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Cdc28 Tyrosine Phosphorylation and the Morphogenesis Checkpoint in Budding Yeast

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A morphogenesis checkpoint in budding yeast delays nuclear division (and subsequent cell cycle progression) in cells that have failed to make a bud. We show that the ability of this checkpoint to delay nuclear division requires the *SWE1* gene, encoding a protein kinase that inhibits the master cell cycle regulatory kinase Cdc28. The timing of nuclear division in cells that cannot make a bud is exquisitely sensitive to the dosage of *SWE1* and *MIH1* genes, which control phosphorylation of Cdc28 at tyrosine 19. In contrast, the timing of nuclear division in budded cells does not rely on Cdc28 phosphorylation, suggesting that the morphogenesis checkpoint somehow turns on this regulatory pathway. We show that *SWE1* mRNA levels fluctuate during the cell cycle and are elevated in cells that cannot make a bud. However, regulation of *SWE1* mRNA levels by the checkpoint is indirect, acting through a feedback loop requiring Swe1 activity. Further, the checkpoint is capable of delaying nuclear division even when *SWE1* transcription is deregulated. We propose that the checkpoint delays nuclear division through post-translational regulation of Swe1 and that transcriptional feedback loops enhance the efficacy of the checkpoint.

INTRODUCTION

In all eukaryotes analyzed to date, entry into mitosis is triggered by activation of a cyclin-dependent kinase (Cdk) by mitotic cyclins of the B class (Nurse, 1990). One mechanism for controlling the timing of activation of the Cdk/cyclin complex is the inhibitory phosphorylation of the Cdk at a conserved tyrosine residue (Dunphy, 1994). In fission yeast, phosphorylation of cdc2 (*Schizosaccharomyces pombe* Cdk1) at Y15 maintains cdc2/cyclin complexes inactive until cells reach a critical size, at which point there is a rapid and complete dephosphorylation of cdc2 Y15 resulting in its activation and consequent entry into mitosis. The wee1 protein kinase and the cdc25 phosphatase directly regulate the phosphorylation state of cdc2 Y15 and act as dose-dependent regulators of entry into mitosis. The abruptness of the G2/M transition in these cells is thought to result from feedback loops whereby cdc2/cyclin activation leads to inactivation of wee1 and activation of cdc25, thus further enhancing cdc2 dephosphorylation and activation (Dunphy, 1994).

In contrast to the control of mitosis in fission yeast, little is known about the mechanisms that determine the timing of mitosis in budding yeast. Activation of Cdc28 (*Saccharomyces cerevisiae* Cdk1) by the Clb1 and Clb2 cyclins is necessary for nuclear division in this organism, and phosphorylation of Cdc28 Y19 can inhibit the activity of Cdc28/Clb2 (and presumably Cdc28/Clb1) complexes (Fitch *et al.*, 1992; Richardson *et al.*, 1992; Booher *et al.*, 1993). Homologues of the fission yeast *wee1* and *cdc25* genes (called *SWE1* and *MIH1*, respectively) have been identified in budding yeast, and all available data suggest that their products possess similar biochemical activities to their fission yeast counterparts (Russell *et al.*, 1989; Booher *et al.*, 1993). However, the timing of mitosis in budding yeast is unaffected by mutation of *CDC28* Y19 to phenylalanine (a nonphosphorylatable residue), or by deletion of *SWE1* or overexpression of *MIH1* (Russell et al., 1989; Amon et al., 1992; Sorger and Murray, 1992;

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Booher et al., 1993). These observations raise two questions: What constitutes the rate-limiting event in promoting nuclear division? What is the role of Cdc28 Y19 phosphorylation?

Nuclear division in budding yeast can be delayed in response to a number of defects in previous cell cycle processes, including incomplete DNA replication, DNA damage, improper spindle assembly, and defective cell morphogenesis (Pringle and Hartwell, 1981; Lew and Reed, 1995a). These delays are thought to be mediated by checkpoint controls: surveillance pathways that monitor specific processes and act to halt cell cycle progression if those processes have not been adequately completed (Hartwell and Weinert, 1989). Although the mechanism for checkpoint-mediated arrest of the cell cycle is in most cases obscure, we recently proposed that the cell morphogenesis checkpoint delayed nuclear division through regulation of Cdc28 Y19 phosphorylation (Lew and Reed, 1995a).

Bud formation requires sustained polarization of the actin cytoskeleton, beginning at the end of G1 (Lew and Reed, 1995b). A number of environmental perturbations (including sudden increases in the temperature or osmolarity of the medium) trigger a stress response involving transient depolarization of the actin cytoskeleton (Chowdhury *et al.*, 1992; Lillie and Brown, 1994). During the depolarized period (generally 1 h, similar to the post-G1 duration of the cell cycle in lab conditions) bud formation is compromised. We have shown that a "morphogenesis checkpoint" acts to delay nuclear division in cells that cannot polarize growth (Lew and Reed, 1995a). This allows sufficient time for the actin cytoskeleton to recover from transient depolarizations and build a bud before nuclear division, thus preventing cells from becoming binucleate. This delay in nuclear division was almost eliminated by mutations that prevent Cdc28 tyrosine phosphorylation (i.e., *CDC28*^{Y19F} or *MIH1* overproduction), indicating that under these stressed conditions Cdc28 Y19 phosphorylation is critical for correct control of mitosis (Lew and Reed, 1995a).

In this report, we show that the *SWE1* kinase is responsible for delaying mitosis in cells that cannot polarize growth. In these cells, *SWE1* acts as a dose-dependent inhibitor, and *MIH1* as a dose-dependent activator, of nuclear division, just as they do in fission yeast. We also show that *SWE1* is transcriptionally regulated during the cell cycle, and we identify two transcriptional feedback loops (controlling *CLB2* and *SWE1* transcription) that help the morphogenesis checkpoint produce the delay in nuclear division. However, promoter replacement experiments suggest that the checkpoint can function in the absence of *SWE1* transcriptional regulation, and we suggest that the checkpoint works primarily through post-translational activation of Swe1.

