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The G_1 Cyclin Cln3 Promotes Cell Cycle Entry via the Transcription Factor Swi6

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In *Saccharomyces cerevisiae* (budding yeast), commitment to cell division in late G_1 is promoted by the G_1 cyclin Cln3 and its associated cyclin-dependent kinase, Cdc28. We show here that all known aspects of the function of Cln3 in G_1 phase, including control of cell size, pheromone sensitivity, cell cycle progress, and transcription, require the protein Swi6. Swi6 is a component of two related transcription factors, SBF and MBF, which are known to regulate many genes at the G_1-S transition. The Cln3-Cdc28 complex somehow activates SBF and MBF, but there was no evidence for direct phosphorylation of SBF/MBF by Cln3-Cdc28 or for a stable complex between SBF/MBF and Cln3-Cdc28. The activation also does not depend on the ability of Cln3 to activate transcription when artificially recruited directly to a promoter. The amino terminus and the leucine zipper of Swi6 are important for the ability of Swi6 to respond to Cln3 but are not essential for the basal transcriptional activity of Swi6. Cln3-Cdc28 may activate SBF and MBF indirectly, perhaps by phosphorylating some intermediary protein.

Eukaryotic cells generally enter the cell cycle at a commitment point in late G_1 (45, 48). In *Saccharomyces cerevisiae* (budding yeast) this point is called Start. Start occurs when G_1 cells reach a certain critical cell size and have a sufficiently high rate of protein synthesis. Start coincides with a peak in the transcription of over 200 genes, including the G_1 cyclin genes *CLN1* and *CLN2*, the S cyclin genes *CLB5* and *CLB6*, the *HO* endonuclease gene, and a large number of genes with roles in DNA synthesis and repair, budding, cell wall synthesis, and spindle pole body duplication (7, 57).

A large part of the transcriptional program at Start depends upon two transcription factors, SBF and MBF (1, 34). Many of the genes induced at Start have multiple binding sites for SBF and MBF in their promoters (57), and indeed, SBF or MBF has been shown to bind to many of these genes (24, 56). SBF and MBF are related, and both contain the protein Swi6 as a subcomponent. They differ in that SBF contains Swi4 as the DNA-binding protein, while MBF contains a related DNA-binding protein called Mbp1. Swi4 and Mbp1 each have an amino-terminal DNA binding domain, a central region containing ankyrin repeats, and a carboxy-terminal region important for binding Swi6. Swi6 does not have a DNA binding domain, but it does have ankyrin repeats, a leucine zipper, and a Swi4/Mbp1 interaction domain at its carboxyl terminus. SBF and MBF have distinct preferences for DNA binding (4). The empirically derived consensus binding sequences for SBF and MBF are, respectively, the Swi4/6 cell cycle box element PuNNPyCACGAAAA (41) and the *Mlu*I cell cycle box element ACGCGTNA (27). SBF and MBF can, however, act on each other's recognition sequences to some extent (14). Based on more recent genome-wide studies, the consensus sequences for the Swi4/6 and *Mlu*I cell cycle box elements can be updated to, respectively, CRCGAAA and ACGCGN (24, 57).

A *swi4 mbp1* double mutant (which thus lacks both SBF and MBF) is inviable, with a terminal cell cycle arrest in G_1 (28), confirming the crucial role of SBF and MBF in mediating cell cycle entry. A very similar phenotype is found for the *swi4 swi6* mutant, probably because Mbp1 is inactive in the absence of Swi6 (28). However, both *swi6* and *mbp1 swi6* mutants are viable, because Swi4 has residual activity as a transcription factor even in the absence of Swi6 (28, 43).

The activity of SBF and MBF is regulated at many levels. Expression of *SWI4* (but not *MBP1* or *SWI6*) varies throughout the cell cycle, with a peak at the M-G_1 transition (5). The subcellular localization of Swi6, and therefore likely also SBF and MBF, is regulated by phosphorylation (54, 59). The nuclear localization signal of Swi6 contains a phosphorylatable serine residue. Phosphorylation of this residue, Ser160, inactivates the nuclear localization signal. Since this phosphorylation occurs in a cell cycle-regulated manner from the end of G_1 until late mitosis, Swi6 is cytoplasmic from S phase to late mitosis but is nuclear during G_1 (54). However, this cell cycle-regulated subcellular localization of SBF and MBF is not sufficient to explain their activation in late G_1, since in vivo footprinting and chromatin immunoprecipitation experiments have shown that SBF and MBF are bound to the promoters of their target genes in early G_1 phase and yet do not induce expression of these genes (9, 22, 29). Expression of SBF/MBF target genes at Start involves at least three other genes, *CLN3*, *BCK2*, and *CTR9* (12, 30, 60). These genes encode putative activators of SBF and MBF, and they may be responsible for the timing of the transcriptional program at Start.

Cln3 is the most prominent activator of SBF and MBF. The

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CLN3 gene was originally identified by virtue of its roles in the regulation of cell size and pheromone sensitivity (10, 40). *CLN3* transcription is only mildly cell cycle regulated, with a peak in late M or early G_1, and Cln3 protein can be detected throughout the cell cycle (37, 60). Cln3 is a highly unstable protein that localizes to the nucleus (15, 38, 61). Changes in the level of Cln3 expression result in closely correlated changes in the transcription of several hundred Start-specific genes (13, 57, 58, 60). In fact, all known transcriptional targets for SBF and MBF can be induced by overexpression of *CLN3*, even in the absence of passage through Start (57). Furthermore, the activation of SBF and MBF in late G_1 has been shown to be dependent upon Cdc28 (29, 36), strongly suggesting that the Cln3-Cdc28 complex is responsible. Cosma and colleagues have recently shown that this Cdc28-mediated activation coincides with the recruitment of PolII, TFIIB, and TFIIH to SBF/MBF-regulated promoters (9).

In addition to its role in promoting transcription of Start-specific genes, Cln3 has been shown to function as a dosage-dependent regulator of cell size, of pheromone sensitivity, of budding, and of the initiation of S phase (10, 40, 60). *cln3* mutants are characterized by a large cell size, an increased sensitivity to mating pheromone, and a delay at the G_1-S transition (10, 40). It is not clear to what extent these phenotypes are downstream effects of the role of Cln3 as an activator of the transcription factors SBF and MBF and to what extent they are separate effects of Cln3; this is one of the major issues we address.

Cln3 is not the only activator of SBF and MBF. In a *cln3* null mutant, the expression of SBF- and MBF-regulated genes is delayed, but there is enough residual, *CLN3*-independent, SBF/MBF activity to allow cell cycle progression and cell viability. The difference between the phenotype of a *cln3* mutant (which is viable) and of a *swi4 mbp1* mutant (which is inviable) indicates the existence of additional activators of SBF and MBF. *BCK2* and *CTR9* encode activators of SBF and MBF that are essential for viability in the absence of *CLN3*; that is, *bck2 cln3* and *ctr9 cln3* double mutants are each inviable (12, 17, 30).

Although a role for Cln3 in the activation of SBF/MBF-mediated transcription has been recognized in previous studies, it has remained unclear to what extent the various aspects of Cln3 function depend on SBF and MBF. In addition, little or nothing is known about the mechanism by which Cln3 activates SBF and MBF. The work presented here has been directed at determining whether Cln3 acts exclusively via SBF and MBF and at uncovering the mechanism by which Cln3 activates SBF and MBF. We found that all known aspects of the function of *CLN3* require *SWI6*. Our work suggests that Cln3-Cdc28 may not activate SBF and MBF by direct phosphorylation or by forming a stable complex with SBF or MBF. Mutational analysis of *SWI6* has allowed us to uncover roles for the amino terminus and the leucine zipper region of Swi6 in responding to Cln3.

MATERIALS AND METHODS

Yeast strains, culture conditions, and plasmids. The yeast strains used in this study are listed in Table 1. We used standard methods for culture and manipulation of yeast (21). Carbon sources were generally used at a combined final concentration of 2% (wt/vol). Synthetic complete (SC) dropout media were prepared by addition of 2.0 g of a mix containing the appropriate amino acids to 5 g ammonium sulfate and 6.7 g of Difco-Bacto yeast nitrogen base lacking amino acids and ammonium sulfate. The final concentrations of amino acids and bases in the media were 50 mg/liter, except for L-tryptophan (80 mg/liter), adenine sulfate (32 mg/liter), and *p*-aminobenzoic acid (5 mg/liter). YAP-based media were made by supplementing YEP (1% yeast extract, 2% peptone)-based media with filter-sterilized adenine to 0.004% (wt/vol). YAPD medium was YAP with 2% D-glucose. Plasmids were created by using a combination of standard restriction fragment cloning methods, PCR, in vitro mutagenesis, and in vivo gap repair cloning (2, 35, 51).

For the experiment shown in Fig. 8, plasmid pHW254 (Ycplac111-*CLN3*^{NotI}) was used to create plasmid pHW364, which contains a full-length *CLN3* gene fused at its carboxyl terminus to the *TUP1* repression domain (encoding amino acids [aa] 74 to 388).

For the studies shown in Fig. 9, plasmid pHW322 (pGBD-C1-*SWI6*$_{1\text{-}803}$ *SWI6*) was used to create a series of *GAL4BD-SWI6* deletion mutants. The resulting mutant plasmids are pHW344 (pGBD-C1-*SWI6*$_{1\text{-}715}$ *SWI6*Δ*C*), pHW350 (pGBD-C1-*SWI6*$_{1\text{-}568}$ *SWI6*Δ*LZC*), pHW359 (pGBD-C1-*SWI6*$_{1\text{-}281}$ *SWI6*Δ*ALZC*), pHW351 (pGBD-C1-*SWI6*$_{1\text{-}193}$ *SWI6*Δ*NALZC*), pHW354 (pGBD-C1-*SWI6*$_{284\text{-}803}$ *SWI6*Δ*N*), pHW353 (pGBD-C1-*SWI6*$_{561\text{-}803}$ *SWI6*Δ*NA*), pHW360 (pGBD-C1-*SWI6*$_{284\text{-}715}$ *SWI6*Δ*N*Δ*C*), pHW363 (pGBD-C1-*SWI6*$_{715\text{-}803}$ *SWI6*Δ*NALZ*), pHW362 (pGBD-C1-*SWI6*$_{561\text{-}715}$ *SWI6*Δ*NA*Δ*C*), pHW374 (pGBD-C1-*SWI6*$_{561\text{-}583/610\text{-}803}$ *SWI6*Δ*NA*Δ*LZ*), and pHW373 (pGBD-C1-*SWI6*$_{284\text{-}583/610\text{-}803}$ *SWI6*Δ*N*Δ*LZ*). A limited series of *SWI6* deletion mutants under control of the native promoter was created in centromeric (YCplac33) and integrating (YIplac204) vectors: pHW197 (YCplac33-*SWI6*$_{1\text{-}803}$ *SWI6*), pHW378 (YCplac33-*SWI6*$_{561\text{-}803}$ *SWI6*Δ*NA*), pHW379 (YCplac33-*SWI6*$_{561\text{-}583/610\text{-}803}$ *SWI6*Δ*NA*Δ*LZ*), pHW206 (YCplac33-*SWI6*$_{1\text{-}561/610\text{-}803}$ *SWI6*Δ*LZ*), pHW377 (YIplac204-*SWI6*$_{1\text{-}803}$ *SWI6*), pHW386 (YIplac204-*SWI6*$_{561\text{-}803}$ *SWI6*Δ*NA*), pHW387 (YIplac204-*SWI6*$_{561\text{-}583/610\text{-}803}$ *SWI6*Δ*NA*Δ*LZ*), and pHW388 (YIplac204-*SWI6*$_{1\text{-}561/610\text{-}803}$ *SWI6*Δ*LZ*).

