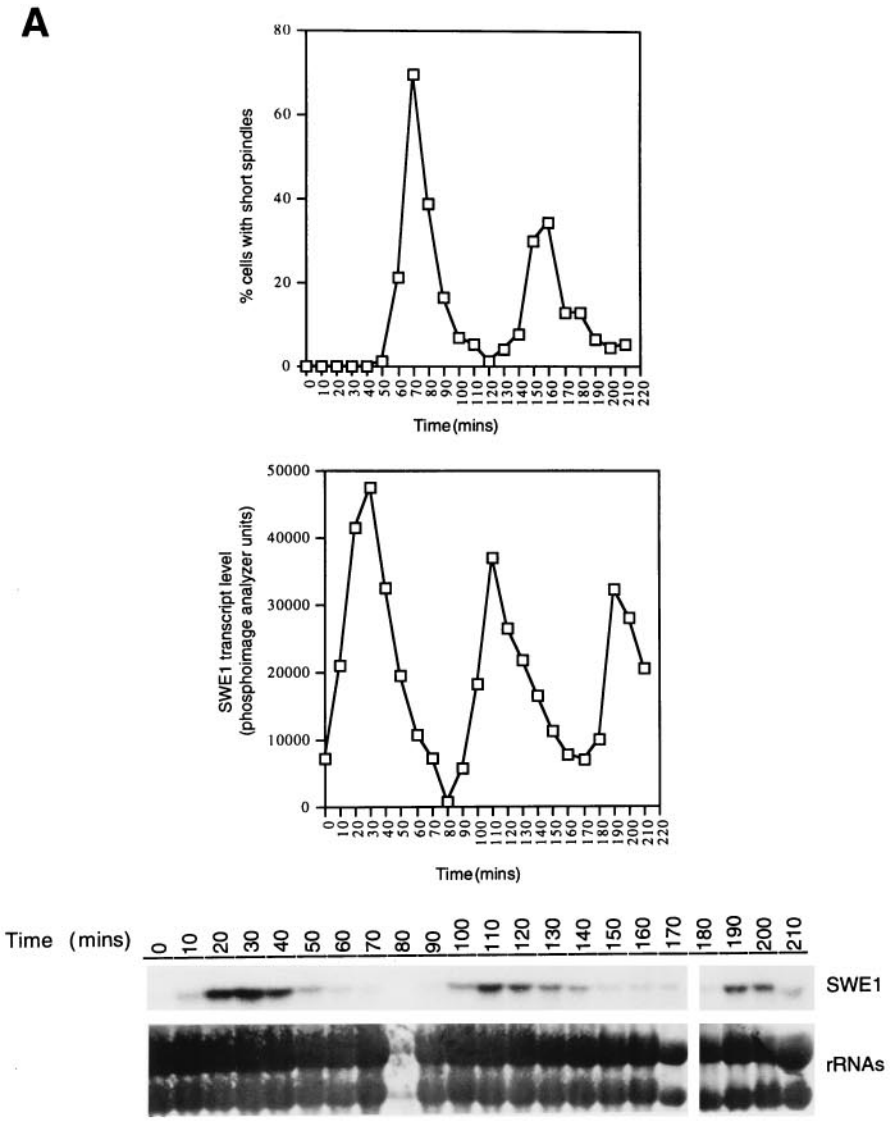


FIG. 9. (A) *SWE1* expression during the cell cycle. Wild-type cells were synchronized in G_1 by α-factor treatment (0.8 μg/ml) and released into glucose medium. Samples were collected at 10-min intervals and analyzed. The top graph shows the proportion of cells containing short spindles at various times. The bottom graph is a quantitative representation of the level of *SWE1* transcript. The amount of total RNA loaded (~30 μg per well) was visualized by methylene blue staining of the rRNAs, and the same filter was used for Northern blot analysis (lower panels). The transcript level was quantitated with a PhosphorImager. (B) Wild-type and *swe1*Δ cells were synchronized in G_1 with α-factor and released into glucose medium. Cells were collected at 10-min intervals and stained with antitubulin antibodies. The graph shows the percentages of cells with no spindles as the cells release from G_1 arrest.

