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Saccharomyces cerevisiae G_1 Cyclins Differ in Their Intrinsic Functional Specificities

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The three budding yeast *CLN* genes appear to be functionally redundant for cell cycle Start: any single *CLN* gene is sufficient to promote Start, while the *cln1 cln2 cln3* triple mutant is Start defective and inviable. Both quantitative and apparently qualitative differences between *CLN* genes have been reported, but available data do not in general allow distinction between qualitative functional differences as opposed to simply quantitative differences in expression or function. To determine if there are intrinsic qualitative differences between Cln proteins, we compared *CLN2*, *CLN3*, and crippled (but still partially active) *CLN2* genes in a range of assays that differentiate genetically between *CLN2* and *CLN3*. The results suggest that different potencies of Cln2, Cln3, and Cln2 mutants in functional assays cannot be accounted for by a simple quantitative model for their action, since Cln3 is at least as active as Cln2 and much more active than the Cln2 mutants in driving Swi4/Swi6 cell cycle box (SCB)-regulated transcription and cell cycle initiation in *cln1 cln2 cln3 bck2* strains, but Cln3 has little or no activity in other assays in which Cln2 and the Cln2 mutants function. Differences in Cln protein abundance are unlikely to account for these results. Cln3-associated kinase is therefore likely to have an intrinsic in vivo substrate specificity distinct from that of Cln2-associated kinase, despite their functional redundancy. Consistent with the idea that Cln3 may be the primary transcriptional activator of *CLN1*, *CLN2*, and other genes, the activation of *CLN2* transcription was found to be sensitive to the gene dosage of *CLN3* but not to the gene dosage of *CLN2*.

The Start transition in the *Saccharomyces cerevisiae* cell cycle requires the activity of one of three cyclin homologs encoded by the *CLN1*, *CLN2*, and *CLN3* gene family, complexed with the cyclin-dependent kinase encoded by the *CDC28* gene. Although the *CLN* genes are functionally redundant for cell cycle Start (39), the *CLN3* gene differs sharply from the *CLN1-CLN2* gene pair in structure and regulation (see reference 7 for a review). Searches for mutations resulting in lethality in strains deficient in *CLN1* and *CLN2* have yielded mutations in a wide range of genes, with widely varying lethal phenotypes. The general conclusion from this result has been that Cln1 and Cln2 are potent at execution of various cell biological processes associated with Start (e.g., cell polarization [4, 12] and septin ring formation [4, 18]), while Cln3 is relatively weak at activating these processes. Mutations in one gene (*BCK2*) are lethal in the absence of *CLN3* but not in the absence of *CLN1* and *CLN2*. The latter defect is significantly but not completely rescued by placing *CLN2* under the control of heterologous promoters (14, 20), as Bck2 is required for efficient transcriptional activation of *CLN1* and *CLN2* in the absence of *CLN3*. This result is consistent with other data which suggest that Cln3 is the main or only physiological activator of *CLN1* and *CLN2* transcription (15, 43, 44). These results have led to a picture in which Cln3 predominantly functions to activate *CLN1* and *CLN2* transcription, while Cln1 and Cln2 carry out various Start-specific processes; Cln3 may be able to carry out these processes only with the aid of various accessory pathways. Other cyclins (the *CLB5-CLB6* pair of B-type cyclins and the *PCL1-PCL2* pair of cyclins that activate the Pho85 kinase) are also essential in the absence of *CLN1* and *CLN2* (see reference 7 for a review), suggesting that they may, like *CLN3*, have accessory or backup roles for the functions normally performed by *CLN1* and *CLN2*.

The results summarized above can be interpreted in two different ways. (i) Cln3-Cdc28 could simply be a weak, low-abundance kinase with no intrinsic difference in specificity compared to other cyclin-Cdc28 complexes; its biological role as an activator of *CLN1,2*, *CLB5,6*, and *PCL1,2* transcription may come about solely because the *CLN3* promoter allows expression in early G_1 at a time when other more potent cyclins are not expressed. (ii) Cln3-Cdc28 could have intrinsic differences in specificity of action compared to Cln1,2-Cdc28 or Clb5,6-Cdc28, resulting in potent activity for a subset of Start-specific processes (probably including transcriptional activation) but an inability to execute other processes. Because most of the experiments performed previously and summarized above have not controlled for differences in expression or abundance of Cln3 and Cln1 or Cln2, it is difficult to choose between these two explanations. Here we investigate the qualitative differences between Cln2 and Cln3 by normalizing their expression and assaying for function both in vivo and in vitro. We also analyze the effects of alteration of *CLN2* and *CLN3* gene dosage on timing of bud emergence and *CLN2* expression.

MATERIALS AND METHODS

Plasmid constructions. The *CLN2* coding sequence with the triple-hemagglutinin (HA) tag (*CLN2C* [44]) was obtained from M. Tyers (MT104). The *CLN2* coding sequence under control of the *CLN3* promoter (*CLN2-3P* [46]) was obtained from C. Wittenberg (pHV104). These constructs were recombined in the low-copy-number vector RS314 (42) as follows. The HV104 *Sal*I-*Spe*I fragment from the *CLN3* promoter to the *Spe*I site in the *CLN2* coding sequence was subcloned into *Sal*I-*Spe*I-digested RS314. The *Bam*HI site at the *CLN3* promoter-*CLN2* junction was converted to a *Cla*I site by *Bam*HI digestion, Klenow fragment fill-in, and ligation. The MT104 *Spe*I-*Hpa*I fragment from the *CLN2C* coding sequence to the *Hpa*I site in the adjacent *LEU2* gene was subcloned into the above plasmid cut with *Not*I, blunted with Klenow fragment, and cut with *Spe*I. The resulting plasmid was named pT411 (*CLN2C* coding sequence under control of the *CLN3* promoter, in a low-copy-number *TRP1* vector). The *CLN3*

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TABLE 1. Strains used[a]

Strain	Genotype
1255-5C	*MAT***a** *CLN1 CLN2 CLN3*
1254-14D	*MAT***a** *cln1-del cln2-del cln3-del leu2*::*LEU2*::*GAL1*::*CLN1*
1456-10	*MAT***a** *cln1-del cln2-del cln3-del swi4*::*LEU2 pURA3*/*GAL1*::*CLN1*
2866-9D	*MAT***a** *cln1-del cln2-del cln3 del swi6*::*LEU2 pURA3*/*GAL1*::*CLN1*
1587-7	*MAT*α *cln1-del cln2-del CLN3*$^+$ *clb5*::*ARG4 clb6*::*ADE1 leu2*::*LEU2*::*GAL1*::*CLN1 arg4*
1238-11A-7	*MAT***a** *cln1-del cln2-del CLN3*$^+$ *URA3*::*GAL1*::*SIC1*
1591-10D	*cln1-del cln2-del cln3-del pcl1*::*HIS3 pcl2*::*URA3 leu2*::*LEU2*::*GAL1*::*CLN1 his3*
1472-24D	*MAT*α *cln1-del cln2-del cln3-del bud2*::*URA3 leu2*::*LEU2*::*GAL1*::*CLN1*
1457-9D	*MAT*α *cln1-del cln2-del cln3-del bck2*::*ARG4 pURA3*/*GAL1*::*CLN1 arg4*
1446-10B	*cln1-del cln2-del cln3-del bck2*::*ARG4 leu2*::*LEU2*::*GAL1*::*CLN3 arg4*

[a] All strains are congenic with BF264-15D: *MAT***a** *trp1 leu2 ura3 ade1 his2.*

coding sequence with the triple-HA tag (*CLN3C* [45]) was obtained from M. Tyers (BF030CW). BF030CW was used as template to amplify the *CLN3C* coding sequence in a thermal cycling reaction using Vent polymerase (New England Biolabs) according to the manufacturer's instructions. Vent polymerase is a high-fidelity DNA polymerase with a reported error rate of less than 1 in 10^4 bp; we therefore consider it unlikely that mutations were introduced during amplification. The resulting product was digested with *Cla*I (introduced upstream of the *CLN3* ATG at the same position as the *Cla*I site in pT411 relative to the *CLN2* ATG) and *Bam*HI (cutting in the epitope tag) and subcloned into pT411 digested with *Bam*HI and *Cla*I, producing pKL001 (identical to pT411 but with *CLN3C* instead of *CLN2C* under control of the *CLN3* promoter). The insert from pKL001 from *Sal*I to *Sac*II (cutting in polylinker) was transferred to *Sal*I-*Sac*II-digested RS424 (5), producing a high-copy-number (2μm origin) *CLN3C* plasmid, pKL034. The *GAL1* promoter was amplified by thermal cycling using plasmid BM272 (from M. Johnston) as the template. The product was digested with *Sal*I (from pBR322 vector sequence) and *Cla*I (introduced in oligonucleotide primer) and subcloned into pKL001 digested with *Sal*I and *Cla*I, to produce pKL002 (*GAL1*::*CLN3C*). *CLN2C* coding sequences were substituted for *CLN3C* coding sequences in pKL002 by digesting pKL002 and pT411 with *Cla*I and *Bam*HI and switching insert fragments to produce pKH100 (*GAL1*::*CLN2C*). Mutant *CLN2* genes containing alanine substitutions for Lys129 or Glu183 were constructed by splice-overlap extension (22) using pT411 as the template and Vent polymerase. Amplified fragments were subcloned into pT411 by *Cla*I-*Bam*HI digestion to produce pKL003 (*cln2-K129A-3P*) and pKL004 (*cln2-E183A-3P*) (identical in structure to pT411 except for the alanine substitutions). The mutations were confirmed by DNA sequencing. The double *cln2-K129A,E183A-3P* mutant (pKL005) was constructed by cutting pKL003 and pKL004 with *Spe*I and *Bam*HI and recombining appropriate fragments. *Cla*I-*Bam*HI fragments containing these mutant *CLN2* genes were transferred into pKL002 to produce *GAL1* promoter-driven derivatives.

