MOLECULAR AND CELLULAR BIOLOGY, Aug. 1996, p. 4573–4583 Vol. 16, No. 8
0270-7306/96/$04.00+0

Dephosphorylation of Threonine 169 of Cdc28 Is Not Required for Exit from Mitosis but May Be Necessary for Start in *Saccharomyces cerevisiae*

HONG HWA LIM, CHONG JIN LOY, SARA ZAMAN, AND UTTAM SURANA*

Institute of Molecular and Cell Biology, National University of Singapore, Singapore 119260

Received 22 February 1996/Returned for modification 28 March 1996/Accepted 20 May 1996

Entry into mitosis requires activation of cdc2 kinase brought on by its association with cyclin B, phosphorylation of the conserved threonine (Thr-167 in *Schizosaccharomyces pombe*) in the T loop, and dephosphorylation of the tyrosine residue at position 15. Exit from mitosis, on the other hand, is induced by inactivation of cdc2 activity via cyclin destruction. It has been suggested that in addition to cyclin degradation, dephosphorylation of Thr-167 may also be required for exit from the M phase. Here we show that *Saccharomyces cerevisiae* cells expressing *cdc28-E169* (a *CDC28* allele in which the equivalent threonine, Thr-169, has been replaced by glutamic acid) are able to degrade mitotic cyclin Clb2, inactivate the Cdc28/Clb2 kinase, and disassemble the anaphase spindles, suggesting that they exit mitosis normally. The *cdc28-E169* allele is active with respect to its mitotic functions, since it complements the mitosis-defective *cdc28-1N* allele. Whereas replacement of Thr-169 with serine affects neither Start nor the mitotic activity of Cdc28, replacement with glutamic acid or alanine renders Cdc28 inactive for Start-related functions. Coimmunoprecipitation experiments show that although Cdc28-E169 associates with mitotic cyclin Clb2, it fails to associate with the G_1 cyclin Cln2. Thus, an unmodified threonine at position 169 in Cdc28 is important for interaction with G_1 cyclins. We propose that in *S. cerevisiae*, dephosphorylation of Thr-169 is not required for exit from mitosis but may be necessary for commitment to the subsequent division cycle.

Cell cycle progression in eukaryotic cells is, in part, regulated by activation and inactivation of cyclin-dependent kinases (CDKs), of which cdc2 is the most widely known prototype. While animal cells need different CDKs to course through the different phases of the division cycle (reviewed in references 36, 37, 42, and 53), the fission yeast *Schizosaccharomyces pombe* requires cdc2 for both the G_1-to-S and G_2-to-M transitions (38). In the budding yeast *Saccharomyces cerevisiae*, three CDKs have been identified, namely, Cdc28, Pho85, and Kin28 (16, 17, 34, 55). Of these, Cdc28 is the main regulator of progression through the cell cycle.

One of the most conserved features of the eukaryotic cell cycle is the way in which the mitotic activity of cdc2 is regulated (2, 10, 25, 32). Monomeric cdc2 is inactive and requires binding of cyclin B as the first step towards its activation as mitotic kinase. The affinity of cyclin binding is further stabilized and enhanced by phosphorylation of residue Thr-167 (Thr-167 in *S. pombe*, Thr-161 in frogs and mammals, and Thr-169 in *S. cerevisiae*) (10, 25, 32). However, phosphorylation of the conserved tyrosine at position 15 (Tyr-15) by the wee1 and mik1 gene products prevents the kinase complex from becoming active until cells are ready to enter mitosis (13, 20, 30). Thus, dephosphorylation of Tyr-15 by a tyrosine phosphatase encoded by the *cdc25* gene constitutes the final step in the activation of mitotic kinase. This event is critical for entry into mitosis in that replacement of Tyr-15 by phenylalanine causes fission yeast cells to attempt mitosis prematurely (23). In human cells, both threonine 14 (Thr-14) and Tyr-15 must be dephosphorylated for full activation of cdc2 kinase (37). Another dimension to the regulation of CDK has been added by the discovery of CDK inhibitors that directly bind to and inhibit CDK-cyclin complexes (31, 39, 51, 54). However, the mechanism by which they inhibit the kinase activity is not known.

In *S. cerevisiae*, Cdc28 interacts with nine different cyclin subunits, including three G_1 cyclins encoded by *CLN* genes and four mitotic cyclins encoded by the *CLB1*, *CLB2*, *CLB3*, and *CLB4* genes, to regulate various aspects of the cell cycle transitions (15, 34, 49, 52, 59). Its mitotic form, like cdc2, is activated by the binding of mitotic cyclins and dephosphorylation of Tyr-19 (equivalent to Tyr-15 of cdc2) (34). While Tyr-19 dephosphorylation appears to be necessary for the onset of mitosis, this event alone is not sufficient in that replacement of tyrosine with phenylalanine does not lead to premature mitosis in *S. cerevisiae* (1, 57).

The crystal structures of nonphosphorylated Cdk2 (7, 41) and the Cdk2-cyclin A complex (28, 43) have provided some rationale for the activation of CDKs by cyclin binding and phosphorylation-dephosphorylation of various amino acid residues. The active site of Cdk2 resides in the cleft of a bilobed structure. In the ATP-bound form, residues Thr-14 and Tyr-15 in the glycine-rich loop containing the ATP-binding site are relatively close to the γ-phosphate of ATP. Therefore, phosphorylation of Thr-14 is predicted to disrupt the conformation of the ATP phosphates. This may explain why dephosphorylation of this residue, and perhaps also Tyr-15, is necessary for complete activation. Cdk2 also has the so-called T loop which contains Thr-160 (equivalent to Thr-167 of cdc2 in *S. pombe* and Thr-169 of Cdc28 in *S. cerevisiae*). The T loop is poorly accessible to the solvent and prevents substrate binding. It has been predicted that the binding of cyclin changes the Cdk2 conformation, permitting phosphorylation of Thr-160, which in turn may stabilize the association of cyclin with Cdk2.

The kinase that phosphorylates the threonine residue in the T loop of Cdk2 is known as CDK-activating kinase (CAK or

* Corresponding author. Mailing address: Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, Singapore 119260. Phone: (65) 774 3612 or (65) 772 6680. Fax: (65) 779 1117.

Cdk7) (4). It is a cdc2-related kinase encoded by the *Xenopus MO15* gene (19, 45, 56). In both HeLa and fission yeast cells, Thr-160–Thr-167 of Cdk2-cdc2 is phosphorylated during the S phase (24, 25); whether this is because CAK activity is cell cycle regulated is not clear. In *Xenopus laevis*, at least, CAK seems to be constitutively active during the cleavage of fertilized eggs (3, 46).

While progression through mitosis requires activation of cdc2/cyclin B kinase, its inactivation is a prerequisite for exit from mitosis and entry into the subsequent division cycle. One way cells can inactivate the mitotic kinase is by reversing the steps involved in its activation. Indeed, mitotic cyclins are rapidly destroyed by proteolytic degradation at the end of mitosis by a ubiquitin-dependent pathway (22), matching a concomitant reduction in the kinase activity. That the inactivation of mitotic kinase is necessary for exit is suggested by the observation that expression of nondestructible cyclin B causes late-anaphase arrest in *S. cerevisiae* (58). Studies of *Xenopus* egg extracts concluded that both cyclin destruction and dephosphorylation of Thr-161 are required to inactivate the cdc2-cyclin B complex (29). In *S. pombe*, cells overexpressing the *cdc2* allele in which Thr-167 has been replaced by glutamic acid accumulate multiple nuclei, become multiseptated, and eventually arrest with short mitotic spindles (24). These findings have led to the suggestion that dephosphorylation of Thr-161–Thr-167 may also be required, in addition to cyclin B destruction, for exit from mitosis (24, 29).

In this report, we describe experiments that suggest that in *S. cerevisiae*, dephosphorylation of Thr-169 in Cdc28 is not required for exit from mitosis. We also show that replacement of threonine with glutamic acid or alanine (approximating either the phosphorylated or the dephosphorylated form of threonine, respectively) renders Cdc28 inactive for Start-related functions. This suggests that an unmodified threonine at position 169 is important for Start. Therefore, the purpose of Thr-169 dephosphorylation at the mitotic exit may be to transform Cdc28 from a mitotically active form into a Start-efficient form.

MATERIALS AND METHODS

Strains and growth media. All of the strains used in this study were derived from wild-type strain W303. The *cdc28-4*, *cdc28-1N*, and *cdc15* mutants had been made isogenic by backcrossing at least three times to the wild-type strain. Cells were routinely grown in yeast extract-peptone (YEP) medium containing adenine (50 mg/liter) supplemented with either glucose or galactose. Raffinose was also added to the galactose medium. In experiments in which expression of the *CDC28* gene or its mutant allele was driven by the *GAL1* promoter, cells were first grown in medium containing raffinose as the sole carbon source before induction by galactose.

Synchronization procedures. For experiments requiring both synchronous release from anaphase and induction of gene expression from the *GAL1* promoter, *cdc15* cells were used. To obtain a culture synchronized in anaphase, cells were first grown for 3 to 4 days on YEP-raffinose plates at 24°C. When >90% of the cells had reached the stationary phase, they were suspended in either raffinose or raffinose-galactose medium at 37°C. After 4 h of growth at 37°C, >80% of the cells showed the late-anaphase arrest phenotype. For release, cells were filtered and allowed to resume cell cycle progression by resuspension in YEP supplemented with raffinose at 24°C. Samples collected at 20-min intervals were used for RNA isolation, preparation of cell extracts, flow cytometric analysis, in situ immunofluorescence, and Western blot (immunoblot) analysis.

In experiments in which an arrest prior to nuclear division was required, *cdc28-4* cells expressing either *GAL-cdc28-E169myc* or *GAL-cdc28-A169myc* were grown to the exponential phase in raffinose medium at 24°C before they were treated with nocodazole (15 μg/ml). After 2 h, one-half of the culture was induced by addition of galactose (2%) for a further 2 h. Cells were harvested, and extracts were prepared for kinase activity measurements.

