A Cell Cycle Checkpoint Monitors Cell Morphogenesis in Budding Yeast

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Abstract. Checkpoint controls are regulatory pathways that inhibit cell cycle progression in cells that have not faithfully completed a prior step in the cell cycle. In the budding yeast *Saccharomyces cerevisiae*, DNA replication and spindle assembly are monitored by checkpoint controls that prevent nuclear division in cells that have failed to complete these processes. During the normal cell cycle, bud formation is temporally coincident with DNA replication and spindle assembly, and the nucleus divides along the mother–bud axis in mitosis. In this report, we show that inhibition of bud formation also causes a dramatic delay in nuclear division. This allows cells to recover from a transient disruption of cell polarity without becoming binucleate. The delay occurs after DNA replication and spindle assembly, and results from delayed activation of the master cell cycle regulatory kinase, Cdc28. Cdc28 activation is inhibited by phosphorylation of Cdc28 on tyrosine 19, and by delayed accumulation of the B-type cyclins Clb1 and Clb2. These results suggest the existence of a novel checkpoint that monitors cell morphogenesis in budding yeast.

SUCCESSFUL cell proliferation requires the events of the cell cycle to be coordinated so that DNA replication is completed before chromosome segregation is attempted, and chromosome segregation is completed prior to cell division. This order of events is controlled by a family of serine/threonine protein kinases called cyclin-dependent kinases (Cdks[1]; reviewed by Norbury and Nurse, 1992; Nasmyth 1993). Cdk activity oscillates during the cell cycle, sequentially triggering DNA replication, chromosome segregation, and cytokinesis. If some perturbation delays completion of either DNA replication or assembly of a microtubule spindle (required for chromosome segregation), regulatory pathways called checkpoint controls act to delay cell cycle progression (reviewed by Hartwell and Weinert, 1989; Murray, 1992). This delay is thought to be mediated by regulation of Cdk activity.

Cdk activity is dependent upon association of the kinase catalytic subunit with positive regulatory subunits called cyclins, and can be regulated by phosphorylation of the catalytic subunit, and by association with inhibitory proteins (reviewed by Solomon, 1993; Dunphy, 1994; Peter and Herskowitz, 1994). This allows Cdk activity to respond with exquisite sensitivity to cues from the cell's environment, as well as signals from within the cell (checkpoint controls). The best-characterized class of checkpoint controls monitors the replicative status and general integrity of the cell's genomic DNA. If the DNA is incompletely replicated or damaged, these checkpoint controls delay chromosome segregation (reviewed by Hartwell and Weinert, 1989; Murray, 1992). Experiments in the fission yeast *Schizosaccharomyces pombe* and the frog *Xenopus laevis* have suggested that this checkpoint prevents entry into mitosis by stimulating the inhibitory phosphorylation of Cdc2, the Cdk that promotes entry into mitosis, on tyrosine 15 (Enoch and Nurse, 1990; Smythe and Newport, 1992). An analogous inhibitory pathway that phosphorylates tyrosine 19 of Cdc28 has been described in budding yeast (Russell et al., 1989; Booher et al., 1993). However, tyrosine phosphorylation of Cdc28 is not required for the operation of checkpoint controls related to genome integrity in budding yeast, and studies to date have not uncovered any biological role for this pathway (Amon et al., 1992; Sorger and Murray, 1992; Stueland et al., 1993).

Like DNA replication and spindle assembly, the reorganizations of the actin-based cytoskeleton that shape the bud are triggered by the cyclin/Cdc28 kinases (Lew and Reed, 1993). There were two reasons to suspect the existence of a checkpoint control that monitored bud formation, and delayed progression to anaphase if budding was defective. First, many environmental stresses, including sudden alterations in the temperature or osmolarity of the growth medium, result in a transient depolarization of the actin cytoskeleton in budding yeast (Chowdhury et al., 1992; Lillie

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1. *Abbreviation used in this paper*: Cdk, cyclin-dependent kinase.

and Brown, 1994). The depolarized period can last up to an hour, or 50–70% of the generation time, during which time cells are unable to direct growth to the bud. Without a concomitant delay of the nuclear cycle, many of these cells would become binucleate. However, binucleate cells do not arise under these conditions (Lew, D., unpublished results; also see Fig. 7). Second, several mutant yeast strains have been described that are specifically defective in elements of the actin cytoskeleton (Welch et al., 1994). Some such mutants (e.g., *tpm1* cells, lacking tropomyosin) have a significantly longer generation time than wild type cells but are fully viable and do not produce significant numbers of binucleate cells, suggesting that the nuclear cycle has slowed down to accommodate the defect in morphogenesis (Liu and Bretscher, 1992). Since there is no reason to suspect that tropomyosin plays a direct role in cell cycle events other than bud formation, this suggests the existence of a checkpoint control that detects budding defects and delays cell cycle progression.

In contrast, temperature-sensitive *cdc24* mutants that are completely unable to polarize the actin cytoskeleton at the restrictive temperature continued the nuclear cycle despite the inability to form a bud (Hartwell et al., 1974; Adams et al., 1990). In this report we have reexamined the effect of *cdc24* and other mutants that prevent budding on cell cycle progression. We find that there is a dramatic cell cycle delay in these mutants, and that this delay is largely due to phosphorylation of tyrosine 19 of Cdc28. These findings identify a new class of checkpoint control responsive to defects in cell morphogenesis, and suggest a role for Cdc28 tyrosine phosphorylation in budding yeast.

Materials and Methods

Yeast Strains

The yeast strains used in this study are listed in Table I. Strains 5011-JH01 and JPT19-H01 (gifts from J. Pringle, University of North Carolina, Chapel Hill, NC) are essentially isogenic to C276 (Adams et al., 1990). All DLY strains are in the BF264-15DU background (*ade1, his2, leu2-3, 112, trp1-1$^{\alpha}$, ura3Dns*), or were constructed by backcrossing *cdc24* alleles (from the C276 background) or the *cdc42-1* allele (from DJTD2-16A; a gift from D. Johnson, University of Vermont, Burlington, VT) into the BF264-15DU background at least five times, and are therefore essentially isogenic. The *cdc28::LEU2* disruption was constructed by replacing a XhoI/SacI fragment of Cdc28 (containing 1/2 the coding region) with a SalI/SacI fragment containing *LEU2* (previously cloned as a SalI/XhoI fragment into the pUC19 SalI site). *GAL1::CLB* and *GAL1::CLN2* plasmids were integrated at the *LEU2* locus as described (Ghiara et al., 1991; Lew et al., 1991; Stueland et al., 1993). p*GAL1::MIH1* and p*GAL1::wee1* (gifts from P. Russell, The Scripps Research Institute, La Jolla, CA) contain the *MIH1* or *wee1* genes (Russell et al., 1989) cloned into YCpG2. p*CDC28^{Y19F}* (a gift from P. Sorger, MIT, Cambridge, MA) is a *TRP1*-marked centromeric plasmid described by Sorger and Murray (1992). Strains were constructed using standard yeast genetic procedures (Sherman et al., 1982).

Cell Synchrony and Flow Cytometry

Cells were grown in yeast nitrogen base (Difco Laboratories Inc., Detroit, MI) supplemented with 2% sucrose or raffinose and 0.5% casaminoacids, to a density of 5–10 × 10^6 cells/ml at 25°C. 1–3 liters of cells were chilled on ice, sonicated, and elutriated as described (Lew and Reed, 1993). G1 daughter cells were harvested by centrifugation and resuspended in YEP (1% yeast extract, 2% bacto-peptone, 0.005% adenine, 0.005% uracil) supplemented with 2% dextrose (YEPD) or galactose (YEPG) as indicated, to a final density of 2–4 × 10^6 cells/ml. After incubation, cells were fixed, stained with propidium iodide, and analyzed by flow cytometry exactly as described (Lew et al., 1992). Proportion of cells with G1, S, and G2 DNA contents were estimated using LYSYS II software in two ways: with histogram markers on FL3 histograms, or with regions on FL3 versus FSC dot plots (see Lew et al., 1992). These gave identical results. For the experiment of Fig. 7, cells were grown on YEPS (YEP + 2% sucrose) at 30°C, and α-factor was added to a final concentration of 50 ng/ml. 1.5 h later galactose was added to 2%, and the cells were incubated for one more hour. The cells were then harvested by centrifugation and resuspended in fresh YEPS + 2% galactose with no α-factor (time zero). DAPI staining was as described by McKinney et al. (1993).

