Genes That Can Bypass the *CLN* Requirement for *Saccharomyces cerevisiae* Cell Cycle START

CHARLES B. EPSTEIN† AND FREDERICK R. CROSS*

The Rockefeller University, New York, New York 10021

Received 15 September 1993/Returned for modification 9 November 1993/Accepted 23 November 1993

Cell cycle START in *Saccharomyces cerevisiae* requires at least one of the three *CLN* genes (*CLN1*, *CLN2*, or *CLN3*). A total of 12 mutations bypassing this requirement were found to be dominant mutations in a single gene that we named *BYC1* (for bypass of *CLN* requirement). We also isolated a plasmid that had *cln* bypass activity at a low copy number; the gene responsible was distinct from *BYC1* and was identical to the recently described *BCK2* gene. Strains carrying *bck2::ARG4* disruption alleles were fully viable, but *bck2::ARG4* completely suppressed the *cln* bypass activity of *BYC1*. *swi4* and *swi6* deletion alleles also efficiently suppressed *BYC1 cln* bypass activity; Swi4 and Swi6 are components of a transcription factor previously implicated in control of *CLN1* and *CLN2* expression. *bck2::ARG4* was synthetically lethal with *cln3* deletion, suggesting that *CLN1* and *CLN2* cannot function in the simultaneous absence of *BCK2* and *CLN3*; this observation correlates with low expression of *CLN1* and *CLN2* in *bck2* strains deprived of *CLN3* function. Thus, factors implicated in *CLN1* and *CLN2* expression and/or function are also required for *BYC1* function in the absence of all three *CLN* genes; this may suggest the involvement of other targets of Swi4, Swi6, and Bck2 in START.

CDC28, CLNs, other cyclins, and START. The cell cycle of *Saccharomyces cerevisiae* initiates at a point called START in late G₁ (35). *CDC28*, the budding yeast homolog of the *cdc2* protein kinase and any one of the three *CLN* genes, *CLN1*, *CLN2*, or *CLN3*, are required for START (8, 30, 36). The *CLN* genes are distant cyclin homologs; cyclins were first described as activators of *cdc2* protein kinase activity at mitosis (19). Other cyclin-like genes may contribute to START (*HCS26* [33], *CLB5* [13], and *CLB6* [38]).

CLN1 and *CLN2*, but not *CLN3*, are cell-cycle-regulated transcriptionally, peaking around the time of START (43, 45). The Swi4/Swi6 transcription factor, originally identified as an activator of *HO* endonuclease transcription (31), has been implicated as a positive regulator of *CLN1* and *CLN2* transcription (32, 33). Cln1 and Cln2 proteins are more abundant than Cln3, and the Cln1/Cdc28 and Cln2/Cdc28 protein kinases appear to be significantly more active than the Cln3/Cdc28 kinase when histone H1 is used as a substrate (43).

Here we characterize genes that can eliminate the requirement for any of the three *CLN* genes at START.

MATERIALS AND METHODS

Yeast strains and mutant isolation. All strains were isogenic to BF264-15D (36). Transformations and tetrad analysis were performed as previously described (4, 39). Two strains with the genotype *cln1::TRP1 cln2::LEU2 cln3Δ* [p*GAL1::CLN3/URA3*] were plated on glucose plates. Survivors (frequency, ~10⁻⁷) were further tested for *CLN* independence by being plated on 5-fluoroorotic acid (selecting against maintenance of the *GAL1::CLN3* plasmid [5]). Approximately 10% of the mutants were resistant to 5-fluoroorotic acid; 16 such mutants were

screened by Southern blotting to ensure that they did not result from rearrangements of the disrupted *CLN* loci (data not shown). One mutant that carried a rearranged copy of *CLN3* was identified. The remaining mutants showed no rearrangement of *cln1::TRP1* or *cln2::LEU2*, and the 5-fluoroorotic acid-resistant *ura3* derivatives showed no *CLN3* hybridization.