Cell extracts, immunoprecipitations, and kinase assays. For determination of Cdc28-Clb2 kinase, cells were harvested by centrifugation at 4°C and washed with ice-cold stop mix (59). The pellet was resuspended in an appropriate amount of lysis buffer and mixed with an approximately equal amount of acid-washed glass beads (0.5-mm diameter; Biospec). Cells were broken by two bursts of vigorous vortexing (IKA-Vibrax-VXR), each lasting 3 min. The glass beads and cell debris were removed, and cell extracts were cleared by centrifugation for 15 min in a microcentrifuge. The resulting supernatant was used to assay mitotic kinase activity. Immunoprecipitation with polyclonal antibodies against Clb2 and kinase assays were performed as described by Surana et al. (58). For immunoprecipitation with a c-myc monoclonal antibody (100 μg/ml; clone Ab-1 from Oncogene Science), a 1:4 dilution was used and kinase assays were performed as described above. Kinase activity was quantitated with a PhosphorImager (Molecular Dynamics).

For determination of Cdc28-Cln2-hemagglutinin (HA) kinase activity, cells were harvested and treated as described above. A 4-μg sample of an anti-HA mouse monoclonal antibody (clone 12CA5 from Boehringer Mannheim) was used to immunoprecipitate the Cdc28-Cln2-HA complex from a cell extract containing 1 mg of total protein. Immunoprecipitations were performed as described by Surana et al. (58) with the following modifications. After the immune complexes were bound to protein A-Sepharose beads, they were washed a total of eight times with lysis buffer and then twice with 25 mM morpholinepropanesulfonic acid (MOPS, pH 7). The composition of the reaction mixture was modified as follows. A final volume of 100 μl was made by mixing 20 μl of 4 mg of histone H1 per ml, 10 μl of 250 mM MOPS, 10 μl of [γ-^{32}P]ATP, and 60 μl of sterile water. A 10-μl volume of this mixture was used for each kinase reaction.

For detection of Cdc28 protein in Cdc28-Cln2-HA and Cdc28-Clb2 complexes, cells were harvested and treated as described by Tyers et al. (60). From 5 to 8 mg of protein was used for immunoprecipitation. The lysate was incubated with a mouse anti-HA monoclonal antibody (clone 12CA5; 0.4 μg/ml) at a 1:10 dilution on ice for 1 h and then incubated with protein A-Sepharose beads on a rotatory wheel at 4°C for 1 to 2 h. The beads were collected by gentle centrifugation and washed five times with buffer 3. The samples were run on 10% acrylamide gels, blotted onto Hybond C-extra (Amersham), and probed with polyclonal anti-Cdc28 antibodies. Western blot analysis was performed with an Enhanced Chemical Luminescence kit (Amersham) in accordance with the manufacturer's instructions.

Genetic manipulations. All DNA manipulations were performed as described by Sambrook et al. (50). Thr-169→Glu, Ala, and Ser mutations in the *CDC28* sequence were generated by PCR amplification with PCR primers containing specific mutations (a, b, and c, respectively, below). The top strand 5′ CGTTA G**ACTTAAG**ACCTGGCCAA 3′ (the *Afl*II site is in boldface at ~478 bp) was used in combination with the following bottom strands (mutated sites are underlined, and the *Sst*I site at ~933 bp is in boldface): a, 5′ TACCTCCG**GAGCTC**TATACCAT AGAGTAACAATTTCATGTTCGT 3′; b, 5′ TACCTCCG**GAGCTC**TATAC CATAGAGTAACAATTTCATG(A/G)GCGT; c, 5′ TACCTCCG**GAGCTC**TAT ACCATAGAGTAACAATTTCATG(A/G)GAGT. The 455-bp *Afl*II-*Sst*I PCR fragments thus obtained were subcloned into the corresponding sites in the *CDC28* sequence. The resulting mutated *CDC28* alleles were isolated as 1.2-kb *Asu*II-*Dra*II sequences and blunt end ligated to the *GAL1* promoter in a *URA3*-selectable *CEN* vector. The clones were sequenced in both orientations over the PCR-amplified region to ensure the absence of other mutations.

For tagging of *CDC28* mutant alleles (*cdc28-A169* and *cdc28-E169*) with *TRP1*, a 1.6-kb *Xho*I-*Dra*II fragment containing the mutant allele was first cloned into the Bluescript vector. By PCR, a *Bam*HI site was introduced close to the *Bsg*I site in the 3′ untranslated region. The *TRP1* gene was cloned into this newly created *Bam*HI site. A 2.4-kb fragment containing the tagged mutant allele was used to transform a wild-type diploid strain to obtain Trp^+ transformants. The heterozygous, Trp^+ transformants (confirmed by Southern blotting) were sporulated and dissected.

For tagging of *CDC28* mutant alleles (*cdc28-A169* and *cdc28-E169* with a triple c-myc epitope (G S S R G E Q K L I S E E D L N G E Q K L I S E E D L N G E Q K L I S E E D L N), a 1.6-kb *Xho*I-*Dra*II fragment containing the mutant allele was first cloned into a Bluescript vector. By PCR, a *Bam*HI site was introduced prior to the termination codon. The triple c-myc tag (*Bam*HI fragment) was cloned into the newly created *Bam*HI site. The resulting tagged mutant *CDC28* alleles (*cdc28-A169myc* and *cdc28-E169myc*) were isolated as 1.3-kb *Asu*II-*Dra*II fragments and blunt end ligated to the *GAL1* promoter in a *URA3*-selectable *CEN* vector. Both DNA strands of the PCR-amplified region were sequenced to ensure the absence of other mutations.

Other techniques. Yeast transformation was done by the lithium acetate method. Total RNA was isolated as described by Cross and Tinkelenberg (6), and Northern (RNA) blot analyses were performed as described by Price et al. (47). The method described by Nasmyth et al. (35) was used for immunofluorescence and photomicroscopy. To analyze DNA distribution by flow cytometry, cells were fixed in 70% ethanol overnight at 4°C, washed once with 0.2 M Tris-HCl (pH 7.5) containing 20 mM EDTA, and resuspended in the same buffer. Cells fixed in this way were treated with RNase (1 mg/ml) for 4 h at 37°C, washed once with phosphate-buffered saline (PBS), resuspended in 0.1 ml of a propidium iodide solution (50 μg/ml in PBS), and incubated overnight at 4°C. The cell suspension was diluted 10 times with PBS and sonicated before fluorescence-activated cell sorter analysis.

RESULTS

Kinase activity of *cdc28-E169* and *cdc28-A169* alleles. The threonine residue that occupies amino acid position 167 in

$p34^{cdc2}$ of *S. pombe* is conserved in many other CDKs (5). Phosphorylation of this residue is essential for the activation of cdc2-cyclin B kinase. An equivalent threonine is present at position 169 in Cdc28. To study the role of Thr-169 dephosphorylation in exit from mitosis, we first replaced Thr-169 with either glutamic acid (E) or alanine (A), which mimics either the constitutively phosphorylated or dephosphorylated state, respectively. To determine the mitotic kinase activity specifically associated with the mutant proteins, both alleles were tagged with a triple c-myc epitope. The tagged mutant alleles, driven by the *GAL1* promoter on a centromeric (*CEN*) vector, were introduced into a *cdc28-4* mutant which is defective in Start-related functions and exhibits low mitotic kinase activity, even at the permissive temperature (24°C). Log-phase cells growing at 24°C in raffinose medium (in which *GAL1* is inactive) were first arrested in mitosis with nocodazole treatment, and then one-half of the culture was induced by addition of galactose for 2 h. These cells, arrested prior to nuclear division, are expected to have Cdc28 predominantly in the form of mitotic kinase. The mutant Cdc28 mitotic kinase was immunoprecipitated from these cells by using either c-myc-specific antibodies or anti-Clb2 antibodies, and the kinase activities of the immunoprecipitates were measured. Whereas Cdc28-A169 showed little kinase activity in the immune complexes obtained with c-myc antibodies, Cdc28-E19 exhibited two- to threefold higher activity after 2 h of induction (Fig. 1A). A similar pattern was observed when total mitotic kinase activity was determined by using anti-Clb2 antibodies; there was no change in the kinase activity in cells expressing *cdc28-A169* after 2 h of induction, but expression of *cdc28-E169* caused a three- to fourfold increase (Fig. 1B). Thus, while replacement of Thr-169 with alanine renders Cdc28 inactive as a kinase, Cdc28-E169 retains its capacity to phosphorylate histone H1, suggesting that phosphorylation of Thr-169 is required for the activity of Cdc28-Clb2 kinase in *S. cerevisiae*. Although Cdc28-E169 exhibited kinase activity, it was a weak kinase in comparison with wild-type Cdc28 (Fig. 1C), implying that glutamic acid is a less-than-perfect substitution for a phosphorylated threonine.

Suppression of the *cdc28-1N* mutation by *cdc28-E169*. To determine if the *cdc28-E169* allele is mitotically active in vivo, we tested its ability to suppress the *cdc28-1N* mutation. *cdc28-1N* mutant cells, although normal with respect to Start, fail to progress through mitosis. When grown at the restrictive temperature, they arrest with a large bud, duplicated DNA, and an undivided nucleus with a short mitotic spindle (40, 59). Although unable to undergo nuclear division, these cells exhibit high H1 kinase activity at their arrest point (59). The *cdc28-A169*, *cdc28-S169*, and *cdc28-E169* alleles, driven either by the *GAL1* promoter or by their native promoter, were introduced into *cdc28-1N* mutants, and the resulting transformants were plated at 37°C. The growth of these cells was compared with that of the parental *cdc28-1N* strain. While expression of the *cdc28-S169* allele allowed the *cdc28-1N* mutant to grow almost as well as wild-type cells, mutant cells expressing Cdc28-A169 failed to grow at the restrictive temperature (Fig. 2A and B). Unlike Cdc28-A169, expression of the *cdc28-E169* allele from its native promoter was able to suppress the mitotic defect of the *cdc28-1N* mutant (Fig. 2B). However, suppression of the *cdc28-1N* mutation by *cdc28-E169* was partial since these cells, although able to divide and form colonies, were bigger and elongated. The extent of suppression was increased when the same allele was overexpressed from the *GAL1* promoter but was still somewhat poor compared with that achieved with the *cdc28-S169* allele (Fig. 2A). Nevertheless, these results suggest that the *cdc28-E169* allele is mitotically active in vivo.