Quantitation of Chromosome Segregation and Cell Viability

Cells stained with propidium iodide were observed using an Axiophot photomicroscope with a 100× objective (Carl Zeiss, Inc.). At least 200 cells were scored for each timepoint as follows: (*a*) wild type cells: first, the percent of cells with segregated chromosomes (two equal propidium stained masses, in mother and bud) was quantitated. Next, the percent of cells that had undergone nuclear division and cell separation was determined by counting cell numbers (for this, separate samples were adjusted to 3.7% formaldehyde, and the number of cells/ml was counted using a hemocytometer) according to the formula:

$$\text{Percent divided cells } (t) = \frac{\text{No. of cells } (t) - \text{No. of cells } (0)}{\text{No. of cells } (0)}$$

The cumulative percent chromosome segregation was then calculated by adding the percent of cells with segregated chromosomes to the percent of

Table I. Yeast Strains

Strain	Relevant Genotype	Source
C276	MATa/MATα	J. Pringle
5011-JH01	MATa/MATα, *cdc24-1/cdc24-1*	J. Pringle
JPT19-HO1	MATa/MATα, *cdc24-4/cdc24-4*	J. Pringle
DLY664	MATaMATα, *cdc24-4/cdc24-4*	This study
DLY668	MATa/MATα, *cdc24-4/cdc24-4, GAL1::CLB1(LEU2)*	This study
DLY669	MATa/MATα, *cdc24-4/cdc24-4, GAL1::CLB2(LEU2)*	This study
DLY670	MATa/MATα, *cdc24-4/cdc24-4, GAL1::CLB3(LEU2)*	This study
DLY672	MATa/MATα, *cdc24-4/cdc24-4, GAL1::CLN2(LEU2)*	This study
DLY673	MATa/MATα, *cdc24-4/cdc24-4, pGAL1::MIH1*	This study
DLY685	MATa/MATα, *cdc24-4/cdc24-4, cdc28::LEU2/cdc28::LEU2 pCDC28^{Y19F}*	This study
DLY129	MATa/MATα, *cdc28::LEU2/cdc28::LEU2, pCDC28^{Y19F}*	This study
DLY681	MATa/MATα, *cdc42-1/cdc42-1*	This study
DLY5	MATa/MATα	D. Lew
DLY1	MATa, *bar1*	D. Lew
DLY344	MATa, *bar1 GAL1::CLB1(LEU2)*	D. Lew
DLY1006	MATa/MATα, *pGAL1::wee1*	D. Lew

divided cells. This index becomes an overestimate at later timepoints when some cells start to enter mitosis for the second time, so data for wild type cells are not plotted beyond 90–120 min. (*b*) Morphogenesis mutants: since these cells do not form a bud or divide, the percent chromosome segregation is simply calculated by the frequency of binucleate cells. At later timepoints in some experiments (where the checkpoint was overridden), significant numbers of tetranucleate cells were observed. Since the percent chromosome segregation is a cumulative measure, these were included. There are three potential sources of error in this measurement: (*i*) If chromosome segregation occurs perpendicular to the plane of focus, a binucleate cell might be scored as mononucleate. Because these cells tend to grow large and the chromosomes are generally well separated, such instances were easily detected by focussing up and down, so this source of error is probably negligible. (*ii*) At later times cell lysis occurred in many cases. Cell bodies without nuclei were not counted in our scoring, so if lysis preferentially affected mono- or binucleate cells, this would significantly skew our estimates. We did, in fact, observe that in many instances the percent chromosome segregation decreased at very late times, suggesting that binucleate cells were preferentially lysing. (*iii*) The third source of error comes from the fact that the starting elutriated populations were contaminated with 2–10% budded cells, depending on the experiment. We excluded budded cells from our counts, but a later timepoints (following cell division of these budded cells) these would contribute an excess of mononucleated cells. To try to avoid significant errors, elutriations were repeated to obtain the purest starting populations possible (<5% budded cells).

These sources of error (particularly lysis) would lead to underestimates of the percent chromosome segregation at late times. In cases where the percent chromosome segregation decreased at late times the data were deemed unreliable and were not plotted. Even so, it should be borne in mind that although the cumulative percent chromosome segregation rarely rose above 70–80% by our measure, the true efficiency of chromosome segregation was probably higher.

Cell viability was assayed as follows: 7 μl of the cell culture was diluted into 2 ml of YEPD on ice and mixed by vortexing. Since the cells had been sonicated prior to elutriation, this step was not repeated. 0.1 ml of the diluted culture was plated out onto YEPD-agar, and the plates were kept for 2 d at room temperature. Duplicate dilutions and platings were performed for each timepoint. Colony numbers per plate were counted, and the averages were normalized to the numbers at time zero, designated 100%.

Analysis of RNA and Protein 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 the entire coding regions of *CLB1-3*, *CLN2*, or *ACT1*) were labeled using random primer labeling kits according to the manufacturer's recommendations (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Cell extracts were made as described (Stueland et al., 1993), and total protein was quantitated using the Bio Rad assay (Bio Rad Laboratories, Richmond, CA). 50 μg of protein was loaded per lane onto 11% polyacrylamide gels and blotted as described (Stueland et al., 1993). Antibodies were as follows: α-PSTAIRE, mouse monoclonal, used at 1:20,000 dilution (Yamashita et al., 1992); α-Clb2, affinity-purified rabbit polyclonal, used at 1:200 dilution (Grandin and Reed, 1993); α-Clb3, affinity-purified rabbit polyclonal, used at 1:200 dilution (Grandin and Reed, 1993); α-phosphotyrosine, mouse monoclonal 4G10 (Upstate Biotechnology Inc., Lake Placid, NY), used at 1:1,000 dilution. HRP-conjugated α-mouse or α-rabbit second antibodies were used at 1:3,000 dilution. ECL kits (Amersham Corp., Arlington Heights, IL) were used according to manufacturer's instructions for detection of HRP activity.

p13 beads were prepared as described (Dunphy et al., 1988), and incubated with 1.3 mg of extract for 30 min at room temperature. Extraction buffers and washing conditions were exactly as described (Kornbluth et al., 1992).

Analysis of Histone H1 Kinase Activity and cdc25 Treatment

Immunoprecipitation and histone H1 kinase assays were performed as described (Stueland et al., 1993), starting with 100 μg of cell extract. Histone bands were excised from the gels and radioactivity was quantitated by Cerencov counting. Recombinant *S. pombe* cdc25 protein (a gift from C. H. McGowan, The Scripps Research Institute, La Jolla, CA) was prepared and used as described (Millar et al., 1991). Mock treatment was identical except that no cdc25 was added.

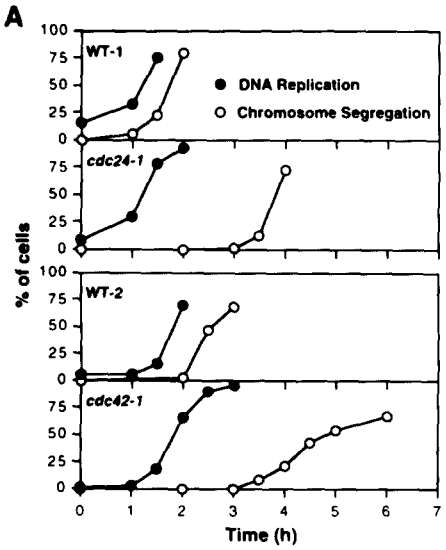
Results

A G2 Delay in the Cell Cycle of Mutants Defective in Cell Morphogenesis

To examine the effects of delayed budding on cell cycle progression we made use of temperature sensitive *cdc24* and *cdc42* strains. *CDC42* encodes a *ras*-related GTPase of the Rho/Rac family (Johnson and Pringle, 1990) and *CDC24* encodes a GDP/GTP exchange factor for Cdc42 (Zheng et al., 1994). The mutants are unable to polarize the actin cytoskeleton or form a bud at the restrictive temperature (Sloat et al., 1981; Adams et al., 1990). These mutant strains and isogenic wild type controls were grown at the permissive temperature, and small G1 daughter cells were isolated by centrifugal elutriation. The synchronized G1 populations were then incubated at the restrictive temperature, and cell cycle progression was monitored by flow cytometry and fluorescence microscopy of propidium iodide stained cells. Compared to the wild type controls, the budding-defective strains were delayed in cell cycle progression, with a delay of about two hours between commencing DNA replication and completing chromosome segregation (Fig. 1 *A*).