MATERIALS AND METHODS

Yeast Strains and Growth Conditions

The yeast strains used in this study are listed in Table 1. The *cdc24-1* allele was crossed into the BF264-15DU background (*ade1, his2, leu2-3, 112, trp1-1*^{a}, *ura3Δns*) at least five times, and all other strains were constructed in the same background. The *swe1::LEU2* (Booher *et al.*, 1993) and *mih1::LEU2* (Russell *et al.*, 1989) disruptions have been described. The *GAP::SWE1* construct was made as follows: a 3-kb *Nco*I-*Bam*HI fragment (Booher *et al.*, 1993) containing the entire *SWE1* open reading frame (with the initiator ATG in the *Nco*I site) was converted to a *Bam*HI fragment by using appropriate linkers and cloned into the *Bgl*II site of YIpGAP2. YIpGAP2 contains the *GAP* promoter and terminator (*Bam*HI-*Hin*dIII fragment from pAB23BXN [Schild *et al.*, 1990]) and *HIS2* gene (2-kb *Hin*dIII fragment) in pUC18. The plasmid was digested with *Hpa*I for integration at the *HIS2* locus. All disruptions and integrations were confirmed with appropriate Southern blots. Cells were grown in rich medium (YEPD: 1% yeast extract, 2% bacto-peptone, 2% dextrose, 0.005% adenine, and 0.005% uracil) at 25°C, except where indicated.

Cell Synchrony, Flow Cytometry, and Fluorescence Microscopy

For pheromone arrest/release experiments, exponentially growing cells (2–5 × 10^6 cells/ml) were incubated in YEPD with 25–50 ng/ml α-factor for 3 h, harvested by centrifugation, and resuspended to the original cell density in prewarmed fresh YEPD at 37°C. For the single-cycle synchrony time courses of Figure 4, Nocodazole was added to 15 μg/ml final concentration. For elutriation synchrony experiments, cells were grown in YEPS (same as YEPD but with sucrose instead of dextrose) and elutriated as described (Lew and Reed, 1993). Small daughter cells were then resuspended in prewarmed fresh YEPD at 37°C at a density of 1–2 × 10^6 cells/ml. Cells were fixed and processed for flow cytometry as described (Lew *et al.*, 1992). To determine the kinetics of S phase, the flow cytometry profiles were quantitated as described (Sundberg *et al.*, 1996); the percentage of cells that had a DNA content greater than the midpoint between G1 and G2 peaks was determined at each time point. Nuclear division was quantitated by fluorescence microscopy of propidium iodide or 4′,6-diamidino-2-phenylindole (DAPI)-stained samples as described (Lew and Reed, 1995a).

Table 1. Yeast strains

Strain	Relevant genotype
DLY1	MAT**a**, *bar1*
DLY657	MAT**a**, *cdc24-1, bar1*
DLY660	MAT**a**/MATα, *cdc24-1/cdc24-1*
DLY690	MAT**a**, *cdc24-1, swe1::LEU2, bar1*
DLY1028	MAT**a**, *swe1::LEU2, bar1*
DLY2659	MAT**a**, YIp*GAP2:SWE1 (HIS2), bar1*
DLY2663	MAT**a**, *cdc24-1*, YIp*GAP2:SWE1 (HIS2), swe1::LEU2, bar1*
DLY2707	MAT**a**, *mih1::LEU2, bar1*
DLY2709	MAT**a**, *cdc24-1, mih1::LEU2, bar1*
RSY31	MAT**a**/MATα, *cdc24-1/cdc24-1, mih1::LEU2/mih1::LEU2*
RSY32	MAT**a**/MATα, *cdc24-1/cdc24-1, mih1::LEU2/MIH1*
RSY41	MAT**a**/MATα, *cdc24-1/cdc24-1, swe1::LEU2/SWE1*
RSY54	MAT**a**/MATα, *cdc24-1/cdc24-1, swe1::LEU2/swe1::LEU2*

Viability Assays

Cell number at the start of the cell synchrony time course was determined with a hemacytometer, and dilutions were made on the basis of this number through the entire time course. Serial fivefold dilutions were spotted onto YEPD plates so that the spots contained (left to right) 2000, 400, 80, and 16 cells. The plates were incubated for 2 d at 23°C, and the colonies formed were documented by photography. We found that bacterial contamination occurred during the elutriation procedure and that bacterial colonies became a problem at later time points. To diminish this problem, we included ampicillin and chloramphenicol (0.1 mg/ml each) in the plates.

Analysis of RNA Levels

Protocols for RNA extraction, formaldehyde-agarose gels, and Northern blotting were as described (Reed *et al.*, 1982; Elder *et al.*, 1983; Sambrook *et al.*, 1989). Probes (DNA fragments containing portions of the coding regions of *SWE1, CLB5, CLB2, CLN2*, or *ACT1*) were labeled with random primer labeling kits according to the manufacturer's recommendations (Boehringer-Mannheim, Indianapolis, IN).

RESULTS

The Role of SWE1 and MIH1 in Cells that Cannot Polarize Growth

In fission yeast, there are two known related protein kinases that can phosphorylate cdc2 Y15: weel and mikl (Lundgren *et al.*, 1991). Swel is related to both weel and mikl, and it is not known whether other kinases exist that can phosphorylate Cdc28 on Y19. It was therefore of interest to determine whether Swel or some other kinase was responsible for Cdc28 phosphorylation and the consequent delay of nuclear division in cells that could not polarize growth. To address this, we used *cdc24* temperature-sensitive mutants that grow normally at 25°C but are unable to polarize the actin cytoskeleton (and hence form a bud) at 37°C (Sloat *et al.*, 1981). We synchronized *cdc24* cells and wild-type controls in G1 with the pheromone α-factor at 25°C and then released them from α-factor arrest into fresh medium at 37°C. As previously reported (Lew and Reed, 1995a), nuclear division was delayed in the *cdc24* cells relative to the wild-type controls (Figure 1A). Note that, compared with the elutriation synchrony protocol used previously (and in Figure 2), the α-factor synchrony protocol leads to a more rapid cell cycle both in wild-type and *cdc24* cells, and the delay in nuclear division is ~1 h. The compression of the cell cycle in this synchrony protocol is probably the result of continued growth during the arrest, priming the cells to undergo a rapid cycle on release.