A list of the plasmids mentioned in this study can be found in Table 2.

Northern analysis. Northern analysis was performed essentially as described previously (63). Northern signals were quantitated using a PhosphorImager and normalized to the *ACT1* signal after subtraction of background signals.

Mutagenesis. Site-directed mutagenesis was performed using materials and protocols of the pAlter system from Promega. Use of the pAlter system required recloning of the targeted sequences into the pAlter vector or one of its derivatives (G. Sherlock and B. Futcher, unpublished results) that contained yeast auxotrophic markers.

Plasmid pHW143 (pYLeuAlter1-*SWI6*) was subject to site-directed mutagenesis to create derivatives with mutations in the Cdc28 consensus phosphorylation sites in the *SWI6* gene. Derivatives of pHW143 were created with a *Not*I restriction site at the *SWI6* stop codon. pHW155 (*SWI6*^{n}) has the carboxy-terminal *Not*I site but no mutations in SP or TP dipeptides, pHW156 (*SWI6*^{s2}) has the *Not*I site and an S-to-A change at position 228, pHW157 (*SWI6*$^{s1\text{-}2}$) is pHW156 with an additional S-to-A change at position 160, pHW179 (*SWI6*$^{s1\text{-}4}$) is pHW157 with a T-to-A mutation at position 179 and an S-to-A mutation at position 238, and pHW180 (*SWI6*$^{s1\text{-}5}$) is pHW179 with a P-to-S change at position 321.

Immunoprecipitation and immunoblot analysis. Yeast extracts for immunoprecipitations and immunoblot analysis were prepared as specified previously (60). Yeast pellets were lysed in a mini-Beadbeater cell disrupter (Biospecs) with 0.5-mm-diameter acid-washed baked zirconium beads in the presence of buffer 3 (0.1% NP-40, 250 mM NaCl, 50 mM NaF, 5 mM EDTA, and 50 mM Tris-HCl [pH 7.5]) and proteinase inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 0.6 mM dimethylaminopurine, 10 μg of soybean trypsin inhibitor per ml, and 1 μg of tosylsulfonyl phenylalanyl chloromethyl ketone per ml). Cell debris was pelleted during a 15-min microcentrifuge spin at maximum speed. Protein concentrations were quantitated using the Bio-Rad dye-binding assay according to the manufacturer's specifications. Immunoprecipitations were carried out with 2 to 6 mg of extract by adding 0.3 μl of ascites fluid containing 12CA5 or 9E10 mouse monoclonal antibody and incubating on ice for 2 h. Next, protein G agarose beads (Pharmacia) were added, and the extracts were rocked at 4°C for 2 h. Beads were washed four times with buffer 3, using pulse spins of $<$1,000 $\times$ *g* in a microcentrifuge. Protein samples (40 μg for immunoblots) were mixed with 2$\times$ loading buffer boiled for 1 to 2 min and subjected to sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis. Protein gels were transferred to nitrocellulose by using a semidry transfer apparatus (Millipore) and probed consecutively with primary (1:10,000 diluted 12CA5 or 9E10 antibody or 1:100 diluted Santa Cruz sc-53 polyclonal antibody) and secondary (1:20,000 Amersham horseradish peroxidase-conjugated sheep anti-mouse or goat anti-rabbit immunoglobulin G) antibodies. Pro-

TABLE 1. *S. cerevisiae* strains

Strain	Background[a]	Relevant genotype	Source
MAV99		*MAT***a** *trp1-901 leu2-3,112 his*Δ*200 ade2-101 gal4*Δ *gal80*Δ *can1*^{r} *cyh2*^{r} *LYS2*::*(GAL1*::*HIS3) GAL1*::*lacZ SPO13*::*10xGAL4site*::*URA3*	M. Vidal
PJ69-4A		*MATa trp1-901 leu2-3,112 ura3-52 his3-200 gal4 gal80 LYS2*::*GAL1-HIS3 GAL2-ADE2 met2*::*GAL7-lacZ*	P. James
RN200-6d		*MAT***a** *cdc34-2 his3 leu2 ura3*	R. Nash
W303a		*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 ssd1-d*	R. Rothstein
W303Va		*MAT***a** *ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 SSD1-v*	K. Arndt
YHW23		*MAT***a** *ade2-101 his3*Δ*200 leu2*Δ*1 lys2-801 ssd1-d ura3-52 swi6*::*HIS3 swi4*::*HIS3* {*YCplac33/GAL1-10-3xHA-SWI4*}	This study
YHW29	W	*MAT***a** *cln3*::*ura3-GAL1-CLN3 trp1*::*MET3-CLN2-TRP1* {*YCp50/S.pADH-SWI4*}	This study
YHW30	W	*MAT***a** *cln3*::*ura3-GAL1-CLN3 trp1*::*MET3-CLN2-TRP1 swi6*::*LEU2* {*YCp50/S.p.ADH-SWI4*}	This study
YHW31		*MAT*α *ade2-101 his3*Δ*200 leu2*Δ*1 lys2-801 ssd1-d ura3-52 swi6*::*HIS3*	This study
YHW95	V	*MAT***a** *swi4*::*HIS3*	This study
YHW97	V	*MAT***a** *swi6*::*LEU2*	This study
YHW204		*MAT*α *ade2 his3-11,15 leu2 trp1 ura3 cln3*::*ura3 bck2*::*TRP1* {*pRS313/MET3-CLN2*}	This study
YHW228		*MAT***a** *ade2-101 his3*Δ*200 leu2*Δ*1 lys2-801 ssd1-d swi6*::*HIS3 swi4*::*HIS3 CLN2*::*MET-CLN2*	This study
YHW446	V	*MAT***a** *swi6*::*LEU2 cln3*::*hisG-URA3-hisG*	This study
YHW541		*MAT***a** *ade2-1his3-11,15 leu2 trp1 ura3 SSD1-v bck2*::*TRP1 swi6*::*LEU2* {*pRS313/MET3-CLN2*}	This study
YHW613		*MAT*α *ade2 his3-11,15 leu2 trp1 ura3 cln3*::*ura3 bck2*::*TRP1 swi6*::*LEU2* {*pRS313/MET3-CLN2*}	This study
YHW623	P	*MAT***a** *cln3*::*hisG*	This study
YHW626	V	*MAT***a** *swi6*::*leu2*::*hisG-URA3-hisG*	This study
YHW665	V	*MAT***a** *cln3*::*hisG-URA3-hisG*	This study
YHW668	P	*MAT***a** *cln3*::*hisG swi6*::*leu2*::*hisG-URA3-hisG*	This study
YHW731	P	*MAT***a** *cln3*::*hisG* {*pGBDU-C2/CDC28*}	This study
YHW838		*MAT***a** *ade2-101 his3*Δ*200 leu2*Δ*1 lys2-801 ssd1-d ura3-52 trp1*::*URA3 swi6*::*HIS3 swi4*::*HIS3 CLN2*::*MET3-CLN2*	This study
YHW888	W	*MAT***a** *cln3*::*ura3-GAL1-CLN3 swi6*::*LEU2 trp1*::*TRP1*	This study
YHW889	W	*MAT***a** *cln3*::*ura3-GAL1-CLN3 swi6*::*LEU2 trp1*::*SWI6-TRP1*	This study

[a] Strains with background P are isogenic to PJ69-4A (25), strains with background V are isogenic to W303Va, strains with background V3 have been backcrossed three times to W303Va, and strains with background W are isogenic to W303a. The difference between W303Va (*SSD1-v*) and W303a (*ssd1-d*) is important for this study, since a *cln1 cln2 SSD1-v* strain is viable but a *cln1 cln2 ssd1-v* strain is inviable (18).

teins were visualized using the Amersham ECL system or the Pierce Supersignal system according to the manufacturer's specifications.

Cell cycle synchrony experiments. Cell cycle synchronizations were performed using centrifugal elutriation as described previously (60). Synchrony was confirmed using microscopic analysis and flow cytometry.

For the experiment shown in Fig. 2, in order to obtain cultures of desirable morphology and starting cell size for elutriation and to control for a possible effect of the *swi6* mutation on *SWI4* expression, strains YHW29 and YHW30 were engineered to contain an integrated *MET3-CLN2* construct and an episomal {*S.pADH-SWI4-URA3-CEN*} plasmid (CB1491). Initial cultures of YHW29 and YHW30 were grown in SC medium lacking uracil (SC−ura) with 2% raffinose and containing 0.001% (wt/vol) methionine, which allows for limited induction of *MET3-CLN2*. Methionine (2 mM final concentration) was added 10 min before harvesting of the cultures for elutriation to repress *MET3-CLN2* during the rest of the experiment.

Flow cytometry. Flow cytometry with a FACSCalibur flow cytometer (Becton Dickinson) was performed on yeast cells stained with propidium iodide. After yeast cells had been harvested, washed, sonicated, and fixed overnight in 70% ethanol at 4°C, they were resuspended in 50 mM sodium citrate, washed in the same buffer, sonicated, treated with RNase A (final concentration, 0.25 mg/ml) for 1 h at 50°C, and treated with proteinase K (final concentration, 1 mg/ml) for an additional hour at 50°C. Before analysis the yeast cells were stained with propidium iodide at a final concentration of 16 μg/ml.

Cell size analysis. Analysis of the cell size distribution of yeast strains was done using cultures in mid-log phase. Samples of the cultures were resuspended in 10 ml of Isoton buffer, briefly sonicated, and immediately analyzed using a Coulter Counter (model ZM; 70-μm aperture) and a Coulter Channelyzer (model 256). Yeast cultures that were to be compared for their cell size distribution were started at the same time in aliquots of the same batch of medium. Cultures were grown to log phase, rediluted at equal densities, and allowed to grow for at least two additional doublings. When cultures reached mid-log phase, as judged by both spectrophotometric analysis and cell count, aliquots were taken for size analysis. For comparison of the cell size profiles of different genotypes, we used strains derived from the same genetic background.

RESULTS

Cln3 functions via the Swi6 subcomponent of SBF and MBF. Cyclin-Cdk complexes are conserved regulators of Start-specific transcription (65). In budding yeast, Cln3-Cdc28 complexes regulate the activity of SBF and MBF (60). We hypothesized that all functions of Cln3 might depend on the Swi6 subcomponent of SBF and MBF on the basis of two published observations: (i) the morphology and growth of *swi6* and *swi6 cln3* mutants are similar, suggesting that Cln3 and Swi6 might function in the same linear pathway (43), and (ii) the ability of *CLN3-1*, a stabilizing allele of *CLN3*, to regulate pheromone sensitivity depends on *SWI6* (43). To determine whether Cln3 acts via Swi6, the effect of varying the *CLN3* dosage was tested in the presence and absence of *SWI6*. All relevant aspects of *CLN3* function were found to be dependent on *SWI6*.