To determine the nature of the cell cycle delay in more detail, G1 populations of wild type and isogenic *cdc24* cells were isolated on the same day using identical elutriation parameters, and monitored as above. These strains initiated and completed DNA replication with indistinguishable kinetics, but chromosome segregation was delayed for two hours in the *cdc24* cells (Fig. 1 *B*). Similar results were obtained with isogenic wild type and *cdc42* strains (not shown). Immunofluorescence microscopy of the *cdc24* cells using anti-β-tubulin antibody showed that during the delay most cells (>80%) had assembled a short intranuclear spindle characteristic of G2 cells, confirming the results of Adams et al. (1990). Thus, *cdc24* and *cdc42* cells display a specific G2 delay in the cell cycle.

The G2 delay observed in these mutants could be due to an independent role for the Cdc24 and Cdc42 proteins in chromosome segregation, in addition to their role in generating cell polarity. To ask whether Cdc24 played such a role, we determined whether budded *cdc24* cells also delayed chromosome segregation at the restrictive temperature. Wild type and *cdc24* cells were arrested at the permissive temperature using the drug hydroxyurea. This drug inhibits DNA replication, and the cells arrest with fully formed mature buds. The cells were then shifted to the restrictive temperature, and one hour later the drug was washed out. Under these conditions, *cdc24* cells progressed through DNA replication and chromosome segregation with the same kinetics as the wild type cells, showing that Cdc24 is not required for timely chromosome segregation in budded cells (Fig. 2). Similar results were obtained with *cdc42* mutants (not shown). This suggests that the G2 delay observed in the unbudded mutants (Fig. 1) was not an independent effect of the mutations, but rather was due to the depolarized cytoskeleton or the absence of a bud.



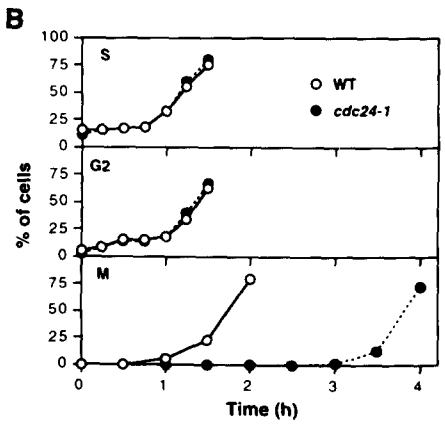


Figure 1. A G2 delay in the cell cycle of mutants defective in cell morphogenesis. (*A*) G1 daughter cells from diploid wild type (WT-1, C276; WT-2, DLY5), *cdc24-1* (5011-JH01, isogenic to WT-1), and *cdc42-1* (DLY681, isogenic to WT-2) strains were isolated by centrifugal elutriation as described in Materials and Methods, and inoculated into YEPD at 36°C (C276, 5011-JH01) or 37.5°C (DLY5, DLY681). Aliquots were taken at the indicated times, stained with propidium iodide, and analyzed by flow cytometry or fluorescence microscopy. (●) Percentage of cells that had initiated DNA replication (DNA content >2C by flow cytometry). (○) Percentage of cells that had completed chromosome segregation (quantitated as described in Materials and Methods). (*B*) Isogenic wild type (C276: ○) and *cdc24-1* (5011-JH01: ●) cells were analyzed as above. (*Top*) Percentage of cells that had initiated DNA replication. (*Middle*) Percentage of cells that had completed DNA replication. (*Bottom*) Percentage of cells that had completed chromosome segregation. Synchrony experiments were performed a minimum of three times for each strain, with consistent results.

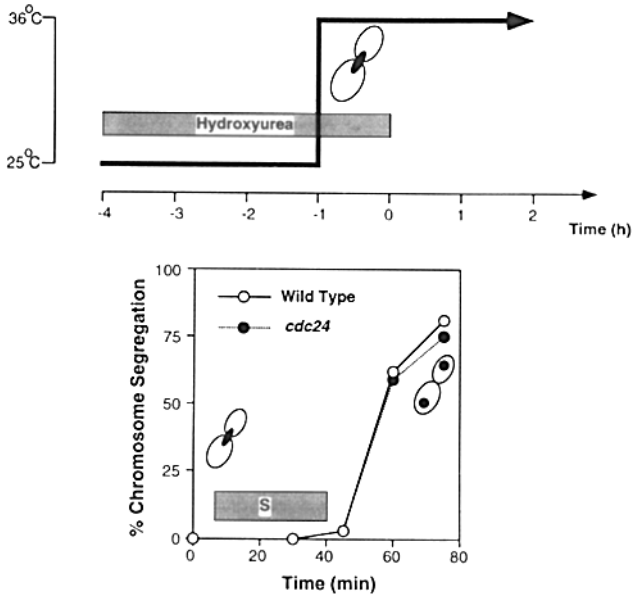


Figure 2. Cdc24 is not required for timely chromosome segregation in budded cells. Wild type (C276: ○) and isogenic *cdc24-1* (5011-JH01: ●) cells were incubated in YEPD supplemented with 0.2 M hydroxyurea for 3 h at 25°C, and then shifted to 36°C for 1 h, as indicated in the diagram (*top*). At this point, 95% of the wild type cells and 93% of the *cdc24* cells had large buds and a single, undivided mass of DNA (assessed by propidium iodide staining). The cells were then harvested by centrifugation and resuspended in prewarmed YEPD at 36°C (time zero in the graph at bottom). Aliquots were taken at the indicated times, stained with propidium iodide, and analyzed by flow cytometry or fluorescence microscopy. Flow cytometry revealed that wild type and *cdc24* cells (>90%) replicated their DNA within 45 min (*shaded bar*), and then underwent chromosome segregation with similar kinetics. This experiment was performed twice, with consistent results.

Activation of Clb2/Cdc28 and Clb3/Cdc28 Kinases Is Delayed in cdc24 Cells

The G2 delay in budding-defective mutants could arise as a checkpoint control of cell cycle progression, or alternatively as a "substrate-product" dependency (Hartwell and Weinert, 1989). For example, the depolarized actin cytoskeleton could result in a mechanical defect in spindle elongation. In this second model, Cdc28 would trigger anaphase with normal kinetics, but translation of this signal into visible chromosome segregation would be delayed because of a defect in spindle function resulting from the depolarized actin cytoskeleton. To determine whether the delayed chromosome segregation in *cdc24* mutants was associated with Cdc28 regulation, we first examined the kinetics of activation of Cdc28 associated with Clb2. Since nuclear division is triggered by the Clb1 and Clb2 pair of cyclins (of which Clb2 is more important: Fitch et al., 1992; Richardson et al., 1992), a G2 delay could come about by inhibition of Clb1,2/Cdc28 activation. Wild type and *cdc24* G1 cells were isolated by centrifugal elutriation as before and incubated at the restrictive temperature. Cell extracts were prepared from samples harvested at 30-min intervals, and Clb2/Cdc28 complexes were immunoprecipitated using Clb2-specific antibodies. The kinase activity associated with these complexes was then assayed in vitro using the standard test substrate, histone H1. In wild type cells Clb2/Cdc28 kinase was induced to a peak at 2.5 h, shortly after DNA replication (Fig. 3, *A* and *B*). However, Clb2/Cdc28 kinase induction in *cdc24* cells was delayed by two hours (Fig. 3 *B*). This delay in kinase induction closely matched the delay in chromosome segregation (Fig. 1 *B*).