Deletion of *SWE1* had no effect on the timing of nuclear division in wild-type cells, but it eliminated the delay in *cdc24* cells (Figure 1B). This indicates that Swel is necessary for the delay in nuclear division in these cells, and there is therefore no need to invoke another kinase. Deletion of *MIH1* also had no effect in wild-type cells, but *cdc24* cells lacking Mihl were further delayed in nuclear division and failed to divide

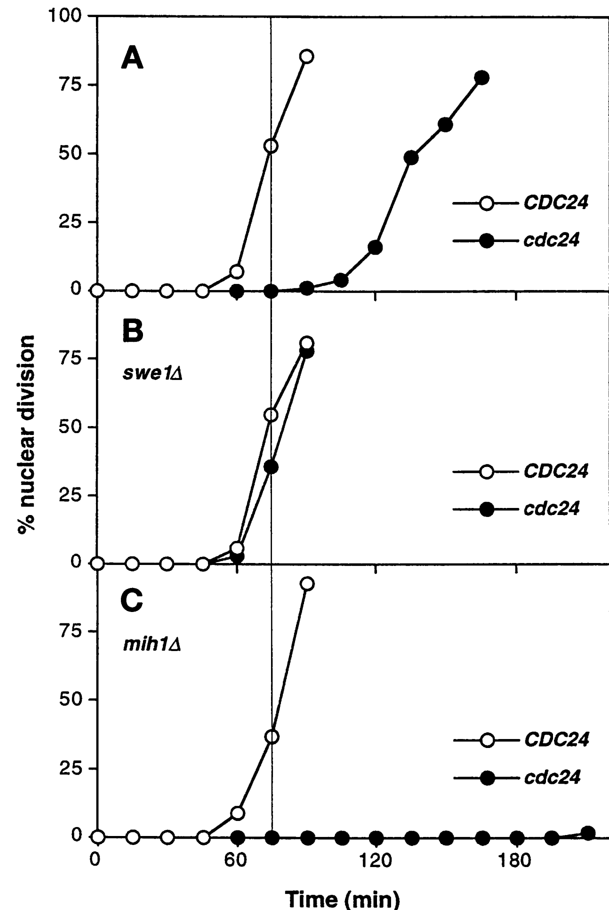

Figure 1. Effect of *SWE1* and *MIH1* on cell cycle kinetics in wild-type and *cdc24* cells. Cells were synchronized with α-factor and released at 37°C. Nuclear division was monitored at 15′ intervals by DAPI staining of fixed cells, and the percentage of cells that had undergone nuclear division is plotted on the Y-axis. (A) *CDC24* (DLY1) vs. *cdc24-1* (DLY657) cells. (B) *CDC24 swel::LEU2* (DLY1028) vs. *cdc24-1 swel::LEU2* (DLY690) cells. (C) *CDC24 mihl::LEU2* (DLY2707) vs. *cdc24-1 mihl::LEU2* (DLY2709) cells.

their nuclei during the time course of the experiment (Figure 1C). The fact that deletion of *MIH1* in wild-type cells failed to produce a significant delay indicates that inhibition of Mihl cannot account for the delay in *cdc24* cells. In addition, the fact that *MIH1* deletion greatly prolonged the delay in *cdc24* cells indicates that Mihl must be active in these cells to promote the eventual override of the Swel-induced arrest.

We wished to determine whether the abundance of Swel or Mihl was an important determinant of mitotic timing in cells that could not polarize growth. Diploid (homozygous *cdc24*) strains were constructed that were homozygous or heterozygous for deletion of *SWE1* or *MIH1*. G1 daughter cells were isolated by centrifugal elutriation from populations growing at 25°C and incubated in fresh medium at 37°C. As seen

above for haploid strains synchronized by α-factor, homozygous *SWE1* deletion abolished the delay in nuclear division, whereas homozygous *MIH1* deletion made the delay permanent (Figure 2). Interestingly, the timing of nuclear division in the heterozygous strains was intermediate between the wild-type (for *SWE1* and *MIH1*) and homozygous deletion strains (Figure 2). This indicates that a twofold difference in