iments were performed with asynchronous cultures in which we found (data not shown) that *SWE1* overexpression resulted in a mixed phenotype in which some cells contain short spindles (~70%) and others do not (~30%). Therefore, we used both immunofluorescence staining and electron microscopy to analyze the effect of Swe1 overexpression in a synchronous culture and found that 3 h after the release from G_1 phase, all cells had arrested with a large bud and 2 N DNA content but >70% of them lacked mitotic spindles and contained paired SPBs (data not shown). Thus, an excess of Swe1 indeed impairs the process of spindle pole segregation. One explanation for the failure of the Swe1-overexpressing cells to segregate SPBs could be that the activity of Clb2/Cdc28 kinase in these cells is inhibited because of phosphorylation of Tyr-19, causing a complete lack of mitotic kinase activity akin to the strain deficient in mitotic cyclins Clb1, -2, -3, and -4, which also fails to separate SPBs (20). Tyrosine phosphorylation by Swe1 of the in vitro-reconstituted Clb2/Cdc28 complex does indeed cause a drastic reduction in its kinase activity (5). Surprisingly, however, our results show that the Clb2/Cdc28 kinase complex immunoprecipitated from cells either overproducing Swe1 or expressing *cdc28-E19* is almost as active as the wild-type complex when tested on the standard substrate histone H1 (Fig. 7). Although we are unable to provide an adequate explanation for this discrepancy, this finding suggests that the inability of Swe1-overexpressing cells to segregate SPB is not due to a reduction in the overall kinase activity of Clb2/Cdc28 complex but could be a result of a change in the substrate specificity of this kinase complex elicited by Tyr-19 phosphorylation.

The observation that Tyr-19 dephosphorylation is required for spindle pole separation has a number of interesting implications. Firstly, as we have confirmed, wild-type cells assemble short mitotic spindles concomitant with the completion of S phase. It follows, then, that during the normal course of cell cycle progression, Cdc28 is not sufficiently dephosphorylated on Tyr-19 to cause SPB separation until after DNA replication. This could imply the existence of controls which negatively regulate Tyr-19 dephosphorylation until the S phase is completed. However, the presence of short spindles in HU-arrested cells clearly indicates that mitotic activation of Cdc28 by Tyr-19 dephosphorylation can occur independently of DNA synthesis. Secondly, though short spindles assemble in cells incapable of completing S phase, they never go on to extend, suggesting that in budding yeast cells, activation of Cdc28 by Tyr-19 dephosphorylation is not sufficient to ensure either elongation of the mitotic spindle or progression through mitosis; activation of other effectors may also be required. Thus, unlike that in *S. pombe*, the initiation of mitotic progression in *S. cerevisiae* could require additional regulatory processes (24). And lastly, cells arrested because of DNA damage-induced checkpoint controls are able to assemble short spindles but do not extend them. This implies that the S-phase-checkpoint controls, if they at all influence the microtubule dynamics, could be affecting not the assembly but the elongation of the mitotic spindle.

What keeps SPBs from separating during S phase? Since Swe1 kinase efficiently phosphorylates Cdc28 on Tyr-19 in vitro (5) and its overproduction can prevent spindle assembly, it is possible that Swe1 plays an important role in determining the rate of these processes. The inverse correlation between the appearance of short spindles and *SWE1* transcript would be consistent with this notion (Fig. 9). However, as our data indicate, deficiency of Swe1 does not alter the kinetics of spindle formation. It is, therefore, not a rate-limiting factor in determining the timing of SPB segregation in wild-type cells. This could be due partly to the presence of other genes which compensate for *SWE1* deficiency. Alternatively, it can also be argued that the regulation of Tyr-19 phosphorylation by Swe1, and in turn the regulation of SPB segregation, is not critical during the normal course of spindle biogenesis but becomes important when nuclear division needs to be delayed in response to disruption of certain cellular events. Such a context has been provided by the work of Lew and Reed (33), which showed that the nuclear division in *S. cerevisiae* is drastically delayed when bud formation is transiently disrupted. It will be of interest to determine whether this delay is a result of a pause in SPB separation and whether it can be abrogated by the deletion of the *SWE1* gene.