Exit from mitosis is not affected by the *cdc28-E169* allele. It has been suggested that in both *S. pombe* and *Xenopus* egg extracts, exit from mitosis requires not only cyclin B destruction but also dephosphorylation of Thr-167–Thr-161 (24, 29). In *S. pombe*, the cdc2 protein, in which Thr-167 has been replaced with glutamic acid (*cdc2-E167*), behaves as a dominant mutation, such that cells expressing both wild-type cdc2 and cdc2-E167 accumulate as multinucleated and multiply septated cells (24). We were prompted to look more closely into the role of Thr-169 dephosphorylation in exit from mitosis by our observation that overexpression of *cdc28-E169* in otherwise wild-type budding yeast cells did not result in inhibition of growth. Although the cells expressing the mutant allele displayed moderately slower growth, no dramatic effect on either overall growth or cell morphology was detected.

We used the *cdc15* mutant to ascertain the effect of cdc28-E169 on exit from mitosis. When grown at the restrictive temperature, *cdc15* mutant cells arrest in late anaphase with a large bud and an elongated spindle stretching between the well-separated nuclei (48). The failure of this mutant to progress beyond telophase and enter the subsequent division cycle is presumably due to its inability to inactivate the mitotic kinase. Upon return to the permissive temperature (25°C), these cells synchronously exit mitosis by destroying the mitotic cyclins (58). *cdc15* cells carrying either *GAL-cdc28-E169* or *GAL-cdc28-A169* tagged with a c-myc epitope were allowed to enter the stationary phase by growth on raffinose medium for 72 h and then released in medium with or without galactose at the nonpermissive temperature. After they had uniformly arrested in telophase, cells were returned to the permissive temperature to resume the cell cycle progression in raffinose medium. The disappearances of anaphase spindles, mitotic cyclin Clb2, and the activity of mutant Cdc28 kinase in immunoprecipitates obtained by using anti-c-myc antibodies were simultaneously monitored as indicators of exit from mitosis. As expected, *cdc15* cells harboring the *cdc28-A169* allele showed no kinase activity throughout the course of the experiment since *cdc28-A169* is an inactive allele and is not anticipated to exhibit kinase activity (Fig. 3A). The anaphase spindles in these cells disappeared at the same rate in both raffinose and galactose media (Fig. 3A). In raffinose-grown cells, the level of Clb2 began to fall 20 min after the release and continued to fall until the cells were well into the next cycle (~80 min). The pattern was similar in galactose-induced cells, except that the disappearance of Clb2 was somewhat less dramatic. The appearance of the *RNR1* transcript, which normally occurs in late G_1 (14), at 40 min indicated that the cells had entered the next cycle (Fig. 3B).

The level of kinase activity in cells carrying *GAL-cdc28-E169* was high in galactose medium at 0 min but began to drop as the cells exited mitosis, reaching its minimum at 60 min (Fig. 4A). The fact that Cdc28-E169 mutant kinase activity was destroyed fairly normally suggests that dephosphorylation of Thr-169 is not necessary for inactivation of Cdc28 kinase activity. Despite the presence of Cdc28-E169, the rates of spindle dissolution, disappearance of Clb2, and reappearance of the *RNR1* transcript in these cells (Fig. 4A and B) were similar, if not identical, to those observed for cells either not expressing Cdc28-E169 because of growth in raffinose medium or expressing cdc28-A169 (Fig. 3A and B). This is also true of the DNA distribution profile (data not shown). However, like overexpression of Cdc28-A169, expression of *cdc28-E169* also seemed to slightly delay the disassembly of the anaphase spindles (Fig. 4A and B). Nevertheless, neither dissolution of the

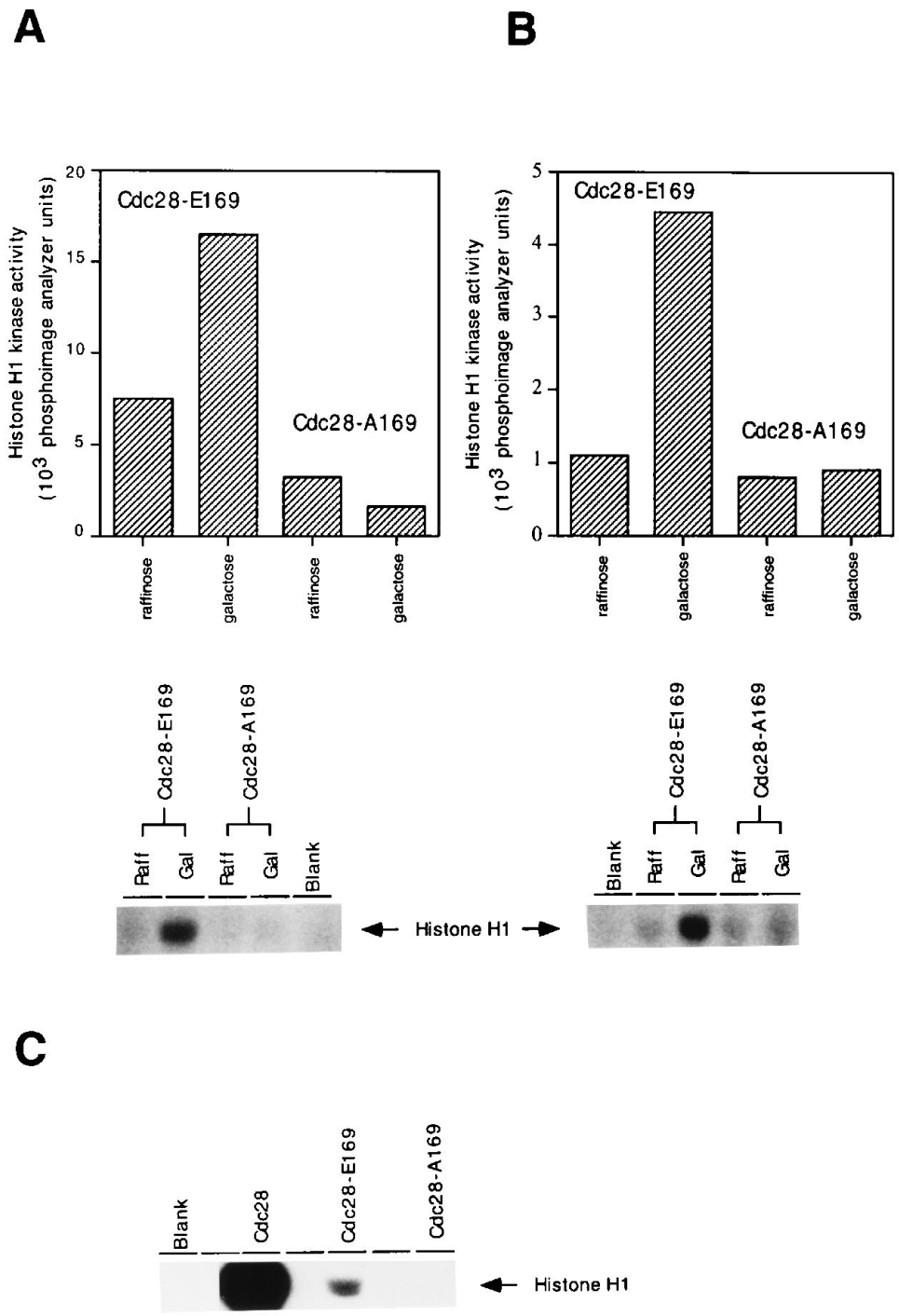

FIG. 1. Mitotic kinase activity of mutant proteins cdc28-A169 and cdc28-E169. A *cdc28-4* strain carrying either the *CDC28-myc*, the *cdc28-A169myc* or the *cdc28-E169myc* allele under the control of the *GAL1* promoter was grown to the exponential phase in YEP-raffinose medium at 25°C and treated with nocodazole (15 μg/ml). After 2 h, one-half of the culture was induced for a further 2 h by addition of galactose. Extracts were prepared, and the mitotic kinase was immunoprecipitated with either anti-c-myc (A) or anti-Clb2 (B) antibodies. The activities of the wild-type and mutant Cdc28 kinases expressed from the *GAL1* promoter and immunoprecipitated with anti-c-myc antibodies are shown for comparison (C). Histone H1 was used as the substrate to measure kinase activity, which was quantitated with a PhosphorImager (Molecular Dynamics).

mitotic spindles nor inactivation of mitotic kinase and re-entry into the next cycle was prevented by constitutive phosphorylation of Thr-169 as mimicked by Cdc28-E169. While alternative explanations exist, one interpretation of these results is that dephosphorylation of residue Thr-169 of Cdc28 is not a prerequisite for the destruction of kinase activity and, hence, exit from mitosis.