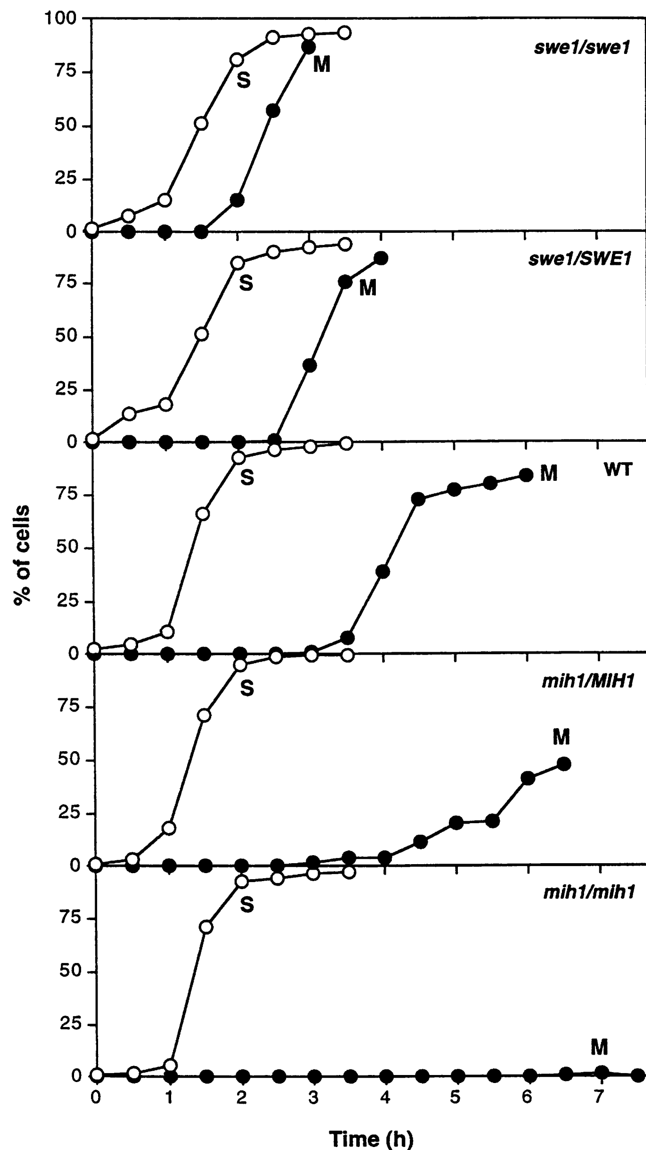

Figure 2. *SWE1* and *MIH1* gene dosage controls the timing of nuclear division in *cdc24* cells. Synchronous G1 *cdc24-1/cdc24-1* daughter cells with the indicated *SWE1* and *MIH1* genotypes were isolated by centrifugal elutriation and incubated at 37°C. Timing of DNA replication (S) and nuclear division (M) were analyzed as described in MATERIALS AND METHODS. Strains were as follows: top panel, RSY54; second panel, RSY41; third panel, DLY660; fourth panel, RSY32; bottom panel, RSY31.

the levels of Swe1 or Mih1 has a significant effect on the timing of mitosis in cells that cannot polarize growth. This is reminiscent of the situation in fission yeast and contrasts greatly with the situation in budding yeast under normal growth conditions, where they are competent to polarize growth and form a bud.

The Checkpoint-induced Cell Cycle Delay Promotes Optimal Viability in Cells that Cannot Polarize Growth

We previously showed that *cdc24* cells retained full viability during the checkpoint-induced cell cycle delay (Lew and Reed, 1995a). To determine how altering the timing of nuclear division might affect cell viability under these conditions, we determined the viability of *cdc24* cells from the experiment of Figure 2 that entered mitosis at different times. The results (Figure 3) showed that either acceleration or *delay* of mitosis in cells that were unable to polarize growth led to a more rapid loss of viability. Although the wild-type (for *SWE1* and *MIH1*) cells began to lose viability at 6 h of incubation at 37°C, the *swe1* cells started to lose viability at 4 h and the *mih1* cells at 5 h. This suggests that accurate timing of mitosis is quite important in these cells. The basis for this surprising observation is considered in DISCUSSION.

Regulation of SWE1 mRNA Levels

The results described above suggest that phosphorylation of Cdc28 Y19 does not occur (or occurs at low stoichiometry) in wild-type cells but that Cdc28 Y19 phosphorylation is induced and becomes the dominant mechanism controlling entry into mitosis in cells that cannot polarize growth. In principle, increased Cdc28 Y19 phosphorylation could be brought about either through an increase in Swe1 activity or a decrease in Mih1 activity (more complex models are also possible). Our data suggest that reduction of Mih1 activity is not a major contributor to the delay in nuclear division, because even a deletion of *MIH1* had no effect in otherwise wild-type cells (note that a small delay was previously reported in *mih1Δ* cells; the apparent conflict with our data is most easily explained by supposing that the cell cycle compression caused by α-factor synchrony obscured this small effect). We therefore turned our attention first to Swe1.

SWE1 mRNA levels were found to vary during the cell cycle and as a result of checkpoint activation (Figure 4, C and D). The experiments shown in Figure 4 represent "single-cycle" cell synchrony analyses, wherein cells synchronized in G1 with α-factor at 25°C were released into fresh medium containing Nocodazole at 37°C. This led to a single synchronous cell cycle, followed by arrest at G2/M. This protocol avoids the breakdown in synchrony that usually oc-

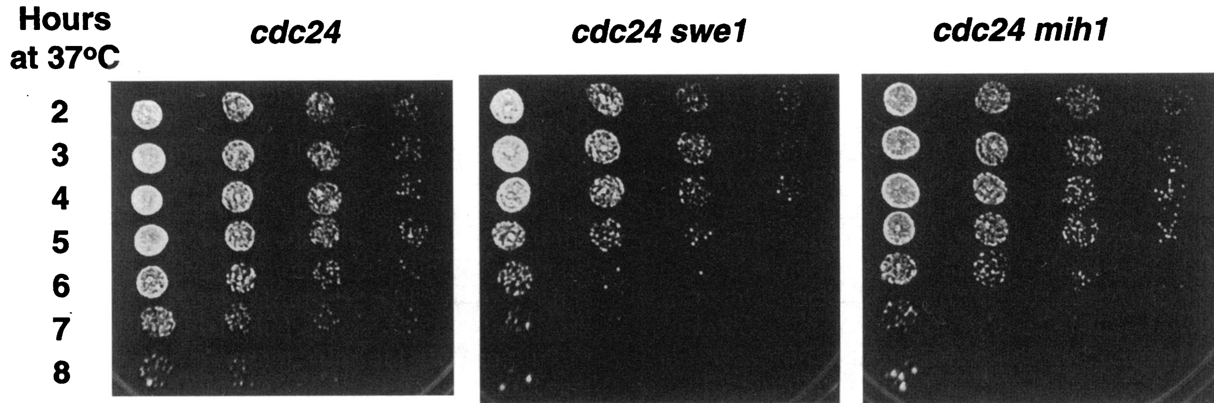

Figure 3. Viability of *cdc24* cells after prolonged incubation at the restrictive temperature. Cells from the experiment of Figure 2 (*cdc24*, DLY660; *cdc24 swe1*, RSY54; *cdc24 mih1*, RSY31) were spotted onto YEPD plates after incubation at 37°C for the periods indicated at the left. Fivefold dilutions of the synchronized cultures were spotted as described in MATERIALS AND METHODS.

curs during the second cycle after α-factor release and allows detailed examination of mRNA behavior through to the end of the first cycle. In addition, it allows direct comparisons between cell cycles in cells that can or cannot polarize growth: because the total cell cycle duration differs in these cells (see Figures 1 and 2 above), attempts to compare later time points in the absence of the Nocodazole block would result in first-cycle *cdc24* cells being matched with second-cycle wild-type cells.