TABLE 2. Plasmids

Name	Vector[a]	Insert	Reference or source
pGBD-C1	pGBD-C1		25
pPC97	pPC97		6
pYLeuAlter1	pYLeuAlter1		G. Sherlock
YCp50	YCp50		49
YCplac111	YCplac111		19
YEplac195	YEplac195		19
YEp352	YEp352		23
C2691	YCplac33	*GAL1-10-3xHA-SWI4*	K. Nasmyth
CB1491	YCp50	*S.p ADH-SWI4*	18
pGBDU-C2-*CDC28*	pGBDU-C2	*CDC28*	G. Sherlock
pML1	pRS313	*MET3-CLN2*	This study
pMT41	YEp24	*GAL1-10-CLN3::3xHA(carboxy-terminal)*	M. Tyers
YEp352#5	YEp352	*CLN3*	R. Nash
YEp352#11	YEp352	*CLN3-1*	R. Nash
pHW126	pRS304	*MET3-CLN2*	This study
pHW136	pRS426	*SWI6*	This study
pHW143	pYLeuAlter1	*SWI6*	This study
pHW155	pYLeuAlter1	$SWI6^{n}$ (*SWI6* with *Not*I site at carboxyl terminus)	This study
pHW156	pYLeuAlter1	$SWI6^{s2}$ ($SWI6^{n}$ with S228→A)	This study
pHW157	pYLeuAlter1	$SWI6^{s1-2}$ ($SWI6^{s2}$ with S160→A)	This study
pHW179	pYLeuAlter1	$SWI6^{s1-4}$ ($SWI6^{s1-2}$ with T179→A and S238→A)	This study
pHW180	pYLeuAlter1	$SWI6^{s1-5}$ ($SWI6^{s1-4}$ with P321→S)	This study
pHW197	YCplac33	*SWI6*	This study
pHW206	YCplac33	*SWI6ΔLZ* (encoding aa 1–561 and 610–803)	This study
pHW254	YCplac111	$CLN3^{NotI}$ (*CLN3* with *Not*I site at carboxyl terminus)	This study
pHW262	YEplac181	*SWI6*	This study
pHW263	YEplac181	*SWI6-6x(myc)*	This study
pHW322	pGBD-C1	*SWI6*	This study
pHW344	pGBD-C1	*SWI6ΔC* (encoding aa 1–715)	This study
pHW350	pGBD-C1	*SWI6ΔLZC* (encoding aa 1–568)	This study
pHW351	pGBD-C1	*SWI6ΔNALZC* (encoding aa 1–193)	This study
pHW353	pGBD-C1	*SWI6ΔNA* (encoding aa 561–803)	This study
pHW354	pGBD-C1	*SWI6ΔN* (encoding aa 284–803)	This study
pHW355	pPC97	*CLN3-1::3xHA*	This study
pHW359	pGBD-C1	*SWI6ΔALZC* (encoding aa 1–281)	This study
pHW360	pGBD-C1	*SWI6ΔNΔC* (encoding aa 284–715)	This study
pHW362	pGBD-C1	*SWI6ΔNAΔC* (encoding aa 561–715)	This study
pHW363	pGBD-C1	*SWI6ΔNALZ* (encoding aa 715–803)	This study
pHW364	YCplac111	*CLN3-TUP1* ($CLN3^{NotI}$ *with TUP1* encoding aa 74-388 at *Not*I site)	This study
pHW373	pGBD-C1	*SWI6ΔNΔLZ* (encoding aa 284–583 and 610–803)	This study
pHW374	pGBD-C1	*SWI6ΔNAΔLZ* (encoding aa 561–583 and 610–803)	This study
pHW377	YIplac204	*SWI6*	This study
pHW378	YCplac33	*SWI6ΔNA* (encoding aa 561–803)	This study
pHW379	YCplac33	*SWI6ΔNAΔLZ* (encoding aa 561–583 and 610–803)	This study
pHW386	YIplac204	*SWI6ΔNA* (encoding aa 561–803)	This study
pHW387	YIplac204	*SWI6ΔNAΔLZ* (encoding aa 561–583 and 610–803)	This study
pHW388	YIplac204	*SWI6ΔLZ* (encoding aa 1–561 and 610–803)	This study

[a] References are as follows: pGBDU-C2, 25; YCp50, 49; YEp24, 3; YCplac111, YCplac33, YEplac181, YEplac195, and YIplac204, 19; pRS304 and pRS313, 55; and pRS426, 8.

Regulation of cell size by *CLN3* depends on *SWI6*. *CLN3* is a dosage-dependent regulator of the length of G_1 and of cell size (40, 48). Whether this control of cell size by *CLN3* depends on *SWI6* was determined by comparing the average cell size of *swi6* mutants with that of *cln3 swi6* mutants. Both mutants have the same cell size (Fig. 1A). Likewise, overexpression of *CLN3* from a multicopy plasmid led to a reduced average cell size in the presence of *SWI6* but not in the absence of *SWI6* (Fig. 1B). Thus, neither under- nor overexpression of *CLN3* affects cell size in the absence of *SWI6*. This effect was specific to *SWI6*, because overexpression of *CLN3* could reduce the average cell size of *swi4* mutants (data not shown).

In a second approach, elutriation was used to obtain small G_1-phase daughter cells from *SWI6* and *swi6* strains carrying *GAL-CLN3*. Cells were grown and G_1 cells were obtained in the absence of galactose (*CLN3* off), and then half of the G_1 cells from each strain were exposed to galactose (*CLN3* on). Budding was assayed as an indicator of Start as the small G_1 cells grew. As expected, the induction of *CLN3* allowed cells to pass through Start (here defined as 50% budding) at a reduced cell size in the *SWI6* cells. However, induction of *CLN3* had little or no effect in the *swi6* mutant cells (Fig. 2).

Regulation of pheromone sensitivity by *CLN3* depends on *SWI6*. In haploid cells, *CLN3* regulates sensitivity to pheromone (10, 40). Haploid cells exposed to pheromone arrest in late G_1 at Start and attempt to conjugate (32). This G_1 arrest

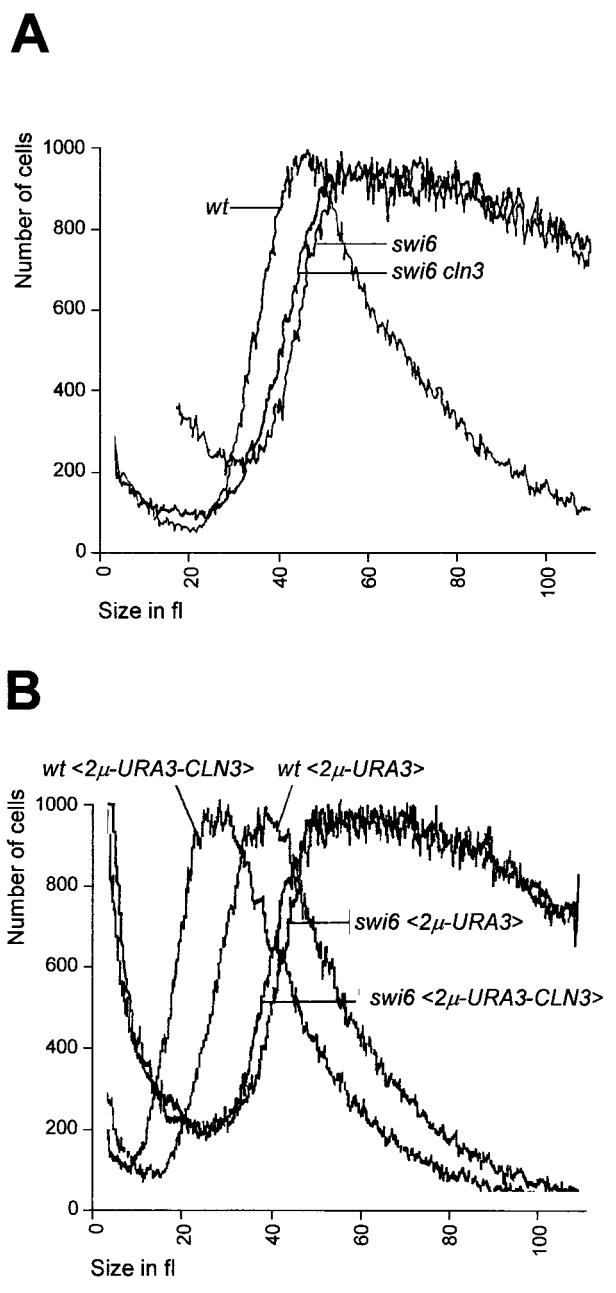
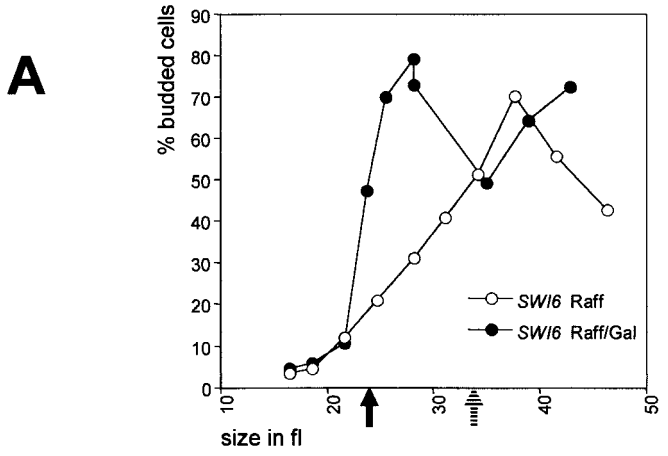


FIG. 1. The effect of *CLN3* on cell size depends on *SWI6*. (A) Isogenic strains W303Va (wild type [wt]), YHW97 (*swi6*), and YHW446 (*swi6 cln3*) were grown to mid-log phase in YAPD medium and analyzed for their cell size.(B) Isogenic strains W303Va (wt), and YHW97 (*swi6*) were transformed with either a high-copy *CLN3* plasmid (YEp352#5) or a vector control (YEplac195). Transformants were grown to mid-log phase in SC−ura with 2% glucose and analyzed for their cell size.

involves the inactivation of Cln-Cdc28 complexes. *CLN1* and *-2* are inhibited at the transcriptional level (16, 62, 64) as well as posttranslationally (47), whereas *CLN3* is inhibited solely at the posttranslational level (16, 26). Part of the transcriptional inhibition of *CLN1* and *CLN2* is likely due to the loss of Cln3-Cdc28 activity. *cln3* mutants have enhanced sensitivity to pheromone, whereas cells with increased Cln3 dosage are more resistant (10, 40). Deletion of *SWI6* leads to an increase in pheromone sensitivity that is comparable to that observed in the absence of *CLN3*. To determine whether Cln3 regulates pheromone sensitivity by regulating SBF and MBF (and thus the expression of *CLN1* and *CLN2*), the effect of *CLN3* dosage on pheromone sensitivity was determined in the presence and absence of *SWI6* (Fig. 3). It was found that high-copy overexpression of *CLN3* caused increased pheromone resistance in the presence of *SWI6* but not in the absence of *SWI6*. Similarly, *CLN3-1* affected pheromone sensitivity preferentially in the presence of *SWI6* (Fig. 3A). *CLN3-1* does increase pheromone resistance slightly even in the absence of *SWI6*, but this appears to be a neomorphic effect associated with the *CLN3-1* allele (see Discussion) (15). Moreover, when *cln3* and *swi6* mutations were combined, no increase in pheromone sensitivity was observed (Fig. 3B), which suggests that these genes may function at different levels in the same linear pathway. These results suggest that *CLN3* regulates pheromone sensitivity upstream of *SWI6* (Fig. 3A) and in the same pathway (Fig. 3). The specificity of these results is illustrated by the ability of high-copy *CLN3* to regulate pheromone sensitivity efficiently in the absence of *SWI4* (Fig. 3A) (when *CLN3* presumably works via *SWI6* and *MBP1*) and the ability of *BCK2*, another regulator of Start, to efficiently reduce pheromone sensitivity in the absence of *SWI6* (63).