Both *cdc28-A169* and *cdc28-E169* alleles are Start inactive. Since our experiments showed that *cdc28-E169* can rescue the mitosis-defective *cdc28-1N* mutant, we wondered whether this

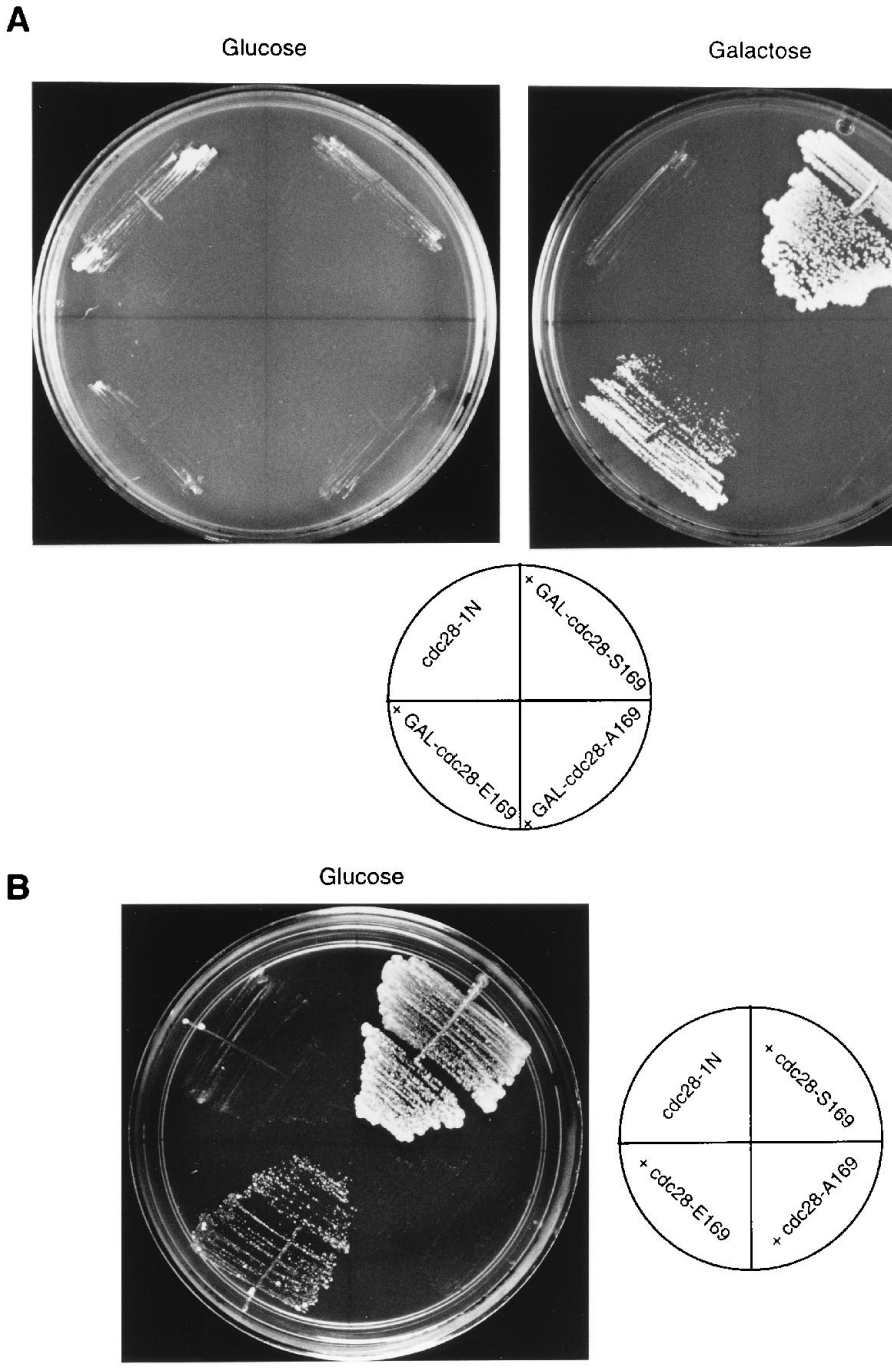

FIG. 2. Suppression of the *cdc28-1N* mutation by the *cdc28-S169*, *cdc28-A169*, and *cdc28-E169* alleles. A mitosis-defective *cdc28-1N* mutant was transformed with the mutant alleles driven either by the *GAL1* promoter (A) or by the native *CDC28* promoter (B). The transformants were plated on glucose- or galactose-containing plates prewarmed to 37°C. The plates were photographed after 2 days at 37°C.

allele can support progression through the entire cell cycle. To test this, we replaced one of the two copies of the *CDC28* gene in a diploid strain with either the *cdc28-A169* or the *cdc28-E169* allele. To monitor segregation during meiosis, the mutant alleles were marked by inserting the *TRP1* gene into the 3′ flanking region. The resulting heterozygous strains containing one wild-type copy and one mutated copy of *CDC28* were sporulated, and the haploid progeny were analyzed by tetrad dissection. In 14 asci dissected from a +/*cdc28-E169* heterozygous diploid, only two spores in each tetrad grew normally; the other two failed to give rise to colonies (data not shown). As all of the surviving segregants were unable to grow on plates lacking tryptophan, we inferred that the nonviable segregants harbored the *TRP1*-marked *cdc28-E169* allele. Identical results were obtained by dissection of the +/*cdc28-A169* diploid. It was difficult to ascertain the cell cycle stage at which the nonviable segregants arrested, since microscopic examination revealed that they had undergone two or three divisions. This

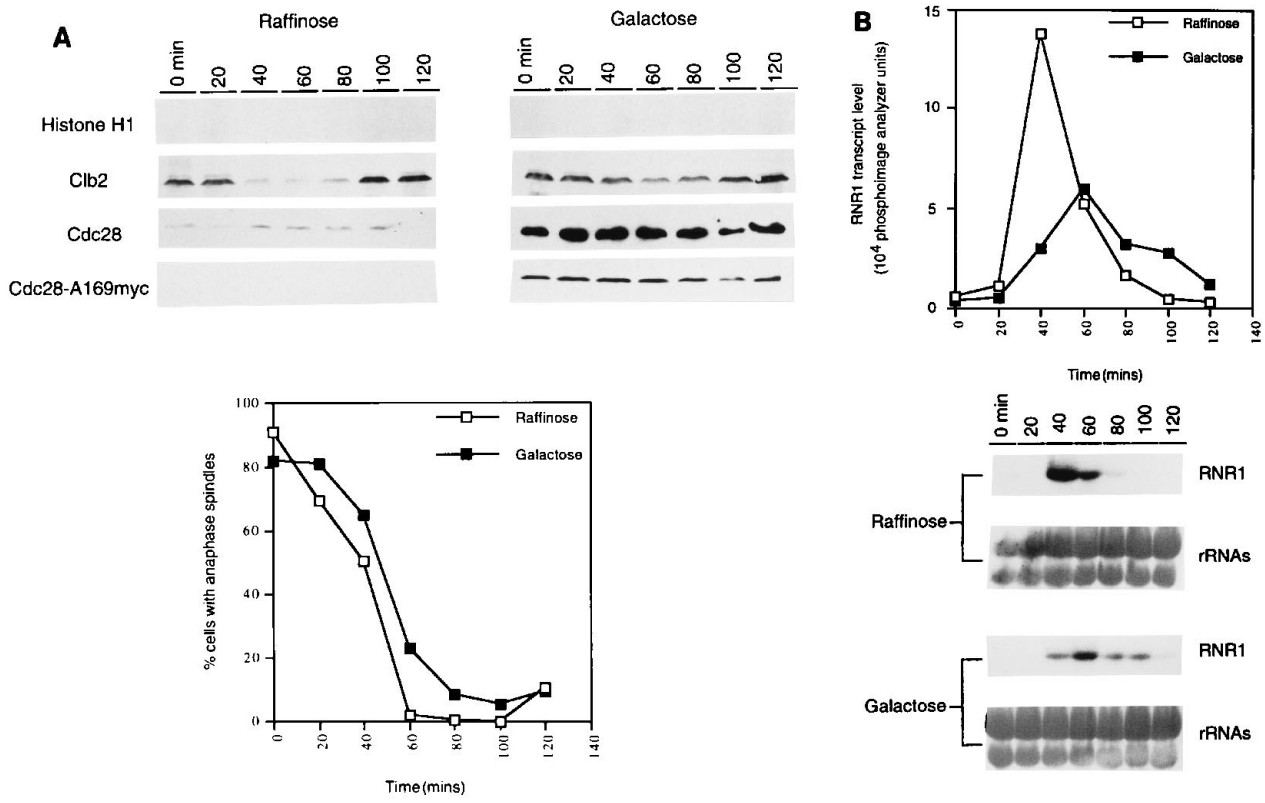

FIG. 3. Kinetics of exit from mitosis in *cdc15* cells expressing *cdc28-A169myc* from the *GAL1* promoter. Stationary-phase cells of the *cdc15* mutant carrying *GAL-cdc28-A169myc* were released into medium containing either raffinose or raffinose-galactose at 37°C. When >80% of the cells had arrested in late anaphase, they were filtered and allowed to resume cell cycle progression by resuspension in raffinose medium at 24°C. Samples were collected at 20-min intervals and analyzed. (A) Cdc28-A169myc kinase activity; amounts of Clb2, Cdc28, and Cdc28-A169myc proteins; and percentage of cells with late-anaphase spindles (graph). (B) *RNR1* transcript measured by Northern blotting in the same samples. A 30-μg sample of total RNA was loaded per well. Both kinase activity and the *RNR1* transcript were quantitated with a PhosphorImager (Molecular Dynamics).

might have been due to the residual wild-type Cdc28 protein inherited from the heterozygous diploid parent. Nevertheless, these results show that neither of the mutant alleles can support progression through the entire cell cycle.

We further tested the ability of the *cdc28-A169* and *cdc28-E169* alleles to complement the Start-defective *cdc28-4* mutant. The mutant alleles, either under the control of the *GAL1* promoter or driven by the *CDC28* promoter, were introduced into *cdc28-4* mutant cells, and the transformants were plated at 37°C. As shown in Fig. 5, while cells carrying the *cdc28-S169* allele grew normally at 37°C, both *cdc28-A169* and *cdc28-E169*, irrespective of the promoter they were expressed from, were unable to complement the *cdc28-4* mutant. Microscopic examination showed that these cells had arrested as unbudded cells, suggesting that mutant alleles *cdc28-A169* and *cdc28-E169* are defective in performing Start-related functions.