We also examined the kinetics of induction of Clb3/Cdc28 kinase activity. Although the Clb3 and Clb4 pair of cyclins are not essential, they can become important for cell cycle progression when other Clb cyclins are compromised (Fitch

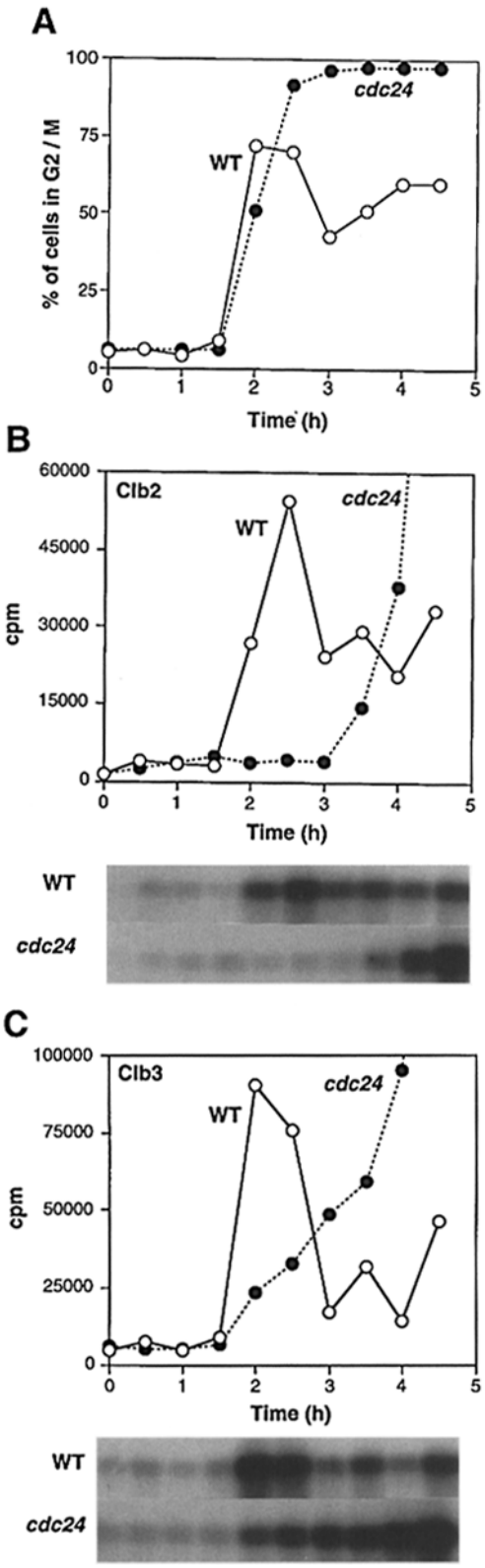


Figure 3. Activation of Clb2/Cdc28 and Clb3/Cdc28 kinases is delayed in *cdc24* cells. (*A–C*) Wild type (C276: ○) and isogenic *cdc24-1* (5011-JH01: ●) G1 daughter cells were isolated by centrifugal elutriation as described in Materials and Methods, and inoculated into YEPD at 36°C. Aliquots taken at the indicated times were processed for flow cytometry, RNA analysis, or protein analysis as described in Materials and Methods. (*A*) The percent of cells with 4C DNA content as measured by flow cytometry is shown. (*B*) Clb2/Cdc28 complexes were immunoprecipitated from cell extracts as described in Materials and Methods, and kinase activity was assayed in vitro with the test substrate histone H1. Autoradiography of the ^{32}P-labeled histone (which runs as a smeary doublet on 11% SDS-PAGE) is shown below, and the histone-associated radioactivity is quantitated in the graph above. (*C*) Clb3/Cdc28 complexes were immunoprecipitated and kinase activity was assayed as in *B*. Extracts and kinase assays were performed with duplicate cell pellets from a single experiment, and gave identical results. Consistent data were obtained in independent experiments with less detailed time-courses.

et al., 1992; Richardson et al., 1992). In agreement with previous studies (Grandin and Reed, 1993), we found that Clb3/Cdc28 kinase activity accumulated slightly earlier than Clb2/Cdc28 kinase activity in wild type cells, peaking at 2 h (Fig. 3 *C*). In *cdc24* cells, however, induction of Clb3/Cdc28 kinase activity was delayed, although not to the same degree as Clb2/Cdc28 kinase activity (compare Fig. 3, *B* and *C*).

Clb/Cdc28 Activity Is Inhibited by Transcriptional and Posttranslational Mechanisms

To determine the basis for the delayed induction of kinase activity, we monitored the accumulation of Clb2 and Clb3 proteins in *cdc24* cells. Since wild type cells enter a second cell cycle following nuclear division, we cannot directly compare kinase activity in wild type (second cycle) versus *cdc24* (first cycle) cells at late time points. Therefore, wild type cells that have been elutriated and incubated for the same period in the presence of the anti-microtubule agent nocodazole were used as a control. These cells accumulate in mitosis of the first cycle, allowing comparison with *cdc24* cells delayed in the first cycle. *cdc24* cells accumulated less Clb2 protein than the controls, which accounted for at least part of the observed lack of Clb2/Cdc28 kinase activity during the G2 delay (Fig. 4 *A*). Comparison of *CLB2* mRNA levels revealed that there was also a delay in *CLB2* mRNA induction (Fig. 4 *B*: scanning densitometry indicated that the *CLB2/ACT1* ratio reached similar levels at 2 h in control cells and 3 h in *cdc24* cells). Although delayed mRNA induction accounts for a part of the delay in Clb2-associated kinase induction, it probably does not account for all of it since mRNA accumulation was only delayed by one hour (Fig. 4 *B*), while kinase induction was delayed by two hours (Fig. 3 *B*).

In contrast to the results with Clb2, the levels of Clb3 protein (Fig. 4 *A*) and mRNA (not shown) were not lower in *cdc24* than control cells. Thus, the approximately twofold lower level of Clb3-associated kinase activity in these cells must be due to posttranslational regulation. Since inhibitory tyrosine phosphorylation of Cdks has been implicated in checkpoint controls in other organisms (see Introduction), we speculated that such a mechanism might account for the inhibition of Clb3-associated kinase activity, and the residual inhibition of Clb2-associated kinase activity, in *cdc24* cells. To determine whether Cdc28 tyrosine phosphorylation occurred in *cdc24* cells, wild type or *cdc24* cells were shifted to the restrictive temperature for 2 or 3 h. Cdc28 was enriched using p13-Sepharose beads (often used as an affinity reagent for purifying cdc2 homologs: Dunphy et al., 1988), and analyzed by Western blotting with anti-phosphotyrosine