Yeast strains and genetic procedures. Standard methods were used for all strain constructions and transformations (21). Mutant alleles used were *CLN* gene deletions described elsewhere (10), *clb5*::*ARG4* (19), *clb6*::*ADE1* (3), *pcl1*::*URA3* and *pcl2*::*HIS3* (30), *swi4*::*LEU2* (9), *swi6*::*LEU2* (provided by B. Andrews), *bck2*::*ARG4* (20), *GAL1*::*SIC1* (13), and *bud2*::*URA3* (4). All mutant alleles used were null alleles (deletion or disruption alleles). All strains were congenic with BF264-15D (39) (Table 1).

Protein extraction, immunoprecipitation, immunoblotting, and protein kinase assays. The procedures were adapted from Cross and Blake (8) and Tyers et al. (44, 45). Yeast cultures (100 ml at an optical density at 660 nm [OD_{660}] of 1.0) were collected by filtration and resuspended in ice-cold LSHN (10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 50 mM NaCl, 10% glycerol). Cells were resuspended in 250 μl of TNN extraction buffer (50 mM Tris [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.1% Nonidet P-40, 10% glycerol) containing 5% aprotinin (Sigma), 0.1 mM phenylmethylsulfonyl fluoride, 10 μg of leupeptin per ml, 10 μg of pepstatin per ml, 10 mM NaPPi (pH 7.4), and 10 mM NaF. Four hundred microliters of acid-washed glass beads (Sigma) was added, and cells were broken by three cycles of 1 min of vortexing in a Vortex-Genie sleeve at top speed. Breakage was performed at 4°C. Between cycles, extracts were microcentrifuged briefly to condense foam, and unbroken cells were resuspended by stirring with a micropipettor tip. Extracts were microcentrifuged, 250 μl was removed, and another 250 μl of extraction buffer was added to cells and beads. After 1 min of vortexing, the sample was microcentrifuged and the extraction buffers were combined. The extracts were clarified by 2 min of microcentrifugation and incubated with 7 μg of monoclonal antibody 12CA5 (from ascites fluid; Babco) for 1 to 2 h on ice. Samples were microcentrifuged for 2 min, and supernatant was added to 30 μl of slurry of protein A-agarose (Repligen) washed in extraction buffer. Samples were rotated at 4°C for 1 to 2 h. Immunoprecipitates were washed with extraction buffer three times for 1 min each and once for 2 min. Immunoprecipitates were washed once in kinase assay buffer (10 mM HEPES [pH 7.4], 10 mM $MgCl_2$, 10% glycerol, 1 mM dithiothreitol) and resuspended in 60 μl of kinase assay buffer, and 15 μl of the suspension was added to 1 μl of histone H1 (2 mg/ml; Boehringer Mannheim)–2 μl of 50 μM ATP–1 μl of water–1 μl of [γ-^{32}P]ATP (NEN). These kinase reaction mixtures were incubated at 30°C for 10 min with shaking every 2 to 3 min. Reactions were stopped by addition of 20 μl of 2× sodium dodecyl sulfate (SDS) sample buffer (2) and heating to 95°C for 5 min. The remainder of the immunoprecipitate was collected by centrifugation, resuspended in 60 μl of 2× SDS sample buffer, heated at 95°C for 5 min, and used for immunoblot analysis. SDS-polyacrylamide gel electrophoresis and transfer to Immobilon were done as described previously (8). Following transfer, radioactivity in histone H1 was quantitated with a PhosphorImager (Applied Biosystems). Blots were blocked in phosphate-buffered saline (PBS) solution containing 0.5% Nonidet P-40, 0.1% Tween 20, 0.5% bovine serum albumin, and 2.3% milk (Carnation). Antibody incubations were in PBS-Tween (0.2%), with 1 to 2% milk included to reduce background. Antibodies used were a polyclonal rabbit anti-HA peptide antibody (Babco) to detect the HA tag and an anti-C-terminal Cdc28 polyclonal rabbit antibody provided by R. Deshaies. Detection was by enhanced chemiluminescence (ECL) using an ECL kit (Amersham). ECL signals were quantitated from autoradiographs by using a DeskScan II scanner (Hewlett-Packard) and ImageQuant software.

Plating efficiency assays. Mutant strains, containing either a *GAL1*::*CLN1* gene essential for viability or the *GAL1*::*SIC1* gene, were transformed with plasmid T411 (*CLN2-3P*), KL004 (*cln2 E183A-3P*), KL003 (*cln2 K129A-3P*), KL005 (*cln2 K129A,E183A-3P*), KL001 (*CLN3-3P*), or KL034 (2μm *CLN3-3P*). For each transformed strain, 10-fold serial dilutions were prepared for two independently isolated transformants, and 5 μl of each dilution was plated onto both a YEP-galactose (YEPGal) plate and a YEP-dextrose (YEPDex) plate. Plates were incubated at 30 or 38°C for 2 to 3 days. The plating efficiencies were determined by counting the colonies at each dilution (where colony density allowed), calculating an average colony count (corrected for dilution) for each transformant, and then calculating the plating efficiency as a fraction of the average colony count on the test plate (YEPDex plate for all strains except YEP Gal for the *GAL1*::*SIC1* strain) to the average colony count on the control plate (YEPGal plate for all strains except YEPDex for the *GAL1*::*SIC1* strain). The plating efficiency assays indicated on the graphs in Fig. 4 represent the averaged values (arithmetic mean) of two independent transformants. The maximum decrease in plating efficiency that can accurately be detected in the assays presented in Fig. 3 and 4 is approximately 1,000-fold. Note that in our strain background, the lethality of *cln1 cln2 cln3 pcl1 pcl2* strains transformed with the *CLN3* plasmid was variable at 30°C, and so the quantitative data presented in Fig. 4 are from incubation at 38°C. However, qualitatively the same order of efficiency of rescue was observed for *pcl1 pcl2* strains at 30°C (data not shown). We also tested a *cln1 cln2 erc10-1* strain, since Cla4 (Erc10) is required for septin ring formation and cytokinesis in the absence of Cln1 and Cln2 (4, 11). We found qualitatively similar results, with the efficiency of rescue *CLN2* > *CLN2-E183A* > *CLN2-K129A* > *CLN2-K129A,E183A* ~ *CLN3*. High-copy-number *CLN3* was no better at rescuing this strain than was low-copy-number *CLN3*. However, the degree of rescue of plating efficiency obtained with the various *CLN* genes was quite variable in this strain for unknown reasons, and so we cannot present quantitative data.

Cell volume assay. Cells of the *cln1 cln2 cln3* (1254-14D) strain containing the *GAL1*::*CLN1* gene, which is essential for viability, were transformed with plasmid T411 (*CLN2-3P*), KL004 (*cln2 E183A-3P*), KL003 (*cln2 K129A-3P*), or KL001 (*CLN3-3P*). All plasmids are episomal centromeric (CEN) (low copy number) and carry the *TRP1* gene. For each transformant strain, stock cultures of two individual transformants were grown in defined (synthetic complete [SC]) medium containing 3% galactose and lacking tryptophan (SCGal-Trp). Stock cultures were used to inoculate YEPDex, and cultures were grown overnight to an OD_{660} of ~1.0. Cells from each culture were fixed in 10% formaldehyde and sonicated to disperse clumps. Cell volume analysis was done with a Coulter Channelyzer 256.

