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Activation of *CLN1* and *CLN2* G_1 Cyclin Gene Expression by BCK2

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The *Saccharomyces cerevisiae* CLN3 protein, a G_1 cyclin, positively regulates the expression of *CLN1* and *CLN2*, two additional G_1 cyclins whose expression during late G_1 is activated, in part, by the transcription factors SWI4 and SWI6. We isolated 12 complementation groups of mutants that require *CLN3*. The members of one of these complementation groups have mutations in the *BCK2* gene. In a wild-type *CLN3* genetic background, *bck2* mutants have a normal growth rate but have a larger cell size, are more sensitive to α-factor, and have a modest defect in the accumulation of *CLN1* and *CLN2* RNA. In the absence of *CLN3*, *bck2* mutations cause an extremely slow growth rate: the cells accumulate in late G_1 with very low levels of *CLN1* and *CLN2* RNA. The slow growth rate and long G_1 delay of *bck2 cln3* mutants are cured by heterologous expression of *CLN2*. Moreover, overexpression of BCK2 induces very high levels of *CLN1*, *CLN2*, and *HCS26* RNAs. The results suggest that *BCK2* and *CLN3* provide parallel activation pathways for the expression of *CLN1* and *CLN2* during late G_1.

The *Saccharomyces cerevisiae CLN1*, *CLN2*, and *CLN3* genes encode G_1 cyclins that interact with the CDC28 protein to form complexes with kinase activity (8, 21, 29, 33, 40). A sufficient level of CLN/CDC28 kinase activity is required during the late G_1 stage of the cell cycle for the execution of Start. Prior to the execution of Start, nutrient limitation or the presence of mating pheromones can arrest the cells in G_1. Once a cell has executed Start, nutrient limitation and mating pheromones do not prevent cell cycle progression (until the following G_1), and the cells are committed to divide.

In early G_1, the cells have very low levels of *CLN1* and *CLN2* RNAs. During late G_1, the levels of *CLN1* and *CLN2* RNAs increase dramatically (42). A large fraction of the late G_1 transcription of *CLN1* and *CLN2* is activated by the SWI4 and SWI6 transcription factors, which bind as a complex to sites in the *CLN1* and *CLN2* promoters (3, 30, 31). In addition, expression of normal levels of *CLN1* and *CLN2* during late G_1 requires the function of the CDC28 kinase and the function of at least one of the three *CLN* genes (*CLN1*, *CLN2*, or *CLN3*) (11, 15). These results have suggested that the accumulation of *CLN1* and *CLN2* RNAs during late G_1 requires CLN-associated kinase activity. It is currently not known what substrate(s) requires phosphorylation by the CLN-associated kinase for *CLN1* and *CLN2* RNA accumulation during late G_1.

In addition to the *CDC28* and *CLN* genes, the *SIT4* serine/threonine protein phosphatase is also required for the normal accumulation of *CLN1* and *CLN2* RNAs during late G_1 (19, 37). By contrast, *SIT4* is not required for normal levels of *CLN3* RNA. During late G_1, the *SWI4* RNA levels increase dramatically and reach their peak just prior to or at the time when *CLN1* and *CLN2* RNA levels reach their peak (5, 19). The requirement for *SIT4* for the normal accumulation of *CLN1* and *CLN2* RNAs during late G_1 is at least partly due to the requirement for *SIT4* for the normal increase in *SWI4* RNA levels during late G_1 (19).

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CLN3 can regulate the rate of accumulation of *CLN1* and *CLN2* RNAs (8, 11, 15, 29). The dominant *CLN3-1* and *CLN3-2* alleles encode hyperactive forms of CLN3 that increase the rate of *CLN1* and *CLN2* RNA accumulation during late G_1. Such cells initiate Start at a smaller than normal critical cell volume and are relatively resistant to α-factor. By contrast, deletion of *CLN3* results in a slower than normal rate of accumulation of *CLN1* and *CLN2* RNAs during late G_1. The Δ*cln3* cells initiate Start at a larger than normal critical cell volume and are more sensitive to α-factor than are wild-type cells. Because the CLN3 protein is unstable (9, 40), the levels of the CLN3 protein might be sensitive to the rate of protein synthesis. Therefore, CLN3 (along with possible additional factors) may set the timing of Start relative to the growth rate of the cell.

The *CLN3* gene is normally dispensable, and Δ*cln3* cells have a normal growth rate (8, 29). However, the defect in *CLN1* and *CLN2* RNA accumulation resulting from the absence of CLN3 becomes apparent when a *cln3* mutation is combined with an additional mutation in a gene that also functions for normal *CLN1* and *CLN2* RNA accumulation. For instance, Δ*swi4* cells have a slow-growth phenotype that is primarily due to a defect in *CLN1* and *CLN2* RNA accumulation (30, 31). Combining a *swi4* mutation with a *cln3* mutation results in a very slow growth rate that is partially cured by high-copy-number *CLN2* (30) or cured by heterologous expression of *CLN2* (3a). Likewise, *sit4* mutants have a defect in *SWI4*, *CLN1*, and *CLN2* RNA accumulation. Combining a *sit4* mutation with a *cln3* mutation results in near lethality. The near lethality of *sit4 cln3* strains is due to a defect in *CLN2* (and probably *CLN1*) RNA accumulation because the synthetic growth rate defect is completely cured by expressing CLN2 at near wild-type levels from a SIT4-independent promoter (19).

To identify possible targets of the SIT4 phosphatase that function for the expression of *CLN1* and *CLN2*, we isolated mutants that (like *sit4* mutants) require *CLN3* for either viability or a normal growth rate. The members of one complementation group of these mutants have mutations in the *BCK2* gene. The *BCK2* gene was originally isolated as a gene that in high copy number can suppress the temperature-sensitive cell lysis defect of mutations in the protein kinase C pathway (24).

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TABLE 1. Yeast strains used

Strain	Genotype	Source or reference
AY925	*MAT***a** *ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-1 ssd1-d2 can1-100*	W303 of R. Rothstein
CY279	*MAT*α Δ*sit4*::*HIS3 SSD1-v ura3 leu2 his3 trp1*	37
CY2446	*MAT*α Δ*cln3*::*HIS3 SSD1-v ura3-52 leu2 his3 trp1 ade2-101 ade3*::*HisG* {*CLN3*/*ADE3*/YCp50}	This study
CY2932	*MAT***a** Δ*cln3*::*HIS3 SSD1-v ura3-52 leu2 his3 lys2 ade2-101 ade3*::*HisG* {*CLN3*/*ADE3*/YCp50}	This study
CY3182	*MAT***a**/*MAT*α Δ*mpk1*::*TRP1*/− *ura3-52*/− *leu2-3,112*/− *his4*/− *trp1-1*/− *can1^r*/*can1^r* homozygous diploid	24
CY3276	*MAT***a** Δ*bck2*::*TRP1 SSD1-v ura3-52 leu2-3,112 his4 trp1-1 can^r*	D. Levin
CY3317	CY3276 {*BCK2*/YCp50}	This study
CY3319	CY3276 {YCp50}	This study
CY3507	AY925 {p*GAL*:*BCK2*/YCp50}	This study
CY3510	AY925 {YCp50/p*GAL* expression plasmid with no insert}	This study
CY3557	*MAT***a** *ssd1-d ura3-52 leu2Δ1 his3Δ200 ade2-101 lys2-801* plus SCB-*lacZ* at *HIS3* locus	B. Andrews
CY3582	*MAT***a** Δ*swi4*::*HIS3* in CY3557 background	J. Ogas
CY3656	*MAT***a** Δ*swi6*::*HIS3* in CY3557 background	B. Andrews
CY3779	*MAT*α Δ*swi6*::*HIS3 ssd1-d ura3-52 leu2Δ1 his3Δ200 ade2-101 lys2-801* {2μm/*URA3*/MCB-*lacZ*}	This study
CY3798	*MAT*α *ssd1-d ura3-52 leu2Δ1 his3Δ200 ade2-101 lys2-801* {2μm/*URA3*/MCB-*lacZ*}	This study
CY4271	CY3507 plus Δ*cln2*::*TRP1*	This study
ΔY79	*MAT***a** Δ*bck2*::*TRP1* p*GAL*:*CLN3* (*LEU2* marked, at *CLN3* locus) *ura3 leu2 his3 trp1 ade2* {YCp50}	This study
ΔY80	*MAT***a** Δ*bck2*::*TRP1* p*GAL*:*CLN3* (*LEU2* marked, at *CLN3* locus) *ura3 leu2 his3 trp1 ade2* {*BCK2*/YCp50}	This study
263	*MAT***a** *ctr7-263* Δ*cln3*::*HIS3 SSD1-v ura3-52 leu2 his3 lys2 ade2-101 ade3*::*HisG* {*CLN3*/*ADE3*/YCp50}	This study

More recently, *BCK2* was isolated as a suppressor of a *cln1 cln2 cln3* strain (18, 20a). Epstein and Cross (18) found that *bck2* cells are dramatically impaired in their growth rate if they also lack *CLN3*. Moreover, these authors found that transcription of a *CLN2* reporter construct in cells arrested in G_1 because of the lack of *CLN* function (which gives low levels of *CLN2* RNA) is about 8.5-fold further reduced in *bck2* cells compared with *BCK2* cells. Here we show that, in the absence of *CLN3*, *BCK2* is required for *CLN1* and *CLN2* RNA accumulation during G_1. Moreover, overexpression of BCK2 causes a large increase in the levels of *CLN1* and *CLN2* RNAs. Genetic interactions between Δ*bck2*, Δ*sit4*, and Δ*cln3* mutations suggest a model whereby BCK2 and SIT4 function in a pathway that activates *CLN1* and *CLN2* expression in parallel with the CLN3 pathway.

MATERIALS AND METHODS

Yeast strains and media. Yeast strains used are listed in Table 1. Yeast cells were grown on either YPD medium (1% yeast extract, 2% peptone, and 2% glucose) or synthetic complete (SC) medium (using, per liter, 0.2 g of leucine and 0.1 g of all other amino acids, 0.1 g of uracil, and 0.075 g of adenine, with the indicated carbon source) (35). For plasmid selection, the appropriate amino acid or uracil was omitted from the SC medium.