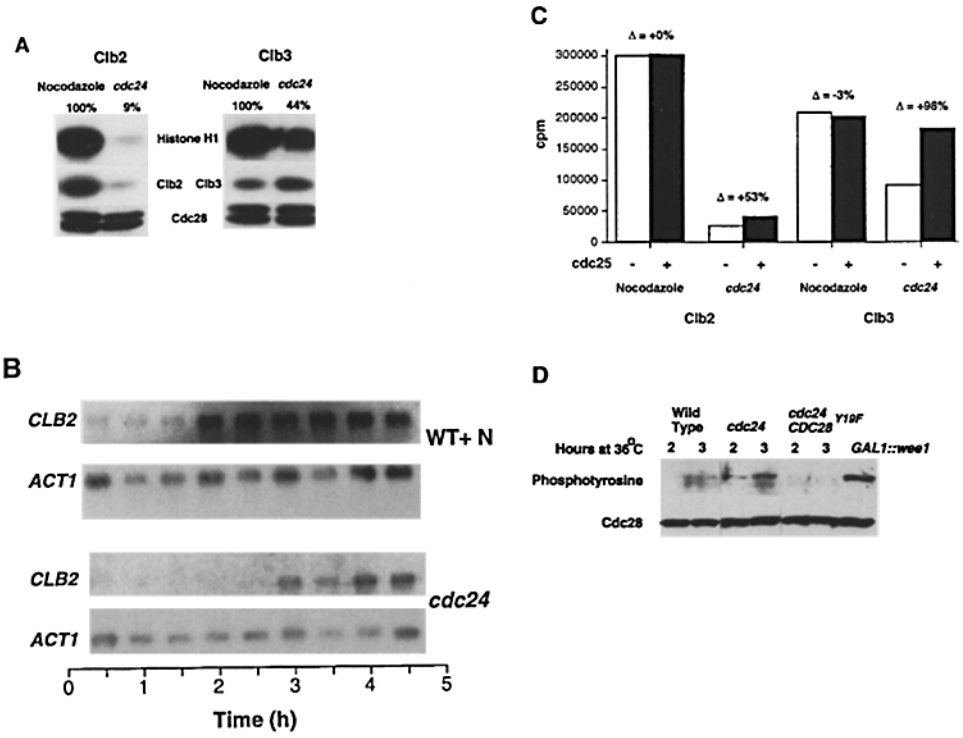


Figure 4. Clb/Cdc28 activity is inhibited by transcriptional and posttranslational mechanisms. (*A*) Wild type (C276) and isogenic *cdc24-1* (5011-JH01) G1 daughter cells were isolated by centrifugal elutriation and incubated as in Fig. 3 except that nocodazole was added to the wild type cells to a final concentration of 15 μg/mL, from a 10 mg/mL stock in DMSO (this was done in parallel with the experiment of Fig. 3). The cells were incubated at 36°C for 2.5 h (at which time all the cells had a 4C DNA content), and processed for protein analysis as described in Materials and Methods. Clb2/Cdc28 (*left*) and Clb3/Cdc28 (*right*) complexes were immunoprecipitated from 100 μg of cell extract and assayed for histone H1 kinase activity (*top*). The percentages shown reflect the radioactivity incorporated into histone, normalized to the nocodazole treated wild type samples (100% = 300,376 cpm for Clb2, 208,193 cpm for Clb3). In parallel, 50 μg of cell extract were separated by SDS-PAGE and immunoblotted with α-Clb2 (*middle, left*), α-Clb3 (*middle, right*), or α-PSTAIRE (*bottom*) antibodies as described in Materials and Methods. The latter recognizes both Cdc28 (*lower band*) and Pho85 (*upper band*) and serves as a normalization control. (*B*) RNA was extracted from the same experiment as above, and processed as described in Materials and Methods. Northern blots were probed with radiolabeled *CLB2* or *ACT1* (to control for RNA loading) probes, as indicated. (*C*) Histone H1 kinase activity in Clb2/Cdc28 or Clb3/Cdc28 complexes from the same samples as in *A* was assayed following mock (−) treatment or cdc25 phosphatase treatment (+) as described in Materials and Methods. The Δ numbers reflect the percent change in kinase activity resulting from cdc25 treatment for each sample. (*D*) Wild type (DLY5), *cdc24* (DLY664), and *cdc24 CDC28^{Y19F}* (DLY685) cells were grown on YEPD and shifted to 36°C for 2 or 3 h, as indicated. Cdc28 was enriched using p13 beads (Materials and Methods), separated by SDS-PAGE, and immunoblotted with α-phosphotyrosine (*top*) or α-PSTAIRE (*bottom*) antibodies. As a positive control, Cdc28 from cells (DLY1006) induced to overexpress weel for 3 h in galactose medium is shown in the right hand lane. This experiment was repeated using four different α-phosphotyrosine antibodies. Apart from variability in signal strength and background bands, all antibodies gave the same result.

antibodies (Fig. 4 *D*). As a positive control, extracts were prepared from cells that overexpressed the *S. pombe* weel kinase previously shown to phosphorylate cdc2 at tyrosine 15 (corresponding to Cdc28 tyrosine 19). As shown in Fig. 4 *D*, Cdc28 from *cdc24* cells gave an elevated signal with the phosphotyrosine antibody. This signal was abolished in control *cdc24* cells that contained a *CDC28^{Y19F}* mutation, suggesting that the signal arose from phosphorylation of tyrosine 19. The cdc25 phosphatase of *S. pombe* has been shown to dephosphorylate tyrosine 15 of cdc2 and activate its kinase (Dunphy and Kumagai, 1991; Gautier et al., 1991; Millar et al., 1991). Assuming that this phosphatase would also be effective on tyrosine 19-phosphorylated Cdc28, we treated the immunoprecipitated Clb3/Cdc28 complexes with recombinant cdc25 protein and then reassayed the histone H1 kinase activity. This treatment had no effect on the kinase from nocodazole-treated wild type cells (or asynchronous wild type cells: cdc25-treated kinase levels were within 5% of untreated controls in six independent experiments), but the Clb3/Cdc28 kinase from *cdc24* cells were activated 2-fold, to levels comparable to the nocodazole control (Fig. 4 *C*). Similarly, Clb2-associated kinase from *cdc24* cells (but not wild type cells) could be stimulated 53% by cdc25 treatment, although given the very low Clb2 abundance in these cells the significance of this result is less clear (Fig. 4 *C*). Since the efficiency of Cdc28 dephosphorylation by the *S. pombe* cdc25 phosphatase is unknown, these results represent minimum estimates of the degree of inhibition caused by tyrosine 19 phosphorylation in *cdc24* cells. These experiments show that Cdc28 tyrosine phosphorylation takes place in *cdc24* cells, and accounts for at least part of the delay in Clb/Cdc28 activation. However, they do not show that Cdc28 tyrosine phosphorylation is important for generating the G2 delay in these cells. Note that Cdc28 phosphorylation was previously shown to occur in hydroxyurea-treated cells, but was not important for the cell cycle block in those cells (Amon et al., 1992).

It has been shown that Cdc28 tyrosine phosphorylation can inhibit Clb2/Cdc28 complexes but not Cln2/Cdc28 complexes (Booher et al., 1993). Our observations extend these findings by showing that Clb3/Cdc28 complexes are also subject to this form of regulation.

Override of the Delay in Chromosome Segregation

The results presented above are consistent with the hypothesis that the G2 delay in mutants that cannot form a bud is due to the delayed induction of Clb/Cdc28 kinases. However, it is also possible that the G2 delay is caused independently (e.g., by mechanical problems with spindle function, as argued above). To resolve this question we constructed a set of isogenic *cdc24* strains carrying various cyclin genes driven by the regulatable *GAL1* promoter (see Materials and Methods). This promoter is off when cells are grown in medium lacking galactose, but is strongly induced upon transfer to galactose containing medium. Note that in this strain background (BF264-15DU), a somewhat longer (3–4 h) G2 delay was observed in *cdc24* cells (Fig. 5). We reasoned that if inhibition of cyclin accumulation were responsible for the G2 delay, then induction of high levels of various Clbs using the *GAL1* promoter might eliminate the G2 delay. In contrast, if the delay resulted from a mechanical defect in spindle elongation, it would not be affected by manipulation of Clb levels. Synchronous G1 populations of isogenic wild type, *cdc24*, and *cdc24 GAL1::CLB* strains were isolated as before and incubated at the restrictive temperature in galactose-containing media. As shown in Fig. 5 *A*, induced expression of Clb1, Clb2, or Clb3 resulted in the complete elimination of the G2 delay, so that *cdc24* cells underwent chromosome segregation at the same time as wild type cells. In contrast, induced expression of the G1 cyclin Cln2 had no effect, as expected. These results imply that the G2 delay is a consequence of Cdc28 regulation rather than a mechanical defect in spindle function, and strongly support the hypothesis that the delay comes about through a regulatory checkpoint control responding to some aspect of the morphogenesis defect.