Plasmids. The isolation of the *BCK2* gene (on pCE116) as a low-copy-number plasmid suppressor of *cln* deficiency has been described (13 [therein called *MPK1* instead of *BCK2*]).

The CE116 insert contained both *BCK2* and at least part of the adjacent *ts352* gene (1, 23; restriction mapping and sequence analysis not shown). *cln* bypass activity of the CE116 insert was mapped to *BCK2* on the basis of testing of deletion derivatives of CE116 (data not shown).

In order to mark a yeast chromosome at the *BCK2* locus, the CE116 insert was subcloned into the integrating plasmid pRS304 (40). This plasmid was linearized inside *BCK2* by *HpaI* digestion and transformed into *S. cerevisiae*.

pCE118 contains the pCE116 insert cloned into RS314 (40). The *BCK2* coding region was disrupted with the *ARG4* gene by linearizing pCE118 with *HpaI* and ligating it to pMLC28Pst6 *ARG4* (41) that had been linearized with *SmaI*. The *bck2::ARG4* allele was excised with *XhoI* and *NotI* and introduced into *arg4/arg4* diploid strain 1036 by one-step gene replacement (4). Deletion alleles of *cln1*, *cln2*, and *cln3* (9, 13, 16, 17) were described previously. The *swi4::URA3* disruption allele was introduced by duplicative integration; this allele duplicates the internal *BamHI* fragment of *SWI4*, resulting in inactivation (1a, 2). The *swi4::LEU2* and *swi6::LEU2* deletion alleles were introduced by one-step gene replacement (4). These plasmids were a gift of B. J. Andrews. The structures of all alleles were confirmed by DNA blot hybridization (data not shown). Installation of *swi4* and *swi6* alleles was performed by J. McKinney and J. Rakonjac.

Other methods. RNA isolation and blot hybridization were performed as previously described (9). Electronic cell volume was determined by using a Coulter Channelyzer as previously described (7).

* Corresponding author. Mailing address: The Rockefeller University, 1230 York Ave., New York, NY 10021. Phone: (212) 327-7685. Fax: (212) 327-7923.

† Present address: Department of Molecular Biology, Princeton University, Princeton, NJ 08544.

RESULTS

Rationale. The requirement for one of three *CLN* genes for cell viability appears to identify a requirement for activation of Cdc28 by a G₁-acting cyclin homolog (30, 37). We wished to identify mutations that relieved this requirement, in the hope of identifying either genes acting downstream of the *CLN* requirement or genes that activate a parallel pathway making *CLN* genes unnecessary.

Isolation of *BYC* mutants. Sixteen mutants that were viable in a *cln1::TRP1 cln2::LEU2 cln3Δ* background were isolated (see Materials and Methods). In crosses to *cln1 cln2 cln3* (*cln*[−]) *GAL1::CLN3* strains, the *Byc* (for bypass of *cln* requirement) phenotype was found to segregate 2:2 by tetrad analysis for 12 of the mutants (data not shown) (12). We found by tetrad analysis of *BYC* strains crossed to *CLN*⁺ strains that the disrupted *cln* loci still caused lethality when segregated away from the *BYC* mutations (data not shown). In tetrad analysis of *BYC* strains crossed to *cln*[−] *GAL1::CLN3* strains, we frequently recovered *cln*[−] strains lacking the *GAL1::CLN3* cassette; such strains were never recovered in control crosses without *BYC* loci (data not shown). This confirmed the conclusion that *cln* bypass was not due to deregulation of the *GAL1::CLN3* cassette. Thus, the *BYC* mutations were single-gene extragenic suppressors of lethality of *cln1::TRP1 cln2::LEU2 cln3Δ*.

cln bypass by *BYC* mutations was distinctly weaker than that provided by any single *CLN* gene. Plating efficiency on restrictive (glucose) versus permissive (galactose) conditions of four *cln*[−] *GAL1::CLN3 BYC1-8* strains was found to be 0.6 ± 0.1 (mean $\pm$ standard error of the mean) (data not shown). The doubling time in liquid of one *BYC1-9 cln*[−] *GAL1::CLN3* strain in glucose medium was found to be 186 min compared with 105 min for a *CLN1 CLN2 CLN3* strain. The *BYC* mutant strains exhibited no obvious cell size or growth rate phenotype when *GAL1::CLN3* was expressed (data not shown).

