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The G1 Cyclin Cln3 Promotes Cell Cycle Entry via the Transcription Factor Swi6

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Received 19 December 2001/Returned for modification 15 February 2002/Accepted 18 March 2002

In Saccharomyces cerevisiae (budding yeast), commitment to cell division in late G1 is promoted by the G1 cyclin Cln3 and its associated cyclin-dependent kinase, Cdc28. We show here that all known aspects of the function of Cln3 in G1 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 G1-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 G1 (45, 48). In Saccharomyces cerevisiae (budding yeast) this point is called Start. Start occurs when G1 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 G1 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 PuNNPyCACGGAAA (41) and the Mbp1 cell cycle box element ACGC GTNA (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 Mbp1 cell cycle box elements can be updated to, respectively, CRCGAAA and ACGC GN (24, 57).

A swi4 mbp1 double mutant (which thus lacks both SBF and MBF) is inviable, with a terminal cell cycle arrest in G1 (28), confirming the crucial role of SBF and MBF in mediating cell cycle entry. A very similar phenotype is found for the swi6 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-G1 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 G1 until late mitosis, Swi6 is cytoplasmic from S phase to late mitosis but is nuclear during G1 (54). However, this cell cycle-regulated subcellular localization of SBF and MBF is not sufficient to explain their activation in late G1, 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 G1 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
**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 G1, and **Cln3** protein can be detected throughout the whole 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 G1 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 PolIII, TFIIIB, 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). **cln3** mutants are characterized by a large cell size, an increased sensitivity to mating pheromone, and a delay at the G1-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/ml, except for L-tryptophan (80 mg/ml), adenine sulfate (32 mg/ml), and p-aminobenzoic acid (5 mg/ml). YAP-based media were made by supplementing YEP (1% yeast extract, 2% peptone)-based media with filter-sterilized adenine to 0.004% (wt/vol). YAP-based medium was YPD with 2% d-glucose. Plasmids were created by using a combination of standard restriction cloning methods, PCR, in vitro mutagenesis, and in vivo gap repair cloning (2, 35, 51).

For the experiment shown in Fig. 8, plasmid pHW254 (YClacpl11-CLN3) was used to create plasmid pHW364, which contains a full-length **CLN3** gene fused to 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-SWI6s1-284 SWI6) was used to create a series of **GAL4BD-SWI6** deletion mutants. The resulting mutant plasmid pHW344 (pGBD-C1-SWI6s1-155 SWI6ΔC), pHW350 (pGBD-C1-SWI6s1-284 SWI6ΔCΔZC), pHW359 (pGBD-C1-SWI6s1-284 SWI6ΔCΔZC), pHW351 (pGBD-C1-SWI6s1-284 SWI6ΔCΔZC), and pHW354 (pGBD-C1-SWI6s1-284 SWI6ΔN), pHW353 (pGBD-C1-SWI6s1-284 SWI6ΔN), pHW360 (pGBD-C1-SWI6s1-284 SWI6ΔNΔCΔZC), pHW361 (pGBD-C1-SWI6s1-284 SWI6ΔNΔCΔZC), and pHW362 (pGBD-C1-SWI6s1-284 SWI6ΔNΔCΔZC). pHW374 (pGBD-C1-SWI6s1-284 SWI6ΔNΔCΔZC and ΔWΔNΔZC), and pHW373 (pGBD-C1-SWI6s1-284 SWI6ΔNΔCΔZC and ΔWΔNΔZC) were created in centromeric (Ycpplac33) and integrating (Yiplac304) vectors: pHW197 (Ycpplac33-SWI6s1-284 SWI6), pHW198 (Ycpplac33-SWI6s1-284 SWI6ΔN), pHW199 (Ycpplac33-SWI6s1-284 SWI6ΔNΔCΔZC), pHW202 (Ycpplac33-SWI6s1-284 SWI6ΔNΔCΔZC), pHW203 (Yiplac304-SWI6s1-284 SWI6), and pHW366 (Yiplac204-SWI6s1-284 SWI6ΔNΔCΔZC), pHW386 (Yiplac204-SWI6s1-284 SWI6ΔNΔCΔZC), and pHW388 (Yiplac204-SWI6s1-284 SWI6ΔNΔCΔZC).

**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 (YPLeuAlter1-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 NorI restriction site at the SWI6 stop codon. pHW155 (Swi6+) has the carboxy-terminal NorI site but no mutations in **SP** or **TP** dipeptides, pHW156 (Swi6+) has the NorI site and an S-to-T mutation at position 225, pHW157 (Swi6ΔC) has an additional S-to-A change at position 160, pHW179 (Swi6ΔCΔZ) is pHW157 with a T-to-A mutation at position 179 and an S-to-A mutation at position 238, and pHW180 (Swi6ΔCΔZ) 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 bsked 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 protease 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 tosylphenylalanil chloride methyl 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 12CAS 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 × g in a microcentrifuge. Protein samples (40 μg for immunoblots) were mixed with 2× 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 dilute 12CAS 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-
Yeast cultures that were to be compared for their cell size distribution were incubated in 0.001% methionine, which allows for limited expression of the MET3-CLN2-TRP1 plasmid (CB1491). Initial cultures of YHW29 and YHW30 were grown in SC medium lacking uracil (SC/URA3) and YHW23 was incubated using 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. Between strains, yeasts 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 Channelizer (model 256).