Regulation of S phase and budding by *CLN3* depends on *SWI6*. *CLN3* is a dosage-dependent activator of Start (40). To confirm that the ability of *CLN3* to regulate the length of G_1 phase fully depends upon SWI6, the effect of altering *CLN3* dosage on S phase and budding was determined in both *SWI6* and *swi6* strains. Cell cycle distributions of asynchronous cultures of wild-type, *cln3*, *swi6*, and *swi6 cln3* yeast strains were assayed by using flow cytometry and propidium iodide staining of DNA (Fig. 4). Deletion of *CLN3* affected cell cycle distribution in the *SWI6* strain but not in the *swi6* strain. The time of budding was determined in the cell cycle synchronization experiment described in Fig. 2 by comparing uninduced and induced *GAL1-CLN3 SWI6* and *GAL1-CLN3 swi6* G_1 cultures for the time when 50% of the cells were budded (Fig. 2C). The time of 50% budding was affected by *CLN3* expression in the presence of *SWI6* but not in the absence of *SWI6*, suggesting that Cln3's sole effect on cell cycle progress is via the activation of Swi6 (Fig. 2C). A similar experiment using a different genetic background is described in Fig. 5. In agreement with the results described in Fig. 2, galactose induction of *GAL1-CLN3* affected the timing of DNA replication (Fig. 5A) and budding (Fig. 5B) only in the presence of *SWI6*.

Induction of transcription by *CLN3* depends on *SWI6*. A genome-wide analysis of gene expression has shown that Cln3 was capable of inducing the expression of over 200 genes with a Start-specific transcription pattern (57). It has not been determined which of these inductions are primary effects, but many of the putative Cln3 targets may be regulated via SBF and MBF. In a previous study we found that the ability of *CLN3* to induce its target genes *CLN1*, *PCL1*, and *RNR1* is eliminated in a *swi4 mbp1* strain, which has no functional Swi6 (63). To determine if Cln3's ability to induce transcription specifically depends on Swi6, we assayed the effect of increased Cln3 expression on the mRNA levels of SBF and MBF target



C

	SWI6 Raff		*SWI6* Raff/Gal		*swi6* Raff		*swi6* Raff/Gal	
	size	time	size	time	size	time	size	time
START	34 fl	120 min	24 fl	65 min	53 fl	100 min	52 fl	105min

FIG. 2. The effect of CLN3 on critical cell size and timing of Start depends on *SWI6*. Synchronous cultures of *SWI6* (YHW29) and *swi6* (YHW30) versions of a strain with a galactose-inducible *GAL-CLN3* allele were obtained by centrifugal elutriation. After growth and elutriation in medium containing raffinose (Raff) (2%), the cultures containing small G_1 daughter cells were split. One half was released into raffinose medium (SC−ura with 2 mM Met and 2% raffinose), whereas the other half was released into medium supplemented with galactose (Gal) (2%) to induce *GAL1-CLN3*. After release, samples were taken every 20 min and average cell size, budding, and DNA content were measured. (A and B) The kinetics of budding as a function of cell size were compared for the raffinose- and raffinose-galactose-grown cultures of YHW29 (*SWI6*) (A) and YHW30 (*swi6*) (B). The size at which 50% of the cells had budded is indicated by an arrow. (C) Estimated cell size and time after release at which 50% budding occurs.

genes in both *SWI6* and *swi6* strains. G_1-phase *SWI6* or *swi6* cells were obtained by elutriation, and the expression of *CLN1*, *RNR1*, and *PCL1* was assayed with and without induction of *GAL1-CLN3* (Fig. 5). Induction of *GAL1-CLN3* shifted the expression of these target genes to a smaller cell size in the presence of *SWI6* but had no effect in the absence of *SWI6* (Fig. 5C to E). (Note that the *SWI6* cells cannot be compared directly to the *swi6* cells on the basis of elapsed time because of an initial difference in cell size [see the legend to Fig. 5].)

The three chosen target genes are regulated in somewhat different ways, and deletion of *SWI6* had a distinct effect on each of these genes. The cell cycle pattern of transcription for *CLN1* and *PCL1* but not *RNR1* persisted in the absence of *SWI6*. Deletion of *SWI6* not only shifted peak expression of

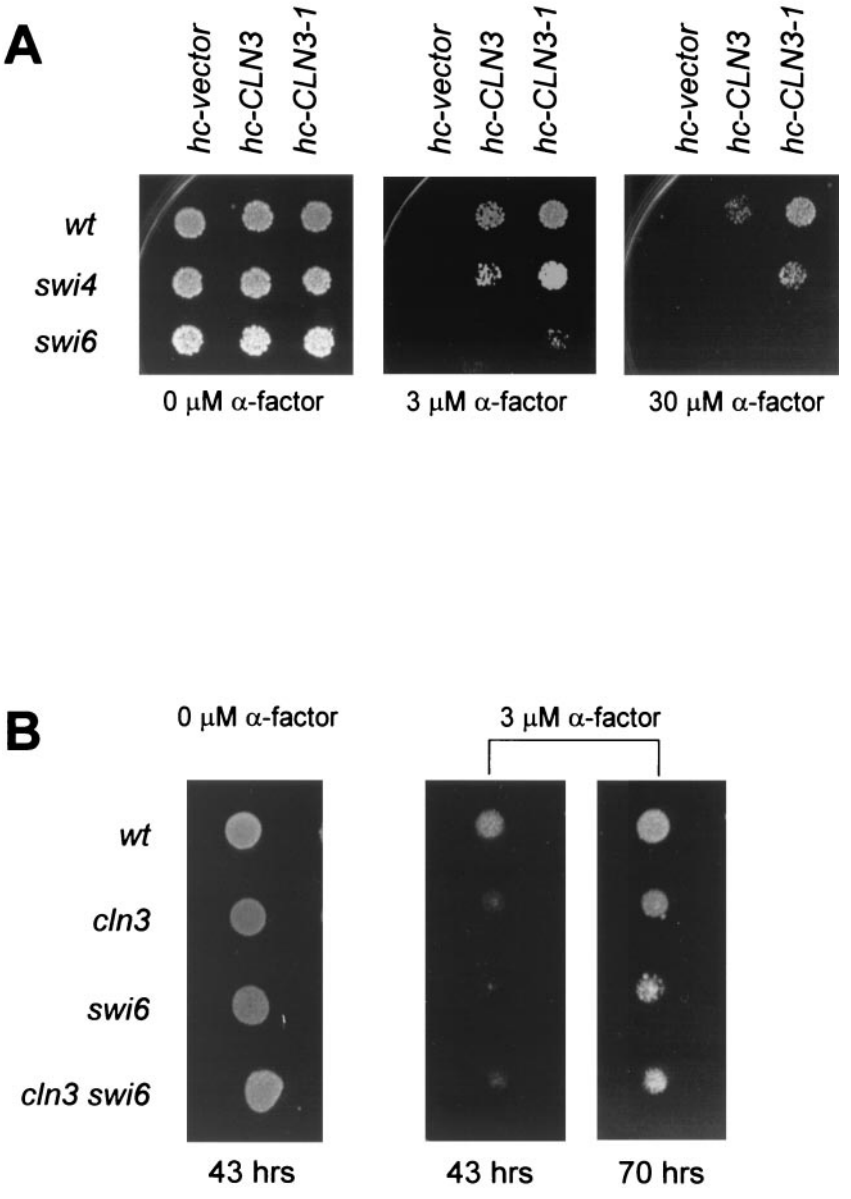


FIG. 3. The effect of CLN3 on pheromone sensitivity depends on *SWI6*. (A) Isogenic strains W303Va (wild type [wt]), YHW95 (*swi4*), and YHW97 (*swi6*) were transformed with high-copy (hc) plasmids containing no insert (YEplac195), *CLN3* (YEp352#5), or *CLN3-1* (YEp352#11). Transformants were grown to mid-log phase in SC−ura with 2% (wt/vol) glucose. Aliquots of 1,000 cells from each culture were spotted on YAPD plates containing 0, 3, or 30 μM α-factor. The picture was taken after 2.5 days of incubation at 30°C. (B) The pheromone sensitivities of isogenic wild-type (W303Va), *cln3* (YHW665), *swi6* (YHW97), and *cln3 swi6* (YHW446) strains were compared by spotting aliquots of 10,000 cells for each of these strains onto YAPD plates containing either 0 or 3 μM α-factor.

both *CLN1* and *PCL1* to a much larger cell size but also specifically delayed the timing of *PCL1* peak expression relative to that of *CLN1* (cf. Fig. 5C and D). The differential effects of *SWI6* deletion on the regulation of the three selected target genes may be explained by their relative dependence on regulation by SBF versus MBF. SBF-regulated genes such as *CLN1* (46) are more likely to benefit from the residual activity of Swi4 (which persists in *swi6* mutants) than predominantly MBF-regulated genes such as *RNR1* (14, 33). SBF-responsive genes that can also be regulated by MBF, such as *PCL1* (63), have an intermediate response to deletion of *SWI6*. Thus, it is particularly striking that in spite of these gene-specific differences, all three genes responded to *CLN3* only in the presence of *SWI6*.

We also induced *GAL1-CLN3* in arrested cultures of *cln3 bck2* or *cln3 bck2 swi6* strains. *GAL1-CLN3* enhanced the expression of *CLN1*, *PCL1*, *RNR1*, and *CLB5* in the *SWI6* strain but had no effect in the *swi6* strain (data not shown).

Regulation of Swi6 by Cln3 does not depend on the consensus Cdc28 phosphorylation sites of Swi6. Having established that Cln3 exerts its function via the Swi6 subcomponent of SBF and MBF, we wanted to address the mechanism by which Cln3 activates Swi6. The biochemical role of Cln3 is to activate the Cdc28 protein kinase, and it is clear that Cln3 requires Cdc28

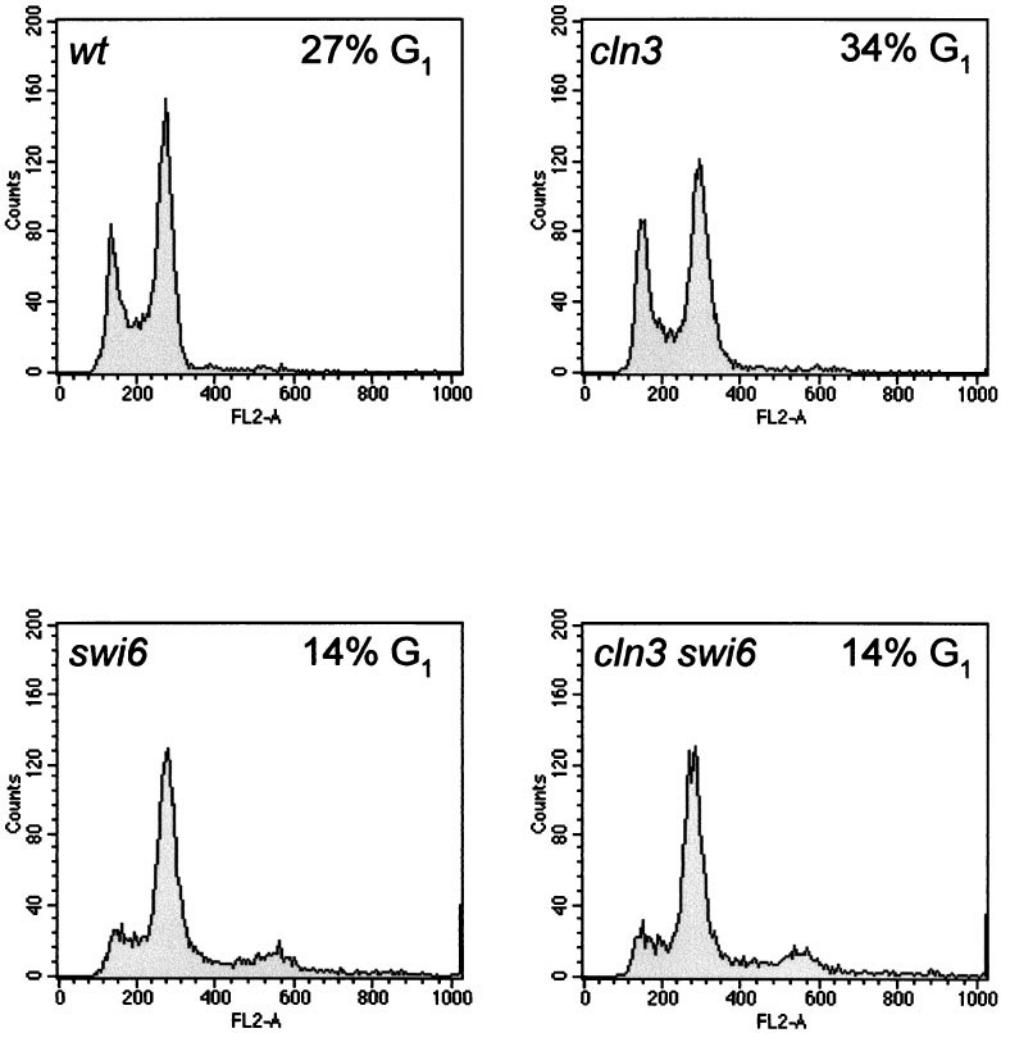


FIG. 4. The effect of CLN3 on cell cycle distribution depends on *SWI6*. Wild-type (W303Va), *cln3* (YHW665), *swi6* (YHW97), and *cln3 swi6* (YHW446) strains of isogenic backgrounds were grown to log phase in YAPD medium at 30°C. The DNA content of the cultures was determined by flow cytometry of propidium iodide-stained samples. The relative number of cells with a 1N DNA content was used as an estimate for the percentage of cells in G_1.