SWE1 mRNA accumulation was periodic during the cell cycle, with peak levels in late G1, in a pattern identical to that of *CLN2* and *CLB5* (Figure 4, A, C, F, and I). Examination of the *SWE1* promoter revealed the presence of SCB motifs (*S*wi4/Swi6 *C*ell cycle *B*oxes) that have been implicated in directing late G1-specific transcription (Koch and Nasmyth, 1994). Similar SCBs are present in the *CLN2* promoter (Figure 4B) and contribute to its periodicity (Stuart and Wittenberg, 1994). The reason that synthesis of Swe1, a regulator of entry into mitosis, should be confined to G1/S is addressed in DISCUSSION.

In *cdc24* cells that could not polarize growth, *SWE1* mRNA levels rose with normal kinetics in late G1 but then persisted for considerably longer than in polarization-competent cells (Figure 4D). In theory, this delayed repression of *SWE1* could lead to elevated Swe1 levels in G2 and hence to induction of Cdc28 Y19 phosphorylation.

Like *SWE1*, *CLN2* mRNA levels remained elevated for a longer time in *cdc24* cells (Figure 4G). Given the similarity in promoter elements between these genes, this suggests that delayed transcriptional repression is responsible for the sustained expression of these transcripts, although we cannot rule out the possibility that mRNA degradation rates are altered. In contrast, *CLB5* transcripts were induced and repressed with identical kinetics in wild-type and *cdc24* cells (Figure 4J). Periodic *CLB5* transcription is controlled by MCB (*Mlu*I *C*ell Cycle *B*ox) elements rather than by SCB elements (Koch and Nasmyth, 1994).

The behavior of *CLN2* and *CLB5* transcripts in *cdc24* cells (delayed repression of *CLN2*, unaltered repression of *CLB5*) is reminiscent of the situation in cells lacking *CLB1-4* (Amon *et al.*, 1993). It has been proposed that Clb/Cdc28 activity is required to repress transcription from SCB-containing promoters but not MCB-containing promoters (Amon *et al.*, 1993). Thus, the delay in *SWE1* and *CLN2* repression that we observed in *cdc24* cells could simply be an indirect consequence of a checkpoint-induced delay in Clb/Cdc28 activation.

These observations can be accommodated by two models on the relationship between *SWE1* expression and the checkpoint-induced delay in Clb/Cdc28 activation. In the first model, the morphogenesis checkpoint directly delays *SWE1* repression, leading to delayed Clb/Cdc28 activation. In the second model, the morphogenesis checkpoint leads to inhibition of Clb/Cdc28 in some other manner, and this inhibition is then responsible for the delay in *SWE1* repression. To distinguish between these possibilities, we examined the behavior of *SWE1* transcripts in *cdc24 swe1::LEU2* cells.

The *swe1::LEU2* allele deletes the C-terminal catalytic domain of *SWE1* (so that no active Swe1 protein is generated) and produces a discrete truncated transcript under control of the *SWE1* promoter. *cdc24 swe1::LEU2* cells at 37°C cannot polarize growth but (in the absence of Swe1) fail to delay Clb/Cdc28 activation and nuclear division (Figure 1). Analysis of the truncated *swe1* transcript in these cells showed that there was no delay in transcriptional repression (Figure 4E). Similarly, *CLN2* mRNA levels were repressed with normal kinetics in these cells (Figure 4H). As expected, *CLB5* expression was also unaffected (Figure