Since induction of *CLB* transcription could override the G2 delay completely, one might speculate that the predominant cause of the G2 delay was the delayed accumulation of *CLB1* and *CLB2* transcripts. However, the level of Clb synthesis from the *GAL1* promoter exceeds the level normally attained during the cell cycle, and it is possible that such overexpression would lead to Clb levels high enough to overwhelm other forms of regulation, such as tyrosine phosphorylation of Cdc28. Indeed, the fact that induction of high levels of *CLB3* mRNA could override the G2 delay (Fig. 5 *A*) suggests that this is the case, since Clb3 accumulation was not delayed in *cdc24* cells (Fig. 4 *A*). In addition, recent findings indicate that induction of *CLB1* and *CLB2* transcripts is largely due to a positive feedback loop whereby Clb1,2/Cdc28 kinase activity stimulates *CLB1,2* mRNA synthesis (Amon et al., 1993). This suggests that the delayed accumulation of *CLB2* transcripts could occur as an indirect effect of post-translational inhibition of Clb/Cdc28 kinases.

To determine whether Cdc28 tyrosine phosphorylation could account for a significant part of the G2 delay, we constructed strains in which the *MIH1* gene (the *Saccharomyces cerevisiae* homology of the *S. pombe cdc25* gene; Russell et al., 1989) was expressed from the *GAL1* promoter. We reasoned that Mih1 overexpression would result in dephosphorylation of Cdc28 tyrosine 19, and that if this was important for the G2 delay, chromosome segregation would be accelerated. This was indeed the case (Fig. 5 *B*): the majority of the G2 delay was abolished by Mih1 induction. To ensure that

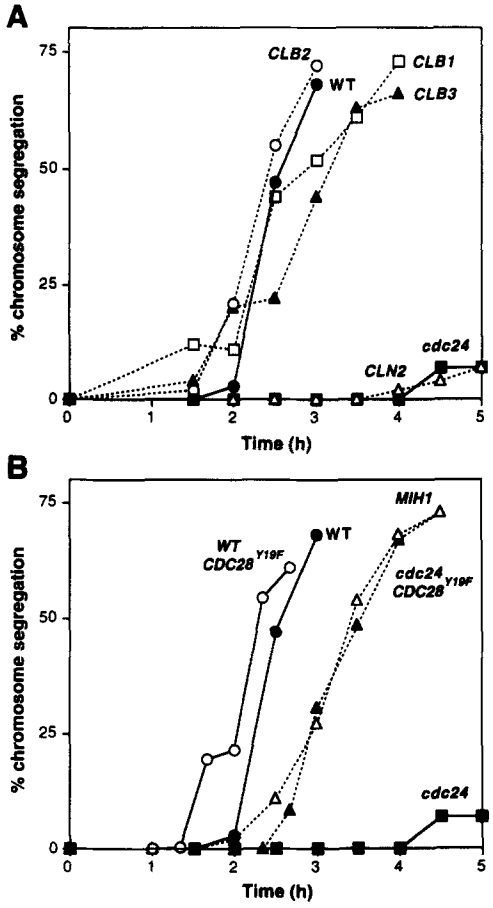


Figure 5. Override of the delay in chromosome segregation. (*A*) Isogenic wild type (DLY5, ●), *cdc24-4* (DLY664, ■), *cdc24-4 GAL1::CLB1* (DLY668, □), *cdc24-4 GAL1::CLB2* (DLY669, ○), *cdc24-4 GAL1::CLB3* (DLY670, ▲), and *cdc24-4 GAL1::CLN2* (DLY672, △) G1 daughter cells were isolated by centrifugal elutriation as described in Materials and Methods, and inoculated into YEPD at 36°C. Aliquots taken at the indicated times were stained with propidium iodide and the percent chromosome segregation was quantitated as described in Materials and Methods. (*B*) Wild type (DLY5, ●), *cdc24-4* (DYL664, ■), *cdc24-4 GAL1::MIH1* (DLY673, △), $CDC28^{Y19F}$ (DLY129, ○), and *cdc24-4* $CDC28^{Y19F}$ (DLY685, ▲) were analyzed as in *A*. Flow cytometry showed that the $CDC28^{Y19F}$ cells replicated their DNA about 15 min earlier than the other cells: variations of this magnitude in the length of G1 were not uncommon between different experiments. Each synchrony experiment was performed at least twice, with consistent results.

tnis effect was due to dephosphorylation of Cdc28 tyrosine 19, rather than some other putative Mih1 substrate, we constructed an isogenic *cdc24* strain in which the *CDC28* gene was replaced with a mutant that changed the tyrosine 19 residue to phenylalanine ($CDC28^{Y19F}$), thus rendering Cdc28 resistant to inhibition by this pathway. Again, the majority of the G2 delay (2.5–3 h) was abolished by this mutation (Fig. 5 *B*). The residual G2 delay (about 45 min relative to wild type) seen in these strains must be due to some other inhibitory mechanism (perhaps the delayed *CLB1,2* mRNA induction; see Discussion). Together, these results show that defects in bud formation trigger a checkpoint control that delays cell cycle progression through Cdc28 regulation, in large part by phosphorylation of Cdc28 tyrosine 19.

cdc24 Cells Remain Viable and Able to Bud during the G2 Delay

Bud formation normally begins at about the G1/S transition, not in G2. If the checkpoint-induced G2 delay is to be an effective defence against conditions that transiently incapacitate cytoskeletal polarization, then cells should be able to initiate bud formation in G2 once budding capacity is restored. To assess the ability of cells to form a bud following a transient block, G1 cells of a *cdc24* strain were incubated for various periods at the restrictive temperature and then shifted down to the permissive temperature. This experiment revealed that cells that were shifted down during the G2 delay period (1–3 h of incubation) budded remarkably rapidly, efficiently and synchronously (Fig. 6, *A* and *B*). If the cells were maintained at the restrictive temperature past the time of chromosome segregation, however, only a fraction of the cells formed buds, and budding was slower and less synchronous (Fig. 6, *A* and *B*). This suggests that the checkpoint does indeed maintain the cells in a state that is highly favorable for efficient bud formation upon recovery from the block.

The cells that budded after nuclear division (4–5 h shift-down) were fixed and stained with DAPI 1–2 h after shift-down (not shown). In most cases, both of the nuclei (i.e., DAPI-stained masses) had divided along the mother–bud axis, yielding tetranucleate cells with binucleate mothers and buds. Significant minorities of cells with two nuclei in the mother and none in the bud, and with three nuclei in the mother and one in the bud, were also observed.

The result that G2 cells were able to bud efficiently was somewhat surprising, since we had previously found that the G1 cyclins, and particularly Cln1 and Cln2, were important to promote cytoskeletal polarization (Lew and Reed, 1993). Given the normal periodicity of Cln1,2 expression, these cyclins would not be expected to be present in G2 cells. To examine this more closely, we monitored *CLN2* mRNA levels in synchronized *cdc24* cultures incubated at the restrictive temperature. Whereas wild type control cells treated with nocodazole underwent a cycle of *CLN2* expression and repression, *CLN2* mRNA levels in *cdc24* cells were induced and continued to rise during the G2 delay (Fig. 6 *C*). Thus, G1 cyclins that promote cytoskeletal polarization accumulate to high levels during the checkpoint-induced G2 delay, a mechanism that may help to maintain the readiness of these cells to bud upon recovery from the *cdc24* block (see Fig. 8).

We then examined cell viability during incubation of synchronous *cdc24* populations at the restrictive temperature. As expected, the cells remained viable during the G2 delay (Fig. 6 *D*). After several hours viability was gradually lost, but this loss occurred about an hour after the cells finally underwent chromosome segregation, indicating that chromosome segregation was not in itself a lethal event. The cause of the loss in viability is unclear, but it was accelerated by *MIH1* overexpression (Fig. 6 *D*) or the *CDC28*^{Y19F} mutation (not shown). Thus, some aspect of continued cell cycle progression is lethal to cells that are unable to polarize the actin cytoskeleton, and one effect of the checkpoint is to protect cells against this lethal event.