Isolation of *ctr* mutants. We prepared *MAT***a** and *MAT*α Δ*cln3*::*HIS3 SSD1-v ade2 ade3 ura3* strains that contained the *CLN3* gene on an *ADE3*/*URA3* centromere plasmid (CY2446 and CY2932; Table 1). When these cells contain the *CLN3*/*ADE3*/*URA3* plasmid, they accumulate a red pigment. Cells that have lost the *CLN3*/*ADE3*/*URA3* plasmid do not accumulate the red pigment. Strains CY2446 and CY2932, which do not require *CLN3*, form red colonies with many white sectors composed of cells that have lost the *CLN3*/*ADE3*/*URA3* plasmid. Also, when replica plated onto 5-fluoro-orotic acid (5-FOA) medium, which allows only Ura^- cells to grow (4), these colonies give rise to a confluent patch of cells because they contain many Ura^- segregants that are able to grow with a normal growth rate. If these starting strains also contain a mutation that causes *CLN3* to be essential, they will give rise to solid red colonies (all viable cells contain the *CLN3*/*ADE3*/*URA3* plasmid) where none of the cells are able to grow when replica plated onto 5-FOA medium. Strains CY2446 and CY2932 were mutagenized to 30% survival with ethyl methylsulfonate and plated for single colonies. From about 80,000 *MAT***a** and 80,000 *MAT*α colonies, we obtained about 400 nonsectoring 5-FOA-negative (Foa^-) colonies. Of these 400 putative *ctr* strains, 114 were placed into 12 complementation groups, using the sectoring and Foa phenotypes.

***CTR7* is the same gene as *BCK2*.** A *ctr7-263 pGAL*:*CLN3* strain was grown on galactose medium and transformed with a YCp50 library containing yeast genomic inserts (34). Two groups of plasmid complemented the slow-growth-rate phenotype of the *ctr7-263 pGAL*:*CLN3* strain grown on glucose. One class of plasmid contained the wild-type *CLN3* gene. The other class of plasmid contained the authentic *CTR7* gene: a *LEU2* marker at the chromosomal locus corresponding to the cloned DNA was 100% linked to *ctr7* in 40 tetrads.

The location of the *CTR7* gene within the yeast DNA sequences was determined by subcloning and complementation analysis. A 3.3-kb *Nsi*I fragment, which gave full *ctr7* complementation, contained sequences from about −550 to +2776 of the *BCK2* gene (24). BCK2 coding sequences are from +1 to +2555, all numbers relative to the A of the *BCK2* putative ATG start codon. Frameshift mutations within *BCK2* coding sequences eliminated complementation.

Deletion alleles. The Δ*cln3* mutation replaces *CLN3* DNA sequences between the *Bst*XI and *Eco*RI sites (removes DNA sequences encoding amino acids 1 through 387, of 581 total amino acids) with either a 1.2-kb *Hin*dIII *URA3* fragment, a 2.2-kb *Xho*I-*Sal*I *LEU2* fragment, or a 1.8-kb *Bam*HI *HIS3* fragment. The Δ*bck2*::*TRP1* mutation, obtained from D. Levin, removes DNA sequences coding for amino acids 338 through 693 of BCK2 (of 851 total amino acids) and replaces them with two tandem copies of the *TRP1* gene (24). The isogenic wild-type, Δ*swi4*, and Δ*swi6* strains were obtained from J. Ogas and B. Andrews and are described in references 31 and 41.

Expression of BCK2 from the *GAL1* promoter. Using oligonucleotide-directed mutagenesis, we created an *Xba*I restriction site 23 bp upstream of the A of the predicted *BCK2* ATG initiation codon. This *Xba*I site was used to place *BCK2* coding sequences under control of the *GAL1* promoter in either a YCp50 or a *LEU2*/*cen* expression plasmid.

***CLN2* promoter deletions.** The 2.5-kb *Nru*I-*Cla*I fragment containing *CLN2* that encodes a CLN2 protein with a triple hemagglutinin epitope tag at the carboxyl terminus, *CLN2C* in reference 39, was blunted into the *Sma*I and *Acc*I sites of pUC118, yielding plasmid pCB1321. Oligonucleotide-directed mutagenesis was used to remove positions −499 to −403 for deletion CC, positions −409 to −318 for deletion DD, or positions −318 to −259 for deletion EE and replace them with a 6-bp *Xho*I site. *Kpn*I-*Pst*I fragments containing *CLN2* (for AA, CC, DD, or EE) or a *Sph*I-*Pst*I fragment containing *CLN2* (for BB) were blunted into the *Hin*dIII site of *LEU2*/*cen* plasmid pAB484 (constructed by replacing the *Sal*I-*Sma*I *URA3*-containing fragment of YCp50 with the 2.2-kb *Xho*I-*Sal*I fragment containing *LEU2*). For orientation 1, the *CLN2*-containing fragment is oriented so that the *CLN2* stop codon is closer to the *LEU2* gene, while for orientation 2, the *CLN2* promoter is closer to the *LEU2* gene.

Northern (RNA) analysis and probes. Total RNA was loaded onto a 1% agarose gel containing 6% formaldehyde, 0.02 M morpholinopropanesulfonic acid, 0.005 M sodium acetate, and 0.001 M EDTA (pH 7.0). The gels were blotted onto BioTrans nylon membranes. The probes were the 1.4-kb *Cla*I-*Not*I fragment of *CLN1*, the 0.75-kb *Xho*I-*Hin*dIII fragment of *CLN2*, the 0.6-kb *Alu*I fragment of *ACT1*, the 2.2-kb *Bam*HI fragment of *SWI4*, the 0.5-kb *Eco*RI fragment of *CLB5*, the 0.9-kb *Eco*RI-*Bam*HI fragment of *HCS26*, the 2.4-kb *Xba*I-*Nsi*I fragment of *MBP1*, the 2.7-kb *Eco*RI fragment of *RNR1*, the 1.36-kb *Hin*dIII-*Eco*RI fragment of *BCK2*, the 1.2-kb *Hin*dIII fragment of *URA3*, and the 2.0-kb *Hin*dIII-*Xho*I fragment of *CLB2*. The blots were washed twice (15 min each) at 24°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) and then twice (15 min each) at 65°C with 0.1× SSC–0.1% SDS.

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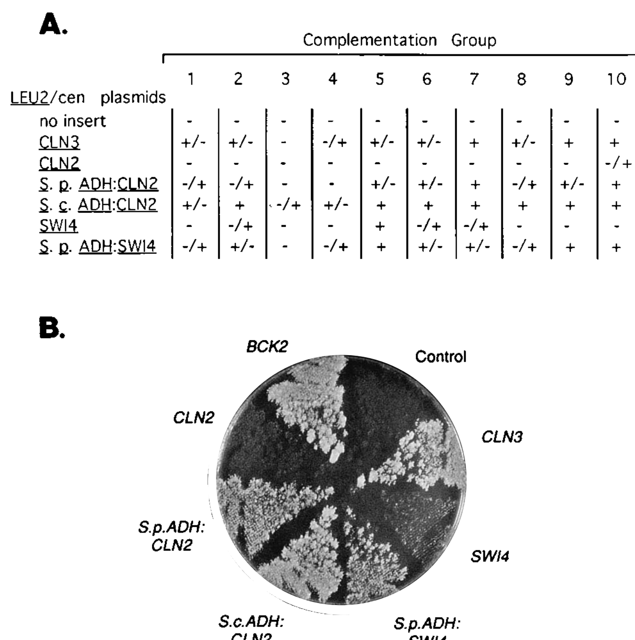
β-Galactosidase assays. To exponentially growing cells (on either SC lacking uracil [SC-uracil] 2% raffinose or SC-uracil,-leucine 2% raffinose medium) at an optical density at 600 nm (OD_{600}) of 0.2 to 0.25, galactose was added to 2% (final concentration) at time zero. Cells were collected at the indicated times. Extracts were prepared by vortexing the cells in breaking buffer (0.1 M Tris [pH 8.0], 20% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) in the presence of glass beads and then centrifuging them for 10 min at 13,000 × *g* to remove cell debris. For the assays, 0.1 ml of extract was mixed with 0.9 ml of Z buffer (0.1 M $NaPO_4$ [pH 7.0], 0.01 M KCl, 1 mM $MgSO_4$, 4 mM 2-mercaptoethanol) and incubated at 28°C. After 5 min, 0.2 ml of *o*-nitrophenylgalactoside (Sigma N-1127; at 4 mg/ml in Z buffer) solution was added. The reactions were stopped by the addition of 0.5 ml of 1 M Na_2CO_3, and the A_{420} was determined. β-Galactosidase units were calculated by the following formula: units = (A_{420})(377.8)/(time, in minutes)(volume of extract in assay = 0.1 ml)(protein concentration of extract, in milligrams per milliliter). For each time point, the assays were performed on at least two separate equivalent cultures, and the average is reported.

RESULTS

Isolation of *BCK2* as a gene that is required in the absence of *CLN3*. Because Δ*cln3* strains have a wild-type growth rate, Δ*cln3* cells containing the *CLN3* gene on a centromere plasmid (a *CLN3/ADE3/URA3* plasmid) grow at about the same rate as Δ*cln3* cells. By contrast, if the Δ*cln3* {*CLN3/ADE3/URA3* plasmid} cells have a mutation (*ctr* [*cln* three requiring]) that causes *CLN3* to be required for a normal growth rate, cells that have lost the *CLN3* plasmid will be inviable or grow very slowly. Using the colony sectoring assay (23), we isolated about 400 *ctr* mutants (see Materials and Methods). Of the 400 putative *ctr* mutants, 114 were placed into 12 complementation groups. The remainder of the putative *ctr* mutants either complemented all *ctr* mutants of the opposite mating type (possible unique mutants), did not complement any of the *ctr* mutants of the opposite mating type (probably resulting from integration of the *CLN3/ADE3/URA3* plasmid), did not mate, or were unstable during storage and propagation.

Another copy of the *CLN3* gene (on a *LEU2/cen* plasmid) was introduced into representative *ctr1* through *ctr10* mutants. These mutants fell into one of three classes (Fig. 1A). For the first class, containing only groups 7, 9, and 10, the *CLN3/LEU2* plasmid transformants were able to grow at wild-type or near wild-type rates in the absence of the *CLN3/ADE3/URA3* plasmid (the strain background has a Δ*cln3* mutation). For the second class, groups 1, 2, 5, 6, and 8, the *CLN3/LEU2* plasmid transformants grew better if they also had the *CLN3/ADE3/URA3* plasmid, which suggests that these mutants grow better if they have two *CLN3* genes. For the third class, groups 3 and 4, the *CLN3/LEU2* plasmid transformants were able to grow only very poorly in the absence of the *CLN3/ADE3/URA3* plasmid.