Yeasts that were used to compare their cell size distribution began at the same time in aliquots of the same batch of medium. Cultures were grown to log phase, rehydrated 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 because of the published observations: (i) the morphology and growth of str w1Δ swi6Δ cln3Δ CLN3+ strains in the presence and absence of Swi6, the effect of varying the Swi6 expression, strains YHW29 and YHW30 were grown in SC medium lacking uracil (SC/URA3) and YHW23 was incubated using 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. Between strains, yeasts were stained with propidium iodide at a final concentration of 16 μg/ml.

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Regulation of cell size by **CLN3** depends on **SWI6**. **CLN3** is a dosage-dependent regulator of the length of G₁ 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 G₁-phase daughter cells from **SWI6** and **swi6** strains carrying **GAL-CLN3**. Cells were grown and G₁ cells were obtained in late G₁ at Start and attempt to conjugate (32). This G₁ arrest

### TABLE 2. Plasmids

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*References are as follows: pGBD-C2, 25; YCp50, 49; YEp24, 3; YCplac33, YEplac181, YEplac195, and YIplac204, 19; pRS304 and pRS313, 55; and pRS426, 8.*
involves the inactivation of Cln-Cdc28 complexes. CLN1 and CLN2 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 G1 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 G1 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 swi6 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 genes.
genes in both SWI6 and swi6 strains. G1-phase SWI6 or swi6 cells were obtained by elutriation, and the expression of CLN1, RNRI, and PCL1 was assayed with and without induction of GALI-CLN3 (Fig. 5). Induction of GALI-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 RNRI persisted in the absence of SWI6. Deletion of SWI6 not only shifted peak expression of
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
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. 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 G1.
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 (SWI6s1-5), and examined the responsiveness of this combination to CLN3-1. 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.

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. 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.

the mode cell size of the CLN3-1 SWI4* SWI6s1-5 CLN3 mbp1 control was 0.81 of the mode cell size of its SWI4* SWI6s1-5 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.
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 G1 (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 G1 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. 6. Swi6 is not activated via its Cdc28 consensus phosphorylation sites. (A) Strain YHW31 (swi6) was transformed with plasmids pHW155 (LEU2-CEN-SWI6N), all five SP or TP sites present) and pHW180 (LEU2-CEN-SWI6N), 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 (SWI6N, no sites mutated), pHW157 (SWI6N, two sites mutated), pHW179 (SWI6N, four sites mutated), pHW180 (SWI6N, five sites mutated), and pHW156 (SWI6N, one site mutated). The resulting strains were grown in SC−leu with 1% rafinose and 1% galactose. Serially diluted aliquots of cells were spotted on either SC−leu with 1% rafinose and 1% galactose (−Leu Raff/Gal) or SC−leu with 2% glucose (−Leu Gluc) plates and were photographed after 3 days at 30°C.
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 (Gal41-147-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 (Gal41-147: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 transactivation 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 227).

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.

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(myo)] (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 [YEp33-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% rafinose. 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°C) or, alternatively, immunoprecipitated (IP) (6 mg 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 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).
FIG. 8. Fusion to the Tup1 transcriptional repression domain does not inhibit Cln3's function. (A and B) Plasmid pHW364 (\(\text{CEN-CLN3-TUP1}\)) was compared to pHW254 (\(\text{CEN-CLN3}\)) and Ycplac111 (\(\text{CEN-vector}\)) 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

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<th>Rescue of bck2 cln3</th>
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*pPC97 (LEU2-CEN-4DH::GAL41-147), pHWS355 (pPC97/CLN3-1::H4), and the specified mutant derivatives of pHWS355 were introduced into strains MAV99 (GAL1::HIS3 SPO13::10xGAL4::URA43) 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.

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
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 pro-
vided an estimate of the location and strength of Swi6’s tran-
scriptional 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 as-
says, i.e., CLN3-dependent rescue of bck2 swi6 and mediation
of CLN3-responsive change in cell size, we identified two re-
As 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
maintaining part of its transcriptional activity. It is not obvious
how the amino terminus and leucine zipper of Swi6 should
have overlapping functions or how Cln3 would act on either of
these two regions.

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, G1 cyclins and their associated CDks activate a tran-
scriptional program associated with cell cycle entry (53). In
mammals, cyclin-Cdk complexes activate the E2F-DP tran-
scription complexes (20), just as Cln3-Cdc28 activates SBF and
MBF (29, 36). Mammalian G1 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.

ACKNOWLEDGMENTS

We thank Martine Lessard for excellent technical assistance; Kim
Nasmyth for communicating unpublished results; and Kim Arndt,
Brenda Andrews, Kim Nasmyth, and members of the Furcher labo-
atory for reagents.

This work was supported by grant RO1 GM39978 from the National
Institutes of Health.

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