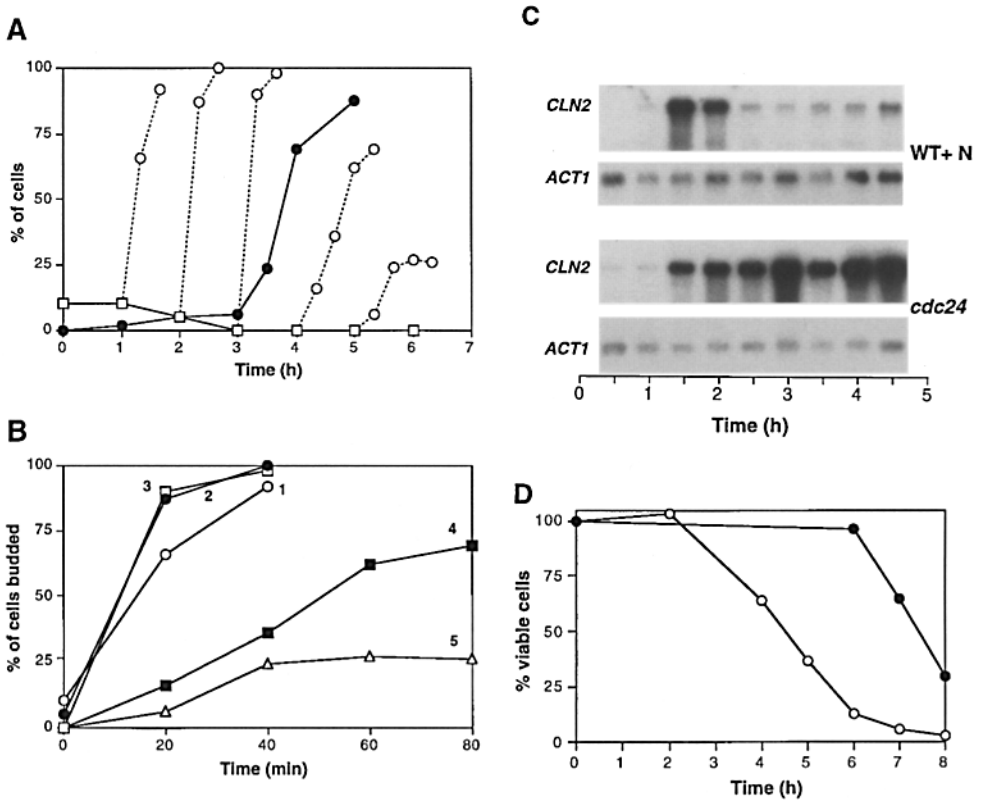


Figure 6. Recovery of *cdc24* cells from a transient arrest. (*A*) *cdc24-4* (JPT19-H01) G1 daughter cells were isolated by centrifugal elutriation as described in Materials and Methods, and inoculated into YEPD at 36°C. At the indicated times, aliquots were shifted down to 25°C and budding was monitored microscopically at 20-min intervals (○, □, percent of cells budded). Chromosome segregation (●) was also monitored in the cells at 36°C. (*B*) The same results as in *A* were replotted on an expanded time axis, with time zero corresponding to the time of temperature shift. The number of hours at 36°C prior to shift-down is indicated by the numbers next to each curve. (*C*) The same Northern blots as in Fig. 4 *B* were probed with *CLN2* or *ACT1* probes as indicated. Quantitation of these samples and samples from an independent experiment indicated that at late times the *cdc24*-arrested cells accumulated 6- to 20-fold higher levels of *CLN2* RNA (normalized to the *ACT1* control) than the peak levels in wild type cells. (*D*) *cdc24-4* (DLY664, ●) and *cdc24-4 GAL1::MIH1* (DLY673, ○) G1 cells were incubated at 36°C and analyzed to determine cell viability as described in Materials and Methods. This experiment was performed three times, with consistent results.

Osmotic Shock and Cell Cycle Progression

The experiments described above were all performed with mutant strains that had defects in cytoskeletal polarity. To investigate the role of the checkpoint in wild type cells, we examined the effects of osmotic shock on the cell cycle. Chowdhury et al. (1992) previously demonstrated that addition of sorbitol (or other solutes) to the growth medium caused a transient depolarization of the actin cytoskeleton. We confirmed this observation, and found that this depolarization delayed bud formation by one hour in a population of cells synchronized by mating pheromone (Fig. 7 *A*). This was accompanied by a cell cycle delay so that the cells did not undergo nuclear division until a bud had been formed, and no binucleate cells were observed (300 cells counted). If this cell cycle delay was generated in the same way as in *cdc24* cells, then elevated expression of *CLB2* should eliminate the delay (see Fig. 5 *A*). To test this, a *GAL1::CLB2* strain was arrested with mating pheromone and transferred to galactose medium. Cells were released from pheromone arrest and subjected to an osmotic shock as before, and at 3 h after release the cells were fixed and stained with DAPI to visualize nuclei. As shown in Fig. 7 *B*, many of these cells (28%: 300 cells counted) were binucleate. Most of the binucleate cells were unbudded or had small buds (40% of cells with buds <1/4 size of mothers were binucleate). Binucleate cells were not observed in identically treated cells lacking the *GAL1::CLB2* gene, or in *GAL1::CLB2* cells that had not received the osmotic shock. The simplest interpretation of this result is that the impaired cell polarity resulting from the osmotic shock triggered a cell cycle delay via the same checkpoint described in *cdc24* cells.

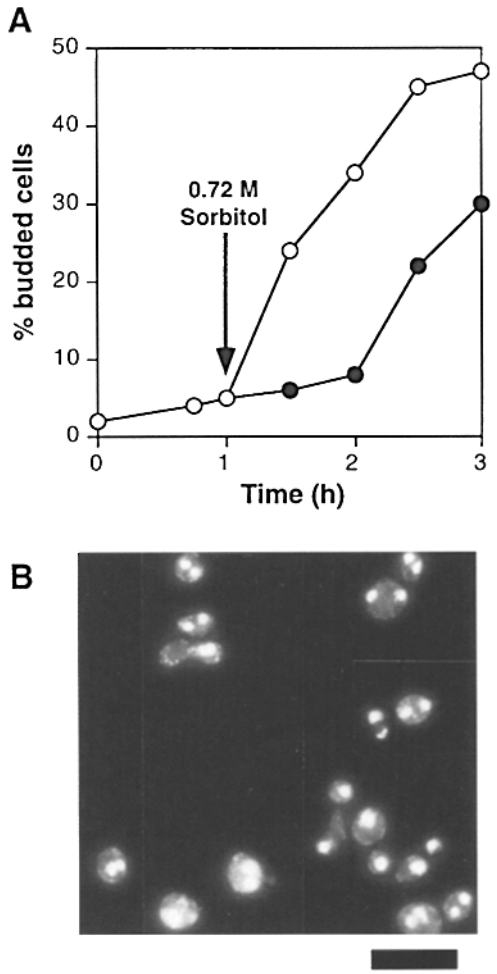


Figure 7. Recovery of wild type cells from osmotic shock. (*A*) Wild type (DLY1) cells were grown in YEPS, arrested with mating pheromone, and released into galactose-containing medium (time zero in graph: see Materials and Methods). The percentage of cells that had formed a bud was quantitated (200 cells counted) in samples fixed at the indicated times. At 1 h, sorbitol was added to one half of the culture (●) to 0.72 M final concentration. *GAL1::CLB1* cells (DLY344) treated identically budded with indistinguishable kinetics and showed the same response to osmotic shock. (*B*) *GAL1::CLB1* cells (DLY344) that had undergone an osmotic shock from the same experiment as in *A* were fixed at 3 h following pheromone release and stained with DAPI. Shown is a picture taken with combined fluorescence and transmitted light, illustrating the high frequency of binucleate cells generated by this treatment. Similar results were obtained in two independent experiments.

Discussion

A Novel Class of Checkpoint Control Responds to Defects in Morphogenesis

Temperature-sensitive *cdc24* and *cdc42* mutants that were unable to polarize the actin cytoskeleton or form a bud at the restrictive temperature displayed a long G2 delay in the cell cycle (Fig. 1). Phenotypes consistent with a long G2 delay have also been reported for other mutants that are unable to form a bud at the restrictive temperature, e.g., *cdc43* (Adams et al., 1990), *myo2* (Johnston et al., 1991), *swi4* (Ogas et al., 1991), *cln1 cln2 bud2* (Benton et al., 1993; Cvrckova and Nasmyth, 1993), *cmd1-233* (Ohya and Botstein, 1994), and *cdc1* (Paidhungat, M., and S. Garrett, personal communication). To our knowledge, the only report consistent with unperturbed cell cycle progression despite the lack of a bud is that of the original *cdc24-1* isolate, 5011 (Hartwell et al., 1973). In that case, subsequent analysis revealed that the cell cycle properties of the heavily mutagenized 5011 strain resulted from additional mutations (Adams et al., 1990). We have shown that the same *cdc24-1* allele in two different strain backgrounds results in a long G2 delay associated with the bud formation defect. We therefore suggest that the additional mutations conferring rapid cell cycle progression in the 5011 strain disrupt a normal checkpoint that links morphogenesis and cell cycle progression (see below).