Some of the spindle pole components and their spatial arrangements are known (30), but little about the regulation and the mechanics of SPB separation is understood. It has been shown that spindle pole separation requires activities of various kinesin-like motor proteins (48). The loss of Cin8 and Kip1 motor functions results in a failure to separate SPBs (49). This phenotype can be partially reversed by deletion of the *KAR3* gene, which encodes a minus-end motor. Furthermore, both Cin8 and Kip1 are located along the entire length of the spindle, including the spindle poles. These observations have led to a simple model (49) according to which the motor proteins preferentially cross-link the microtubules emanating from the two poles. The sliding motion mediated by these motors aligns the microtubules in an antiparallel fashion, eventually positioning the two poles in a face-to-face configuration. A similar cross-linking and sliding role has been proposed for bimC protein in *A. nidulans* (18). The fact that both *cdc28-E19* cells and the cells devoid of Cin8 and Kip1 functions are unable to separate SPBs raises the possibility that the role of Cdc28 kinase in SPB separation is to modulate the activities of the motor proteins. The activation of Cdc28/Clb kinase by Tyr-19 dephosphorylation could, via phosphorylation, either potentiate Cin8 and Kip1 motors or impede antagonistic motor protein Kar3. If this is true, then Cin8 and Kip1 proteins should show very low levels of motor activities in extracts prepared from *cdc28-E19* cells. This prediction has so far not been tested. The question as to what aspect of the motor protein function Cdc28/Clb kinase modulates, if at all, to promote spindle pole separation remains to be resolved. It has been reported that phosphorylation by $p34^{cdc2}$ regulates the localization of human Eg5, a kinesin-related motor, to the mitotic spindle (4).

Structural analysis of the Cdk2-cyclin A complex predicts that phosphorylation of Thr-14 and Tyr-15 hinders phosphate transfer from ATP to the substrate. In this light, it is intriguing that the inability to dephosphorylate Tyr-19 does not affect the Start- and S-phase-related functions of Cdc28 in any drastic way but causes loss of its ability to separate spindle poles. Is spindle extension also dependent on Tyr-19 dephosphorylation? What makes the assembly of mitotic spindles cell cycle stage dependent? Do any of the S-phase-related checkpoint controls prevent nuclear division by modulating spindle dynamics? Answers to these questions will require a detailed investigation of the regulatory aspects of spindle assembly.

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REFERENCES

1. **Althoefer, H., A. Schleiffer, K. Wassmann, A. Nordheim, and G. Ammerer.** 1995. MCM1 is required to coordinate G_2-specific transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**5917–5928.
2. **Amon, A., U. Surana, I. Muroff, and K. Nasmyth.** 1992. Regulation of $p34^{CDC28}$ tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. Nature (London) **355:**368–371.
3. **Atherton-Fessler, S., G. Hannig, and H. Piwnica-Worms.** 1993. Reversible tyrosine phosphorylation and cell cycle control. Semin. Cell Biol. **4:**433–442.
4. **Blangy, A., H. A. Lane, P. d'Herin, M. Harper, M. Kress, and E. A. Nigg.** 1995. Phosphorylation by $p34^{cdc2}$ regulates spindle association of human Eg5, a kinesin-related motor essential for bipolar spindle formation in vivo. Cell **83:**1159–1169.
5. **Booher, R. N., R. J. Deshaies, and M. W. Kirschner.** 1993. Properties of *Saccharomyces cerevisiae* wee1 and its differential regulation of $p34^{CDC28}$ in response to G1 and G2 cyclins. EMBO J. **12:**3417–3426.
6. **Brown, A. J., T. Jones, and J. Shuttleworth.** 1994. Expression and activity of $p40^{MO15}$, the catalytic subunit of CDK-activating kinase, during *Xenopus* oogenesis and embryogenesis. Mol. Biol. Cell **5:**921–932.
7. **Byers, B.** 1981. Cytology of yeast life cycle, p. 59–96. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
8. **Byers, B., and L. Goetsch.** 1974. Behavior of spindles and spindle plaques in the cell cycle and conjugation of *Saccharomyces cerevisiae*. J. Bacteriol. **124:** 511–523.
9. **Byers, B., and L. Goetsch.** 1991. Preparation of yeast cells for thin section electron microscopy. Methods Enzymol. **194:**602–608.