The remainder of this report will focus on the *ctr7* mutants. The *CTR7* gene was cloned and found to be identical to the *BCK2* gene (see Materials and Methods). We will now refer to the *ctr7* mutants as *bck2* mutants. An extra copy of the wild-type *CLN2* gene did not increase the ability of the *bck2* mutants to grow in the absence of *CLN3* (Fig. 1B). By contrast, expression of *CLN2* coding sequences from the *Schizosaccharomyces pombe ADH* promoter (*S.p.ADH*, which expresses CLN2 at levels similar to the CLN2 levels in a wild-type strain [data not shown]) or from the *S. cerevisiae ADH* promoter (*S.c.ADH*, which gives 10- to 20-fold-higher levels of CLN2 [data not shown]) allowed the *bck2* mutants to grow very well in the absence of *CLN3* (Fig. 1B). Related to these findings, Epstein and Cross (18) found that high-level expression of *CLN1* from the *GAL1* promoter can rescue the inviability of a *cln2 cln3 bck2* strain and that expression of *CLN2* coding sequences from the *CLN3* promoter can partially rescue a *cln1 cln3 bck2* strain. These findings suggest that *bck2* mutants



A.

LEU2/cen plasmids	Complementation Group 1	2	3	4	5	6	7	8	9	10
no insert	-	-	-	-	-	-	-	-	-	-
CLN3	+/-	+/-	-	-/+	+/-	+/-	+	+/-	+	+
CLN2	-	-	-	-	-	-	-	-	-	-/+
S. p. ADH:CLN2	-/+	-/+	-	-	+/-	+/-	+	-/+	+/-	+
S. c. ADH:CLN2	+/-	+	-/+	+/-	+	+	+	+	+	+
SWI4	-	-/+	-	-	+	-/+	-/+	-	-	-
S. p. ADH:SWI4	-/+	+/-	-	-/+	+	+/-	+/-	-/+	+	+

B.

FIG. 1. Ability of *ctr* strains to grow in the absence of *CLN3*. (A) Summary of the ability of *ctr1* through *ctr10* strains to grow in the absence of *CLN3* after transformation with the indicated plasmid. We obtained 3 group 1, 14 group 2, 23 group 3, 13 group 4, 29 group 5, 8 group 6, 10 group 7, 5 group 8, 2 group 9, 3 group 10, 2 group 11, and 2 group 12 putative *ctr* mutants. +, full or near full wild-type ability; +/−, partial ability; −/+, slight ability; −, no or almost no ability to grow in the absence of *CLN3*. The ability of the strains to grow in the absence of *CLN3* was determined by both their sectoring and Foa phenotypes. The *ctr5* mutants may be *swi4* mutants because they are complemented by the wild-type *SWI4* gene. *SIT4* does not complement *ctr1* through *ctr10*. (B) Transformants of a *ctr7-263* Δ*cln3* {*CLN3/ADE3/URA3/cen* plasmid} strain (strain 263) with the indicated *LEU2/cen* plasmids were streaked for single colonies on a YPD plate and replica plated onto an SC plate containing 5-FOA (4), on which only Ura^- (that is, without the *CLN3*-containing *URA3/cen* plasmid) cells can grow. Growth is shown after 1.5 days at 30°C. The *S.p.ADH*:*CLN2*, *S.p.ADH*:*SWI4*, and *S.c.ADH*:*CLN2* plasmids were described in reference 19. An independent *ctr7* mutant gave equivalent results. In all cases, the ability to give sectored colonies corresponded to the ability to grow on 5-FOA medium.

cannot grow (or grow very slowly) in the absence of *CLN3* is because of a defect in the expression of *CLN1* and *CLN2* from their normal promoters.

Like the *CLN1* and *CLN2* RNA levels, the *SWI4* RNA levels also peak in late G_1. The periodic expression of *SWI4* may be required for the normal expression of *CLN1* and *CLN2* (5). When the *bck2* mutants contained an extra copy of wild-type *SWI4*, there was a very slight increase in the ability to grow in the absence of *CLN3* (Fig. 1B). When *SWI4* coding sequences were transcribed from the *S.p.ADH* promoter (which gives about 5- to 10-fold-higher levels of *SWI4* RNA than from the wild-type promoter [data not shown]), the *bck2* mutants grew reasonably well in the absence of *CLN3* (Fig. 1B). Thus, overexpression of *SWI4* from a heterologous promoter can partially suppress the slow growth rate of *bck2 cln3* strains.

In the absence of *CLN3*, *BCK2* is required for normal *CLN1* and *CLN2* expression. To determine the effect of a null allele of *BCK2* on the ability to grow in the absence of *CLN3*, we obtained a Δ*bck2*::*TRP1* strain (CY3276) from D. Levin (24). When the Δ*bck2* strain was crossed to a Δ*cln3* strain, viable Δ*bck2* Δ*cln3* progeny were obtained, but they grew very slowly (data not shown). These findings contrast with those of Epstein and Cross (18), who reported that *bck2 cln3* spores are inviable. Perhaps strain background effects or their use of a *bck2*

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disruption allele accounts for these differences. Two hours after glucose was added to a Δ*bck2 pGAL*:*CLN3* strain growing on galactose medium (which repressed *pGAL*:*CLN3* expression), the culture became highly enriched in unbudded uninucleate cells with a 1n DNA content (data not shown), confirming the findings of Epstein and Cross (18). Moreover, for the Δ*bck2 pGAL*:*CLN3* strain on glucose medium, heterologous expression of *CLN2* from the *S.p.ADH* promoter eliminated much of the long G_1 delay and restored a near wild-type growth rate, while heterologous expression of *CLN2* from the *S.c.ADH* promoter eliminated all of the G_1 delay and gave a wild-type growth rate (data not shown).

Previously, Epstein and Cross (18) showed that the RNA levels of a *CLN2* reporter in cells arrested in G_1 due to the lack of *CLN* function (conditions which give low *CLN2* expression because most *CLN2* transcription requires *CLN* function [11, 15]) is about 8.5-fold lower in *bck2* cells than in *BCK2* cells. To determine the rate and level of *CLN1* and *CLN2* RNA accumulation during G_1 in the absence *BCK2* and *CLN3* function in an otherwise wild-type cell, we used the procedure described in the legend to Fig. 2A. For the *BCK2 pGAL*:*CLN3* cells (effectively *BCK2 cln3*), the levels of *CLN1*, *CLN2*, *SWI4*, and *CLB5* RNAs increased and reached maximal levels at about 1 h after α-factor release (after which most of the cells formed a bud) (Fig. 2A). This rate of increase for the *CLN1*, *CLN2*, *SWI4*, and *CLB5* RNA levels is somewhat slower than for wild-type cells because of the absence of normal *CLN3* function. By contrast, when the Δ*bck2 pGAL*:*CLN3* cells (effectively *bck2 cln3*) were released from the α-factor arrest, the levels of *CLN1*, *CLN2*, and *CLB5* RNA remained very low and the cells did not form a bud, even up to 72 min after the release (Fig. 2A). The *SWI4* RNA levels for the Δ*bck2 pGAL*:*CLN3* cells increased, but not quite to the levels as for the *BCK2 pGAL*:*CLN3* cells. The very low *CLN1* and *CLN2* RNA accumulation in *bck2 cln3* cells, along with the finding that the long G_1 delay and the growth defect of *bck2 cln3* cells is cured by heterologous expression of *CLN2*, suggests that in the absence of *CLN3*, *BCK2* is most likely required for significant levels of *CLN1*, *CLN2*, and *CLB5* RNA accumulation. That the defect in *SWI4* RNA accumulation is relatively modest compared with the very strong defects in *CLN1* and *CLN2* RNA accumulation raises the possibility that *bck2 cln3* cells are defective in the ability of SWI4 to stimulate *CLN1* and *CLN2* RNA accumulation (or alternatively in the expression of SWI4 protein from *SWI4* RNA).

Effect of Δ*bck2* and high-copy-number *BCK2* in the presence of *CLN3*. In the presence of *CLN3*, deletion of *BCK2* does not result in any significant decrease in the growth rate of the cell (18, 24). However, the absence of *BCK2* caused a modest decrease in the accumulation rate (peak levels were delayed about 10 min) and a modest decrease in the peak levels of *CLN2* and *HCS26* RNAs during late G_1 (Fig. 2B). Therefore, like the loss of *CLN3* function in an otherwise wild-type strain, deletion of *BCK2* in an otherwise wild-type strain results in a modest decrease in the rate and levels of *CLN2* RNA accumulation. In contrast to the *CLN2* and *HCS26* RNA levels, the *SWI4* RNA levels are more similar (and have about a 5-min delay) in the Δ*bck2* strain compared with the wild-type strain, suggesting that the ability of SWI4 to activate *CLN2* expression is partially defective in the absence of BCK2.

As a consequence of the alteration in *CLN1* and *CLN2* RNA accumulation, Δ*cln3* cells are more sensitive to α-factor and initiate Start at a larger than normal cell volume. Moreover, cells containing hyperactive alleles of *CLN3* are more resistant to α-factor and initiate Start at a smaller than normal cell volume (8, 29). Therefore, we determined the α-factor sensitivity and cell size of Δ*bck2*, wild-type, and high-copy-number *BCK2* strains. The Δ*bck2* cells were more sensitive to α-factor, while high-copy-number *BCK2*-containing cells were more resistant to α factor (Fig. 3A). The increased sensitivity of Δ*bck2* cells to α-factor was due to a defect in *CLN1* and *CLN2* RNA accumulation because expression of *CLN2* coding sequences from the *S.p.ADH* promoter restored normal α-factor sensitivity to the Δ*bck2* cells (data not shown). In addition, Δ*bck2* cells also had a larger than normal cell volume (confirming the findings of Epstein and Cross [18]), while high-copy-number *BCK2*-containing cells had a slightly smaller than normal cell volume (Fig. 3B). Together, these findings indicate that even in the presence of CLN3, BCK2 has a role at Start.