in order to induce SBF- or MBF-dependent transcription (11, 60). Thus, the most obvious model is that the Cln3-Cdc28 complex directly phosphorylates Swi6, or perhaps Swi6 and also Swi4 and Mbp1, and that this causes activation of transcription. Work by Sidorova and colleagues (54) showed that Swi6 is regulated in part by phosphorylation. This particular phosphorylation, however, does not reflect activation by Cln3-Cdc28 but rather reflects inactivation of the nuclear localization signal of Swi6 by an unspecified kinase (54).

Swi6 has five SP or TP sites (i.e., the minimal recognition motif for Cdc28-cyclin complexes). To test the idea that phosphorylation of Swi6 by Cln3-Cdc28 was important for transcriptional activation, we made a quintuple *SWI6* mutant lacking all five sites. Four of the sites were changed to AP. For the fifth site, which resides in one of Swi6's ankyrin repeats and has a conserved threonine, the proline residue was mutated to serine. The resulting quintuple phosphorylation site mutant (as well as various other combinations of phosphorylation site mutations) was tested for function in several assays. As shown in Fig. 6, cells bearing the quintuple *SWI6* mutant have exactly the same cell size distribution as cells with wild-type *SWI6*. In addition, we assayed the ability of the quintuple mutant to respond to the presence of *CLN3* by measuring cell size in the presence and absence of *CLN3*. The quintuple mutant *SWI6* was fully responsive to *CLN3* (data not shown). In addition, the quintuple mutant *SWI6* was indistinguishable from wild-type *SWI6* in its ability to rescue *swi4 swi6* and *bck2 swi6* mutants. The quintuple mutant *SWI6* and wild-type *SWI6* were also indistinguishable with regard to cell cycle distribution and cell morphology, even in an *mbp1* background (data not shown).

The results described above suggest that Cln3-Cdc28 does not work solely by phosphorylating Swi6 on SP or TP sites. We therefore also examined the role of potential phosphorylation sites in Swi4. There are many SP and TP sites in Swi4, but M. Neuberg and K. Nasmyth (personal communication) previously mapped cell cycle-regulated phosphopeptides on Swi4, discovered two Cdc28-dependent phosphorylations, and mapped

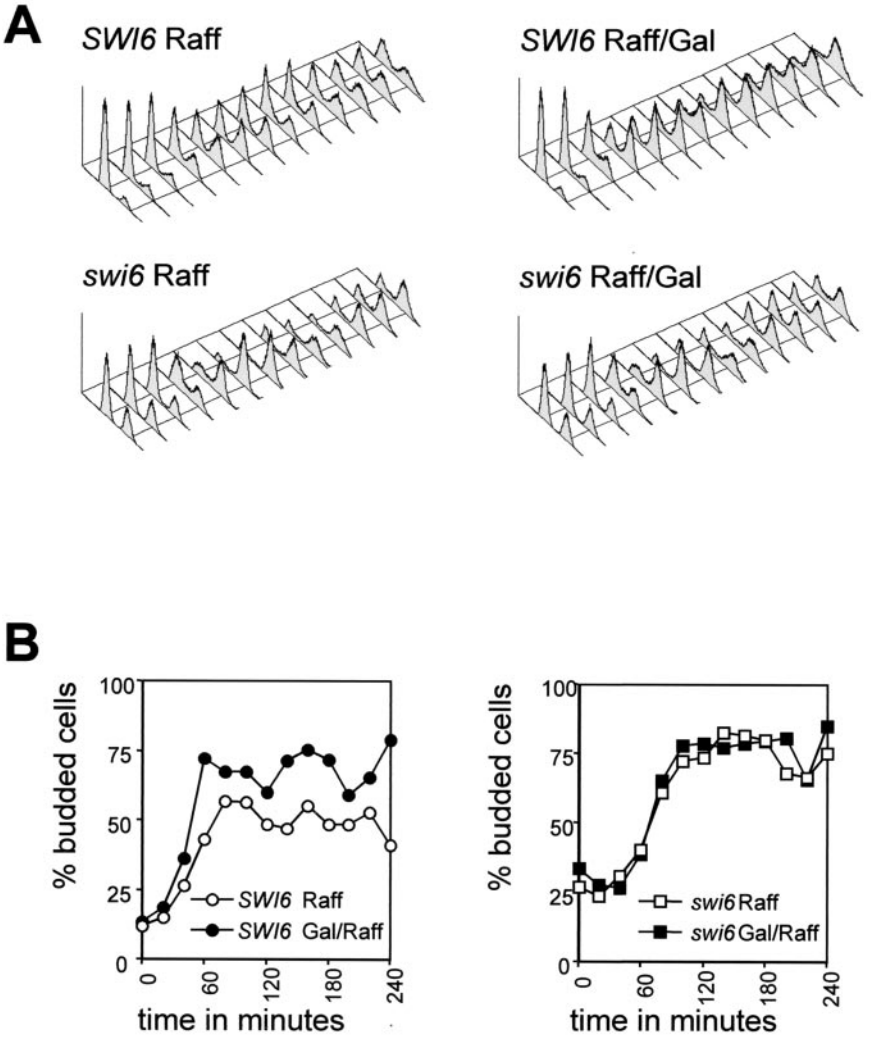


FIG. 5. Effect of *CLN3* on cell cycle progress and Start-specific transcription in *SWI6* and *swi6* cells. After growth and centrifugal elutriation in YAP with 2% raffinose, *cln3*::*GAL1-CLN3 SWI6* (YHW889) and *cln3*::*GAL1-CLN3 swi6* (YHW888) cells were released into either YAP–2% raffinose (Raff) or YAP–2% raffinose–2% galactose (Raff/Gal) medium. Because the *swi6* mutation affects cell size at cytokinesis, elutriated cells from the *swi6* strain were larger than elutriated cells from the *SWI6* strain; thus, the left and right panels cannot be directly compared on the basis of elapsed time. Samples were taken every 20 min, and DNA content (A), budding index (B), and cell size were measured. (A) Histograms of DNA content from samples taken 20 min apart. (B) Budding as a function of time. (C to E) The same samples were also processed for Northern analysis. Blots were hybridized with DNA fragments corresponding to the *ACT1*, *CLN1*, *PCL1*, and *RNR1* genes, and hybridization signals were quantitated using a Fuji PhosphorImager. The signals for the Start-specific transcripts *CLN1*, *PCL1*, and *RNR1* were normalized to *ACT1* (loading control). Expression of *CLN1*, *PCL1*, and *RNR1* is shown as a function of time in, respectively, panels C, D, and E. Peak expression levels of *CLN1* and *PCL1* corresponded to the following approximate average cell sizes in each of the four conditions: *SWI6* Raff, *CLN1* peak at a volume of ~50 fl and *PCL1* peak at ~55 fl; *SWI6* Raff/Gal, *CLN1* peak at ~45 fl and *PCL1* peak at ~45 fl; *swi6* Raff, *CLN1* peak at ~85 fl and *PCL1* peak at ~100 fl; and *swi6* Raff/Gal, *CLN1* peak at ~85 fl and *PCL1* peak at ~100 fl.

them to S159 and T799. We obtained the *SWI4* S159A T799V double mutant (*SWI4**) from Neuberg and Nasmyth, combined it with the quintuple *SWI6* mutant (*SWI6*$^{s1\text{-}5}$), and examined the responsiveness of this combination to *CLN3-1*. Because the presence of Mbp1 could potentially interfere with interpretation, we did these experiments in an *mbp1* background. Experiments were done in the presence of the resident copy of *CLN3*, with or without an added copy of *CLN3-1*. The results showed that the *SWI4** mutation had no effect on cell size and also no effect on the ability of *CLN3-1* to affect cell size. For example, the mode cell size of the *CLN3-1 SWI4 SWI6*$^{s1\text{-}5}$ *CLN3 mbp1* strain was 0.80 of the mode cell size of its *SWI4 SWI6*$^{s1\text{-}5}$ *CLN3 mbp1* control, while the mode cell size of the *CLN3-1 SWI4* SWI6*$^{s1\text{-}5}$ *CLN3 mbp1* strain was 0.81 of the mode cell size of its *SWI4* SWI6*$^{s1\text{-}5}$ *CLN3 mbp1* control. These two ratios, 0.80 and 0.81, are not significantly different. Thus, we can find no evidence that Cln3-Cdc28 exerts its effect by phosphorylating any component of SBF. We acknowledge that, formally, we cannot rule out the possibility that other, untested sites on Swi4 or non-SP/TP sites on Swi6 could be involved (although no such sites were noted by Sidorova et al. [54]).