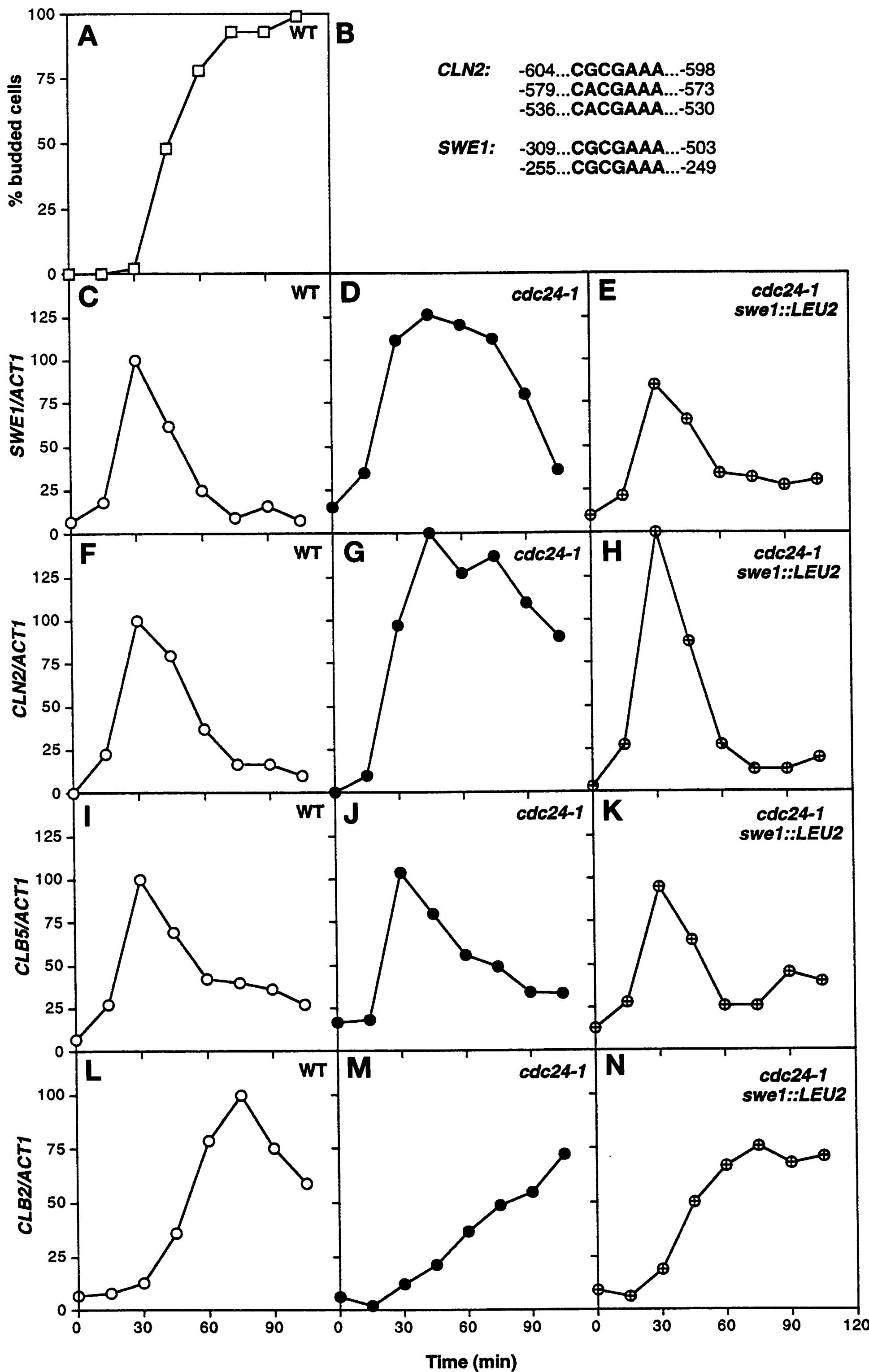

Figure 4. Analysis of cyclin and *SWE1* mRNA levels during a single synchronized cell cycle in wild-type or *cdc24* cells. Wild-type (DLY1: panels A, C, F, I, and L), *cdc24-1* (DLY657: panels D, G, J, and M), and *cdc24-1 swe1::LEU2* (DLY690: panels E, H, K, and N) cells were synchronized with α-factor and released into Nocodazole at 37°C, as described in MATERIALS AND METHODS. Samples were taken at the indicated times and processed for Northern analyses, as described in MATERIALS AND METHODS. Cells were also fixed in formaldehyde to monitor the kinetics of bud formation (A). Northern blots were first probed with *SWE1* (C–E), *CLN2* (F–H), *CLB5* (I–K), or *CLB2* (L–N) and then reprobed with *ACT1*. Signals were quantitated on a PhosphorImager, and the ratio between the *SWE1* or cyclin probes and *ACT1* for the same lane was calculated to control for loading. For each probe, this number was then normalized so that the peak signal in the wild-type synchrony was 100. (B) A comparison of SCB sequences in the *CLN2* promoter (Ogas *et al.*, 1991) and the *SWE1* promoter. Numbering is upstream from the translation initiation ATG in each case.

4K). These results suggest that the second model is correct. The checkpoint does not act directly on *SWE1* transcription; rather, it delays Clb/Cdc28 activation (with the aid of Swe1 protein), which indirectly affects

SWE1 transcript levels. This model implies the existence of a feedback loop that contributes to the delay in nuclear division (Figure 6 and DISCUSSION).

SWE1 Transcriptional Regulation Is Not Essential for Checkpoint Function

The results above show that Swe1 levels are an important determinant of the length of the delay in nuclear division (Figure 2) and that a transcriptional feedback loop leads to elevated *SWE1* mRNA levels late in the cell cycle of cells that cannot polarize growth (Figure 4). How important is *SWE1* transcriptional regulation for generating the delay in nuclear division?

To address this question, we constructed a fusion of *SWE1* coding and downstream sequences to the glyceraldehyde 3-phosphate dehydrogenase (*GAP*) promoter. Integration of this fusion into yeast cells led to the production of an easily detectable mRNA that did not vary in abundance as a function of the cell cycle or in *cdc24* cells (our unpublished results). The fusion junction creates an unfavorable Kozak sequence upstream of the initiator ATG, predicting that the mRNA would be poorly translated and therefore produce little Swe1 protein. We have been unable to detect Swe1 by Western blotting with an anti-Swe1 antibody either in wild-type or GAP::SWE1 cells (our unpublished results). The *GAP::SWE1* construct was introduced into wild-type or *cdc24* cells that were also *swe1::LEU2*, and cell cycle progression was monitored in cultures synchronized by α-factor and released at 37°C.

The *GAP::SWE1* gene did not cause any change in the cell cycle of wild-type cells (Figure 5A), but in *cdc24* cells it was able to partially restore the delay in nuclear division (Figure 5B). This experiment shows that, although Swe1 is essential for the checkpoint to delay nuclear division, transcriptional regulation of *SWE1* is not. The fact that the delay was smaller in the *GAP::SWE1* cells suggests that the *GAP::SWE1* construct produces less Swe1 protein than the endogenous *SWE1* gene in *cdc24* cells.

Regulation of CLB2 mRNA Levels

We have shown that part of the delay in Clb2/Cdc28 activation in cells that cannot polarize growth is due to delayed *CLB2* mRNA accumulation (Lew and Reed, 1995a). As with *SWE1* above, this could arise in one of two ways. In the first model, the checkpoint delays *CLB2* mRNA accumulation directly, thus helping to delay nuclear division. In the second model, the delayed *CLB2* mRNA accumulation is an indirect consequence of the Clb/Cdc28 inhibition. This second model would be consistent with the finding that *CLB2* mRNA accumulation was delayed in cells lacking Clb/Cdc28 activity (Amon *et al.*, 1993). Examination of *CLB2* mRNA levels in *cdc24 swe1::LEU2* cells revealed that there was no delay in *CLB2* induction despite the inability to polarize growth (Figure 4N). This result supports the second model and suggests the presence of a second feedback loop that contributes to the delay in nuclear division (Figure 6 and DISCUSSION). It is difficult to tell from these data whether the feedback loop accounts for 100% of the delay: the possibility remains that a small fraction of the delay was caused directly by the checkpoint.