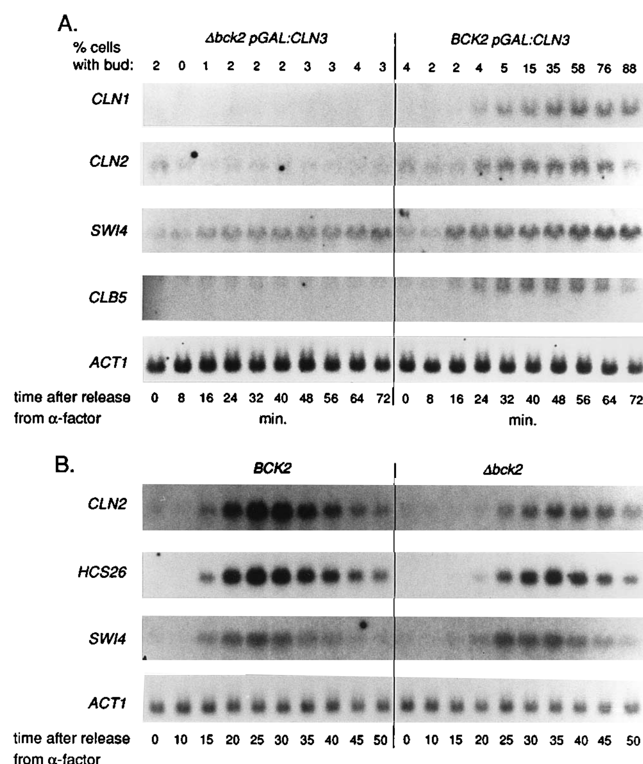

FIG. 2. Effect of *BCK2* on *CLN1* and *CLN2* expression in the absence or presence of CLN3. (A) The absence of CLN3. Strains ΔY79 (Δ*bck2* p*GAL*:*CLN3* {YCp50}) and ΔY80 (Δ*bck2 pGAL*:*CLN3* {*BCK2*/YCp50}) were grown exponentially at 30°C in SC-uracil 2% galactose medium to an OD_{600} of 0.08. The cells were arrested in G_1 by addition of α-factor (to a final concentration of 0.012 mM), which represses *CLN1* and *CLN2* expression. After 2.75 h with α-factor, the cells arrested in late G_1 by the criteria of budding index, DNA content (flow cytometry), and the presence of schmoo-shaped cells. Next, *CLN3* expression was repressed by addition of glucose to 2% (final concentration) (*CLN3* RNA and protein are unstable so that their levels decrease rapidly [9, 40]). After 0.5 h in glucose, the cells were released from the α-factor arrest by filtering, washing, and resuspending in fresh SC-uracil 2% glucose medium. All procedures were performed in parallel for the two cultures. (B) The presence of CLN3. Isogenic strains CY3317 (*BCK2*) and CY3319 (Δ*bck2*) were grown exponentially in SC-uracil 2% glucose medium at 30°C to an OD_{600} of 0.22, at which time α-factor was added to 0.01 mM (final concentration). After 2.5 h, the cells were released (in parallel) from the α-factor arrest by filtering, washing, and resuspending in fresh SC-uracil 2% glucose medium. For both panels, total RNA was prepared from cells collected at the indicated times after the α-factor release; 5 μg of total RNA was loaded onto each lane of a 1% agarose gel, and the Northern blots were prepared as described in Materials and Methods.

Overexpression of BCK2 stimulates the expression of *CLN1* and *CLN2*. The results presented above suggest that *BCK2* and *CLN3* provide parallel activation pathways for the expression of *CLN1* and *CLN2*. Moreover, the *BCK2* gene on a high-copy-

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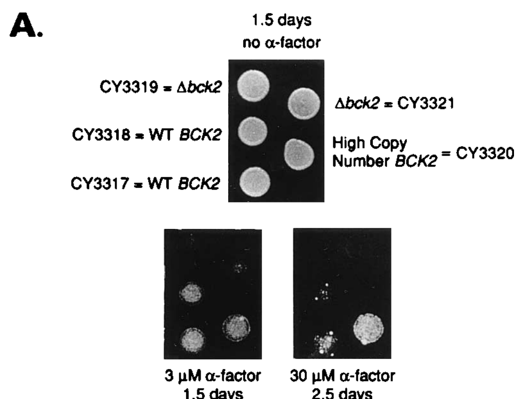

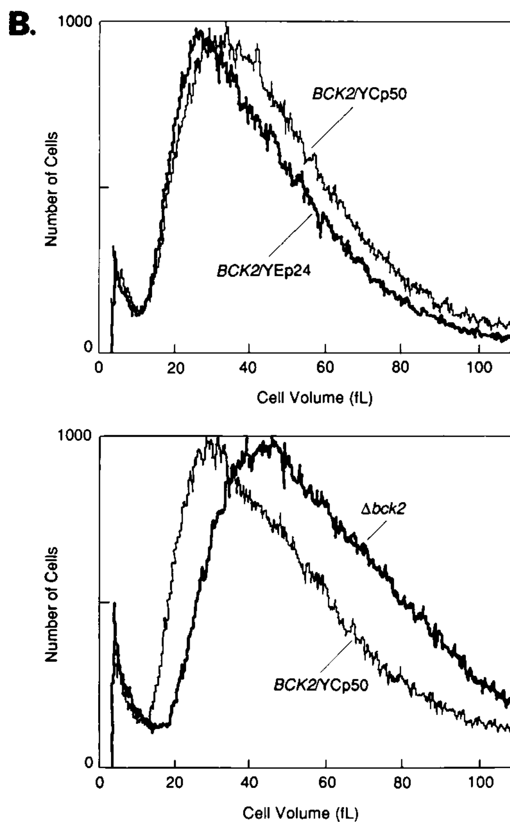

FIG. 3. Phenotypes due to the absence of BCK2 or the overexpression of BCK2. (A) α-Factor sensitivity. Equal numbers of exponentially growing cells (about 1,000 cells, grown in SC-uracil or SC-leucine 2% glucose medium) of strains CY3319 (Δ*bck2* {YCp50}), CY3317 and CY3318 (Δ*bck2* {*BCK2*/YCp50}), CY3321 (Δ*bck2* {YEp13}), and CY3320 (Δ*bck2* {*BCK2*/YEp13}) were spotted onto YPD plates containing the indicated concentration of α-factor. All of the strains are transformants of strain CY3276. The plates were incubated at 30°C for the indicated times. WT, wild type. (B) Cell size. Shown are Coulter Channelyzer plots (29) of cell volume distributions for cells growing exponentially in SC-uracil 2% glucose medium at 30°C. The strains were CY3319 (Δ*bck2* {YCp50}), CY3317 (Δ*bck2* {*BCK2*/YCp50}), and CY3368 (Δ*bck2* {*BCK2*/YEp24}). Strain CY3370 (Δ*bck2* {YEp24}) had a cell volume distribution indistinguishable from that of CY3319 (Δ*bck2* {YCp50}) (data not shown). All of the strains are transformants of CY3276.

number plasmid stimulated the growth rate of a *CLN1* Δ*cln2* Δ*cln3* strain (which has a moderate slow-growth phenotype due to lower than normal levels of G_1 cyclins [13]) and also stimulated the slow growth rate of a Δ*swi4* strain (the slow growth rate of Δ*swi4* strains is due to a defect in G_1 cyclin expression; see reference 31) (Table 2). In contrast, the slow growth rate of a Δ*swi6* strain, which is not stimulated by low- or high-level heterologous expression of *CLN2* (data not shown), is not stimulated by the *BCK2* gene on a high-copy-number plasmid (Table 2).

TABLE 2. Summary of genetic interactions of *BCK2*

Strain	Growth rate	Plus high-copy-number *BCK2*	Plus Δ*bck2*
Δ*cln3*	wt[a]	ND[b]	Viable, but grows very slowly[c]
Δ*cln1*	wt	ND	No effect
Δ*cln2*	wt	ND	No effect
Δ*cln1* Δ*cln2*	Near wt[d]	ND	Slightly slower than Δ*cln1* Δ*cln2*
Δ*cln2* Δ*cln3*	Moderate	Stimulates	ND
Δ*swi4*	Slow	Stimulates	Much slower than Δ*swi4* alone[e]
Δ*swi6*	Slow	No effect	Lethal[f]

[a] wt, wild type.
[b] ND, not determined.
[c] Heterologous expression of *CLN2* from either the *S.p.ADH* or the *S.c.ADH* promoter stimulated the growth rate of *bck2 cln3* strains to near wild-type levels.
[d] Both strains for this cross had *SSD1-v* genetic backgrounds because Δ*cln1* Δ*cln2 CLN3 ssd1-d* strains grow very slowly (12).
[e] Heterologous expression of *CLN2* from either the *S.p.ADH* or the *S.c.ADH* promoter stimulated the growth rate of *bck2 swi4* strains, but not quite to that of *swi4* strains.
[f] Heterologous expression of *CLN2* from either the *S.p.ADH* or the *S.c.ADH* promoter cured the lethality of *bck2 swi6* strains, but the resulting strains did not grow as well as *swi6* strains.

To look directly at the ability of overexpressed BCK2 to activate transcription of *CLN1* and *CLN2*, we prepared Northern blots with RNA isolated from a strain containing *BCK2* coding sequences expressed from the *GAL1* promoter (see Materials and Methods). Twenty minutes after galactose was added to a p*GAL*:*BCK2* strain, the levels of *BCK2* RNA increased dramatically (Fig. 4A). The increase in BCK2 levels subsequently (by 40 min) induced very high level expression of *CLN1* and *CLN2* (whose promoters contain SWI4/SWI6-dependent cell cycle box [SCB]-like and *Mlu*I-containing cell cycle box [MCB]-like elements [10, 31, 36]) and *CLB5* (whose promoter contains MCB-like elements [17]) (Fig. 4A). Transcription from SCB and MCB promoter elements is cell cycle dependent and reaches maximal levels during late G_1. The increase in *CLN1*, *CLN2*, and *CLB5* RNA levels by overexpressed BCK2 was not due to accumulation of the cells in G_1, as determined by the budding index and flow cytometry analysis of the cultures (data not shown). Interestingly, overexpression of BCK2 only slightly increased *SWI4* RNA levels in this strain (Fig. 4B and data not shown). Therefore, overexpression of BCK2 can induce high-level accumulation of *CLN1* and *CLN2* RNAs by a mechanism that does not require a large increase in *SWI4* transcription. Further demonstration of specificity in the ability of overexpressed BCK2 to induce high levels of a given cell cycle-regulated RNA is the finding that overexpressed BCK2 did not increase the levels of histone *HTA1* RNA (Fig. 4A).