The finding that many different mutants that cannot form a bud all experience a long G2 delay suggests that the delay is a cellular response to the budding defect. We have shown that the delay arises through regulation of Cdc28, primarily at the level of tyrosine phosphorylation (Fig. 8), and can be eliminated by mutations that abrogate this regulatory pathway (Fig. 5). Thus, the dependence of timely chromosome segregation on bud formation can be relieved by inactivating a cellular function (Cdc28 tyrosine phoshorylation). This suggests that the G2 delay is caused by a novel class of checkpoint control, triggered by the morphogenesis defect.

We were first encouraged to search for a morphogenesis checkpoint by the observation that the polarity of the actin cytoskeleton was very sensitive to perturbations of the temperature or osmolarity of the growth medium. Such perturbations would be expected to occur rather frequently in cells growing outside of the protected lab environment. If cells

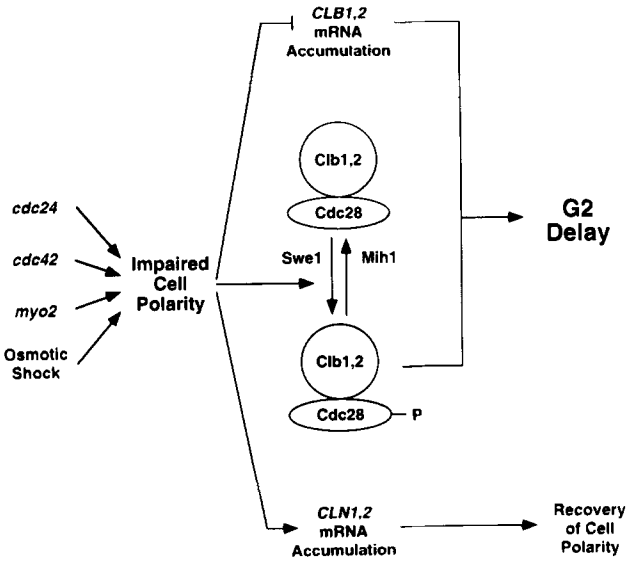


Figure 8. Summary of the effects of the morphogenesis checkpoint on Cdc28 and cyclins. See text for details.

really use a morphogenesis checkpoint to slow the cell cycle during recovery from such perturbations, then overriding the checkpoint should result in the generation of binucleate cells as the cell cycle continues unfettered in cells that cannot bud. Indeed, a large fraction of wild type cells exposed to an osmotic shift became binucleate upon override of the checkpoint (Fig. 7). Since osmotic shock provides a very different way of perturbing morphogenesis (compared to *cdc24* or *cdc42* mutants), this result validates our conclusions from the mutant studies.

The molecular nature of the defect(s) sensed by the novel checkpoint is unknown. It seems likely that either the presence of a bud, the size of the bud, or some other manifestation of cell polarity is monitored. However, we cannot rule out the possibility that the checkpoint monitors some very indirect consequence of the disturbed cell polarity. Conceivably, such indirect effects might even encompass defects in chromosome structure or spindle properties, but these defects must be distinct from those monitored by the other known checkpoints, since the effects of triggering these checkpoints on Cdc28 activity are very different (see below).

The G2 delay observed in *cdc24* and *cdc42* mutants is transient, despite the fact that the morphogenesis defect (at the restrictive temperature) is permanent. It seems likely that the checkpoint functions in wild type cells to adjust for transient lapses in cell polarity, which (at least in lab conditions) rarely last longer than an hour. Thus, the 2–4-h checkpoint delay may be more than sufficient for this purpose.

The existence of a mechanism that can monitor some aspect of the actin cytoskeleton or cell morphology has long been postulated for mammalian cells (Curtis and Seehar, 1978). Signals arising from such a "mechanical" (rather than "chemical") sensor are thought to generate important inputs into proliferative and differentiation decisions in many cell types during development (Ingber, 1993). It is tempting to speculate that the molecular basis for such mechanical signalling pathways in mammals could be related to the yeast morphogenesis checkpoint identified here. The availability of powerful molecular genetic tools in the yeast system should make it possible to test this hypothesis in the not too distant future.

A Role for Cdc28 Tyrosine Phosphorylation in Budding Yeast

A regulatory pathway that could inhibit Cdks by tyrosine phosphorylation was first described in the fission yeast *S. pombe* (Gould and Nurse, 1989), and shown to play a critical role in regulating entry into mitosis (Dunphy, 1994). Fission yeast cells that are unable to phosphorylate cdc2 at tyrosine 15 enter mitosis prematurely, even if the DNA is not fully replicated, leading to "mitotic catastrophe" (Enoch and Nurse, 1990; Lundgren et al., 1991). This regulatory pathway has now been characterized in many species including frogs, mammals, fruit flies, and budding yeast (reviewed in Dunphy, 1994). Studies in metazoans have confirmed the importance of this pathway in regulating entry into mitosis, but until now budding yeast has seemed to provide the exception to the rule. Mutation of *CDC28* tyrosine 19 to phenylalanine had no effect on cell cycle progression even under conditions that induced DNA damage (Amon et al., 1992; Sorger and Murray, 1992), raising the question of what role, if any, was played by the Cdc28 tyrosine phosphorylation pathway in budding yeast.

In this paper, we showed that cells that were unable to polarize the actin cytoskeleton or form a bud inhibited activation of Clb/Cdc28 kinases, thus delaying chromosome segregation. Mutation of *CDC28* tyrosine 19 to phenylalanine, or overexpression of the Mih1 phosphatase, abolished most of the delay in chromosome segregation, demonstrating that tyrosine phosphorylation of Cdc28 was important for the delay in vivo. These data show that phosphorylation of Cdc28 tyrosine 19 does indeed play an important role in regulating cell cycle progression, in cells that experience defects in morphogenesis (Fig. 8).

In addition to tyrosine phosphorylation of Cdc28, cells with morphogenesis defects displayed a delayed induction of *CLB2* mRNA, and a sustained hyperinduction of *CLN2* mRNA (Fig. 8). Recent studies have shown that *CLB2* transcription is subject to a positive feedback loop whereby Clb/Cdc28 kinase activity stimulates *CLB2* mRNA accumulation (Amon et al., 1993). Thus, it is possible that the inhibition of Clb/Cdc28 kinase by tyrosine phosphorylation was responsible for the delay in *CLB2* mRNA induction. Further, Clb/Cdc28 kinase activity was shown to play a role in repression of *CLN2* mRNA in G2 (Amon et al., 1993). Thus, the sustained induction of *CLN2* mRNA could also be due to the inhibition of Clb/Cdc28 kinase by tyrosine phosphorylation. However, the fact that reversing the inhibition due to Cdc28 tyrosine phosphorylation still left a residual G2 delay (of about 45 min) indicates that the morphogenesis checkpoint does not work solely by this mechanism.

The effects of the morphogenesis checkpoint on Cdc28 and cyclins are very different from the effects of the other known checkpoints. Treatment of cells with hydroxyurea (which inhibits DNA replication) or nocodazole (which inhibits assembly of the microtubule spindle) causes a large-budded arrest with undivided nuclei and moderate (hydroxyurea) or high (nocodazle) Clb/Cdc28 kinase activity. To date, no manipulation of Cdc28 or cyclins has overcome the arrest

caused by these agents (Stueland et al., 1993). Thus, the morphogenesis checkpoint is the first checkpoint in budding yeast for which we understand at least part of the mechanism whereby cell cycle progression is delayed.

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