To determine whether the failure of mutant alleles to complement the *cdc28-4* mutant is due to their inability to form active G_1 kinase, a *cdc28-4* mutant carrying the *CLN2* gene tagged with an HA epitope was transformed with either wild-type *CDC28*, *cdc28-E169*, or *cdc28-A169* driven either by the native *CDC28* promoter or the *GAL1* promoter. The transformants were grown at either 24 or 37°C, and the Cln2-associated G_1 kinase was immunoprecipitated by using antibodies to the HA epitope. The kinase activity of the immunoprecipitates was measured by using histone H1 as the substrate. As expected, cells carrying the *CDC28* wild-type gene and *cdc28-S169* continued to grow and showed G_1 kinase activity at both temperatures, although the level of the activity was low at 37°C (Fig. 6A). Cells expressing the *cdc28-E169* and *cdc28-A169* alleles, on the other hand, arrested as unbudded G_1 cells at 37°C; fluorescence-activated cell sorter analysis showed that these cells had 1N DNA content (data not shown). Consistent with their arrest phenotype, the level of Cln2-associated G_1 kinase activity in these cells was very low. Cells overexpressing mutant alleles from the *GAL1* promoter also arrested in G_1 and showed very low levels of G_1 kinase activity (Fig. 6B). Taken together, these results strongly suggest that both the *cdc28-E169* and *cdc28-A169* alleles are defective in Start-related functions. It is surprising that both substitutions that mimic either the dephosphorylated or the phosphorylated form of Thr-169 are Start inactive. It is possible that threonine, in the unmodified form, at position 169 is important for Start execution. The fact that *cdc28-S169* complements the *cdc28-4* mutant well indicates that serine can serve the same function as threonine in the activation of G_1 kinase.

Cdc28-E169 is defective in its interaction with G_1 cyclin Cln2. It is puzzling that Cdc28-E169 exhibits detectable G_2 kinase activity but a very low level of G_1 kinase activity. One reason for this atypical characteristic may be that phosphorylation of Thr-169 (i.e., replacement of Thr-169 with glutamic acid in the present context) affects the interaction of Cdc28

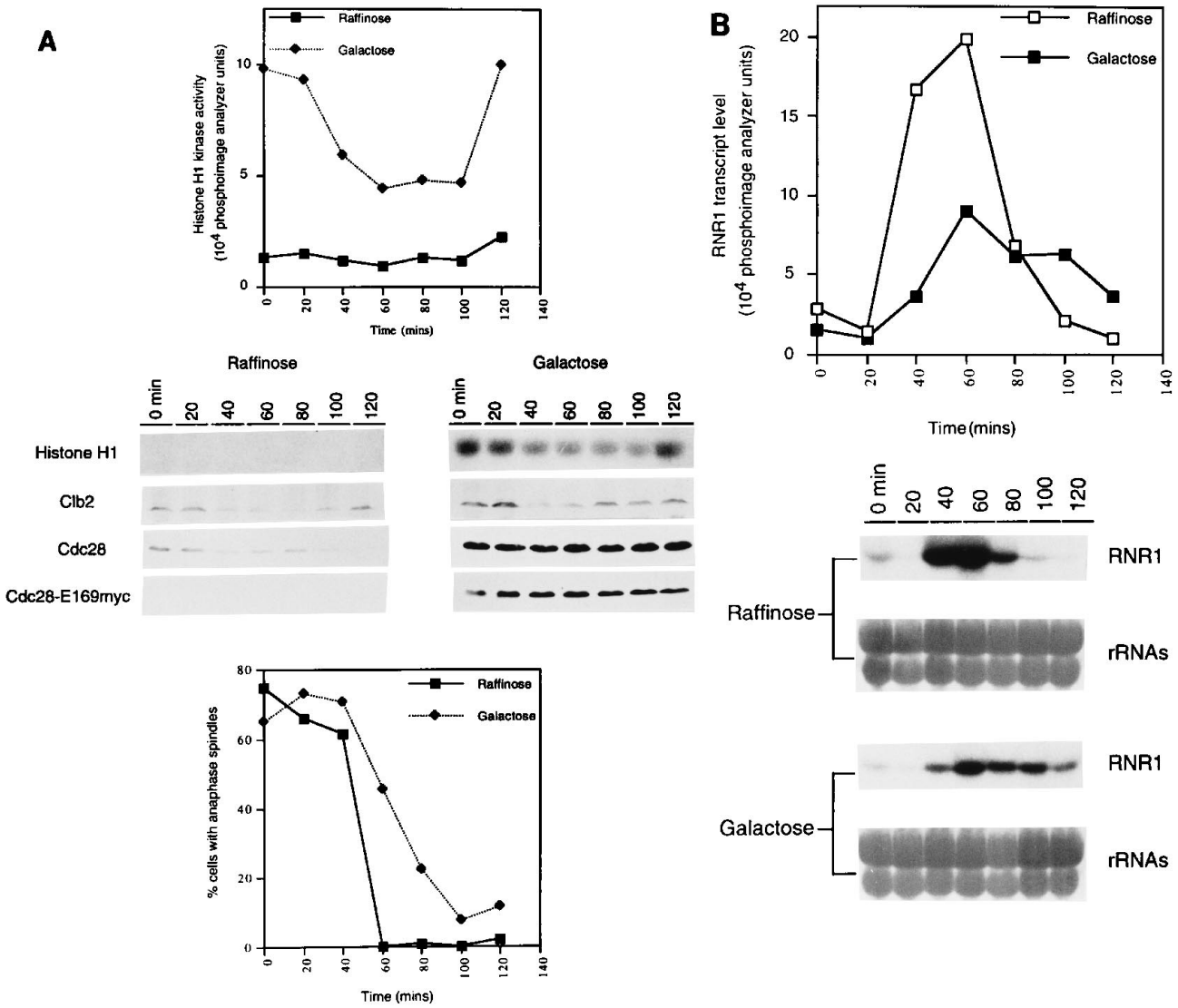

FIG. 4. Kinetics of exit from mitosis by *cdc15* cells expressing *cdc28-E169myc* from the *GAL1* promoter. *cdc15* mutant cells carrying *GAL-cdc28-E169myc* were grown and analyzed as described in the legend to Fig. 3. (A) Cdc28-E169myc kinase activity (upper graph); amounts of the Clb2, Cdc28, and Cdc28-E169myc proteins; and percentage of cells with late-anaphase spindles (lower graph). (B) *RNR1* transcript measured by Northern blotting in the same samples.

with G_1 cyclins but not with mitotic cyclins. To test this possibility, a single-copy vector carrying either *GAL-CDC28*, *GAL-cdc28-E169*, or *GAL-cdc28-A169* was introduced into a *cdc28-4* strain expressing HA-tagged Cln2. Cell extracts were prepared from strains grown in raffinose or galactose medium at 24°C, and Cdc28-Cln2-HA and Cdc28-Clb2 kinase complexes were immunoprecipitated by using anti-HA and anti-Clb2 antibodies, respectively. To determine whether Cdc28, Cdc28-E169, or Cdc28-A169 had coprecipitated with the G_1 or G_2 cyclins, components of the immunoprecipitates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with anti-Cdc28 antibodies. Very little Cdc28 is detected in the G_1 or mitotic kinase complexes isolated from cells grown in raffinose, suggesting that Cdc28-4 protein is defective in binding to both Cln2 and Clb2 cyclins (Fig. 7, raffinose lanes). While substantial Cdc28 was detected in the G_1 kinase complex isolated from cells expressing wild-type Cdc28 (Fig. 7, top and middle), little was detected in the immunoprecipitates from cells expressing Cdc28-E169 (Fig. 7, top panel). However, in the G_2 kinase complexes obtained by using anti-Clb2 antibodies, Cdc28 was coprecipitated from cells expressing either Cdc28-E169 or wild-type Cdc28 (Fig. 7, bottom). These data suggest that Cdc28-E169 is grossly defective in its interaction with Cln2 but not in its interaction with mitotic cyclin Clb2. This is consistent with the observation that Cdc28-E169 showed moderate mitotic kinase activity but no Cln2-associated G_1 kinase activity. The fact that Cdc28-A169 mutant kinase can bind Cln2 is also consistent with the notion that Thr-169 phosphorylation may interfere with the binding of Cdc28 to Cln2 (Fig. 7, middle). The ability of Cdc28-A169 to bind Clb2 (Fig. 7, bottom) is unexpected, since phosphorylation of T-loop threonine is proposed to be necessary for the association of CDKs with cyclins A and B (32, 43). Although able to interact with Clb2 and Cln2, this mutant kinase fails to