Assay of expression from a Swi4/Swi6 cell cycle box (SCB)-*lacZ* plasmid. Plasmid BD53 (*URA3*-3×-SCB-*lacZ*) was obtained from Linda Breeden. This plasmid gave expression of *lacZ* (measured as described elsewhere [2]) that was genetically dependent on *SW14* and *SW16* as expected (data not shown) (see

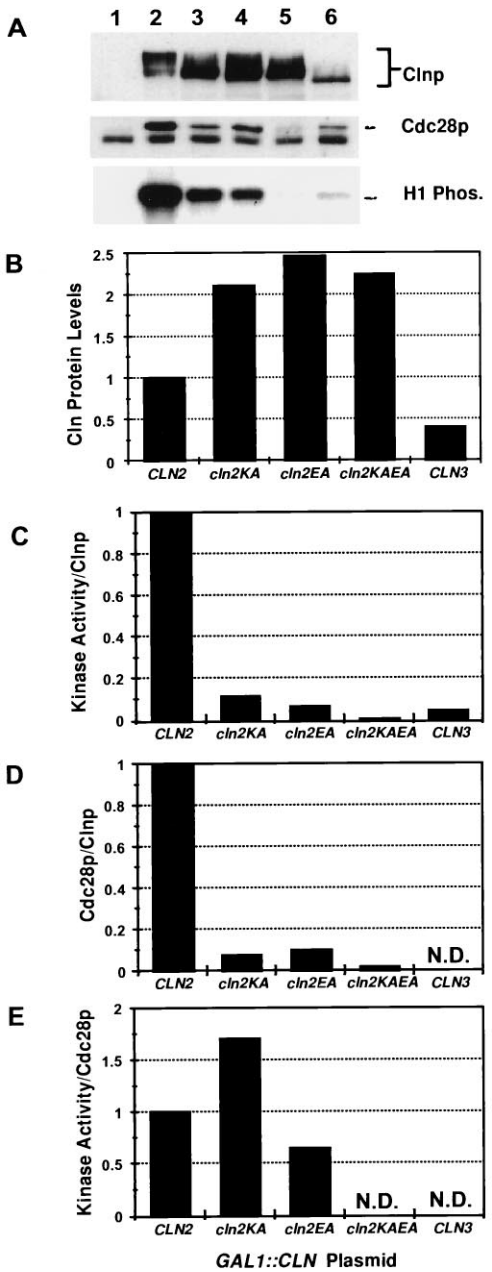

FIG. 1. Comparison of associated kinase activity, associated Cdc28 protein, and associated Cdc28-specific kinase activity for HA-tagged Cln proteins expressed from the *GAL1* promoter. Wild-type cells (1255-5C) were transformed with plasmid pRS414, KH100 (*GAL1*::*CLN2*), KL029 (*GAL1*::*cln2 K129A*), KL030 (*GAL1*::*cln2 E183A*), KL031 (*GAL1*::*cln2 K129A,E183A*), or KL002 (*GAL1*::*CLN3*) (lanes 1 to 6 in panel A). All plasmids are episomal CEN (low copy number) and carry the *TRP1* gene. Cultures of each transformant strain were grown overnight at 30°C in SCGal-Trp. HA-tagged Cln proteins were isolated from log-phase cultures, and kinase assays were performed. Cln proteins and Cln-associated Cdc28 protein were analyzed by immunoblotting. All protocols were as described in Materials and Methods. (A) Anti-HA and anti-Cdc28 immunoblots and H1 kinase blot. (B) Relative Cln protein levels. (C) Relative Cln-associated histone H1 kinase activities. Values obtained by standardizing histone H1 radioactivity to Cln protein levels. (D) Relative Cln-associated Cdc28 protein. Values were obtained by standardizing associated Cdc28 protein levels to Cln protein levels. (E) Relative Cln-associated Cdc28-specific kinase activity. Values were obtained by standardizing H1 radioactivity to Cln-associated Cdc28 protein levels. Bar graphs show relative values, standardized to wild-type Cln2 values, and represent the averaged values of samples prepared in duplicate. For unknown reasons, the levels of Cln3-associated Cdc28 protein and Cln3-associated Cdc28-specific kinase activity were not reproducible between experiments and are therefore reported as not determined (N.D.) (see text). In this particular experiment, the Cdc28/Cln3 ratio was 0.224, and the kinase activity/Cdc28 activity ratio for Cln3 was 0.185. The Cdc28 protein and specific kinase activities for the *cln2KAEA* samples were too low to be accurately quantified and are also indicated as N.D.

reference 27 for a review). This plasmid was transformed into *cln1 cln2 cln3 leu2*::*LEU2*::*GAL1*::*CLN3 bck2*::*ARG4* strains (20), and the resulting strain was secondarily transformed with plasmid KL001 (*CLN3-3P*), T411 (*CLN2-3P*), KL004 (*cln2 E183A-3P*), KL003 (*cln2 K129A-3P*), or pRS414. Transformant strains were grown overnight in defined medium containing 3% galactose and lacking both tryptophan and uracil (SCGal-Trp-Ura). Strains were shifted from galactose-containing medium to dextrose-containing medium (SCDex-Trp-Ura) and grown for an additional 24 h; this prolonged incubation in dextrose medium (*GAL1*::*CLN3* off) was needed to minimize the background activity observed in the pRS414 transformant strain, presumably arising from inheritance of stable β-galactosidase from pregrowth in galactose medium (*GAL1*::*CLN3* on). After 24 h, the cultures were each diluted in SCDex-Trp-Ura and allowed to grow to mid-log phase. β-Galactosidase liquid assays were carried out as described previously (2).

Centrifugal elutriation and RNA hybridization analysis. The analyses were carried out as described previously (35). Strains used were diploid strains varying in *CLN2* and *CLN3* gene dosage. The tandem integrated 3× *CLN3* allele was described previously (34). This tandem integration of the *CLN3* gene at the *TRP1* locus results in approximately threefold elevation of *CLN3* mRNA and protein production (8a). All diploid strains contained *GAL1*::*CLN3* cassettes, but these were inactive since all culture growth for this experiment was in rich glucose-containing medium (YEPDex).

RESULTS

Construction of reduced-function alleles of *CLN2*. To begin sorting out the relative contributions of qualitative and quantitative differences between *CLN2* and *CLN3*, we constructed alleles of *CLN2* with reduced function. We relied on the cyclin A-Cdk2 crystal structure and the sequence homology between cyclin A and *CLN2*. Lys266 and Glu295 of cyclin A hydrogen-bond to each other and to the backbone of the critical PSTAIRE helix of Cdk2 (24). We mutated the corresponding Lys129 and Glu183 of *CLN2* to alanines, separately or in combination.

Cln2-, Cln3-, and mutant Cln2-associated Cdc28 and kinase activity. We used the triple-HA-epitope-tagged versions of the *CLN2* and *CLN3* coding sequence described by Tyers et al. (44, 45). These coding sequences were placed under the control of the *CLN3* or the *GAL1* promoter on episomal CEN (low-copy-number) plasmids. We used the *GAL1* promoter-driven constructs of *CLN2*, *CLN3*, and the *CLN2* mutants to examine Cdc28 association and kinase activity, since this gave us more easily quantitated data than did the *CLN3* promoter-driven constructs. We found an approximately 20-fold reduction in Cln3-associated kinase activity compared to Cln2-associated kinase activity when kinase activity was standardized to Cln protein (Fig. 1 and data not shown). Therefore, the defect in recovery of Cln3-associated kinase activity compared to Cln2-associated kinase activity under these conditions (44) is due to an intrinsic defect in Cln3-associated kinase activity toward histone H1, not solely to low recovery of Cln3 compared to Cln2. Although we reproduced the qualitative result of Cdc28 binding to Cln3 (45), we were unable to obtain a reproducible quantitative comparison between the efficiency of Cln2 and Cln3 binding to Cdc28 for unknown reasons. (We cannot exclude the possibility that the presence of the HA tag, or the constitutive overexpression of the proteins from the *GAL1* promoter, perturbs these results relative to the wild-type situation.)