10. **Clarke, P. R.** 1995. CAK-handed kinase activation. Curr. Biol. **5:**40–42.
11. **Cross, F., and A. H. Tinkelenberg.** 1991. A positive feedback loop controlling *CLN1* and *CLN2* gene expression at the START of yeast cell cycle. Cell **65:**875–883.
12. **De Bond, H. L., J. Rosenblatt, J. Jancarik, H. D. Jones, D. O. Morgan, and S. H. Kim.** 1993. Crystal structure of cyclin-dependent kinase 2. Nature (London) **363:**595–602.
13. **Draetta, G.** 1993. cdc2 activation: the interplay of cyclin binding and Thr161 phosphorylation. Trends Cell Biol. **3:**287–289.
14. **Dunphy, W. G.** 1994. The decision to enter mitosis. Trends Cell Biol. **4:**202–207.
15. **Elledge, S. J., and R. W. Davis.** 1990. Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. Genes Dev. **4:**740–751.
16. **Endow, S. A., S. J. Kang, L. L. Satterwhite, M. D. Rose, V. P. Skeen, and E. D. Salmon.** 1994. Yeast Kar3 is a minus-end microtubule motor protein that destabilizes microtubules preferentially at the minus ends. EMBO J. **13:** 2708–2713.
17. **Enoch, T., and P. Nurse.** 1990. Mutation of fission yeast cell cycle control gene abolishes dependence of mitosis on DNA replication. Cell **60:**665–673.
18. **Enos, A. P., and N. R. Morris.** 1990. Mutation of a gene that encodes a kinesin-like protein blocks nuclear division in *A. nidulans*. Cell **60:**1019–1027.
19. **Fesquet, D., J. C. Labbe, J. Derancourt, J. P. Capony, S. Galas, F. Girard, T. Lorca, J. Shuttleworth, M. Doree, and J. C. Cavadore.** 1993. The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. EMBO J. **12:**3111–3121.
20. **Fitch, I., C. Dahman, U. Surana, A. Amon, K. Nasmyth, L. Goetsch, B. Byers, and B. Futcher.** 1992. Characterization of four B-type cyclin genes of the budding yeast *Saccharomyces cerevisiae*. Mol. Biol. Cell **3:**805–818.
21. **Fleig, U. N., and K. L. Gould.** 1991. Regulation of cdc2 activity in *Schizosaccharomyces pombe*: the role of phosphorylation. Semin. Cell Biol. **2:**195–204.
22. **Ghiara, J. B., H. E. Richardson, K. Sugimoto, M. Henze, D. J. Lew, C. Wittenberg, and S. I. Reed.** 1991. A cyclin B homolog in *S. cerevisiae*: chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. Cell **65:**163–174.
23. **Glover, D. M., M. H. Leibowitz, D. A. McLean, and H. Parry.** 1995. Mutations in *aurora* prevent centrosome separation leading to the formation of monopolar spindles. Cell **81:**95–105.
24. **Gould, K., and P. Nurse.** 1989. Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature (London) **39:**39–45.
25. **Guacci, V., E. Hogan, and D. Koshland.** 1994. Chromosome condensation and sister chromatid pairing in budding yeast. J. Cell Biol. **125:**517–530.
26. **Hagan, I., J. Hayles, and J. S. Hyams.** 1990. Intramitotic controls in the fission yeast Schizosaccharomyces pombe: the effect of cell size on spindle length and the timing of mitotic events. J. Cell Biol. **110:**1617–1621.
27. **Hagan, I., and M. Yanagida.** 1992. Kinesin-related cut7 protein associates with mitotic and meiotic spindles in fission yeast. Nature (London) **356:**74–76.
28. **Hoyt, M. A., H. Ling, K. K. Loo, and W. S. Saunders.** 1992. Two *Saccharomyces cerevisiae* kinesin-related gene products required for mitotic spindle assembly. J. Cell Biol. **118:**109–120.
29. **Jeffrey, P. D., A. A. Russo, K. Polyak, E. Gibbs, J. Hurwitz, J. Massague, and N. P. Pavletich.** 1995. Mechanism of CDK activation revealed by the structure of a cyclin A-CDK2 complex. Nature (London) **376:**313–320.