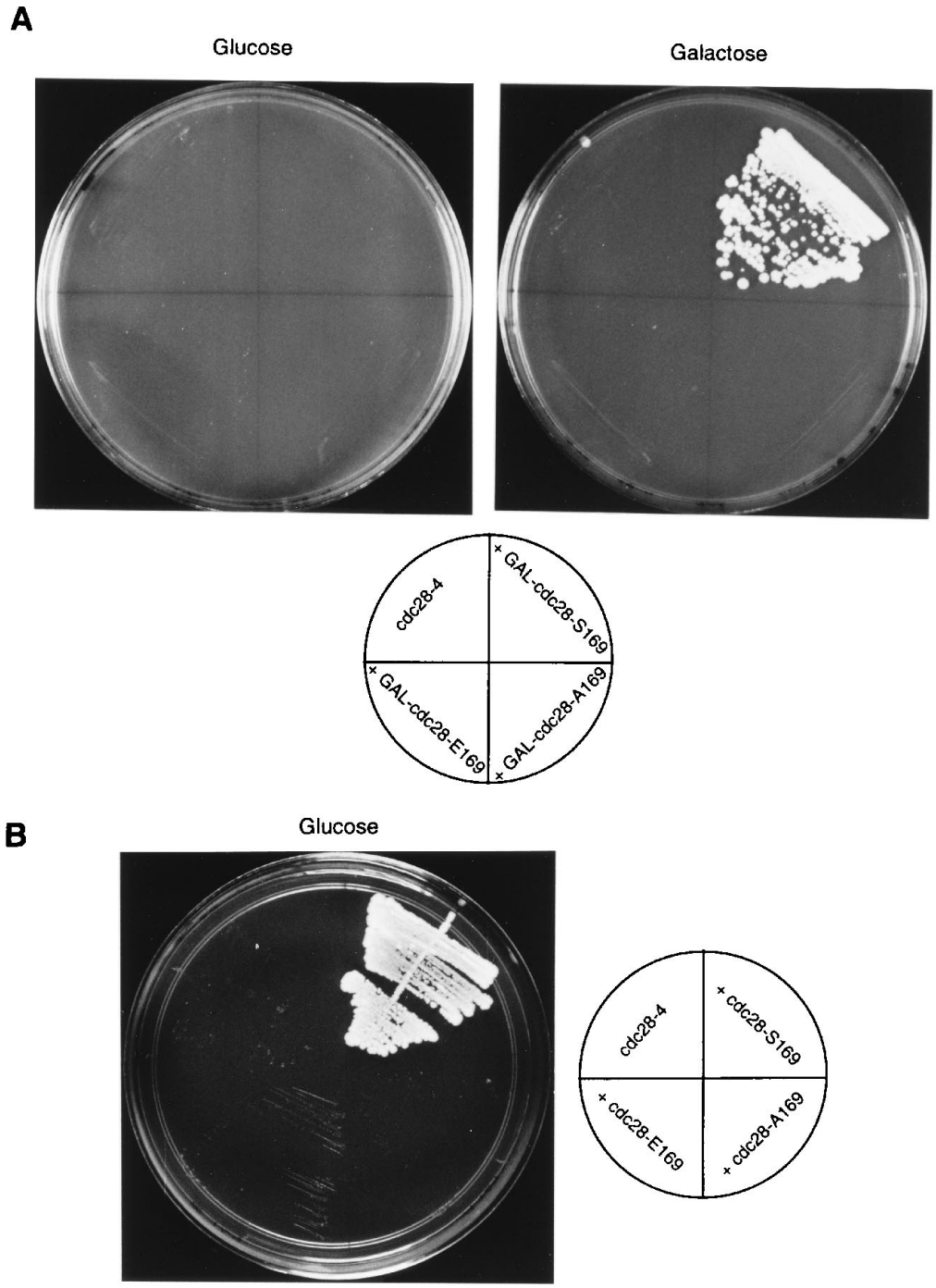

FIG. 5. Suppression of the *cdc28-4* mutation by the *cdc28-S169*, *cdc28-A169*, and *cdc28-E169* alleles. A Start-defective *cdc28-4* mutant was transformed with the mutant alleles driven either by the *GAL1* (A) or by the native *CDC28* (B) promoter. The transformants were plated on glucose- or galactose-containing plates prewarmed to 37°C. The plates were photographed after 2 days at 37°C.

exhibit any kinase activity (Fig. 1 and 6), implying that in addition to the physical association of Cdc28 with G_1 or mitotic cyclins, the appropriate state of the threonine residue at position 169 is critical for the kinase activity.

DISCUSSION

The monomeric form of cdc2, the prototype of CDKs, is inactive as a protein kinase. Its activation requires cyclin binding, which is then stabilized by phosphorylation of Thr-167 by CAK (9, 42). In the budding yeast *S. cerevisiae*, the role of Thr-169 phosphorylation in the activation or inactivation of Cdc28-Clb kinase has not been extensively examined. In one study, it was shown that Cdc28 is phosphorylated on Thr-169 as cells progress through the cell cycle but this phosphorylation is not dependent on the activity of Kin28, a putative homolog of the mammalian CAK (4). Since this residue in the T loop is highly conserved, it has been generally assumed that Thr-169

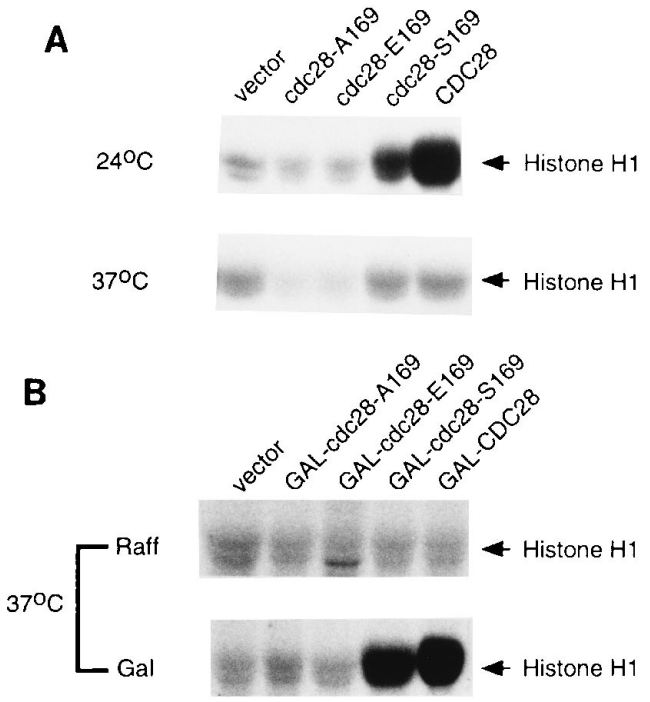

FIG. 6. G_1 kinase activity in a *cdc28-4* mutant expressing the *cdc28-A169*, *cdc28-E169*, or *cdc28-S169* allele. (A) *cdc28-4* mutant expressing Cln2-HA transformed with *cdc28* mutant alleles driven by the native *CDC28* promoter. The cells were grown in YEP medium plus glucose (YEPD) at 25°C until the log phase. One-half of the culture was then transferred to 37°C for 3 h, after which cells were harvested for preparation of cell extracts. The Cdc28-Cln2-HA complex was immunoprecipitated with anti-HA antibodies. Kinase assays were performed as described in Materials and Methods. (B) *cdc28-4* mutant expressing Cln2-HA transformed with *cdc28* mutant alleles driven by the *GAL1* promoter. The cells were grown in raffinose or galactose medium at 25°C until the log phase. The cultures were then transferred to 37°C for 3 h, after which the cells were harvested for preparation of cell extracts. Immunoprecipitation of the Cdc28-Cln2-HA complex and kinase assays were performed as described in Materials and Methods. The extracts from cells carrying the wild-type *CDC28* gene under the control of either the native or the *GAL1* promoter were used as the positive control.

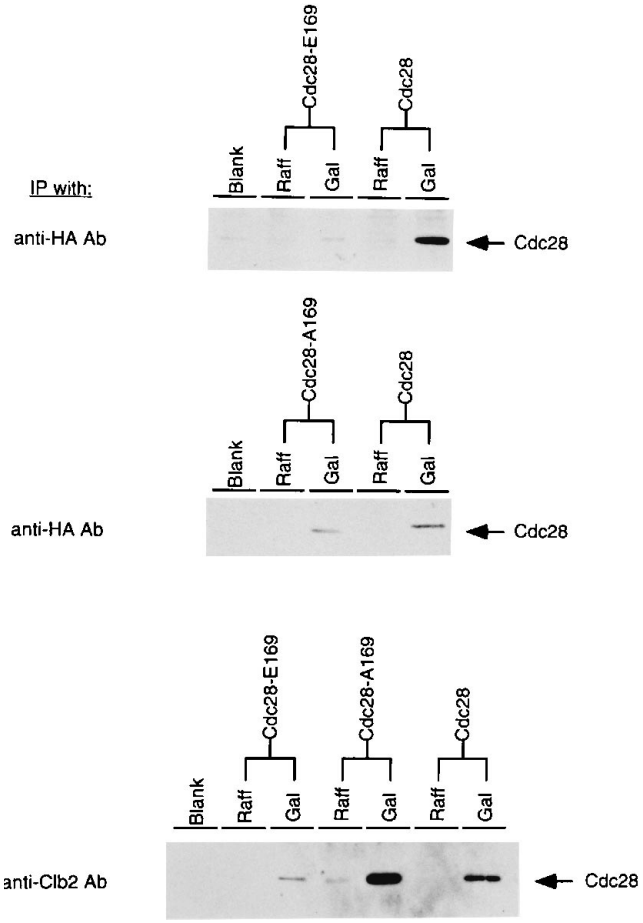

FIG. 7. Coimmunoprecipitation (IP) of Cdc28, Cdc28-E169, and Cdc28-A169 with Cln2-HA and Clb2 cyclins. *cdc28-4* cells expressing *CLN2-HA* and carrying either *GAL-CDC28*, *GAL-cdc28-E169*, or *GAL-cdc28-A169* were grown in raffinose (Raff) medium at 25°C until the log phase. One-half of the culture was then induced for 4 h by addition of galactose (Gal) (2%), and cell extracts were prepared. G_1 and mitotic kinase complexes were immunoprecipitated by using anti-HA (top and middle) and anti-Clb2 (bottom) antibodies (Ab), respectively. The immunoprecipitates were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The separated proteins were transferred to a nitrocellulose membrane and probed with anti-Cdc28 antibodies. The bands corresponding to Cdc28 are shown.

phosphorylation serves the same function in Cdc28 as that served by Thr-167 in cdc2 kinase. In this study, we investigated the effect of replacing Thr-169 with either alanine or glutamic acid on Cdc28 activity and exit from mitosis. Our data show that replacing Thr-169 with alanine renders Cdc28 completely inactive, since the *cdc28-A169* allele neither exhibits histone H1 kinase activity nor can it complement the *cdc28-4* and *cdc28-1N* mutants at the nonpermissive temperature (Fig. 1, 2, and 5). The *cdc28-E169* allele, on the other hand, shows histone H1 kinase activity and is capable of complementing a mitosis-defective *cdc28-1N* mutant (Fig. 1, 2, and 5). Consistent with the findings on *S. pombe*, these results imply that Thr-169 phosphorylation is important for the mitotic activity of Cdc28. It should be pointed out that although the *cdc28-E169* allele complements the *cdc28-1N* mutant, it does so rather poorly, suggesting that glutamic acid is not a perfect substitute for a phosphorylated threonine.