Compared to wild-type Cln2, the Cln2 mutant proteins had significantly decreased associated kinase activity and Cdc28 binding (Fig. 1). The K129A,E183A double mutant was almost inactive in these assays. These residues are surface residues in

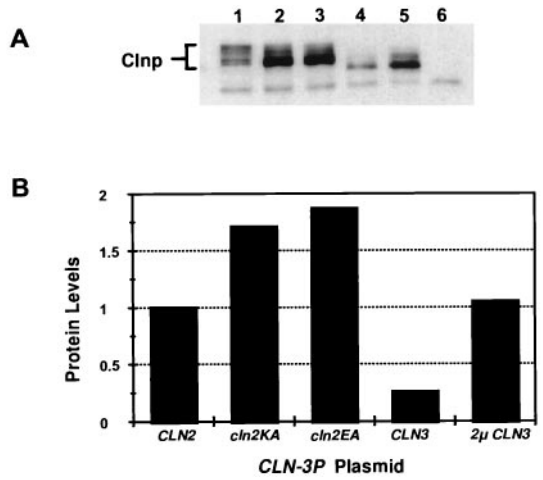

FIG. 2. Comparison of HA-tagged Cln protein levels when expressed from the *CLN3* promoter. Wild-type cells (1255-5C) were transformed with plasmid T411 (*CLN2-3P*), KL003 (*cln2 K129A-3P*), KL004 (*cln2 E183A-3P*), KL001 (*CLN3-3P*), KL034 (2μm *CLN3-3P*), or pRS414 (lanes 1 to 6 in panel A). All plasmids carry the *TRP1* gene, and except for the 2μm *CLN3* (high copy number) plasmids, all are episomal CEN (low copy number). Cultures of each transformant strain were grown overnight at 30°C in SC medium containing 2% dextrose and lacking tryptophan. Log-phase cultures (100 ml at an OD_{660} of ~1.0) were extracted, and protein concentration of lysates was determined by the Bradford Assay (Bio-Rad) to ensure roughly equivalent concentrations for each sample (between 25 and 30 μg/μl for this experiment). HA-tagged Cln proteins were isolated and analyzed by immunoblot assay, and ECL signals were quantified as described in Materials and Methods. (A) Anti-HA immunoblot. (B) Bar graph shows relative Cln protein levels, standardized to wild-type Cln2 levels. Values shown represent the averaged values of samples prepared in duplicate.

cyclin A (and presumably in Cln2), and so we consider it likely that these mutations specifically affect Cdc28 binding rather than altering overall folding of Cln2. Therefore, we expect that these mutant proteins are essentially quantitatively reduced for function rather than generally misfolded. Consistent with this interpretation, we note that the specific activity of kinase associated with K129A or E183A was similar to wild-type activity, suggesting that the reduced amount of Cdc28 that associated with the mutant Cln2 proteins was in the active conformation.

Cln2, Cln3, and mutant Cln2 abundance. To evaluate the protein levels of Cln2, Cln3, and the Cln2 mutants when expressed from the *CLN3* promoter (the plasmid constructs used in the functional assays described below), protein extracts from transformants of a wild-type strain containing these plasmids were immunoprecipitated and immunoblotted to determine the relative levels of Cln protein. Following the reasoning of Tyers et al. (44), we assume that signal intensity will likely reflect protein abundance since both proteins are tagged identically at their C termini (with the caveat that we must assume identical accessibility of the tag to antibody). We found that wild-type Cln2 was apparently about fivefold more abundant than Cln3 when both were expressed from the *CLN3* promoter (Fig. 2 and data not shown). (The abundance of Cln2 protein when expressed from the *CLN3* promoter was roughly equivalent to its abundance when expressed from the wild-type *CLN2* promoter [data not shown].) A direct test of stability of Cln2 and Cln3 (by turning off transcription from the *GAL1* promoter in strains containing *GAL1*::*CLN2C* or *GAL1*::*CLN3C* in parallel) revealed short half-lives (about 10 min) in each case (data not shown). Hence, we do not know the molecular basis for this difference in abundance between Cln2 and Cln3. The K129A and E183A mutant Cln2 proteins were somewhat more abundant than wild-type Cln2.

In an attempt to equalize the protein levels of wild-type Cln2 and Cln3, we transferred the *CLN3* gene from the low-copy-number vector (RS314) used in the analysis described above to the high-copy-number vector RS424 (5). The Cln3 protein levels in transformants containing this high-copy-number plasmid were roughly equivalent to those of wild-type Cln2 when expressed from the low-copy-number plasmid, about four- to fivefold higher than the levels of Cln3 in transformants containing the low-copy-number plasmid (Fig. 2).

Functional assays for *CLN2* and *CLN3*. The basic assay for *CLN* function is complementation of a *cln1 cln2 cln3* mutant strain (39). When expressed under the control of the *CLN3* promoter, *CLN2*, *CLN3*, *CLN2-E183A*, and *CLN2-K129A* are positive in the *cln1 cln2 cln3* rescue assay. *CLN2-K129A* is detectably weaker (Fig. 3A) and is also temperature sensitive (Fig. 4). *CLN2-K129A,E183A* is essentially negative for rescue even at 30°C (Fig. 3A and 4).

In addition to the complementation of a *cln1 cln2 cln3* strain, the efficiency of *CLN* rescue can be assessed by the cell volume of rescued strains, since *CLN* overexpression drives bud emergence and reduces cell volume (6, 15, 32, 39). When tested for cell volume, the apparent order of potency of the genes was *CLN2* > *CLN3* > *CLN2-E183A* > *CLN2-K129A* (Fig. 5).

To further evaluate *CLN2*, *CLN3*, and the *CLN2* mutants under the control of the *CLN3* promoter, we made use of a series of mutant strains that functionally differentiate between *CLN2* and *CLN3*. As summarized in Fig. 4, mutant strains were transformed with the *CLN2*, *CLN3*, and *CLN2* mutant plasmids and tested for viability with the plasmid-encoded *CLN* gene as the only functional *CLN* gene in the cell. As predicted from published work (see the introduction and reference 7 for fuller discussions), *CLN3* is unable to rescue *cln1 cln2 cln3* strains that are also deficient in the *SWI4* or *SWI6* genes encoding components of the transcription factor SCB-binding factor (SBF) (see reference 27 for a review), the *CLB5-CLB6* pair of B-type cyclins (41), the *PCL1-PCL2* pair of cyclin homologs (30, 36), and the *BUD2* gene (because cell polarization is defective) (4, 12). In contrast, *CLN2* under the control of the *CLN3* promoter rescues all of these mutant backgrounds (Fig. 4; see Fig. 3B for the example of the *swi4* assay). This result formally proves that the coding sequence of *CLN2* rather than its promoter is specifically responsible for its greater activity in these assays. (In contrast, *cln1 cln2 rad27* strains [defective in S-phase completion {38, 47}] are much better rescued by *CLN2* coding sequences under control of the *CLN2* promoter compared to the *CLN3* promoter [data not shown].)

In the absence of *CLN1* and *CLN2*, overexpression of the Sic1 inhibitor of B-type cyclin-Cdc28 kinases (31, 40) is lethal (43a). This may reflect reduced ability of Cln3-Cdc28 compared to Cln1- or Cln2-Cdc28 to trigger Sic1 degradation (40), although this has not been established as yet. We transformed a *cln1 cln2 CLN3 GAL1*::*SIC1* strain with the various *CLN* genes under control of the *CLN3* promoter. We found at least a 1,000-fold-lower plating efficiency for *CLN3* compared to *CLN2* (Fig. 4).

The *CLN2-K129A* mutant was reduced in activity compared to the wild type in these assays (Fig. 4), while the *CLN2-E183A* mutant displayed lesser but detectable defects. The *CLN2-K129A,E183A* mutant was negative in these assays. These results are consistent with a simple linear ordering of strength of these alleles: *CLN2* > *CLN2-E183A* > *CLN2-K129A* > *CLN2-K129A,E183A*. *CLN3* is at most slightly more active than the *CLN2-K129A,E183A* in these assays (Fig. 4).