Promoters containing multiple SCB or multiple MCB elements are activated about twofold by overexpression of BCK2. Overexpression of BCK2 could increase *CLN1* and *CLN2* RNA levels either by increasing the rate of transcription or by increasing the stability of the RNAs. Although overexpressed BCK2 greatly increased *CLN2* RNA levels when *CLN2* was expressed from the wild-type promoter, it had no effect when

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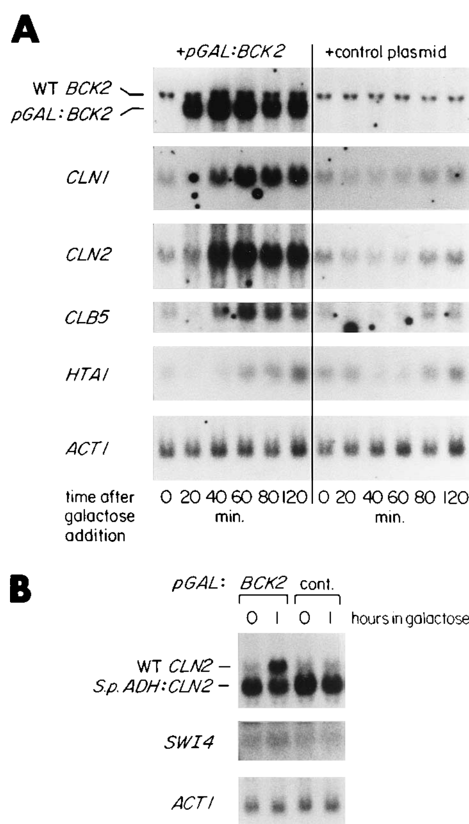

FIG. 4. Overexpressed BCK2 can activate *CLN1*, *CLN2*, and *CLB5* transcription. (A) Strains CY3507 (p*GAL*:*BCK2*/YCp50) and CY3510 (YCp50/p*GAL* expression plasmid with no insert) were grown exponentially at 30°C on SC-uracil 2% raffinose medium. Both strains are transformants of strain AY925 (which contains a wild-type [WT] *BCK2* gene). At an OD_{600} of 0.20, galactose was added to 2% (final concentration), and cells were collected at the indicated times. (B) Strains CY3507 and CY3510 were transformed with a *LEU2*/*cen* plasmid containing the *S.p.ADH*:*CLN2* gene. The *S.p.ADH*:*CLN2* gene gives a *CLN2* RNA shorter than the wild-type *CLN2* RNA because it is missing about 100 bases of 5′ *CLN2* RNA sequences. The strains were grown exponentially at 30°C on SC-uracil,-leucine 2% raffinose medium. At an OD_{600} of 0.20, galactose was added to 2% (final concentration), and cells were collected at the indicated times. cont., control. For both panels, total RNA was prepared and Northern analysis was performed as described in Materials and Methods.

CLN2 was expressed from the *S.p.ADH* promoter (Fig. 4B). Therefore, sequences upstream of the *CLN2* start codon are required for induction by overexpressed BCK2.

In vitro, SWI4/SWI6 complexes are the major factors that bind to SCB elements, while MBP1/SWI6 complexes are the major factors that bind to MCB elements (1, 2, 14, 22, 26, 38). Recently, it has been shown that deletion of a 101-bp *Nru*I-*Sph*I restriction fragment from the *CLN2* promoter, which removes or destroys three SCB-like sequences and two CGCG core MCB-like sequences, causes a dramatic reduction in *CLN2* RNA levels (10, 36). We placed the *Nru*I-*Sph*I *CLN2* promoter fragment into the *Δ52-HIS4:lacZ* reporter gene on YCp50 (28). The *Δ52* mutation removes the *HIS4* upstream activating sequences so that this reporter, without an insert, gave 1 U of β-galactosidase activity, and p*GAL*:*BCK2* had no effect on these levels. In a control strain (no *pGAL*:*BCK2*), the reporter containing the *Nru*I-*Sph*I *CLN2* promoter fragment was not activated when galactose was added to the culture (Table 3). By contrast, in a *pGAL*:*BCK2* strain, the reporter containing the *Nru*I-*Sph*I *CLN2* promoter fragment was activated 1.74-fold when galactose was added to the culture (Table 3). These findings suggest that BCK2-responsive elements are at least partially within the *Nru*I-*Sph*I *CLN2* promoter fragment, which contains two intact SCB-like sequences and one intact CGCG core MCB-like sequence.

TABLE 3. Overexpressed BCK2 can activate transcription from a *CLN2* promoter fragment[a]

Strain	β-Galactosidase activity (U)[b]		Ratio[c]
	Raffinose	+Galactose	
Control	53 (8.3)	52 (1)	0.98
pGAL:BCK2	46 (4.5)	80 (7.2)	1.74

[a] All strains were transformants of AY925 that contained the *Δ52-HIS4:lacZ*/YCp50 reporter plasmid (28) containing the *Nru*I-*Sph*I *CLN2* promoter fragment in the wild-type orientation and either a control plasmid or a *pGAL:BCK2* plasmid (*LEU2* marker). The reverse orientation of the *CLN2* promoter fragment gave 1.84-fold induction with *pGAL:BCK2* and 0.99-fold induction with the control plasmid. The transformants were grown exponentially at 30°C on SC-uracil-leucine 2% raffinose medium. At time zero, galactose was added to 2% (final concentration), and the cultures were grown for 4 h. Whole cell extracts were prepared and β-galactosidase assays were performed as described in Materials and Methods.

[b] Standard deviations (from three or four independent assays) are in parentheses.

[c] Units on galactose divided by units on raffinose.

To assay transcription from only SCB promoter elements, we used a strain containing a *lacZ* reporter gene whose transcription was activated by four synthetic SCB elements (SCB sequence from the *HO* promoter [31]; strain provided by B. Andrews). Relative to the control vector with no insert, overexpression of SWI4 from the *pGAL* promoter greatly stimulated transcription from the multiple synthetic SCB elements (Fig. 5A). Overexpression of BCK2 also stimulated transcription from the multiple synthetic SCB elements, to levels slightly higher than those obtained from overexpression of CLN3-1 (which encodes a hyperactive form of CLN3) and modestly higher than those obtained from overexpression of wild-type CLN3 (by 4 h, *pGAL*:*CLN3* gives 75 U [data not shown]). Therefore, like overexpressed CLN3 or CLN3-1, overexpressed BCK2 can induce transcription from SCB elements.

Overexpressed BCK2 might activate transcription from the multiple synthetic SCB elements indirectly. For instance, overexpressed BCK2 might activate the expression of *CLN1* or *CLN2* via a non-SCB promoter element, and the higher levels of CLN1 and CLN2 would then activate transcription from the multiple synthetic SCB elements (the same argument is also possible for the activation of SCB elements by overexpressed CLN3). If overexpressed BCK2 activates *CLN1* or *CLN2* expression, which in turn activates SCB-driven transcription, then overexpression of CLN1 or CLN2 from the *pGAL* promoter should induce SCB-driven transcription. Neither *pGAL*:*CLN1* (Fig. 5A) nor *pGAL*:*CLN2* (data not shown) caused an increase in the β-galactosidase levels expressed from a *lacZ* gene whose transcription is driven by multiple synthetic SCB elements. Therefore, in this assay, overexpressed CLN3, but not CLN1 or CLN2, activated SCB-dependent transcription. These findings suggest that CLN1 and CLN2 are not nearly as effective as CLN3 for activating SCB-dependent transcription. In addition, we looked at the ability of overexpressed BCK2 to activate transcription from the multiple synthetic SCB elements in the absence of SWI4 or SWI6. Unlike transcription from the *CLN1* and *CLN2* promoters (which are transcribed at low levels in the absence of SWI4 [10, 31, 36]), transcription from the promoter containing multiple synthetic SCB elements is completely SWI4 dependent (in the presence of SWI6) or

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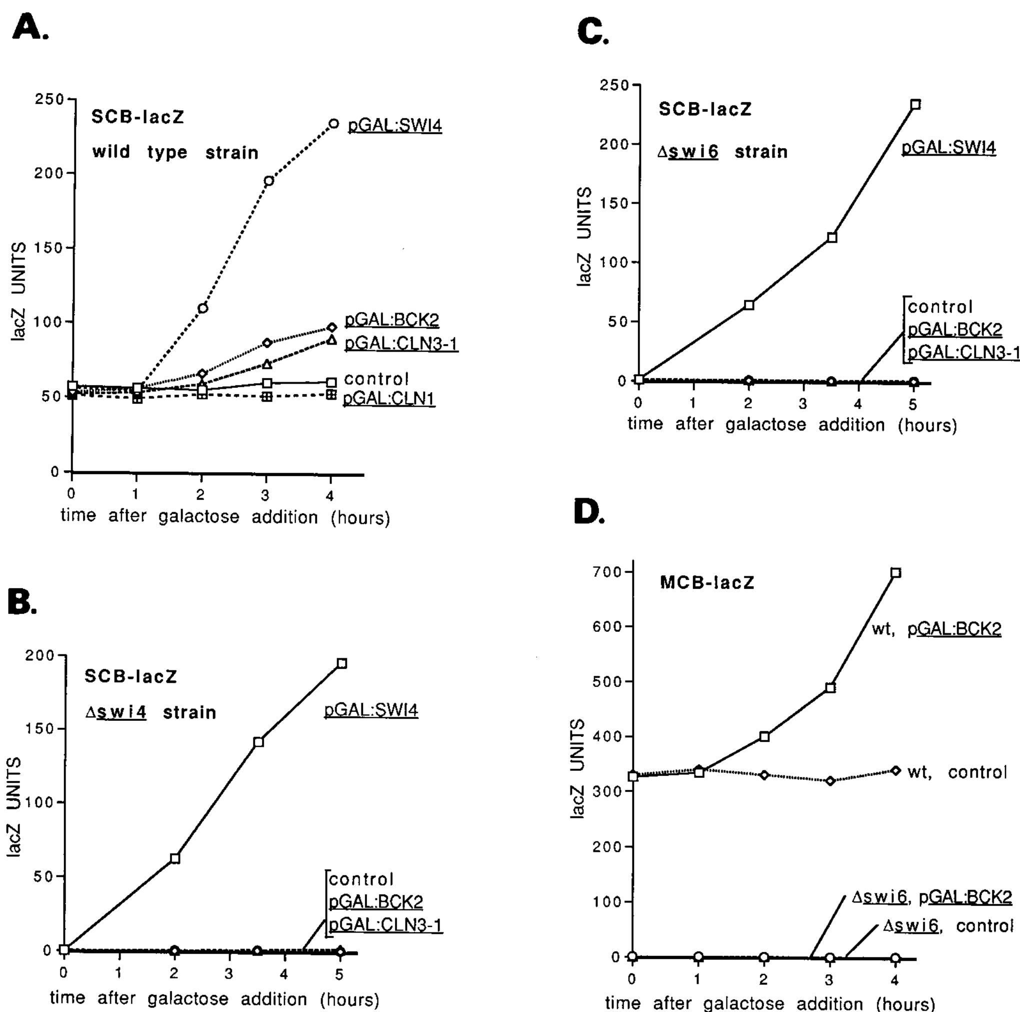

FIG. 5. Overexpressed BCK2 can activate transcription from SCB and MCB promoter elements. (A to C) Strains CY3557 (wild type), CY3582 (Δ*swi4*), and CY3656 (Δ*swi6*) were isogenic and contained the *lacZ* gene under the control of four tandem synthetic SCB elements integrated at the *HIS3* locus, termed *his3Δ124lacZ* (31). These strains were transformed with the indicated *URA3/cen* p*GAL* expression plasmid. The transformants were grown exponentially at 30°C on SC-uracil 2% raffinose medium, and at time zero, galactose was added to 2% (final concentration). (D) Strains CY3798 (wild type [wt]) and CY3779 (Δ*swi6*), both isogenic and containing a 2μm *URA3* plasmid with the *lacZ* gene under the control of four tandem synthetic MCB elements (41), were transformed with the indicated *LEU2/cen* p*GAL* expression plasmid. The transformants were grown exponentially at 30°C on SC-uracil,-leucine 2% raffinose medium, and at time zero, galactose was added to 2% (final concentration). For all panels, extracts were prepared and β-galactosidase assays were performed as described in Materials and Methods.