While activation of cdc2 by cyclin binding and Thr-167 phosphorylation promotes cell entry into and progression through the M phase, its inactivation is a prerequisite for exit from mitosis. One way cells inactivate mitotic kinase at the end of mitosis is by inducing rapid proteolytic destruction of cyclins associated with cdc2 (11, 18, 21, 26, 33). Studies on *S. pombe* and *Xenopus* egg extracts raised the possibility that in addition to cyclin degradation, dephosphorylation of Thr-167–Thr-161 may also be required for exit from mitosis (24, 29). For *S. pombe*, this conclusion was based on two separate observations: (i) that cdc2 undergoes Thr-167 dephosphorylation as cells progress through mitosis and (ii) that expression of the *cdc2-E167* allele, which mimics the cdc2 protein constitutively phosphorylated on the Thr-167 residue, leads to elongated, multiseptated, and multinucleated cells which eventually arrest with short spindles. Fission yeast cells expressing the human *cdc2-E161* allele also formed multiple septa (12). The chromosomes segregated abnormally and remained condensed in these cells. In *X. laevis*, it was demonstrated that cdc2 undergoes Thr-161 dephosphorylation following cyclin degradation. It was also shown that prevention of dephosphorylation by inhibition of type 1 and 2A phosphatases did not prevent cyclin destruction but interfered with the inactivation of maturation-promoting factor (29). The notion that both cyclin destruction and dephosphorylation of Thr-167–Thr-161 are required for exit from mitosis seems logical, as reversing the activation steps would be an effective way to inactivate the mitotic kinase.

However, in this report, we describe experiments which show that budding yeast cells expressing the *cdc28-E169* allele inactivate mitotic kinase, exit mitosis, and re-enter the subsequent cycle normally. *cdc28-E169*, although mitotically active, is deficient in Start-related functions, such as formation of G_1 kinase and bud emergence. The fact that the Cdc28-E169 kinase can be inactivated fairly normally suggests that dephosphorylation of Thr-169 is not a prerequisite for the destruction of mitotic kinase activity. In light of the studies on *S. pombe* and *Xenopus* egg extracts, our observation that the inability to dephosphorylate Thr-169 does not interfere with exit from mitosis in *S. cerevisiae* is intriguing. One explanation for this discrepancy could be that while major mitotic controls are well conserved in various organisms, *S. cerevisiae* may differ in some aspects. Such differences are exemplified by the behavior of the constitutively active *cdc28-F19* allele which, unlike in *S. pombe*, does not lead to premature mitosis in *S. cerevisiae* (1, 57).

On the other hand, this apparent disparity may stem from the criteria we used to monitor exit from mitosis. Since *S. cerevisiae* cells can proceed to a new division cycle prior to the completion of cytokinesis (48), we used the kinetics of Clb2 degradation, loss of Cdc28-Clb2 kinase activity, and disappearance of anaphase spindles as indicators of exit from mitosis. Reappearance of the *RNR1* transcript was taken to indicate entry into the subsequent cycle. The multinucleated phenotype of *S. pombe* cells expressing *cdc2-E167* (24) suggests that these cells had undergone multiple cell cycle rounds without cytokinesis. The presence of short spindles when these cells finally arrested is suggestive of a defect prior to nuclear division. Thus, the multinucleated phenotype caused by the expression of *cdc2-E167* in *S. pombe* could be interpreted as being due not to the failure of cells to exit mitosis per se and re-enter the next cycle but to their inability to complete some aspects of nuclear division and cytokinesis.

Expression of mutant *cdc28* alleles in *S. cerevisiae* does seem to affect the rate of cyclin destruction, since the Clb2 destruction in cells expressing these alleles was somewhat less dramatic (Fig. 3A and 4A, compare galactose and raffinose samples). It is not clear what aspect of cyclin destruction is affected by the mutant proteins. However, this moderate extent of Clb2 degradation appears to be sufficient to inactivate the mitotic kinase, permitting cells to exit mitosis, although the activity of other players, such as the CDK inhibitor Sic1, which is known to act at this stage, may also contribute (8). Overexpression of the mutant alleles also caused a delay in the maximum expression of *RNR1* (Fig. 3B and 4B), suggesting that the mutant kinase interferes in some way with the Start activity of wild-type Cdc28.

Both *S. pombe* and *Xenopus* studies show that Thr-167–Thr-161 is dephosphorylated either during or at the end of mitosis (23, 29). Given the extensive similarity in the modes of cdc2 and Cdc28 regulation, it is not unreasonable to expect that similar changes in phosphorylation of Thr-169 of Cdc28 occur during the cell cycle. If Thr-167–Thr-161 dephosphorylation is not required for exit from mitosis, as our results imply, then what purpose does it serve? One possibility is that the association of Cdc28 with G_1 cyclins after the completion of mitosis requires dephosphorylation of Thr-167–Thr-161. We found that the *cdc28-E169* allele, although mitotically active, failed to complement the Start-defective *cdc28-4* mutant. *cdc28-4* cells expressing *cdc28-E169* arrested as unbudded G_1 cells and exhibited very low Cln2-associated G_1 kinase activity (Fig. 6). Furthermore, our coimmunoprecipitation experiments suggest that Cdc28-E169, although normal with respect to its interaction with Clb2, is defective in its association with Cln2 (Fig. 7). These findings imply that Cdc28 phosphorylated at Thr-169 may be Start incompetent because of its failure to interact effectively with G_1 cyclins. The Start inactivity of this allele indicates that phosphorylation of Thr-169, while necessary for mitotic kinase activity, may interfere with the formation of an active Cdc28-Cln complex. Given that cyclins A and B are more similar to each other than to G_1 cyclins (27), it is possible that the nature of the interactions that lead to CDK activation by G_1 cyclins is different from that suggested by the structure of the CDK2-cyclin A complex (or predicted for the cdc2-cyclin B complex) (29, 45).

Thus, the purpose of the Thr-169 dephosphorylation that occurs upon completion of the cell cycle may be to convert Cdc28 from a mitotically active form to a Start-efficient form. If this is true, then G_1 cyclin-associated cdc2-Cdc28 must remain unphosphorylated on Thr-167–Thr-169, unlike the mitotic form. This prediction has not been tested. It had been shown that KAP (also called Cdi1), a Cdk-associated phosphatase, can dephosphorylate Thr-160 in monomeric Cdk2 (44). It is conceivable that a similar phosphatase could dephosphorylate cdc2-Cdc28 Thr-169 following Clb2 proteolysis, preventing any formation of an active mitotic kinase complex until after cells have traversed Start. Although there is some evidence that Thr-160 phosphorylation on Cdk2 increases during the S and G_2 phases (25), the state of threonine phosphorylation in G_1-S kinase is not known. It is also unknown if CAK is necessary for activation of G_1-S kinase. Only detailed analysis of the CDK-G_1 cyclin complex will provide insight into the role dephosphorylation of the T-loop threonine plays after cells have exited mitosis.

ACKNOWLEDGMENTS

We thank Pamela Ong for excellent technical assistance; Paramjeet Singh, Bor Luen Tang, and Jin Ngee Chia for oligonucleotide syntheses; Bruce Futcher for providing the *CLN2-HA* gene; John Kilmartin for anti-tubulin antibodies; and Catherine Pallen for critical reading of the manuscript. We are grateful to Marcel Doree for valuable discussion and advice.

This work was supported by the National Science and Technology Board, Singapore.

REFERENCES

1. **Amon, A., U. Surana, I. Muroff, and K. Nasmyth.** 1992. Regulation of $p34^{CDC28}$ tyrosine phosphorylation is not required for entry into mitosis in *S. cerevisiae*. Nature (London) **355:**368–371.
2. **Atherton-Fessler, S., G. Hannig, and H. Piwnica-Worms.** 1993. Reversible tyrosine phosphorylation and cell cycle control. Semin. Cell Biol. **4:**433–442.
3. **Brown, A. J., T. Jones, and J. Shuttleworth.** 1994. Expression and activity of $p40^{MO15}$, the catalytic subunit of CDK-activating kinase, during *Xenopus* oogenesis and embryogenesis. Mol. Biol. Cell **5:**921–932.
4. **Cismowski, M. J., G. M. Laff, M. J. Solomon, and S. I. Reed.** 1995. *KIN28* encodes a C-terminal domain kinase that controls mRNA transcription in *Saccharomyces cerevisiae* but lacks cyclin-dependent kinase-activating kinase (CAK) activity. Mol. Cell. Biol. **15:**2983–2992.
5. **Clarke, P. R.** 1995. CAK-handed kinase activation. Curr. Biol. **5:**40–42.
6. **Cross, F., and A. H. Tinkelenberg.** 1991. A positive feedback loop controlling CLN1 and CLN2 gene expression at the START of yeast cell cycle. Cell **65:**875–883.
7. **De Bond, H. L., J. Rosenblatt, J. Jancarik, H. D. Jones, D. O. Morgan, and S. H. Kim.** 1993. Crystal structure of cyclin-dependent kinase 2. Nature (London) **363:**595–602.
8. **Donovan, J. D., J. H. Toyn, A. L. Johnson, and L. H. Johnston.** 1994. $P40^{SDB25}$, a putative CDK inhibitor, has a role in the M/G1 transition in *Saccharomyces cerevisiae*. Genes Dev. **8:**1640–1653.
9. **Doree, M., and S. Galas.** 1994. The cyclin-dependent protein kinases and the control of cell division. FASEB J. **8:**1114–1121.
10. **Draetta, G.** 1993. cdc2 activation: the interplay of cyclin binding and Thr161 phosphorylation. Trends Cell Biol. **3:**287–289.
11. **Draetta, G., F. Luca, J. Westerndorf, L. Brizuela, J. Ruderman, and D. Beach.** 1988. cdc2 protein kinase is complexed with both cyclin A and B: evidence for proteolytic inactivation of MPF. Cell **56:**829–838.
12. **Ducommum, B., P. Brambilla, M.-A. Felix, B. R. Franza, Jr., E. Karsenti,**