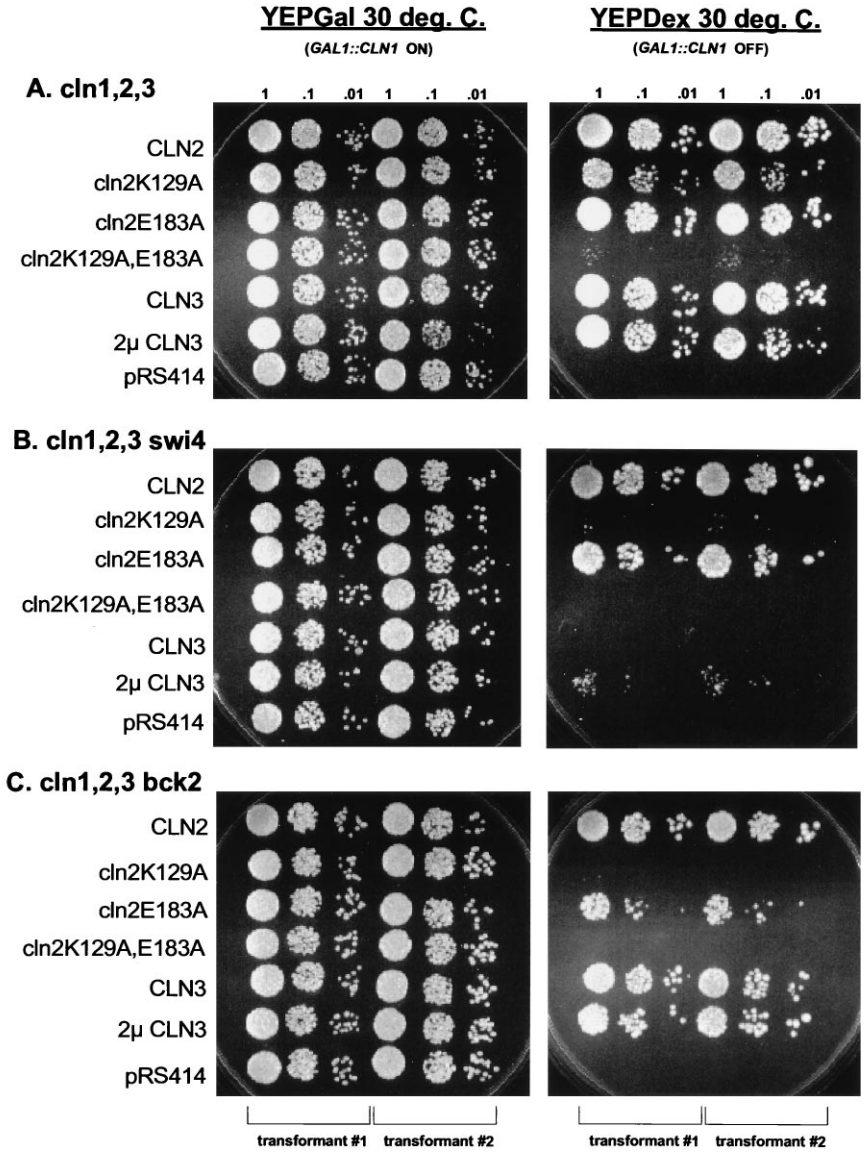

FIG. 3. Plating efficiency assays for *CLN* gene function. (A) *cln1 cln2 cln3* (1254-14D); (B) *cln1 cln2 cln3 swi4* (1456-10); (C) *cln1 cln2 cln3 bck2* (1457-9D). Mutant strains, each containing a *GAL1*::*CLN1* gene essential for viability, were transformed with plasmid T411 (*CLN2-3P*), KL003 (*cln2 K129A-3P*), KL004 (*cln2 E183A-3P*), KL001 (*CLN3-3P*), KL034 (2μm *CLN3-3P*), or pRS414. Except for the 2μm *CLN3* (high-copy-number) plasmid, all plasmids are episomal CEN (low copy number). For each transformed strain, 10-fold serial dilutions were prepared for two independently isolated transformants, and 5 μl of each dilution was plated onto both a YEPGal (*GAL1*::*CLN1* on) and YEPDex (*GAL1*::*CLN1* off) plate. Plates were incubated at 30°C for 2 days.

When we tested rescue of a *cln1 cln2 cln3 bck2* strain, the results were quite different. The order of strength of the genes tested became *CLN2* ≧ *CLN3* > *CLN2-E183A* ≫ *CLN2-K129A* ≧ *CLN2-K129A,E183A* (Fig. 3C and 4). Note that this order is similar to the order derived from *cln1 cln2 cln3* rescue and cell volume assays (Fig. 3A, 4, and 5). The *CLN2-E183A* mutant, which was nearly as active as *CLN2* in most of the assays in which *CLN3* was negative, was reproducibly reduced about 10-fold in plating efficiency in the *cln1 cln2 cln3 bck2* background.

As mentioned, we detected about a fivefold reduction in Cln3 abundance compared to Cln2 when expressed from the *CLN3* promoter (Fig. 2). To see if reduced abundance of Cln3 could account for its deficit in many of these assays, we repeated these assays using transformants containing the high-copy-number *CLN3* plasmid described above. Increasing *CLN3* copy number significantly improved rescue of the *cln1 cln2 cln3 swi4* and *cln1 cln2 cln3 clb5 clb6* backgrounds, which were rescued with reasonable efficiency by the high-copy-number *CLN3* gene at 30°C but not at 38°C. Increasing *CLN3* copy number did not improve rescue of *cln1 cln2 cln3* strains containing *bud2*, *swi6*, or *pcl1 pcl2* mutations. The failure of *CLN3* to rescue many of these genetic backgrounds thus cannot be attributed simply to low Cln3 protein levels compared to Cln2, since the high-copy-number *CLN3* plasmid yielded Cln3 protein levels similar to those of Cln2 expressed from the low-copy-number *CLN2* plasmid (Fig. 2). (As a caveat, it should be noted that Cln protein levels expressed from these plasmids [Fig. 2] were evaluated in a *CLN1 CLN2 CLN3* wild-type strain rather than in these mutant backgrounds. We cannot exclude an effect of the mutant background or of selection for function on Cln protein levels in these contexts; moderate plasmid copy

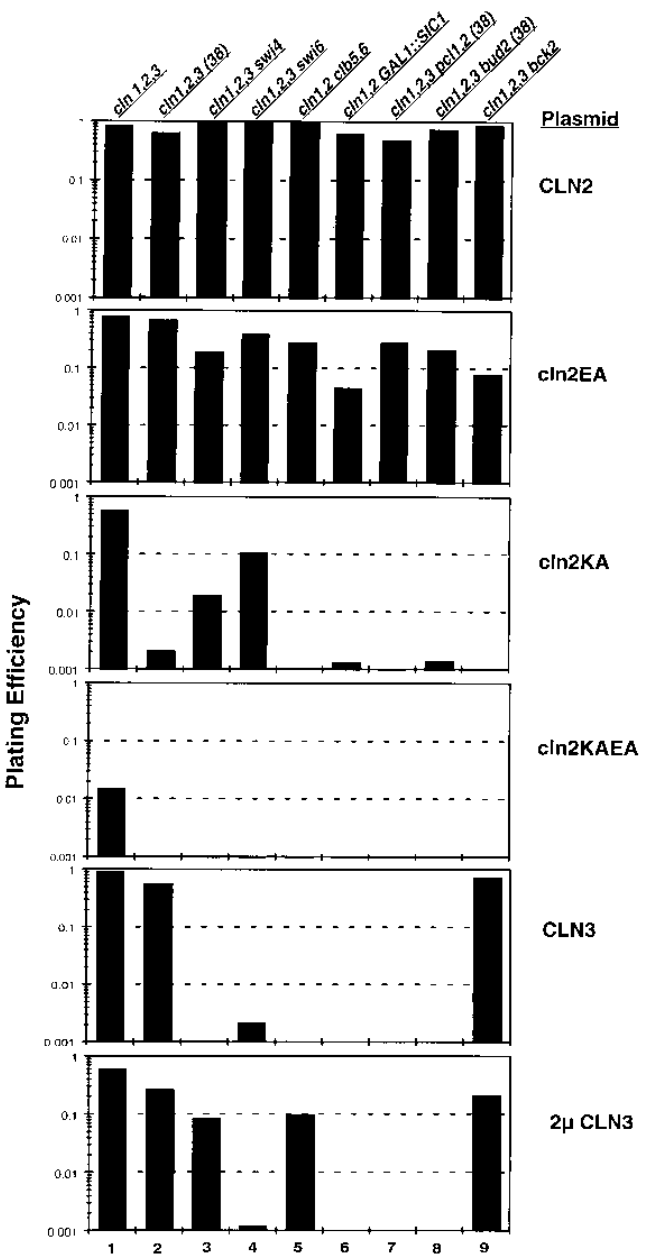

FIG. 4. Plating efficiency assays for differential aspects of *CLN* gene function. Plating efficiency assays were carried out as described in Materials and Methods. Each graph shows the plating efficiencies of the following transformed mutant strains and conditions: *cln1 cln2 cln3 leu2*::*LEU2*::*GAL1*::*CLN1* (1254-14D) at 30°C, *cln1 cln2 cln3 leu2*::*LEU2*::*GAL1*::*CLN1* (1254-14D) at 38°C, *cln1 cln2 cln3 swi4 pURA3*/*GAL1*::*CLN1* (1456-10) at 30°C, *cln1 cln2 cln3 swi6 pURA3*/*GAL1*::*CLN1* (2866-9D) at 30°C, *cln1 cln2 CLN3 clb5 clb6 leu2*::*LEU2*::*GAL1*::*CLN1* (1587-7) at 30°C, *cln1 cln2 CLN3 GAL1*::*SIC1* (1238-11A-7) at 30°C, *cln1 cln2 cln3 pcl1 pcl2 leu2*::*LEU2*::*GAL1*::*CLN1* (1591-10D) at 38°C, *cln1 cln2 cln3 bud2 leu2*::*LEU2*::*GAL1*::*CLN1* (1472-24D) at 38°C, and *cln1 cln2 cln3 bck2 pURA3*/*GAL1*::*CLN1* (1457-9D) at 30°C (bars 1 to 9). The different *CLN* gene plasmids, indicated to the right of each graph, are as follows (top to bottom): T411 (*CLN2-3P*), KL004 (*cln2 E183A-3P*), KL003 (*cln2 K129A-3P*), KL005 (*cln2 K129A,E183A-3P*), and KL001 (*CLN3-3P*), KL034 (2μm *CLN3-3P*). Except for the 2μm *CLN3* (high-copy-number) plasmid, all plasmids are episomal CEN (low copy number). A representative experiment for the strains represented by bars 1, 3, and 9 is shown in Fig. 3. The plating efficiencies indicated on each graph represent the averaged values (arithmetic mean) of two independent transformants. Note that the *bud2* and *pcl1 pcl2* assays were done at 38°C to tighten the restriction of rescue by *CLN3* (reference 4 and data not shown). To control for this, rescue of a *cln1 cln2 cln3 BUD2 PCL1 PCL2* strain (1254-14D) at 38°C was also determined (bars 2).