SWI6 dependent (in the presence of SWI4) (Fig. 5B and C). Moreover, overexpression of BCK2 (or CLN3 or CLN3-1) cannot bypass the requirement of SWI4 or SWI6 for the activation of transcription from the multiple synthetic SCB elements (Fig. 5B and C) (overexpressed BCK2 did not give any induction of the low *SWI4*- or *SWI6*-independent expression of 1 U).

Because the *CLN1* and *CLN2* promoters also contain core MCB-like CGCG elements (SWI4 can bind in vitro to MCB elements [22]) and because overexpressed BCK2 was also able to stimulate the transcription of *CLB5* (whose promoter contains MCB-like elements [17]), we determined the ability of overexpressed BCK2 to stimulate transcription from a promoter containing four synthetic MCB elements (plasmid pBA487 in reference 41; provided by B. Andrews). Interestingly, overexpressed BCK2 stimulated expression from the multiple synthetic MCB elements, and this stimulation was completely dependent on SWI6 (Fig. 5D). Overexpression of CLN2 (from the *GAL1* promoter) was not able to stimulate MCB-dependent expression in this assay (data not shown). Therefore, the ability of overexpressed BCK2 to stimulate transcription from the multiple synthetic MCB elements is not due to the increased levels of *CLN2* RNA.

Roles of SWI4 and SWI6 in BCK2-induced transcription from promoters containing SCB-like and MCB-like elements. In contrast to the promoter containing the multiple synthetic SCB elements (whose transcription is completely SWI4 or SWI6 dependent), the *CLN1* and *CLN2* promoters are expressed at low levels in the absence of SWI4 or in the absence of SWI6. Interestingly, some of the transcriptional activation of *CLN1*, *CLN2*, and *HCS26* (an additional cyclin whose promoter contains SWI4/SWI6 binding sites [31]) by overexpressed BCK2 was not dependent on SWI4 (in the presence of SWI6) or dependent on SWI6 (in the presence of SWI4). The fold induction by overexpressed BCK2 was similar for the Δ*swi4* or Δ*swi6* strain compared with the wild-type strain. However, overexpressed BCK2 induced *CLN1*, *CLN2*, and *HCS26* to higher levels in the presence of SWI4 and SWI6 (Fig. 6) (the

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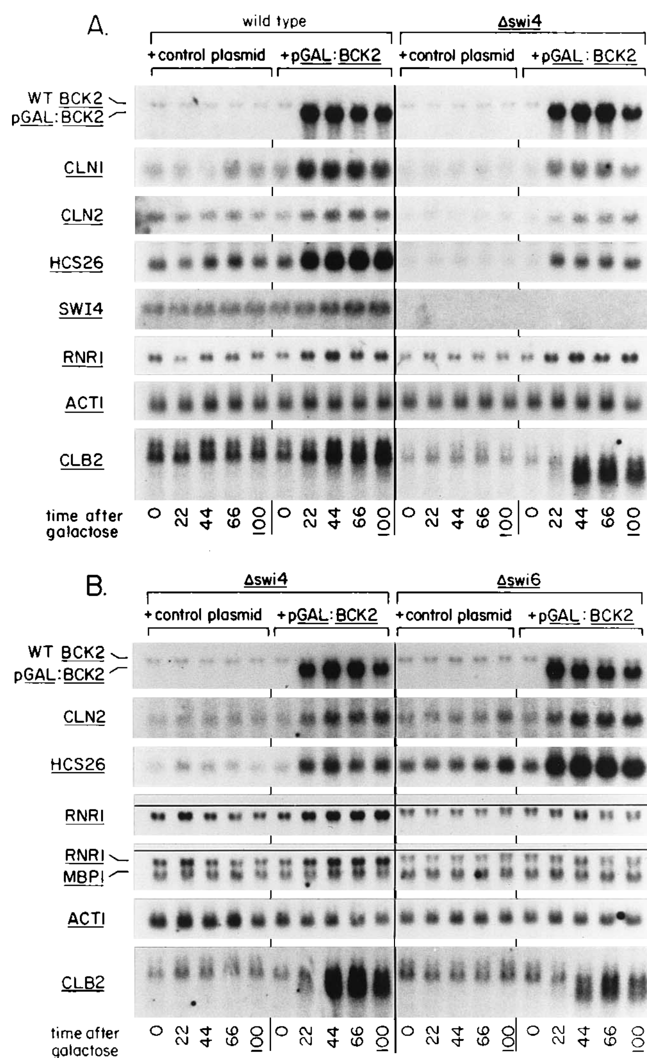

FIG. 6. Roles of SWI4 and SWI6 in BCK2-induced transcription. Isogenic strains CY3574 (wild type [WT], YCp50/p*GAL* expression plasmid with no insert), CY3575 (wild type, p*GAL*:*BCK2*/YCp50), CY3594 (Δ*swi4*, YCp50/p*GAL* expression plasmid with no insert), CY3595 (Δ*swi4*, p*GAL*:*BCK2*/YCp50), CY3671 (Δ*swi6*, YCp50/p*GAL* expression plasmid with no insert), and CY3672 (Δ*swi6*, p*GAL*:*BCK2*/YCp50) were grown exponentially at 30°C on SC-uracil 2% raffinose medium. Strains CY3574 and CY3575 are transformants of CY3557, strains CY3594 and CY3595 are transformants of CY3582, and strains CY3671 and CY3672 are transformants of CY3656. At an OD_{600} of 0.20, galactose was added to 2% (final concentration), and the cells were collected at the indicated times. Total RNA was prepared and Northern analysis was performed as described in Materials and Methods. Because the gel only had 20 lanes, identical Δ*swi4* samples were loaded onto two separate gels, so that the RNA levels of the Δ*swi6* cells can be directly compared with those in the Δ*swi4* cells and, by comparison with the other gel, with those in the wild-type cells. Like *ACT1*, the *URA3* RNA lengths and levels were not affected by overexpression of BCK2 (data not shown).

level of induction of the *GAL1* promoter is not as high for the isogenic wild-type, Δ*swi4*, and Δ*swi6* strains used for Fig. 5 and 6 as for the strains used in Fig. 4; note also that the level of *CLN2* induction was lower for the strains in Fig. 6 than for those in Fig. 4).

Therefore, although maximal stimulation of *CLN1* and *CLN2* transcription by overexpressed BCK2 required SWI4 or SWI6, some of the overexpressed BCK2-induced levels of *CLN1* and *CLN2* RNA resulted from either a promoter element that is normally SWI4 or SWI6 independent (an element unrelated to SCB or MCB elements to which SWI4 and SWI6 would not interact) or from a SWI4- or SWI6-independent mode of activation through promoter elements that are normally largely SWI4 or SWI6 dependent (such as SCB- or MCB-like elements). If the stimulation of *CLN1* and *CLN2* expression by overexpressed BCK2 is due to promoter elements that are normally largely SWI4 or SWI6 dependent, overexpressed BCK2 would have to function differently at the natural *CLN1* and *CLN2* SCB-like and MCB-like elements compared with the multiple synthetic SCB and MCB elements (where activation by overexpressed BCK2 is completely SWI4 or SWI6 dependent [Fig. 5]).

Overexpression of BCK2 in wild-type strains also stimulated *CLB5* expression and *RNR1* (16) expression, and this stimulation was completely independent of *SWI4* (Fig. 4, Fig. 6, and data not shown). Both the *CLB5* promoter (17) and the *RNR1* promoter (15a) contain multiple MCB-like sequences. Normal periodic transcription of certain MCB-driven genes requires both MBP1 and SWI6 (14, 22, 25, 27). MBP1 is a SWI4-related transcription factor that binds, in a complex with SWI6, to MCB promoter elements (22). In a Δ*swi6* strain, the normal length *RNR1* RNA is reduced in abundance, while a longer *RNR1* RNA is present (Fig. 6) (because this length change is small, we include two panels for *RNR1*, each from a different gel). This effect of a *swi6* mutation on *RNR1* RNA has been seen previously (14). Importantly, overexpression of BCK2 stimulated *RNR1* expression in the absence of *SWI6*, and this *BCK2*-induced *RNR1* RNA had a normal or very near normal length (Fig. 6). This *SWI6*-independent stimulation of *RNR1* transcription is not due to the ability of overexpressed BCK2 to stimulate *MBP1* RNA levels (Fig. 6) (in these cultures, the overall *MBP1* RNA levels are independent of *SWI4* or *SWI6* [Fig. 6] and do not vary in the cell cycle [22]).

We also determined the effect of overexpressed BCK2 on *CLB2* expression. *CLB2*, which encodes a B-type cyclin, is expressed during S and G_2 phases (20, 32). Overexpression of BCK2 increased *CLB2* RNA levels in a wild-type strain (Fig. 6). The levels of *CLB2* RNA were low for the Δ*swi4* cells, possibly because the culture is enriched in G_1 cells as a result of the defect in *CLN1* and *CLN2* expression. Interestingly, overexpression of BCK2 in a Δ*swi4* strain or a Δ*swi6* strain (but not a wild-type strain) stimulated the expression of a shorter *CLB2* RNA (Fig. 6). The nature of the *CLB2* promoter element responsible for this effect is not known.

For the *CLN2* promoter, the UAS2 region is required for maximal induction by overexpressed BCK2. Overexpressed BCK2 caused about a twofold activation of transcription from the *CLN2 Nru*I-*Sph*I promoter fragment, which contains two intact SCB-like sequences and one intact CGCG core MCB-like sequence (Table 3). This fold activation is similar to the approximately twofold activation of multiple synthetic SCB or MCB elements by overexpressed BCK2 (Fig. 5). By contrast, for the chromosomal *CLN1* and *CLN2* genes in the W303 strain background (Fig. 4A), overexpressed BCK2 increased the RNA levels much more than twofold.