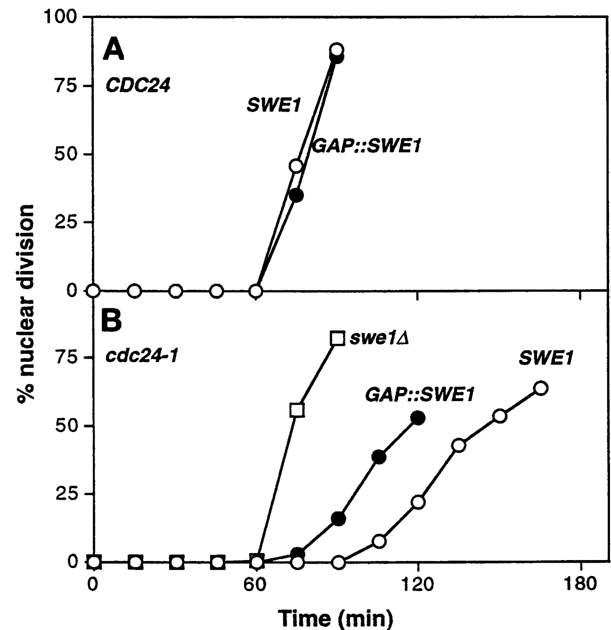

Figure 5. Transcriptionally deregulated *SWE1* is able to provide a delay in nuclear division in *cdc24* cells. Cells were synchronized with α-factor and released at 37°C. Nuclear division was monitored at 15′ intervals by DAPI staining of fixed cells, and the percentage of cells that had undergone nuclear division is plotted on the Y-axis. (A) Wild-type cells with (filled circles, DLY2659) or without (open circles, DLY1) an integrated copy of *GAP::SWE1*. (B) *cdc24-1* cells with the endogenous *SWE1* gene (open circles, DLY657), a deleted *SWE1* gene (open squares, DLY690), or a deleted *SWE1* gene plus an integrated copy of *GAP::SWE1* (filled circles, DLY2663).

DISCUSSION

In agreement with previous studies, we have found that phosphorylation of Cdc28 on Y19 plays no discernible role in the unperturbed cell cycle of budding yeast but becomes an important determinant of mitotic timing in cells that cannot polarize growth to form a bud. We have proposed that such cells possess a morphogenesis checkpoint that detects the defect in bud formation and acts to delay nuclear division (Lew and Reed, 1995a). The results reported here represent a first step in characterizing the basis for the checkpoint-promoted delay in nuclear division and provide several novel insights.

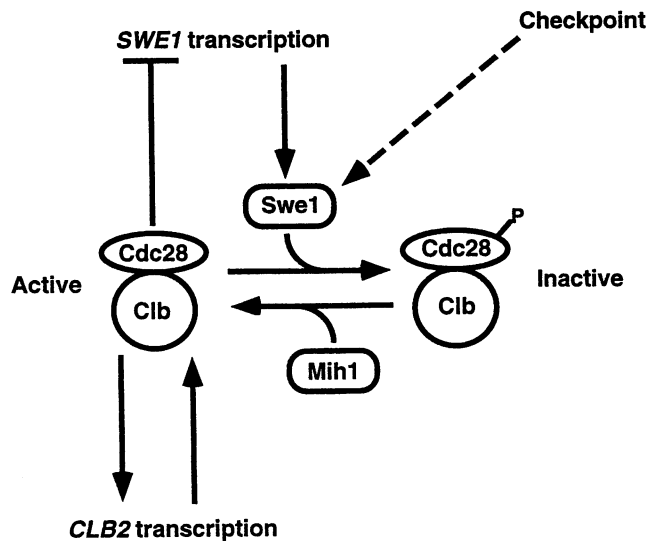

Figure 6. Model for the mechanism by which the morphogenesis checkpoint delays nuclear division. We propose that the primary effect of the checkpoint is to regulate Swe1 post-translationally by altering its activity, stability, or localization to make it more effective in Cdc28 phosphorylation. When Clb1-4/Cdc28 complexes begin to appear, this enhanced Swe1 would inhibit their activity, and the delay in Clb/Cdc28 activation would then result in a delay in *CLB2* transcriptional induction and a delay in *SWE1* transcriptional repression. These transcriptional effects would both contribute to prolonging the delay in Clb/Cdc28 activation.

SWE1 accounted for all of the observed delay in nuclear division. Thus, unlike the situation in fission yeast in which two related kinases (wee1 and mik1) phosphorylate cdc2 Y15, a single kinase apparently suffices for checkpoint-induced regulation of Cdc28 Y19 phosphorylation in budding yeast.

In previous studies (Lew and Reed, 1995a), we found that there was a slight residual delay in nuclear division even in cells that were unable to phosphorylate Cdc28 because of a $CDC28^{Y19F}$ mutation, whereas in this report we found no residual delay in *swe1* mutant cells. At least two possible explanations can account for this apparent discrepancy. One is that the difference arises from the different synchronization protocols used in the two studies. As mentioned above, the pheromone synchrony protocol of Figure 1 compresses the cell cycle after release, and it is possible that a small delay would be undetectable in the compressed cycle. Alternatively, it is possible that Swe1 contributes to the delay in nuclear division through other mechanisms in addition to Cdc28 phosphorylation. Data from Booher *et al.* (1993) suggest that even the Y19F mutant of Cdc28 is susceptible to inhibition by direct binding of Swe1 in vitro. If such a mechanism were operative in vivo, it could account for the small delay in the $CDC28^{Y19F}$ mutant.