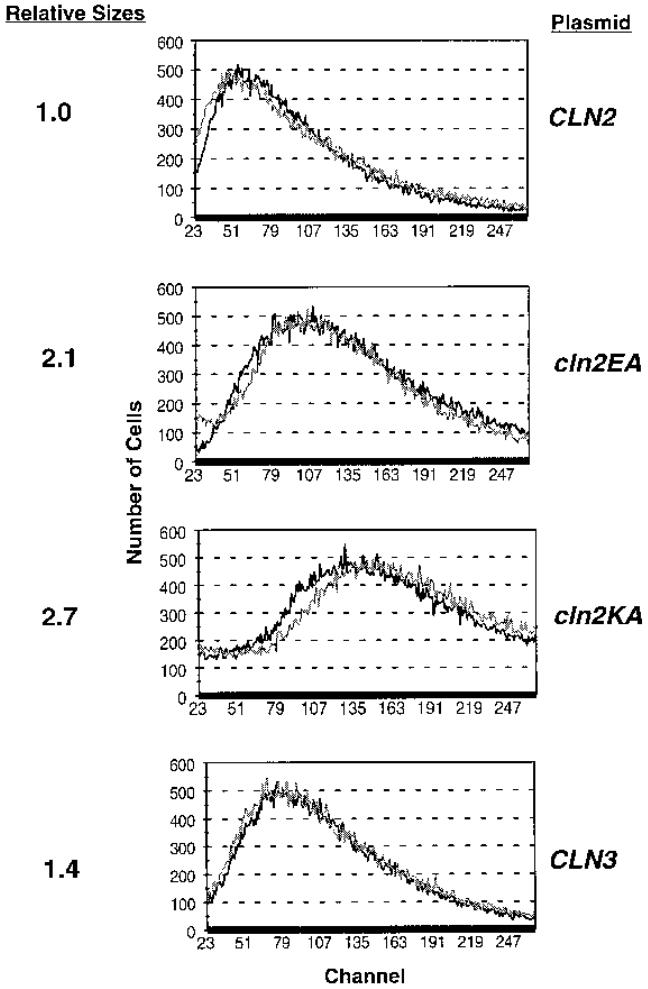

FIG. 5. Cell volume assay for *CLN* gene function. Cells of the *cln1 cln2 cln3* (1254-14D) strain containing the *GAL1*::*CLN1* gene, which is essential for viability, were transformed with plasmid T411 (*CLN2-3P*), KL004 (*cln2 E183A-3P*), KL003 (*cln2 K129A-3P*), or KL001 (*CLN3-3P*). All plasmids are episomal CEN (low copy number) and carry the *TRP1* gene. Cell volume analysis was carried out as described in Materials and Methods. Data points for each of two transformants are shown together on each graph. The average modal volume for each strain was calculated, and relative sizes standardized to the *CLN2* transformants are indicated.

number variation also could play a role, even though these plasmids contain a CEN conferring reasonable stability of low copy number [2].)

Activation of Bck2-independent SCB-dependent transcription. Bck2 may encode a transcriptional activator of *CLN1*, *CLN2*, and other genes (14, 20); it contains a potent transcriptional activation domain (22a). One way to explain the strong differences in rescue for the different *CLN* genes with the *bck2* assay compared to the other assays would be to suppose that this reflects Cln3-dependent, Bck2-independent transcriptional activation of a target gene(s) required for Start (14, 20). As a test of this, we compared the abilities of *CLN3*, *CLN2*, *CLN2-E183A*, and *CLN2-K129A* expressed from the *CLN3* promoter to activate transcription from an SCB reporter gene when these genes were the only functional *CLN* genes present in the cell. The SCB sequence is responsive to Swi4/Swi6 control (see reference 27 for a review), and both Cln3 and Bck2 may activate expression independently through this sequence

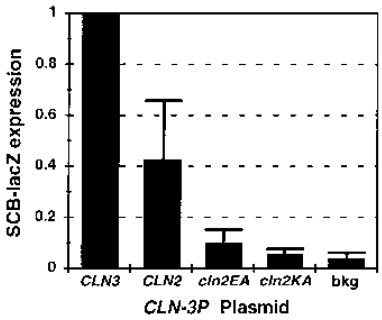

FIG. 6. Bck2-independent SCB-dependent transcription assay for *CLN* gene function. A *cln1 cln2 cln3 bck2* strain (1446-10B), containing a *GAL1*::*CLN3* gene essential for viability, was cotransformed with both the BD53 plasmid (*URA3*-3×-SCB-*lacZ*) and one of the following plasmids: KL001 (*CLN3-3P*), T411 (*CLN2-3P*), KL004 (*cln2 E183A-3P*), KL003 (*cln2 K129A-3P*), or pRS414. These strains were analyzed for *lacZ* expression as described in Materials and Methods. Values shown in graph represent the averaged values of three independent experiments; two to five samples of each transformant strain were analyzed for each experiment. Error bars indicate standard errors of the means. bkg, background.

(14, 20, 43). In a *BCK2* background, a moderate level of *CLN*-independent expression was observed as expected (reference 20 and data not shown), making it difficult to accurately determine the effects of the *CLN* genes. Therefore, we made *cln1 cln2 cln3 bck2 GAL1*::*CLN3* strains carrying an SCB-*lacZ* reporter and also containing vector, *CLN2*, *CLN3*, *CLN2-K129A*, or *CLN2-E183A* (all under control of the *CLN3* promoter) and shifted these strains from galactose to glucose medium to turn off the *GAL1*::*CLN3* gene. After 24 h, we analyzed β-galactosidase activity. *CLN3* was more active than *CLN2*, *CLN2-E183A* showed a further reduction in efficiency, and *CLN2-K129A* was little more effective than the vector control at this induction (Fig. 6). An important caveat to the interpretation of this assay, however, is the possibility that the greater activity of *CLN3* than of *CLN2* is at least in part due to *CLN2*'s greater ability to promote transcriptional inactivation of SCB-dependent gene expression (15, 43).

Dosage studies of *CLN2* and *CLN3* support a primary role for Cln3 in activating *CLN2* transcription. Stuart and Wittenberg (43) and Dirick et al. (15) presented evidence that the potential positive feedback loop for activating *CLN1* and *CLN2* transcription (10, 16) was likely not to be of much or any significance in dictating timing of *CLN1* and *CLN2* transcription compared to Cln3 activation of transcription of these genes. These studies relied on fairly drastic manipulations (medium shifts to inactivate conditional *CLN* gene overexpression and elutriation of large volumes of chilled culture followed by reinoculation into warm medium of very small G_1-phase cells) and on comparison of wild-type with *cln1 cln2* strains, which are known to have many defects in cell cycle initiation and progression (see the introduction). We wanted to test this idea in a less manipulative way. We reasoned that if positive feedback were important in the onset of *CLN1* and *CLN2* transcription, then doubling the *CLN2* gene dosage should dramatically affect *CLN2* transcription by providing twice the amount of "substrate" for positive feedback to feed back upon. We therefore compared the relationship of cell size, bud emergence, and *CLN2* and histone H2A transcript levels in diploid strains containing one or two copies of the *CLN2* gene (and no functional *CLN1* gene and only one *CLN3* gene, to increase sensitivity to effects of *CLN2*). This analysis was performed simply by chilling and size fractionating log-phase cultures, and so manipulation was minimal; also, the strains compared differed only in *CLN2* or *CLN3* gene dosage, rather than in the presence or complete absence of *CLN* genes as in the previous studies (15, 43). We found (Fig. 7A) that in strong contrast to the expectation of positive feedback, doubling the *CLN2* gene dosage somewhat less than doubled the level of *CLN2* transcription and had little or no effect on the cell volume at which *CLN2* transcription initiated. Also, we found that *cln1 CLN2*/*cln2*::*LEU2 CLN3*/*cln3-del* diploids executed bud emergence at a cell volume identical to that of isogenic *CLN2*/*CLN2* strains, suggesting that Cln2 (once activated) is in excess for driving bud emergence. In contrast, increasing the number of *CLN3* genes in a diploid of genotype *cln1-del CLN2*/*cln2*::*LEU2* from one to four significantly decreased the cell volume of budding, peak *CLN2* transcription, and peak histone H2A transcription in parallel (Fig. 7B). (An increase in *CLN3* gene dosage from one to two had a somewhat lesser effect [data not shown].) In a similar analysis, we found that *cln1 cln2* strains were significantly delayed in bud emergence even after activating a high level of reporter *cln2* transcription, consistent with previous reports (15, 43) (data not shown). These results clearly confirm previous conclusions (15, 43, 44) that Cln3 is the primary initiator of *CLN2* transcription, that *CLN2* has no obvious role in driving its own transcription, and that Cln2 (and not Cln3) is highly active in driving bud emergence.