To determine the *CLN2* promoter sequences that are responsible for this greater activation by overexpressed BCK2, we prepared *CLN2* promoter deletions (Fig. 7A). The *CLN2-AA* gene lacks sequences upstream of the *Nru*I site, which removes or destroys potential MCB CGCG core elements at −644 and −603 and the SCB-like element at −603. Compared with the chromosomal *CLN2* gene, *CLN2-AA* on the *LEU2*/*cen* plasmid gave about twofold-lower levels of *CLN2* RNA when BCK2 was overexpressed (Fig. 7B and data not shown). When UAS1 was also absent (*CLN2-BB*), the level of *CLN2* expression was extremely low and was no longer

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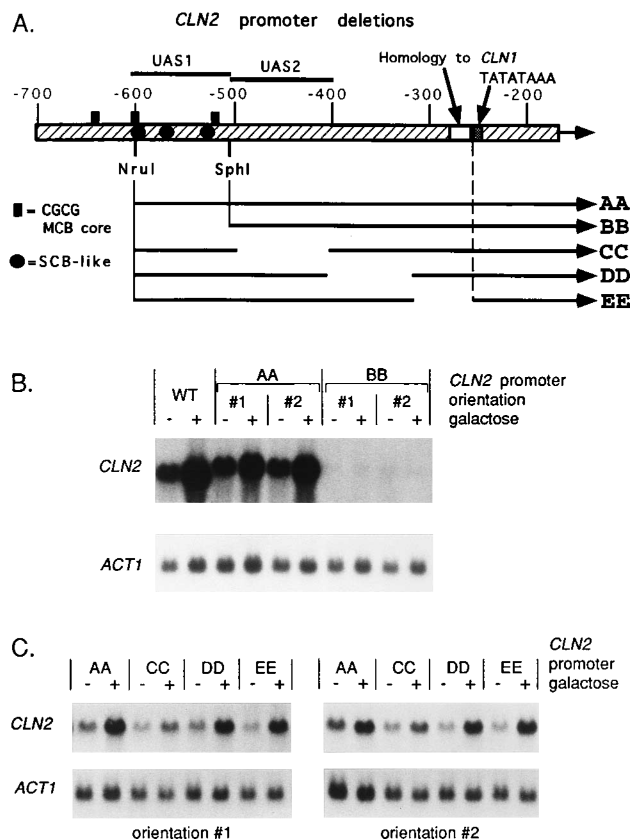

FIG. 7. *CLN2* promoter sequences required for activation by overexpressed BCK2. (A) The *CLN2* promoter sequences present in each deletion (see Materials and Methods) are indicated by a horizontal line. All numbers are relative to the A of the predicted *CLN2* ATG initiation codon. UAS1 and UAS2 of Stuart and Wittenberg (36) are shown. The *Nru*I site is at position −601, and the *Sph*I site is at position −505. For the three indicated SCB-like elements, the element at position −603 has a 6-of-8-bp (6/8), the element at position −578 has a 7/8, and the element at position −526 has a 7/8 match to the *HO* consensus SCB sequence CACGAAAA. However, additional possible SCB-like sequences with a 6/8 match to the SCB consensus sequence are present in the *CLN2* promoter (forward orientation 6/8 sequences occur at positions −621, −615, −526, −361, and −355, while reverse orientation 6/8 sequences occur at positions −471, −403, and −302). (B) In the absence of sequences upstream of the *Sph*I site (−505), *CLN2* RNA levels are extremely low and are not induced by overexpressed BCK2. The wild-type (WT) chromosomal *CLN2* strain (CY3507) was grown on SC-uracil 2% raffinose medium. The strains containing *CLN2* expressed from promoters AA or BB (in either orientation on a *LEU2/cen* plasmid; see Materials and Methods) are isogenic to CY3507 except that they also have a near complete deletion of chromosomal *CLN2* coding sequences (CY4271). The strains containing *CLN2* expressed from the AA or BB promoters were grown on SC-uracil,-leucine 2% raffinose medium. Cells, grown at 30°C, were collected just prior to (−) or 55 min after (+) the addition of galactose to 2% (final concentration). The *CLN2* RNAs expressed from the *CLN2* AA and BB promoters are slightly longer than wild-type *CLN2* RNA because the *CLN2* promoter deletions were prepared by using a *CLN2* gene with sequences encoding a triple hemagglutinin epitope tag at the carboxyl terminus (*CLN2C* in reference 39). The *CLN2* panel is overexposed to show the very low levels of *CLN2* RNA expressed from the BB promoter. (C) The UAS2 region is required for maximal activation of *CLN2* by overexpressed BCK2. *CLN2* was expressed from promoter AA, CC, DD, or EE (on a *LEU2/cen* plasmid) in strain CY4271. The strains were grown at 30°C in SC-uracil,-leucine 2% raffinose medium. Cells were collected just prior to (−) or 55 min after (+) the addition of galactose to 2% (final concentration).

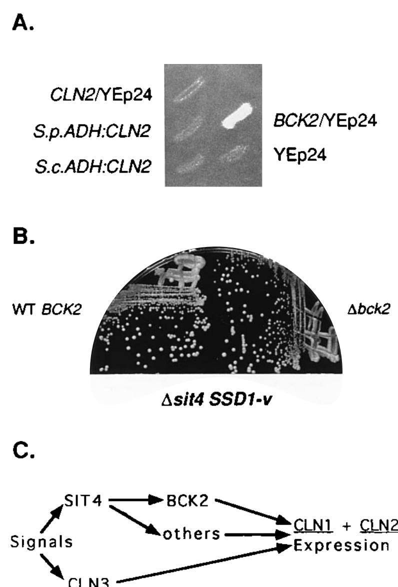

FIG. 8. Genetic interactions of *BCK2* with *SIT4* and *MPK1*. (A) Low- or high-level heterologous expression of CLN2 does not cure the temperature-sensitive phenotype of a Δ*mpk1* strain. Strain CY3182 (Δ*mpk1*) was transformed with the indicated plasmids. Transformants were patched onto SC-uracil 2% glucose plates, grown for 1 day at 24°C, and replica plated onto YPD plates. Growth is shown after 1.5 days at 37°C. (B) Unlike Δ*cln3* Δ*sit4 SSD1-v* strains, which are nearly inviable, Δ*bck2* Δ*sit4 SSD1-v* strains grow at the same rate as Δ*sit4 SSD1-v* strains. Strain CY3276 (Δ*bck2 SSD1-v*) was crossed to strain CY279 (Δ*sit4 SSD1-v*), and representative progeny (from 20 tetrads) of the indicated genotype were streaked onto YPD plates. Growth is shown after 4.5 days at 30°C. WT, wild type. (C) A model of BCK2 function relative to that of CLN3 and SIT4. The placement of SIT4 upstream of BCK2 is arbitrary.

increased by overexpressed BCK2 (Fig. 7B). Therefore, activation of *CLN2* transcription by overexpressed BCK2 requires promoter elements within UAS1 (probably the SCB-like and MCB-like sequences). Also, UAS2 (36) by itself is not sufficient for activation by overexpressed BCK2.

We also determined if UAS2 sequences or other sequences downstream of UAS1 could modulate the ability of BCK2 to induce transcription from UAS1. The activation of *CLN2-DD* and *CLN2-EE* transcription by overexpressed BCK2 was similar to that of *CLN2-AA* (Fig. 7C). By contrast, the absence of UAS2 sequences (*CLN2-CC*) caused a substantial reduction in the ability of overexpressed BCK2 to induce *CLN2* transcription (Fig. 7C). Therefore, sequences contained in the UAS2 region are required for maximal induction of *CLN2* transcription by overexpressed BCK2.

Relationship of *BCK2* to *PKC1* and *MPK1*. The *BCK2* gene was originally isolated by its ability, when present on a high-copy-number plasmid, to suppress the temperature-sensitive phenotype of a Δ*mpk1* strain (24). *MPK1* encodes a mitogen-activated protein kinase homolog that functions downstream of the protein kinase C homolog encoded by the *PKC1* gene. That overexpression of BCK2 stimulated *CLN1* and *CLN2* expression raised the possibility that the suppression of a Δ*mpk1* strain by *BCK2* was due to the ability of *BCK2* to stimulate G_1 cyclin expression. Although the *BCK2* gene on a high-copy-number plasmid suppressed, neither normal-level (*S.p.ADH*:*CLN2*) or high-level (*S.c.ADH*:*CLN2*) heterologous expression of CLN2 (Fig. 8A) nor high-copy-number *MBP1* (data not shown) suppressed the temperature-sensitive phenotype of the Δ*mpk1* strain. We suggest that high-copy-number *BCK2* suppresses a Δ*mpk1* strain by stimulating the expression of some gene(s) other than *CLN1* and *CLN2*. In addition, although high-copy-number *BCK2* partially suppressed the temperature-sensitive phenotype of an *mpk1* strain (and also partially suppressed the slow-growth-rate phenotype at 24°C), neither *CLN3* nor *CLN3-1* on a high-copy-number plasmid suppressed the Δ*mpk1* strain (data not shown). Therefore,

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although BCK2 and CLN3 can each separately activate *CLN1* and *CLN2* transcription, only BCK2 would be able to stimulate the transcription of the putative gene(s) that suppresses the defects of an *mpk1* strain.

Relationship of BCK2 to SIT4. We isolated mutations that result in lethality or cause a very slow growth rate in the absence of *CLN3* in order to obtain genes that function in the *SIT4* pathway for the activation of *CLN1* and *CLN2* expression. The absence of both the *SIT4* pathway and the *CLN3* pathway for the activation of *CLN1* and *CLN2* expression results in an extremely slow-growth phenotype (7 days for a *sit4 cln3* strain to form microcolonies) that is due to a defect in *CLN1* and *CLN2* expression (the synthetic growth defect is completely cured by heterologous expression of *CLN2* from the *S.p.ADH* promoter) (19). The absence of both the *BCK2* pathway and *CLN3* pathway for the activation of *CLN1* and *CLN2* expression also results in a slow-growth phenotype that is due to a defect in *CLN1* and *CLN2* expression. If *BCK2* functions within the *SIT4* pathway for the activation of *CLN1* and *CLN2* expression, then a Δ*bck2* Δ*sit4* strain should not grow any more slowly than a Δ*sit4* strain (viability of Δ*sit4* strains requires *SSD1-v* genetic backgrounds [19, 37]). If, like *CLN3*, *BCK2* functions in a pathway parallel to *SIT4* for the activation of *CLN1* and *CLN2* expression, then a Δ*bck2* Δ*sit4* strain (like a Δ*cln3* Δ*sit4* strain) should grow much more slowly than a Δ*sit4* strain. From a cross of a Δ*bck2* strain to a Δ*sit4* strain, the Δ*bck2* Δ*sit4* progeny grew at the same rate as the Δ*sit4* progeny (Fig. 8B). This finding suggests that BCK2 and CLN3 activate *CLN1* and *CLN2* expression by different pathways and that BCK2 may function within the *SIT4* pathway for the activation of *CLN1* and *CLN2* expression (Fig. 8C). Consistent with this model is the finding that, like a Δ*sit4* Δ*swi6* strain which is inviable (19), a Δ*bck2* Δ*swi6* strain is also inviable (Table 2). Because a Δ*sit4* strain accumulates *CLN1* and *CLN2* RNA at a rate much slower than that of a Δ*bck2* strain, the pathway proposed in this model cannot be linear but must include factors in addition to BCK2 within the SIT4 pathway.