The duration of the checkpoint-induced delay in nuclear division was significantly altered by twofold changes in the levels of Swe1 or Mih1, the kinase and phosphatase that control Cdc28 Y19 phosphorylation. This sensitive control of mitotic timing is similar to that found in fission yeast, in which entry into mitosis is triggered by cdc2 Y15 dephosphorylation on attainment of a critical cell size (Russell and Nurse, 1986, 1987).

It is not clear why the checkpoint provides only a limited delay in nuclear division when (in the *cdc24* cells) the bud formation defect is effectively permanent. As expected, we found that shortening the delay (by reducing or eliminating Swe1) caused a more rapid loss of viability in cells exposed to depolarizing conditions for prolonged periods. Surprisingly, however, lengthening the delay in nuclear division (by eliminating Mih1) also caused a more rapid loss of viability. This suggests that the transient delay produced by the checkpoint is in fact optimized for survival under conditions that perturb bud formation for extended periods.

We found that unbudded *cdc24* cells retained good viability for some time even after undergoing nuclear division and becoming binucleate (compare Figure 2 with Figure 3). Microscopic examination of these cells after shift-down to the permissive temperature revealed that many were able to form buds and undergo a cell cycle culminating in nuclear division of both nuclei. In some cases both nuclei divided along the mother–bud axis to produce binucleate daughters, whereas in other cases one nucleus divided entirely within the large mother cell, yielding a trinucleate mother and a mononucleate (i.e., normal) daughter. In *cdc24 mih1* cells shifted down to the permissive temperature at comparable times, nuclear division had not occurred, and the G2 cells proceeded to form tiny buds. However, bud growth was not observed, and DAPI staining revealed that most cells lost stainable material shortly after shift-down, suggesting that budding was rapidly followed by cell lysis (our unpublished observations). We speculate that very prolonged cell cycle delay leads to defective cell wall construction on resumption of bud formation and, hence, lysis and loss of viability. Thus, the benefits of a checkpoint-induced cell cycle delay (preventing formation of binucleate cells) are eventually outweighed by its drawbacks (defective cell wall construction).

SWE1 mRNA accumulation was periodic during the cell cycle, with a peak in late G1. Two observations suggest that this is the result of transcriptional, rather than post-transcriptional, control: 1) SCB promoter elements in the *SWE1* promoter resemble those in the *CLN2* promoter, which is transcribed with similar periodicity. 2) Replacement of the *SWE1* promoter with the *GAP* promoter eliminated the periodicity of *SWE1* mRNA accumulation (although a possible caveat here is that this construct did not produce an identical mRNA). Why synthesis of Swe1, a regulator of entry

into mitosis, should be confined to G1/S is an interesting question. If Swe1 is an unstable protein (which has yet to be determined), then Swe1 protein would only be present during a brief interval of the cell cycle, before accumulation of the Clb/Cdc28 complexes that are its regulatory target. This would explain why, in the unperturbed cell cycle, Cdc28 Y19 phosphorylation does not significantly affect cell cycle kinetics.

G1/S is also the time during which the cytoskeleton becomes polarized and bud emergence takes place. We speculate (Figure 6) that, if polarization of growth were compromised during this period, the morphogenesis checkpoint would induce the activity and/or stability of the Swe1 present at that time. Thus, when Clb1-4 cyclins begin to accumulate and bind to Cdc28, the complexes would be inactivated by Cdc28 Y19 phosphorylation, leading to a delay in nuclear division. However, if polarization of growth were to be compromised later in the cell cycle when bud construction was already well underway, there would be no Swe1 protein for the checkpoint to regulate, and nuclear division would occur on schedule. In this model, restraining *SWE1* transcription to the G1/S interval provides a limited window of the cell cycle in which morphogenesis defects can effectively delay cell cycle progression. Before this time, growth is unpolarized, whereas after this time a bud is present: in neither case would cells need the checkpoint. This model makes the testable prediction that depolarization of growth in G2 cells will not cause a *SWE1*-dependent delay of nuclear division.

We have documented three perturbations in cell cycle-regulated transcription (delayed repression of *SWE1* and *CLN2*, delayed induction of *CLB2*) in *cdc24* cells that cannot polarize growth. In all cases, the perturbation was Swe1 dependent, implying that it was a result of Clb/Cdc28 inhibition. Because two of the perturbations (elevated *SWE1* and decreased *CLB2*) also contribute to lowering Clb/Cdc28 activity, these results suggest the presence of feedback loops in Clb/Cdc28 activation (Figure 6). These data are fully consistent with an earlier study that proposed the existence of similar transcriptional feedback loops on the basis of the phenotypes of *clb* knockout mutants (Amon *et al.*, 1993). Such positive feedback loops are generally considered in the context of speeding the transition from low to high Clb/Cdc28 activity: accumulation of a little Clb/Cdc28 would rapidly autoamplify by promoting synthesis of Clb2 and repression of Swe1. However, our results emphasize another, equally valid, consequence of such feedback loops. If some perturbation (e.g., a checkpoint-induced activation of Swe1) prevents activation of the small initial amount of Clb/Cdc28, then the feedback loops would effectively stabilize the "low Clb/Cdc28 activity" status by delaying Clb2 synthesis and maintaining high Swe1 levels. In this context, the feedback loops act to prolong the checkpoint-induced delay.

Although the *SWE1* transcriptional feedback loop presumably contributes to the length of the checkpoint-induced cell cycle delay, constitutive *SWE1* transcription still allowed a significant delay in nuclear division in *cdc24* cells. Thus, although Swe1 is essential for the checkpoint, its transcriptional regulation is not. We propose that the checkpoint acts to stabilize and/or activate Swe1 post-translationally (Figure 6) to produce a cell cycle delay. Post-translational regulation of wee1 by phosphorylation has been documented in frog egg extracts (Dunphy, 1994). We are currently testing this model in budding yeast.

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