These results fit well with the genetic studies reported above showing that Cln3 may be specialized for transcriptional activation.

DISCUSSION

Is Cln3-Cdc28 a "weak" kinase? Tyers et al. (44) observed very low recovery of both Cln3 protein and Cln3-associated kinase activity compared to Cln1 and Cln2. Their results did not address whether the low Cln3-associated kinase activity was simply a reflection of the relatively low amount of Cln3 protein or resulted from additional defects in Cln3-associated specific activity. We have confirmed the observation that Cln3 is significantly less abundant than Cln2 (Fig. 2 and data not shown). After standardizing for amount of Cln protein, we could still detect a significant defect in Cln3-associated kinase activity relative to Cln2 (Fig. 1). It seems likely that Cln3-associated kinase is indeed much less active than Cln2-associated kinase under these conditions and using histone H1 as the substrate. However, the implication from the results reported here, that Cln3-Cdc28 may be specialized for a narrower range of substrates than Cln2-Cdc28, means that a fair comparison of the two enzyme activities must await identification of appropriate physiological substrates. (A differential adverse effect of the epitope tag on Cln3-associated and Cln2-associated kinases also cannot be ruled out at present.)

Is Cln3 specialized for transcriptional activation? Recent experiments (15, 43, 44) have suggested that Cln3 is the primary in vivo activator of transcription of *CLN1*, *CLN2*, *PCL1*, *PCL2*, *CLB5*, and *CLB6*; while Cln1 and Cln2 can activate transcription of these genes, it has been suggested that they do not contribute significantly to this activation under physiological conditions. These conclusions were confirmed in a different analysis (Fig. 7). However, since *CLN3* transcription is less tightly regulated and initiated earlier than that of *CLN1* and *CLN2* (4a, 34, 44), the apparently specialized role of Cln3 in activating transcription could simply reflect its time of expression rather than any higher activity. Indeed, Stuart and Wittenberg (43) suggested that when placed under control of the *CLN3* promoter, Cln2 was at least as good at activating transcription from the *CLN2* promoter as was Cln3. Our results, obtained by using a simple reporter construct (SCB-*lacZ*)

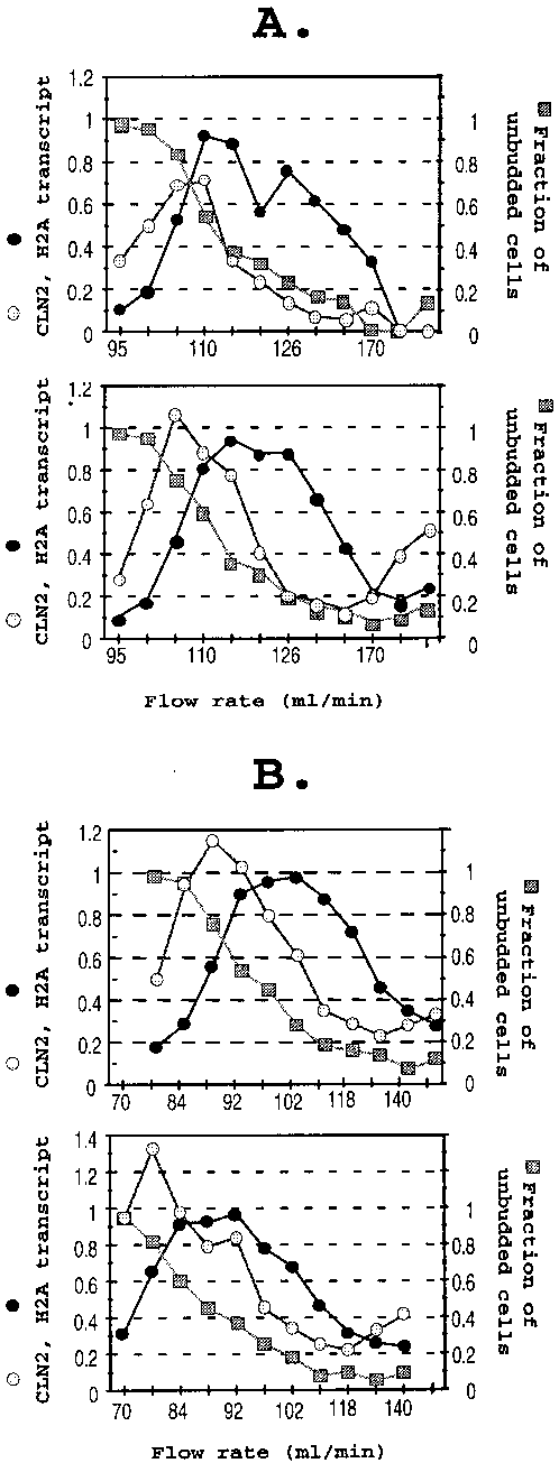

FIG. 7. *CLN* gene dosage and cell cycle initiation. Diploid strains were constructed with various *CLN2* and *CLN3* gene dosages. In these diploid strains, *CLN1* was homozygously deleted to enhance detection of effects of *CLN2* and *CLN3* dosage. Strains were grown to log phase in YEPD, and cultures were quick-chilled on ice, collected by centrifugation, sonicated, and fractionated by centrifugal elutriation at 0 to 4°C as described in Materials and Methods (spun at 3,000 rpm in panel A and at 2,800 rpm in panel B). Fractions were analyzed for proportion of unbudded cells and for *CLN2*, *H2A*, and *TCM1* RNA content by RNA hybridization analysis followed by PhosphorImager quantitation. *CLN2* and histone H2A transcript levels were normalized to transcript levels for *TCM1* to standardize loading as described previously (35). Squares, fraction of unbudded cells; light circles, *CLN2*/*TCM1*; dark circles, histone H2A/*TCM1*. Flow rate was the pump speed eluting fractions from an elutriating rotor. Pump speed correlates linearly with cell volume as determined by a Coulter Channelyzer (data not shown). (A) The top graph is analysis of a strain of genotype *cln1-del CLN2*/*cln2*::*LEU2 CLN3*/*cln3-del*. The *cln2*::*LEU2* allele does not result in a transcript that is scored in this analysis (data not shown). The bottom graph is analysis of a strain of genotype *cln1-del CLN2*/*CLN2 CLN3*/*cln3-del*. The samples for the top and bottom graphs were run and quantitated in parallel, and so all values are comparable. (B) The top graph is analysis of a strain of genotype *cln1-del CLN2*/*cln2*::*LEU2 CLN3*/*cln3-del.* The *cln2*::*LEU2* allele does not result in a transcript that is scored in this analysis (data not shown). The bottom graph is analysis of a strain of genotype *cln1-del CLN2*/*cln2*::*LEU2* 4× *CLN3* (this strain contains one copy of *CLN3* at its normal chromosomal location and approximately three copies tandemly integrated at the *TRP1* locus). The samples for the top and bottom graphs were run and quantitated in parallel, and so all values are comparable.

rather than the more complex *CLN2* promoter, show that Cln3 is probably at least as active as Cln2 at activating SCB-dependent transcription (given the caveat discussed above concerning the possibility that Cln2 could be more active than Cln3 at inducing shutoff of SCB-dependent transcription; this issue is thoroughly discussed in reference 43). This occurs with both *CLN2* and *CLN3* coding sequences under control of the *CLN3* promoter, conditions under which Cln2 accumulates to about fivefold-higher levels than does Cln3 (Fig. 2 and data not shown). The *CLN2-E183A* and *CLN2-K129A* mutants have greatly reduced ability to induce SCB-dependent transcription, despite their retaining significant biological activity (much higher than *CLN3*) in many functional assays. These proteins also accumulate to higher levels than either Cln2 or Cln3 (Fig. 2), and so low protein levels do not account for their defect in transcriptional activation. It is striking that these mutants are most defective in the transcriptional activation assay, which is the function most clearly comparable between wild-type *CLN2* and *CLN3*. This result is inconsistent with the idea that *CLN3* is equivalent to a weak version of *CLN2*; rather, it is likely specialized to activate transcription even when expressed at low levels. The effects of alteration of *CLN2* and *CLN3* gene dosage on timing of *CLN2* transcription described above (Fig. 7) also fit with this conclusion.