and G. Draetta. 1991. cdc2 phosphorylation is required for its interaction with cyclin. EMBO J. **11:**3311–3319.
13. **Dunphy, W. G.** 1994. The decision to enter mitosis. Trends Cell Biol. **4:**202–207.
14. **Elledge, S. J., and R. W. Davis.** 1990. Two genes differentially regulated in the cell cycle and by DNA-damaging agents encode alternative regulatory subunits of ribonucleotide reductase. Genes Dev. **4:**740–751.
15. **Epstein, C. B., and F. Cross.** 1992. CLB5: a novel B type cyclin from yeast with a role in S phase. Genes Dev. **6:**1695–1706.
16. **Espinoza, F. H., J. Ogas, I. Herskowitz, and D. Morgan.** 1994. Cell cycle control by a complex of the cyclin HCS26(PCL1) and the kinase PHO85. Science **266:**1388–1395.
17. **Feaver, W. J., J. Q. Svejstrup, N. L. Henry, and R. D. Kornberg.** 1994. Relationship of CDK-activating kinase and RNA polymerase II CTD kinase TFIIH/TFIIK. Cell **79:**1103–1109.
18. **Felix, M. A., J. C. Labbe, M. Doree, T. Hunt, and E. Karsenti.** 1990. Triggering of cyclin degradation in interphase extracts of amphibian eggs by cdc2 kinase. Nature (London) **346:**379–382.
19. **Fesquet, D., J. C. Labbe, J. Derancourt, J. P. Capony, S. Galas, F. Girard, T. Lorca, J. Shuttleworth, M. Doree, and J. C. Cavadore.** 1993. The MO15 gene encodes the catalytic subunit of a protein kinase that activates cdc2 and other cyclin-dependent kinases (CDKs) through phosphorylation of Thr161 and its homologues. EMBO J. **12:**3111–3121.
20. **Fleig, U. N., and K. L. Gould.** 1991. Regulation of cdc2 activity in *Schizosaccharomyces pombe*: the role of phosphorylation. Semin. Cell Biol. **2:**195–204.
21. **Ghiara, J. B., H. E. Richardson, K. Sugimoto, M. Henze, D. J. Lew, C. Wittenberg, and S. I. Reed.** 1991. A cyclin B homolog in *S. cerevisiae*: chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. Cell **65:**163–174.
22. **Glotzer, M., A. W. Murray, and M. Kirschner.** 1991. Cyclin is degraded by ubiquitin pathway. Nature (London) **349:**132–138.
23. **Gould, K., and P. Nurse.** 1989. Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature (London) **39:**39–45.
24. **Gould, K. L., S. Moreno, D. J. Owen, S. Sazar, and P. Nurse.** 1991. Phosphorylation at Thr167 is required for *Schizosaccharomyces pombe* $p34^{cdc2}$ function. EMBO J. **10:**3297–3309.
25. **Gu, Y., J. Rosenblatt, and D. O. Morgan.** 1992. Cell cycle regulation of CDK2 activity by phosphorylation of Thr160 and Tyr15. EMBO J. **11:**3995–4005.
26. **Holloway, S. L., M. Glotzer, R. W. King, and A. W. Murray.** 1993. Anaphase is initiated by proteolysis rather than by the inactivation of maturation-promoting factor. Cell **73:**1393–1402.
27. **Hunt, T.** 1991. Cyclins and their partners: from a simple idea to complicated reality. Semin. Cell Biol. **2:**213–222.
28. **Jeffrey, P. D., A. A. Russo, K. Polyak, E. Gibbs, J. Hurwitz, J. Massague, and N. P. Pavletich.** 1995. Mechanism of CDK activation revealed by the structure of a cyclin A-CDK2 complex. Nature (London) **376:**313–320.
29. **Lorca, T., J. C. Labbe, A. Devault, D. Fesquet, J. P. Capony, J. C. Cavadore, F. Le Bouffant, and M. Doree.** 1992. Dephosphorylation of cdc2 on threonine 161 is required for cdc2 inactivation and normal anaphase. EMBO J. **11:**2381–2390.
30. **Lundgren, K., N. Walworth, R. Booher, M. Dembski, M. Kirschner, and D. Beach.** 1991. mik1 and wee1 cooperate in the inhibitory tyrosine phosphorylation of cdc2. Cell **64:**1111–1122.
31. **Mendenhall, M. D.** 1993. An inhibitor of p34CDC28 protein kinase activity from *Saccharomyces cerevisiae*. Science **259:**216–219.
32. **Morgan, D. O.** 1995. Principles of CDK regulation. Nature (London) **374:**131–134.
33. **Murray, A. W., M. Solomon, and M. Kirschner.** 1989. The role of cyclin synthesis and degradation in the control of maturation promoting factor activity. Nature (London) **339:**280–286.
34. **Nasmyth, K.** 1993. Control of the yeast cell cycle by the Cdc28 protein kinase. Curr. Opin. Cell Biol. **5:**166–179.
35. **Nasmyth, K., G. Adolf, D. Lydall, and A. Seddon.** 1990. The identification of a second cell cycle control on the *HO* promoter in yeast: cell cycle regulation of SWI5 nuclear entry. Cell **62:**631–647.
36. **Nigg, E. A.** 1993. Cellular substrates of p34cdc2 and its companion cyclin-dependent kinases. Trends Cell Biol. **3:**296–301.
37. **Norbury, C., and P. Nurse.** 1993. Animal cell cycles and their controls. Annu. Rev. Biochem. **61:**441–470.
38. **Nurse, P., and Y. Bisset.** 1981. Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. Nature (London) **292:**558–560.
39. **Peters, M., and I. Herskowitz.** 1994. Direct inhibition of the yeast cyclin-dependent kinase Cdc28-Cln by Far1. Science **265:**1388–1395.
40. **Piggott, J. R., R. Rai, and B. L. A. Carter.** 1982. A bifunctional gene product involved in two phases of the yeast cell cycle. Nature (London) **298:**391–393.
41. **Pines, J.** 1993. Clear as crystal? Curr. Biol. **3:**544–547.
42. **Pines, J.** 1994. Protein kinases and cell cycle control. Semin. Cell Biol. **5:**399–408.
43. **Pines, J.** 1995. Confirmational change. Nature (London) **376:**294–295.
44. **Poon, R. Y. C., and T. Hunter.** 1995. Dephosphorylation of Cdk2 Thr160 by the cyclin-dependent kinase-interacting phosphatase KAP in the absence of cyclin. Science **270:**90–93.
45. **Poon, R. Y. C., K. Yamashita, J. P. Adamczewski, T. Hunt, and J. Shuttleworth.** 1993. The cdc2-related protein $p40^{MO15}$ is the catalytic subunit of a protein kinase that can activate $p33^{Cdk2}$ and $p34^{cdc2}$. EMBO J. **12:**3123–3132.
46. **Poon, R. Y. C., K. Yamashita, M. Howell, M. A. Ershler, A. Belyavsky, and T. Hunt.** 1994. Cell cycle regulation of the $p34^{cdc2}$/$p33^{Cdk2}$-activating kinase $p40^{MO15}$. J. Cell Sci. **107:**2789–2799.
47. **Price, C., K. Nasmyth, and T. Schuster.** 1991. A general approach to the isolation of cell cycle-regulated genes in the budding yeast *Saccharomyces cerevisiae*. J. Mol. Biol. **218:**543–556.
48. **Pringle, J. R., and L. H. Hartwell.** 1981. The *Saccharomyces cerevisiae* cell cycle, p. 97–142. *In* J. N. Strathern, E. W. Jones, and J. R. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: life cycle and inheritance. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
49. **Richardson, H. E., C. Wittenberg, F. Cross, and S. Reed.** 1989. An essential G1 function for cyclin-like proteins in yeast. Cell **59:**1127–1133.
50. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
51. **Schwob, E., T. Bohm, M. D. Mendenhall, and K. Nasmyth.** 1994. The B-type cyclin kinase inhibitor p40SIC1 controls the G1 to S transition in *S. cerevisiae*. Cell **79:**233–244.
52. **Schwob, E., and K. Nasmyth.** 1993. CLB5 and CLB6, a new pair of B cyclins involved in S phase and mitotic spindle formation in *S. cerevisiae*. Genes Dev. **7:**1160–1175.
53. **Sherr, C. J.** 1993. Mammalian G1 cyclins. Cell **73:**1059–1065.
54. **Sherr, C. J., and J. Roberts.** 1995. Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. **9:**1149–1163.
55. **Simon, M., S. Bertrand, and G. Faye.** 1986. *Kin28*, a yeast split gene coding for a putative protein kinase homologue to *CDC28*. EMBO J. **5:**2697–2701.
56. **Solomon, M., J. Wade-Harper, and J. Shuttleworth.** 1993. CAK, the $p34^{cdc2}$ activating kinase, contains a protein identical or closely related to $p40^{MO15}$. EMBO J. **12:**3133–3142.
57. **Sorger, P. K., and A. W. Murray.** 1992. S-phase feedback control in budding yeast is independent of tyrosine phosphorylation of $p34^{CDC28}$. Nature (London) **355:**365–368.
58. **Surana, U., A. Amon, C. Dowzer, J. McGrew, B. Byers, and K. Nasmyth.** 1993. Destruction of the CDC28/CLB mitotic kinase is not required for the metaphase to anaphase transition in budding yeast. EMBO J. **12:**1969–1978.
59. **Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A. B. Futcher, and K. Nasmyth.** 1991. The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. Cell **65:**145–161.
60. **Tyers, M., G. Tokiwa, R. Nash, and B. Futcher.** 1992. The Cln3-Cdc28 kinase complex of *S. cerevisiae* is regulated by proteolysis and phosphorylation. EMBO J. **11:**1773–1784.