Stable complexes between Cln3 and Swi6 cannot be detected. Activation of Swi6 by Cln3-Cdc28 could involve a physical interaction between Cln3-Cdc28 and SBF or MBF. If Cln3-Cdc28 did bind to SBF or MBF, the resulting complex would

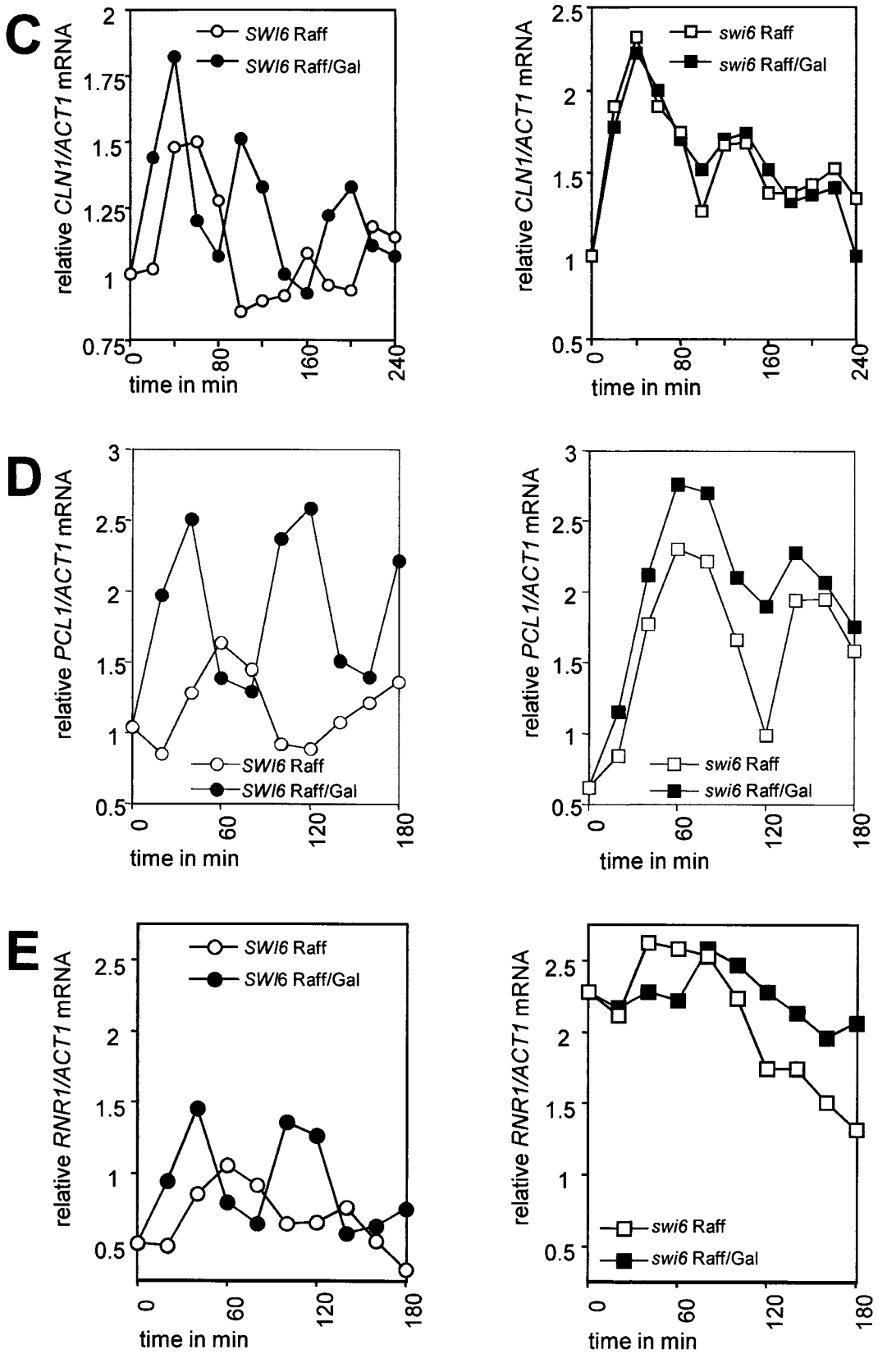


FIG. 5—*Continued.*

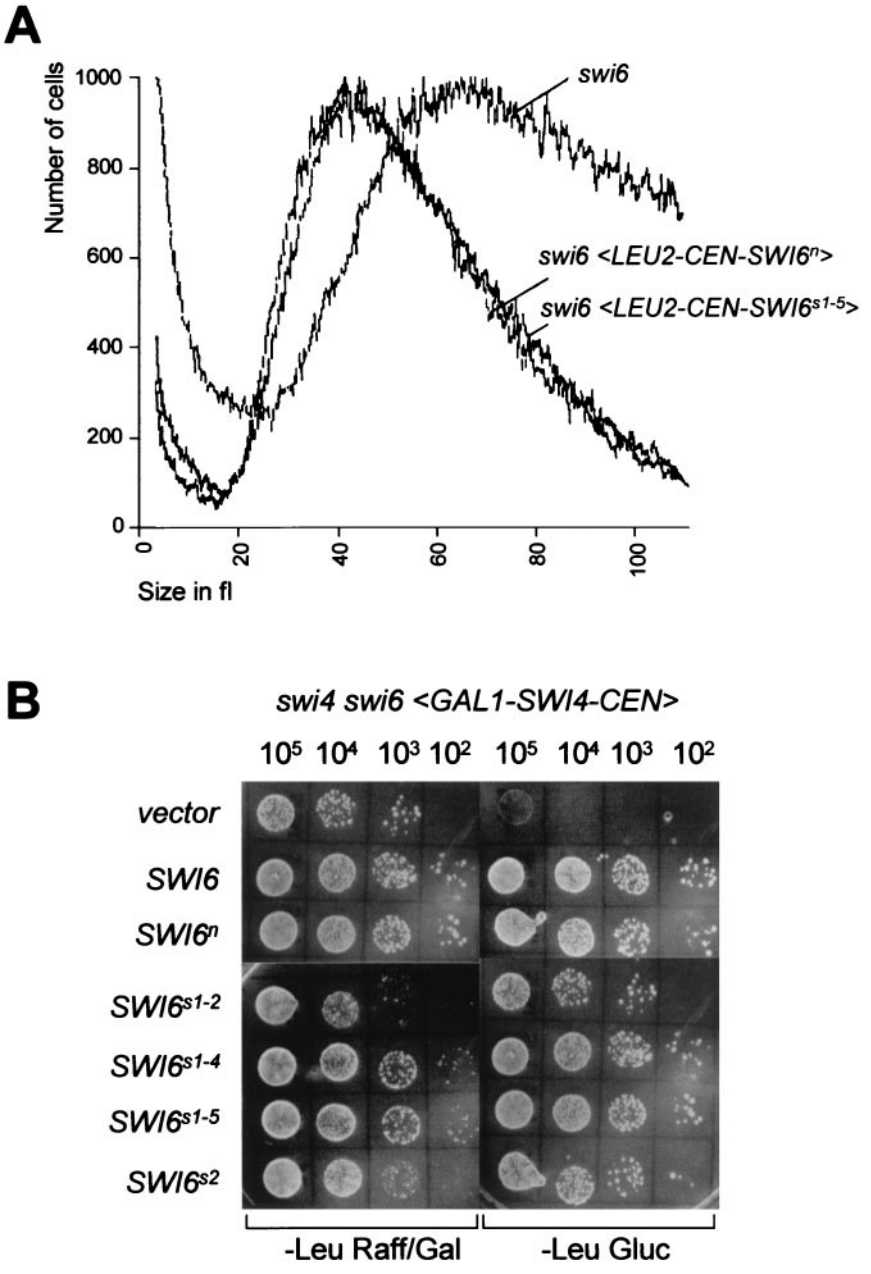


FIG. 6. Swi6 is not activated via its Cdc28 consensus phosphorylation sites. (A) Strain YHW31 (*swi6*) was transformed with plasmids pHW155 ({*LEU2-CEN-SWI6*^{n}}, all five SP or TP sites present) and pHW180 ({*LEU2-CEN-SWI6*$^{s1\text{-}5}$}, all five SP or TP sites mutated [see Materials and Methods]). Transformants were grown to log phase in SC−leu with 2% glucose and analyzed for cell size. As a control, cell size was measured for a culture of untransformed YHW31 (*swi6*) grown in the same medium supplemented with 0.01% leucine. (B) Strain YHW23 (*swi4 swi6* {*GAL1-SWI4-CEN*}) was transformed with pYLeuAlter1 (vector) and its derivatives pHW143 (*SWI6*), pHW155 (*SWI6*^{n}, no sites mutated), pHW157 (*SWI6*$^{s1\text{-}2}$, two sites mutated), pHW179 (*SWI6*$^{s1\text{-}4}$, four sites mutated), pHW180 (*SWI6*$^{s1\text{-}5}$, five sites mutated), and pHW156 (*SWI6*^{s2}, one site mutated). The resulting strains were grown in SC−leu with 1% raffinose and 1% galactose. Serially diluted aliquots of cells were spotted on either SC−leu with 1% raffinose and 1% galactose (−Leu Raff/Gal) or SC−leu with 2% glucose (−Leu Gluc) plates and were photographed after 3 days at 30°C.

likely be found at the promoters of SBF and MBF target genes, because in vivo footprinting and chromatin immunoprecipitation assays have shown that SBF is found on the promoters of its target genes throughout G_1 (9, 22, 29). We looked for stable complexes between Cln3-Cdc28 and SBF or MBF by immunoprecipitating epitope-tagged versions of Swi6 and looking for coprecipitation of Cdc28 or epitope-tagged Cln3. Even when both Cln3 and Swi6 were overexpressed in cultures with a large fraction of G_1 cells, no such coprecipitation was seen (Fig. 7). In the same experiments, Cln3-Cdc28 complexes and Swi4-Swi6 complexes were readily detected by coimmunoprecipitation (Fig. 7).

We have also attempted chromatin cross-linking immunoprecipitation experiments to see if hemagglutinin (HA)-tagged

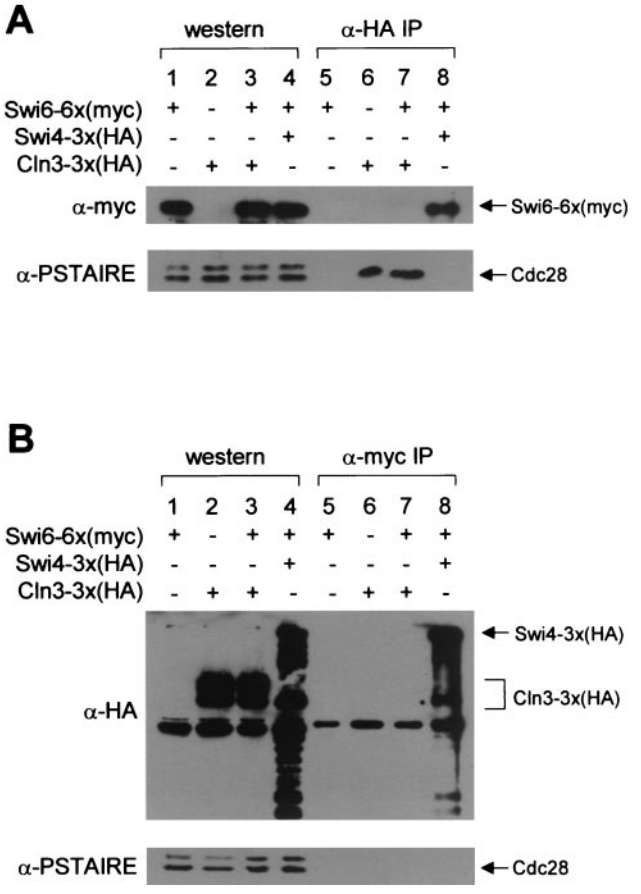


FIG. 7. Cln3 and Swi6 do not coimmunoprecipitate. Strain RN200-6d (*cdc34-2*) was transformed with the following plasmid combinations: (i) YEplac195 and pHW263 [YEplac181-*SWI6-6x*(*myc*)] (lanes 1 and 5), (ii) pMT41 [YEp24-*GAL1-CLN3-3x*(*HA*)] and pHW262 [YEplac181-*SWI6*] (lanes 2 and 6), (iii) pMT41 and pHW263 (lanes 3 and 7), and (iv) C2691 [Ycplac33-*GAL1-SWI4-3x*(*HA*)] and pHW263 (lanes 4 and 8). Doubly transformed strains were grown to early log phase at 24°C in SC−ura−leu with 2% raffinose. Galactose was added to 2%, and after another hour of growth at 24°C, cells were harvested for analysis. Flow cytometry indicated that about half of the cells in each strain had unreplicated DNA. Protein extracts were analyzed on immunoblots directly (40 μg per sample) or, alternatively, immunoprecipitated (IP) (6 mg per sample) with monoclonal antibodies 12CA5 (anti-HA [α-HA]) (A) and 9E10 (α-myc) (B) before immunoblotting. Immunoblots were performed with antibodies 9E10 (α-myc) (A), 12CA5 (α-HA) (B), and sc-53 (Santa Cruz Biotechnology; polyclonal α-PSTAIRE). The α-PSTAIRE antibody recognized a number of bands; in the immunoblot sections shown, the lower band corresponds to Cdc28. The α-HA antibody recognized a nonspecific band in all samples as well as epitope-tagged versions of Cln3 and Swi4 in the corresponding lanes (B). The ladder of bands below the signal corresponding to full-length epitope-tagged Swi4 presumably represents degradation products of epitope-tagged Swi4 (panel B, lane 4). Because Swi4 was epitope tagged at its amino terminus and its interaction with Swi6 occurs at the carboxyl terminus, most of these truncated forms are not coimmunoprecipitated with epitope-tagged Swi6 (panel B, lane 8).