It remains unclear how Cln-Cdc28 activity results in activation of expression of Swi4-Swi6- and Mbp1-Swi6-dependent genes (27). Shutoff of Swi4-regulated genes correlates with activation of Clb-Cdc28 complexes and coincident displacement of Swi4 from its in vivo binding sites (28); however, Swi4 binding is regained upon exit from mitosis, before Swi4-dependent and Cln-dependent gene expression occurs. Mbp1 DNA binding through the cell cycle has not been characterized. Mbp1 becomes essential only in the absence of Swi4 (26). Unlike *SWI4*, *MBP1* can be disrupted with no effect on viability of *cln1 cln2 cln3* strains transformed with the *CLN2*, *CLN3*, or mutant *CLN2* constructs used in these experiments (data not shown).

Cyclin specificity. The genetic data discussed above showed that Cln2 (even when crippled) is more active than Cln3 in some assays, but Cln3 is as active as Cln2 and more active than crippled Cln2 in others. In formal terms, *CLN3* cannot be fit in an allelic series with these mutant *CLN2* genes, meaning that it probably differs qualitatively rather than (only) quantitatively. These results are most easily interpreted by assuming that Cln3 has a different in vivo substrate specificity than Cln2.

The logic of this interpretation requires assuming the crippled Cln2 proteins to be quantitatively reduced in function, rather than themselves having altered substrate specificity compared to wild-type Cln2. While we cannot prove this assumption, the observation that the mutants show strong reductions in Cdc28 binding, but little or no defect in specific activity

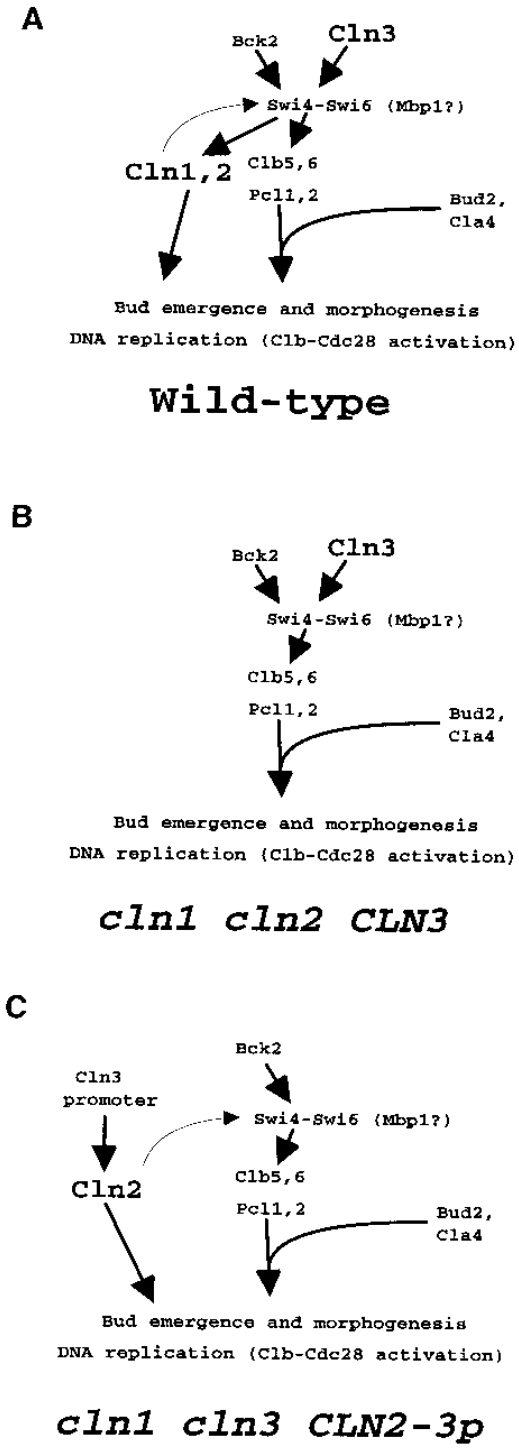

FIG. 8. Model for the action of G_1 cyclins in driving cell cycle initiation. See Discussion for justification.

of the kinase normalized using bound Cdc28, is consistent with the idea that the mutants are quantitatively reduced in function strictly due to a Cdc28 binding defect. However, the *CLN2-K129A* mutant has significantly greater in vivo defects than the *CLN2-E183A* mutant, and we cannot account for these differences on the basis of measurement of in vitro associated kinase activity. This inconsistency weakens the logic of the interpretation. However, a stronger effect of the *K129A* mutant than of the *E183A* mutant can be rationalized on the basis of the cyclin A-Cdk2 structure, since the equivalent lysine residue K266 makes two hydrogen bonds to the PSTAIRE helix, while the equivalent glutamic acid residue E295 makes only one (24). The in vitro measurements reflect only the amount of Cdc28 that remains bound to Cln2 following immunoprecipitation and multiple washes and therefore may not exactly reflect the amount bound in vivo, especially for mutant cyclins that bind more weakly.

The crystal structure of cyclin A-Cdk2 shows that cyclin is bound to approximately the opposite side of Cdk2 from the presumed substrate binding region. This fact could interfere with speculation as to the mechanism by which cyclins could contribute to substrate selectivity. Possible mechanisms could include (i) interaction of cyclin with substrates at regions distant from the site of phosphorylation (possible example from a mammalian system: cyclin D prefers Rb as a target and interacts with Rb in the pocket region [17, 25]), (ii) altered subcellular localization of different cyclins controlling substrate access (as suggested for cyclin B1 and cyclin B2 [23]), and (iii) altered conformation of the substrate binding cleft due to allosteric effects of different modes of binding of cyclins to the kinase. Several cases of substrate preference or specificity for mammalian cyclins, with the cyclins complexed to the same cyclin-dependent kinase, have been reported (37, 48). Substrate specificity has been inferred from strong biological specificity of cyclin action in situations in yeast where substrates have not yet been identified (1, 19, 29, 34, 41).

Cln2 and Cln3 are "functionally redundant" proteins that probably have quite different mechanisms of action. Our interpretation of the roles of Cln1 and Cln2 compared to Cln3 in wild-type and mutant cells is outlined in Fig. 8. In wild-type cells (Fig. 8A), Cln1 and Cln2 directly activate pathways leading to the processes of bud emergence, morphogenesis, and DNA replication. Cln3, perhaps specialized for transcriptional activation, acts through the Swi4 and Swi6 (and possibly Mbp1) transcription factors to activate the transcription of *CLN1*, *CLN2*, *PCL1*, *PCL2*, *CLB5*, and *CLB6* (27). Bck2 is also involved in the transcription of these genes, probably in a Cln-independent manner (14, 20). Bud2 and Cla4 are factors facilitating budding and cell morphogenesis (4, 11, 12). In cells containing *CLN3* as the only functional *CLN* gene (Fig. 8B), the pathway leading to bud emergence, morphogenesis, and DNA replication is dependent on the activities of the Swi4 and Swi6 transcription factors, the Clb5-Clb6 and Pcl1-Pcl2 cyclin pairs, and the Bud2 and Cla4 budding factors (4, 11, 12, 30, 33, 41). Bck2 plays a nonessential role in the Cln3-activated pathway. In *cln1 CLN2-3P cln3* cells (Fig. 8C), the transcription of *CLN2* from the *CLN3* promoter is not dependent on the activities of Bck2, Swi4, or Swi6. The Cln2 pathway leading to bud emergence, morphogenesis, and DNA replication is not dependent on the activities of the Clb5-Clb6 or Pcl1-Pcl2 cyclin pair or the Bud2 and Cla4 budding factors. Thus, once the requirement for Cln3 to efficiently activate *CLN2* transcription is eliminated, it becomes clear that Cln2 directly activates many events that Cln3 can activate only via a complex pathway.

The probable biological specialization of Cln3 for transcriptional activation, as indicated by physiological and genetic studies (see reference 7 for a review), is shown here to be accompanied by intrinsic functional specialization of the kinase, possibly due to selective targeting. Cln2-Cdc28, in contrast, may perform multiple roles in driving the Start transition (4, 7, 11, 12). How many independent specificities or targeting functions are required to account for the apparently diverse

activities of Cln2 revealed by these genetic studies remains to be determined.

ACKNOWLEDGMENTS

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