The *BYC* loci are dominant mutations, most likely in the same gene. *BYC* mutations conferred *cln* bypass in heterozygous diploids that were homozygous *cln*[−], although bypass was weaker than in the haploids. A triploid *cln*[−] strain that was *BYC1-8/+* exhibited *cln* bypass activity (data not shown). Thus, the *BYC* mutations are either gain-of-function mutations or, in a highly dosage-sensitive gene, loss-of-function mutations.

We constructed diploid strains that were *cln*[−] *GAL1::CLN3* and heteroallelic for *BYC* alleles. In tetrad analysis, no glucose-sensitive progeny were recovered, indicating that recombination between the different *BYC* loci was not observed (Table 1). The most likely explanation of this result is that all of the mutations are in the same gene. The rest of the experiments described here were performed with the *BYC1-8* allele, since it appeared to have the strongest phenotype (data not shown).

In an attempt to identify the *BYC1-8* gene, we tested for linkage of various genes to *BYC1-8*. *BYC1-8* segregates independently of *CLN1*, *CLN2*, *CLN3*, *CDC28*, *CLB1*, *CLB2*, *CLB3*, *CLB4*, *CLB5*, *HCS26*, *SWI4*, and *SWI6*. It is also unlinked to *LYS2*, implying that it is probably unlinked to *CKS1* (17). Lack of linkage to *CLB1* implies that *BYC1-8* is not an allele of *CLB6* (38). *BYC1-8* is not centromere linked.

***BYC1-8* is a *cln* bypass suppressor but does not suppress other forms of START arrest.** To eliminate the possibility that *BYC1-8* worked by allowing utilization of the disrupted *cln* loci, we confirmed the ability of *BYC1-8* to suppress lethality due to simultaneous deletion of *cln1*, *cln2*, and *cln3* (data not shown) (see Materials and Methods).

We tested the possibility that *BYC1-8* might confer overall

TABLE 1. Crosses between *BYC-9* and other *BYC* alleles^a

Mating partner	Cross	No. Glu ^r /total no. of progeny
<i>BYC-3</i>	1021	13/13
<i>BYC-4</i>	1034	16/16
<i>BYC-6</i>	1046	22/22
<i>BYC-7</i>	1057	18/18
<i>BYC-8</i>	1067	39/39
<i>BYC-9</i>	1076	49/49
<i>BYC-10</i>	1077	19/19
<i>BYC-11</i>	1078	14/14
<i>BYC-12</i>	1079	32/32
<i>BYC-13</i>	1080	11/11
<i>BYC-14</i>	1081	10/10
<i>BYC-18</i>	1084	47/47

^a Diploids were constructed with mutant strains following at least two backcrosses to the wild type. All diploids were homozygous *cln1 cln2 cln3* and carried the *GAL1::CLN3/URA3* plasmid. Progeny were scored for the presence of the *GAL1::CLN3* plasmid, indicated by a Ura⁺ phenotype, and for the ability to propagate on glucose medium. Recombination between two *BYC* loci would be predicted to generate Ura⁺ glucose-sensitive progeny. Note that Ura[−] progeny are obligatorily *BYC* mutants, and therefore their presence is irrelevant to detection of recombination. If two *BYC* loci were unlinked, one-fourth of the Ura⁺ progeny should be glucose sensitive. *BYC-18* was separated from a dominant sporulation defect before analysis (12). Meiotic recombination at other loci segregating in these crosses was observed (12).