Cln3 could be specifically cross-linked to an SBF-regulated promoter. Results have been negative, but because of the high background in these experiments (i.e., the presence of DNA from the target promoter in immunoprecipitates from the untagged control), we place no weight on these negative results.

Is Cln3 a direct transcriptional activator? In the course of other work, Cln3 was fused to a DNA-binding protein for the purpose of a two-hybrid screen. It was found that under this artificial condition, Cln3 is a powerful transcriptional activator (data not shown). We considered the possibility that this activity reflected a relevant aspect of Cln3 function. Perhaps Cln3-Cdc28 is targeted to SBF- and MBF-dependent promoters (possibly by a weak interaction with SBF and MBF) and, once at the promoter, Cln3 operates relatively directly as a transcriptional activator. To test this possibility, we fused full-length Cln3 to the Tup1 repression domain (aa 74 to 388). Whereas wild-type Cln3 increased transcription of a reporter gene when recruited to its promoter by a Gal4BD-Cdc28 fusion protein ($Gal4_{1-147}$-Cdc28), the Cln3-Tup1 fusion protein had no such effect (Fig. 8C), showing that the Tup1 repression domain was effective in this context. That is, in contrast to Cln3, Cln3-Tup1 is not a transcriptional activator. However, Cln3 and Cln3-Tup1 were equally effective in rescuing the viability of *cln1 cln2 cln3* or *bck2 cln3* strains and were equally effective in promoting Start, as assayed by cell size and cell cycle distribution (Fig. 8A and B and data not shown). (These results also weakly suggest that Cln3 does not form a long-lived complex with SBF and MBF at target promoters; if it did, then the Tup1 repression domain would likely have interfered with transcription of these targets.)

In a second approach, we mutagenized Cln3 in an attempt to separate its normal cell cycle role from its ability to activate transcription when fused to a DNA binding domain. We started with a *CLN3* fusion construct that lacked the PEST region previously associated with part of *CLN3*'s transactivating activity ($GAL4_{1-147}$::*CLN3-1*::*3xHA*; pHW355) (50). The fusion protein expressed by this construct provides Cln3 function and also activates transcription at Gal4-driven promoters (Table 3). We then identified point mutations in the *CLN3* portion of the fusion gene that eliminated the transactivation activity of the fusion protein. We found that there was no correlation between the behavior of fusion proteins in assays for Cln3 function (rescue of *bck2 cln3*) and in assays for transactivation at Gal4-driven promoters (Table 3). Therefore, the direct transcriptional activation activity of *CLN3* is dispensable for its normal cell cycle function. Thus, both lines of experimentation suggest that the ability of Cln3 to promote Start has no correlation with its ability to activate transcription when artificially recruited to the promoter of a reporter gene.

Mutational analysis of *SWI6*. To better understand the role of Swi6 as a component of SBF and MBF and as a mediator of Cln3 function, a mutational analysis of *SWI6* was performed. Various domains of Swi6 were deleted in the context of a Gal4BD-Swi6 fusion protein. These deletion constructs were then tested for various aspects of Swi6 function by using a variety of assays, including the ability to activate transcription of Gal4-driven reporter genes, the ability to rescue *swi4 swi6*, *bck2 swi6*, and *bck2 cln3 swi6* strains, and responsiveness to *CLN3* (Fig. 9). The results of these assays are summarized in Fig. 9B. The ability of the various *GAL4BD-SWI6* mutants to activate Gal4-driven reporter genes suggested that Swi6 has at least two domains that can activate transcription: a powerful transactivating domain between aa 561 and 715 (probably excluding the leucine zipper region from aa 583 to 610) and a minor transactivating domain at its amino terminus (aa 193 to

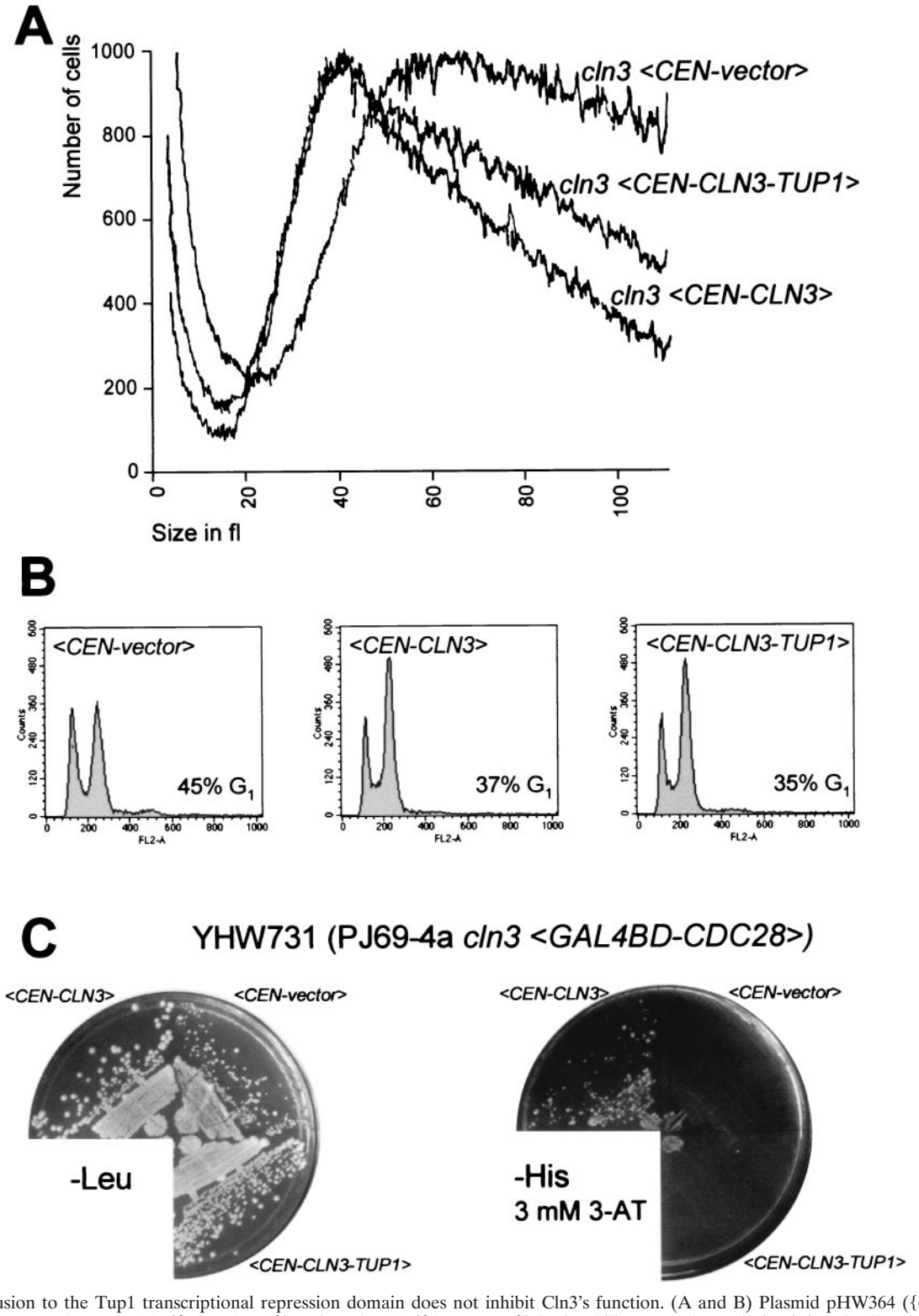


FIG. 8. Fusion to the Tup1 transcriptional repression domain does not inhibit Cln3's function. (A and B) Plasmid pHW364 ({*CEN-CLN3-TUP1*}) was compared to pHW254 ({*CEN-CLN3*}) and Ycplac111 ({*CEN-vector*}) for its ability to regulate cell size (A) and cell cycle distribution (B) in strain YHW623 (PJ69-4A *cln3*). Transformants of YHW623 were grown to log phase in SC−leu with 2% glucose and examined for cell size (A) and DNA content (B). (C) The same plasmids were introduced into strain YHW731 (PJ69-4A *cln3* {*GAL4BD-CDC28*}). Transformants of YHW731 were streaked on plates of SC−his with 2% glucose and 3 mM 3-amino-1,2,4-triazole (3-AT) to test for induction of the two-hybrid *HIS3* reporter gene of YHW731. As a control, plates of SC−leu with 2% glucose were used. The pictures were taken after 4 days of incubation at 30°C.

TABLE 3. Analysis of *GAL4*::*CLN3* constructs for transactivation and function[a]

Construct	Mutation(s)	Induction of Gal4-driven reporter genes on:				Rescue of *bck2 cln3*
		−His with:			−Ura	
		10 mM 3-AT	30 mM 3-AT	50 mM 3-AT		
pPC97		−	−	−	−	−
pHW355		++	++	++	++	++
pHW355	C380stop	+	−	−	−	++
pHW355	L42S C180R	−	−	−	−	+
pHW355	H67R K199R F220L L309S	++/+	+/−	−	−	+/−

[a] pPC97 (*LEU2-CEN-ADH*::$GAL4_{1-147}$), pHW355 (pPC97/*CLN3-1*::*3xHA*), and the specified mutant derivatives of pHW355 were introduced into strains MAV99 (*GAL1*::*HIS3 SPO13*::*10xGAL4site*::*URA3*) and YHW204 (*bck2 cln3* {pRS313/*MET3-CLN2*}). Reporter gene expression was estimated by assessing growth of the MAV99 transformants on SC−leu−his with 10, 30, or 50 mM 3-amino-1,2,4-triazole (3-AT) and SC−leu−ura. Rescue of *bck2 cln3* was assayed by growth of the YHW204 transformants on SC−leu with 2mM Met.

281) (Fig. 9A and B). The ankyrin repeats of Swi6 are not required for transcriptional activation (Fig. 9A and B). Unlike the construct carrying full-length *SWI6,* none of the deletion mutants generated were capable of rescuing the viability of the *swi4 swi6* strain (Fig. 9A and B). A number of mutants, however, were shown to be partially functional in other assays. For example, *GAL4BD-SWI6ΔNA* ($GAL4_{1-147}$-$SWI6_{561-803}$), *GAL4BD-SWI6ΔNAΔLZ* ($GAL4_{1-147}$-$SWI6_{561-583/610-803}$), and *GAL4BD-SWI6ΔNΔLZ* ($GAL4_{1-147}$ -$SWI6_{284-583/610-803}$) were all capable of rescuing viability in a *bck2 swi6* strain (Fig. 9A and B).

Strikingly, deletion mutants simultaneously lacking both the N-terminal domain and the leucine zipper (*GAL4BD-SWI6ΔNAΔLZ* and *GAL4BD-SWI6ΔNΔLZ*) were able to rescue a *bck2 swi6 cln3* strain; that is, they did not require *CLN3* in order to promote transcription (Fig. 9A and B). In addition, these two alleles of *GAL4BD-SWI6* were defective in modulating cell size in response to *CLN3* (Fig. 9A and B). Thus, these two versions of Swi6 behave as if they have lost the ability to respond to *CLN3*, and they no longer require *CLN3* for transactivation. By comparison, two similar alleles that retain the leucine zipper, *GAL4BD-SWI6ΔNA* and *GAL4BD-SWI6,* are both capable of mediating cell size control by Cln3 and are less effective in rescuing *bck2 cln3 swi6* (Fig. 9A and B). Thus, it appears that the leucine zipper of Swi6 may have a role in mediating the activation of SBF and MBF by Cln3.