30. **Kilmartin, J. V.** 1994. Genetic and biochemical approaches to spindle function and chromosome segregation in eukaryotic microorganisms. Curr. Opin. Cell Biol. **6:**50–54.
31. **Kilmartin, J. V., and A. E. M. Adams.** 1984. Structural arrangements of tubulin and actin during the cell cycle of yeast Saccharomyces. J. Cell Biol. **98:**922–933.
32. **Koch, C., and K. Nasmyth.** 1994. Cell cycle regulated transcription in yeast. Curr. Opin. Cell Biol. **6:**451–459.
33. **Lew, D. J., and S. I. Reed.** 1995. A cell cycle checkpoint monitors cell morphogenesis in budding yeast. J. Cell Biol. **129:**739–749.
34. **Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirschner, and D. Beach.** 1991. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell **64:**1111–1122.
35. **Meluh, P. B., and M. D. Rose.** 1990. KAR3, a kinesin-related gene required for yeast nuclear fusion. Cell **60:**1029–1041.
36. **Morgan, D. O.** 1995. Principles of CDK regulation. Nature (London) **374:** 131–134.
37. **Nasmyth, K.** 1993. Control of the yeast cell cycle by the Cdc28 protein kinase. Curr. Opin. Cell Biol. **5:**166–179.
38. **Nigg, E. A.** 1993. Cellular substrates of p34cdc2 and its companion cyclin-dependent kinases. Trends Cell Biol. **3:**296–301.
39. **Norbury, C., and P. Nurse.** 1993. Animal cell cycles and their controls. Annu. Rev. Biochem. **61:**441–470.
40. **Ohkura, H., I. M. Hagan, and D. M. Glover.** 1995. The conserved *Schizosaccharomyces pombe* kinase plo1, required to form a bipolar spindle, the actin ring and septum, can drive septum formation in G1 and G2 cells. Genes Dev. **9:**1059–1073.
41. **Piggott, J. R., R. Rai, and B. L. A. Carter.** 1982. A bifunctional gene product involved in two phases of the yeast cell cycle. Nature (London) **298:**391–393.
42. **Pines, J.** 1993. Clear as crystal? Curr. Biol. **3:**544–547.
43. **Pines, J.** 1995. Confirmational change. Nature (London) **376:**294–295.
44. **Price, C., K. Nasmyth, and T. Schuster.** 1991. A general approach to the isolation of cell cycle-regulated genes in the budding yeast *Saccharomyces cerevisiae*. J. Mol. Biol. **218:**543–556.
45. **Pringle, J. R., and L. H. Hartwell.** 1981. The *Saccharomyces* cell cycle, p. 97–142. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
46. **Roof, D. M., P. B. Meluh, and M. D. Rose.** 1992. Kinesin-related proteins required for assembly of the mitotic spindle. J. Cell Biol. **118:**95–108.
47. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
48. **Saunders, W. S.** 1993. Mitotic spindle pole separation. Trends Cell Biol. **3:** 432–436.
49. **Saunders, W. S., and M. A. Hoyt.** 1992. Kinesin-related proteins required for structural integrity of the mitotic spindle. Cell **70:**451–458.
50. **Schwob, E., and K. Nasmyth.** 1993. *CLB5* and *CLB6*, a new pair of B cyclins involved in S phase and mitotic spindle formation in *S. cerevisiae*. Genes Dev. **7:**1160–1175.
51. **Solomon, M., J. Wade-Harper, and J. Shuttleworth.** 1993. CAK, the $p34^{cdc2}$ activating kinase, contains a protein identical or closely related to $p40^{MO15}$. EMBO J. **12:**3133–3142.
52. **Sorger, P. K., and A. W. Murray.** 1992. S-phase feedback control in budding yeast is independent of tyrosine phosphorylation of $p34^{CDC28}$. Nature (London) **355:**365–368.
53. **Surana, U., A. Amon, C. Dowzer, J. McGrew, B. Byers, and K. Nasmyth.** 1993. Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. EMBO J. **12:**1969–1978.
54. **Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A. B. Futcher, and K. Nasmyth.** 1991. The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. Cell **65:**145–161.
55. **Tyers, M., G. Tokiwa, and B. Futcher.** 1993. Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. EMBO J. **12:**1955–1968.