DISCUSSION

Cells containing a deletion of either the *BCK2* gene or the *CLN3* gene have a modest defect in *CLN1* and *CLN2* expression that causes the cells to be more sensitive to α-factor and to execute Start at a larger than normal cell volume. Therefore, BCK2 and CLN3 have similar effects on Start regulation. The defect in *CLN1* and *CLN2* expression in the absence of either BCK2 or CLN3 is modest: the cells have a wild-type growth rate, and the levels of *CLN1* and *CLN2* RNA increase to near normal levels during late G_1, but with a modest delay. Therefore, in the absence of CLN3, BCK2 (*BCK2* RNA levels do not vary in the cell cycle [12a]) can effectively activate the periodic expression of *CLN1* and *CLN2* during late G_1. Likewise, in the absence of BCK2, CLN3 (*CLN3* RNA levels vary two- to threefold during the cell cycle [19]) can effectively activate the periodic expression of *CLN1* and *CLN2* during late G_1. However, in the absence of both BCK2 and CLN3, the cells cannot effectively accumulate *CLN1* and *CLN2* RNAs during late G_1. This defect in *CLN1* and *CLN2* RNA expression is so severe that it causes Δ*bck2* Δ*cln3* cells to have a slow growth rate.

In contrast to Δ*bck2* Δ*cln3* cells, Δ*bck2* Δ*cln1* cells and Δ*bck2* Δ*cln2* cells grow at about the same rate as wild-type strains (reference 18 and Table 2). Moreover, Δ*bck2* Δ*cln1* Δ*cln2* strains grow only slightly more slowly than Δ*cln1* Δ*cln2* strains (Table 2). Although a cell is viable if it has any one of the three *CLN* genes (*CLN1*, *CLN2*, or *CLN3*) (33), the *CLN* genes are not equivalent. Unlike *CLN3*, the normal expression of *CLN1* and *CLN2* requires *SWI4*, *SWI6*, and *SIT4* (19, 30, 31). Moreover, CLN3 has been proposed to be a regulator of Start via its effects on the accumulation of *CLN1* and *CLN2* RNA levels (39). Whether or not CLN3 can mediate Start directly (in which case the CLN3/CDC28 complex would phosphorylate the same Start substrates as the CLN1/CDC28 and CLN2/CDC28 complexes) is not currently known.

There are two possible explanations for the near normal growth rate of *bck2 cln1 cln2* cells in contrast to the very slow growth rate of *bck2 cln3* cells. For the first explanation, CLN3 can directly activate Start. In this case, in the absence of *CLN1*, *CLN2*, and *BCK2* (BCK2 is not required for *CLN3* expression [data not shown]), CLN3 would mediate the direct activation of Start. For the other explanation, CLN3 cannot directly activate Start but is a regulator of the expression of *CLN1*, *CLN2*, and other G_1 cyclins that are the direct activators of Start (39). In this case, CLN3, compared with CLN1 and CLN2, would be a much more potent activator of the expression of *CLN1* and *CLN2* and other G_1 cyclins. In the absence of the BCK2 activation pathway, CLN3 becomes almost essential for activating the expression of *CLN1*, *CLN2*, and other G_1 cyclin genes.

The late G_1 activation of *CLN1* and *CLN2* expression is due to multiple pathways. The major pathway for the activation of the *CLN1* and *CLN2* promoters is via SWI4 (10, 31, 36). SWI4 most likely binds to SCB-like and possibly MCB-like elements in the *CLN1* and *CLN2* promoters (22, 31). Because the great majority of *CLN1* and *CLN2* transcription is SWI4 dependent, BCK2 (at normal levels) is required in the absence of CLN3 for the SWI4-dependent activation of *CLN1* and *CLN2* transcription during late G_1.

Another way to assay the function of BCK2 is via the effects due to its overexpression. Overexpression of BCK2 greatly stimulates *CLN1*, *CLN2*, and *HCS26* expression (the extent of *CLN2* induction is strain dependent; compare Fig. 4 and 6). Overexpressed BCK2 also stimulates *CLB5*, *RNR1*, *SWI4*, and *CLB2* expression, but to a lesser extent. For these genes, a large fraction of the BCK2-induced RNA levels is dependent on either SWI4 or SWI6. However, in the absence of SWI4 (in the presence of SWI6) or in the absence of SWI6 (in the presence of SWI4), overexpression of BCK2 is still able to increase the levels of *CLN1*, *CLN2*, and *HCS26* RNAs, suggesting that BCK2 can also induce *CLN1*, *CLN2*, and *HCS26* transcription by a SWI4-independent mechanism (in the presence of SWI6) or by a SWI6-independent mechanism (in the presence of SWI4).

Unlike the reporter gene containing multiple synthetic SCB elements (whose expression is completely SWI4-dependent and cannot be induced by overexpressed BCK2 in the absence of SWI4), *CLN1* and *CLN2* have SWI4-independent pathways of transcriptional activation (they are transcribed at low levels in the absence of SWI4 [references 10 and 36 and Fig. 6]). It has been reported that the SWI4-independent expression of *CLN2* is dependent on MBP1, that MBP1 can bind in vitro to SCB elements, and that SWI4 can bind in vitro to MCB elements (22). MBP1 might be able to activate *CLN1* and *CLN2* transcription via the SCB-like or MCB-like elements present in these promoters (but MBP1 cannot activate transcription from the promoter containing multiple synthetic SCB elements). One possibility is that the ability of overexpressed BCK2 to stimulate *CLN1*, *CLN2*, and *HCS26* transcription in the absence of SWI4 (but to lower levels than when SWI4 is present) is due to the ability of BCK2 to stimulate MBP1-dependent activation from the SCB-like and/or MCB-like elements present in these promoters. Alternatively, overexpressed BCK2 might be able to stimulate *CLN1* and *CLN2* transcription via two different types of promoter elements; one type

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would be an SCB- or MCB-like SWI4/SWI6/MBP1-dependent element, and a second type would be a non-SCB/MCB-related element (which would be present in the *CLN1*, *CLN2*, and *HCS26* promoters).

A large fraction of the expression of *CLN1* and *CLN2* during late G_1 is dependent on SWI4 and, for *CLN2*, requires the SCB-like and/or MCB-like elements present in the *CLN2* promoter (10, 30, 31, 36). Therefore, in the absence of CLN3, normal levels of BCK2 are required for the SWI4-dependent expression of *CLN1* and *CLN2*, possibly for regulating SWI4's ability to activate transcription via SCB-like and/or MCB-like elements. The large activation of *CLN2* transcription by overexpressed BCK2 seems to be due to two pathways; one pathway is the about twofold induction inherent within UAS1 (probably via the SCB- and MCB-like elements), a second pathway is via the ability of UAS2 to modulate activation by UAS1. It is not known if the sequences within UAS2 through which BCK2 modulates activation by UAS1 are the same sequences that give UAS2 the ability, by itself, to very weakly activate transcription during late G_1 (36).

Overexpression of BCK2 also stimulates the transcription of *CLB5* and *RNR1*, two genes suggested to be activated by MCB-like elements, and the transcription of a promoter containing multiple synthetic MCB elements. In vitro, the major binding activity to MCB elements is due to MBP1/SWI6 complexes (22). In a Δ*swi6* strain, in which the normal length *RNR1* RNA is reduced in abundance while a lower amount of a longer *RNR1* RNA is present, overexpression of BCK2 stimulates the transcription of the normal- or near-normal-length *RNR1* RNA. Therefore, at the *RNR1* promoter, overexpressed BCK2 can substitute for the function of SWI6. A model whereby BCK2 would regulate the function of both SWI4- and MBP1-containing complexes to modulate transcription at SCB-like and MCB-like elements is consistent with our findings. However, BCK2 may also be able to activate transcription from sequences other than SCB/MCB-like elements. The effect of overexpressed BCK2 on *CLB2* expression in the absence of SWI4 or SWI6 cannot be interpreted within this model until more is known about the factors that regulate the periodic transcription of *CLB2*. Interestingly, although the *CLB2* promoter region does not contain apparent SCB elements, it does contain two CGCG core MCB-like sequences at −827 and −660.

BCK2 has been isolated previously by two different suppression schemes. In one scheme, high-copy-number *BCK2* was isolated by its ability to suppress the temperature-sensitive phenotype of a Δ*mpk1* strain (24). Because low- or high-level heterologous expression of *CLN2* did not cure the temperature-sensitive phenotype of the Δ*mpk1* strain, we suggest that overexpression of BCK2 suppresses the temperature-sensitive phenotype of a Δ*mpk1* strain by stimulating the expression of some gene(s), other than G_1 cyclin genes, whose expression is defective in the absence of MPK1 or whose increased expression can bypass MPK1 function.

BCK2 was also isolated by its ability, on low- or high-copy-number plasmids, to suppress the lethality due to the absence of *CLN1*, *CLN2*, and *CLN3* function (18, 20a). One possibility is that overexpressed BCK2 can activate the expression of additional cyclins (such as HCS26) that can substitute for the function of CLN1, CLN2, and CLN3. Alternatively, because overexpressed BCK2 can stimulate the expression of two different MCB-driven genes (*CLB5* and *RNR1*) and the expression driven by multiple synthetic MCB elements, overexpressed BCK2 might be able to bypass *CLN* function for the activation of MCB-driven genes.

We isolated *bck2* mutations because BCK2 is required for significant *CLN1* and *CLN2* transcription in the absence of CLN3. The accumulation of *CLN1* and *CLN2* RNA during late G_1 probably functions as an integrator of growth and mating signals from various sources. Proteins such as FAR1 are induced by the α-factor pathway to repress the accumulation of CLN1 and CLN2 (6, 7). Because the CLN3 protein is very low in abundance and is unstable (9, 40), the levels of CLN3 might reflect the rate of protein synthesis. As such, CLN3 could set the rate of *CLN1* and *CLN2* RNA accumulation, and hence the timing of Start, relative to the rate of protein synthesis (39). Like CLN3, BCK2 can also activate *CLN1* and *CLN2* expression, and the importance of BCK2 for Start is uncovered when the cells lack the CLN3 activation pathway. It remains to be determined what growth signals, if any, regulate the ability of BCK2 to stimulate G_1 cyclin accumulation.

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