The roles of the leucine zipper and the amino terminus of Swi6 were further analyzed by creating more specific deletions in otherwise wild-type *SWI6* (Fig. 9A and C). These mutants were tested for the ability to rescue *bck2 swi6* and *swi4 swi6* mutants and for the ability to mediate Cln3's control of cell size. The results of this analysis (Fig. 9A and C) support the previous results obtained with mutants in the *GAL4BD-SWI6* context. Moreover, by including mutant *SWI6ΔLZ* ($SWI6_{1-561/610-803}$) in this analysis, the effect of deleting the leucine zipper region could be determined in an otherwise wild-type context. Surprisingly, *SWI6ΔLZ* could mediate size control by Cln3 (Fig. 9A and C). Thus, it is possible to delete either the amino-terminal 561 aa (including the nuclear localization signal and ankyrin repeats) or the leucine zipper of Swi6 without blocking Cln3 from regulating cell size, but if these two deletions are combined, the resulting Swi6 protein becomes incapable of mediating this Cln3 function.

DISCUSSION

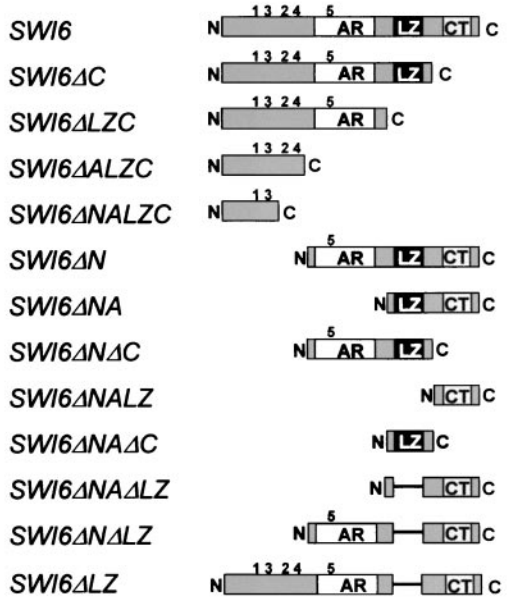
Cln3 acts via Swi6. The experiments described here have shown that all known aspects of Cln3's function, including control of cell size, pheromone sensitivity, cell cycle progress, and transcription, require the Swi6 component of SBF and MBF. Consistent with the epistasis of *swi6* to *cln3,* the effect of disrupting the *SWI6* gene in various genetic backgrounds (*cln1 cln2* [43], *swi4* [43], *bck2* [12], and *sit4* [18]) is at least as severe as that of a *cln3* mutation.

In a *swi6* mutant, expression of *CLN3* at wild-type levels has no detectable effect whatsoever. However, very high levels of expression of *CLN3* or the stabilized allele *CLN3-1* do have some SWI6-independent effects on pheromone sensitivity and the timing of DNA replication (Fig. 3) (H. Wijnen and B. Futcher, unpublished results). At artificially high levels, Cln3 can probably mimic the role of Cln1 and Cln2 and phosphorylate some of their substrates. Mislocalization to the cytoplasm, which is expected to occur at high expression levels, has been shown to allow Cln3 to partially substitute for Cln1 and Cln2 (15, 38, 39). A Swi6-independent effect of Cln3 overexpression on the timing of DNA replication could be explained by direct phosphorylation and subsequent degradation of the replication inhibitor Sic1. It is unlikely, however, that Cln3 plays a Swi6-independent role in the degradation of Sic1 under wild-type conditions, because *swi6* and *swi6 cln3* mutants have similar cell cycle defects and because Cln2 is much more efficient than Cln3 at suppressing the phenotype of Sic1 overexpression (31).

Spellman and coworkers (57) noted that *CLN3* seems to have some repressive effect on genes expressed late in the cell cycle (see Fig. 6 of reference 57). We have not tested whether this putative repressive mitotic function of *CLN3* requires *SWI6*.

The effect of *CLN3* on SBF/MBF-mediated transcription appears to be indirect. Cln3 requires Cdc28 for its ability to activate SBF and MBF, but it remains unclear what substrates need to be phosphorylated for this activation. Neither Swi4 nor Swi6 appears to be activated by Cln3-dependent phosphorylation. Mutation of putative Cdc28 phosphorylation sites in Swi6 as described in this study and elsewhere (54) does not block the responsiveness of Swi6 to *CLN3*. Although we cannot exclude the possibility that Cln3 activates Swi6 via phosphorylation of a site that does not match the Cdc28 recognition consensus, this appears to be unlikely because phosphopeptide analysis

A



B

	trans-activation	rescue of *swi4 swi6*	rescue of *bck2 swi6*	rescue of *bck2 cln3 swi6*	mediation of Cln3's cell size control
SWI6	++++	++	++++	++	++++
SWI6ΔC	++++	-	-		-
SWI6ΔLZC	-	-	-		-
SWI6ΔALZC	++	-	-		-
SWI6ΔNALZC	-	-	-		-
SWI6ΔN	+	-	-		-
SWI6ΔNA	+++	-	++	-	++
SWI6ΔNΔC	++++	-	-		-
SWI6ΔNALZ	-	-	-		-
SWI6ΔNAΔC	+++	-	-		-
SWI6ΔNAΔLZ	+++	-	++++	++++	-
SWI6ΔNΔLZ	++	-	++++	+++	-

C

	rescue of *swi4 swi6*	rescue of *bck2 swi6*	mediation of Cln3's cell size control
SWI6	++	++++	++++
SWI6ΔLZ	-	++	+++
SWI6ΔNA	-	++	++
SWI6ΔNAΔLZ	-	++	-

FIG. 9. Functional analysis of *swi6* deletion mutants. (A) Domain structures of the proteins encoded by wild-type *SWI6* and a series of deletion mutants. Swi6 contains five matches (1, 3, 2, 4, and 5) to the minimal consensus (S/T-P) for a Cdc28 phosphorylation site. In addition, Swi6 contains a domain with four ankyrin repeats (AR), a leucine zipper domain (LZ), and a carboxy-terminal domain for interaction with Swi4 and Mbp1 (CT). (B) Analysis of *GAL4BD-SWI6* deletion mutants. The *GAL4BD-SWI6* mutants described in panel A were tested for their ability to activate transcription of Gal4-driven reporter genes (transactivation); their ability to rescue the growth defects of *swi4 swi6*, *bck2 swi6*, and *bck2 cln3 swi6* strains, and their ability to mediate Cln3's control of cell size. Strain YHW668 (PJ69-4A *cln3 swi6*) was used to assay the abilities of the mutants to activate the *GAL1-HIS3* and *GAL2-ADE2* reporter genes. The same strain was used together with the empty vector control YCplac111 or the single-copy *CLN3* plasmid pHW254 (YCplac111-$CLN3^{Not}$) to assay the ability of the mutants to modulate cell size in response to *CLN3*. Reporter gene activation was scored using serial dilutions on plates of SC−ade with 2% glucose and of SC−his with 2% glucose containing various amounts of 3-amino-1,2,4-triazole (3-AT) (transactivation). Cell size was measured using a Coulter Channelyzer. Strains YHW838 (*swi4 swi6 CLN2*::*MET3-CLN2*), YHW541 (*bck2 swi6* {*MET3-CLN2-CEN*}), and YHW613 (*bck2 cln3 swi6* {*MET3-CLN2-CEN*}) were transformed with the set of *GAL4BD-SWI6* mutant plasmids, and suppression of their growth defects was estimated from the ability of the transformants to grow on plates of SC with 2 mM Met and 2% glucose plates (rescue). (C) Functional analysis of *SWI6* deletion mutants. The *swi6* deletion mutants shown in panel A were tested for their abilities to rescue the growth defects of *swi4 swi6* and *bck2 swi6* strains and for their ability to mediate regulation of cell size by Cln3. The YCplac33-based deletion mutant plasmids pHW197, pHW206, pHW378, and pHW379 were used to transform YHW228 (*swi4 swi6 CLN2*::*MET3-CLN2*) and YHW541 (*bck2 swi6* {*MET3-CLN2-CEN*}). Suppression of the growth defects of YHW228 and YHW541 was tested on plates of SC with 2 mM Met and 2% glucose (rescue). The YIplac204-based deletion mutant plasmids pHW377, pHW386, pHW387, and pHW388 were integrated at the *trp1* loci of isogenic strains YHW97 (*swi6*) and YHW446 (*cln3 swi6*). Log-phase YAPD-grown cultures of the resulting integrants were used to analyze the effect of the mutations on Cln3-mediated cell size control.

has failed to detect cell cycle-regulated phosphorylation outside of serine 160 (54). Similarly, we cannot exclude the possibility that phosphorylation of one of the many SP or TP sites in Swi4 other than S159 or T799 is involved, but again, such phosphorylation has not been detected.

Stable complexes containing both Cln3 and Swi6 have not been detected, even under conditions where both proteins are active and expressed at high levels. The possibility that activation of SBF and MBF by Cln3 involves a temporary and weak physical interaction is difficult to rule out. Our results suggest, however, that Cln3 is not directly involved in activating transcription at SBF/MBF-driven promoters. Manipulation of the *CLN3* gene to delete its putative transcriptional activation motifs or to add a transcriptional repression domain did not alter its function.

The amino terminus and leucine zipper of Swi6 play a role

in Cln3 function. Our mutational analysis of *SWI6* has provided an estimate of the location and strength of Swi6's transcriptional activation domains that is largely in agreement with previously reported results (52). More importantly, however, our analysis has uncovered features of Swi6 that allow it to mediate Cln3's function. By employing two independent assays, i.e., *CLN3*-dependent rescue of *bck2 swi6* and mediation of *CLN3*-responsive change in cell size, we identified two regions of Swi6 with a role in mediating Cln3's function: (i) the amino terminus (aa 1 to 284) and (ii) the leucine zipper (aa 583 to 610). Either of these two regions can be deleted without blocking Cln3 function, but if both are deleted simultaneously, the resulting mutant Swi6 becomes insensitive to Cln3 while maintaining part of its transcriptional activity. It is not obvious why the amino terminus and leucine zipper of Swi6 should have overlapping functions or how Cln3 would act on either of these two regions.

One possible model is that both the amino terminus and the leucine zipper of Swi6 contribute to the interaction of SBF or MBF with another protein and that this interaction is somehow the target of Cln3. More specifically, some repressive protein or protein complex could bind to SBF, using interactions with both the amino terminus and leucine zipper of Swi6, and this repressive protein would mask the native transcriptional activity of SBF. Cln3-Cdc28 could interfere with the binding of the repressive protein to SBF, thus freeing SBF as an activator.

An attractive feature of this model is that it is analogous to the situation in mammalian cells. The function of cyclin-Cdk complexes in regulating cell cycle entry has been conserved throughout eukaryotes (42, 44). In both budding yeast and mammals, G_1 cyclins and their associated Cdks activate a transcriptional program associated with cell cycle entry (53). In mammals, cyclin-Cdk complexes activate the E2F-DP transcription complexes (20), just as Cln3-Cdc28 activates SBF and MBF (29, 36). Mammalian G_1 cyclin-Cdk complexes work by phosphorylating and neutralizing the pocket proteins Rb, p107, and p130, which bind to and inhibit E2F-DP transcription complexes (20). Similarly, we propose that Cln3-Cdc28 may neutralize a protein that binds to and inhibits SBF.

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