START bypass, by constructing *cln*[−] *BYC1-8 GAL1::CN3* strains that were either *bar1* (so that sensitivity to START arrest by α -factor could be tested) or *cdc28-13* (so that the Cdc28 requirement for START could be tested). We found that *BYC1-8* did not bypass α -factor sensitivity or the Cdc28 requirement for START, since *BYC1-8* strains arrested as unbudded cells in response to α -factor treatment or in response to inactivation of Cdc28 (Table 2 and data not shown). Thus, *BYC1-8* activity is specific to *cln* bypass.

An attempt to clone *BYC1-8* yielded another gene, *BCK2*. We attempted to clone *BYC1*, assuming that its dominant phenotype could allow recovery of the mutant gene on a plasmid in a *cln*-deficient strain (13). This attempt yielded two low-copy-number suppressors, *CLB5* (characterized previously [13]) and another gene (referred to as *MPK1* in reference 13; this name will not be used in this report because it has been published elsewhere and refers to a different gene [24]). Neither gene corresponded to *BYC1*, since marked alleles of each recombined freely with *BYC1* in tetrad analysis (data not shown). The gene previously referred to as *MPK1* and here called *BCK2* was

TABLE 2. *BYC1-8* does not bypass the *CDC28* requirement at START^a

Genotype at:		% Unbudded cells after 6 h in:			
		Galactose		Glucose	
<i>BYC1</i>	<i>CDC28</i>	30°C	38°C	30°C	38°C
+	+	42	34	96	95
<i>BYC1-8</i>	+	47	33	46	52
+	<i>cdc28-13</i>	61	97	91	96
<i>BYC1-8</i>	<i>cdc28-13</i>	57	98	38	98

^a All strains were *cln*[−] *GAL1::CLN3*. Log-phase cultures (in yeast extract-peptone-galactose medium) of strains of the indicated genotypes at *BYC1* and *CDC28* were transferred to the indicated carbon source and temperature for 6 h. Cells were collected, fixed with formaldehyde, sonicated to disperse clumps, and analyzed microscopically for the percentage of unbudded cells, a morphological indicator of G₁, pre-START cells (28).

identified as a *cln* deficiency suppressor by two other groups (13a, 42a).

Sequence and restriction analysis (data not shown) revealed identity of the novel *cln* suppressor to the *BCK2* gene (14a, 23, 24a). *BCK2* was identified as a high-copy-number plasmid suppressor of lethality due to MAP kinase deletion or to protein kinase C deletion; *BCK2* is nonessential (23).

Our isolate of *BCK2* contained *BCK2* open reading frame fused to the cloning vector. A plasmid containing a full-length *BCK2* sequence (23) provided *cln* bypass activity (data not shown), so the potential N-terminal truncation of *BCK2* in our clone is not required for *cln* bypass.

***bck2* is synthetically lethal with *cln3*.** A *bck2::ARG4* disruption allele (*ARG4* was introduced at the *HpaI* site in the middle of the gene [23]) had only a very mild phenotype: strains with the mutation were completely viable and had normal doubling times, but modal electronic cell volume was increased by about 30% (data not shown). However, *bck2 cln3* mutants were inviable in tetrad analysis. Pooling dissection data from four diploids, viability of *BCK2 CLN3*, *BCK2 cln3*, *bck2 CLN3*, and *bck2 cln3* spores was 48 of 48, 38 of 40, 60 of 64, and 0 of 72, respectively (data not shown). Examination of tetrad dissection agar at the positions where *bck2 cln3* strains were expected revealed single, very large, round, unbudded cells (data not shown), suggesting failure of first-bud emergence. In contrast, the *bck2* disruption allele was readily combined with *cln1* and *cln2* deletion alleles alone or in combination in tetrad analysis; these multiple mutants had essentially normal growth rates (data not shown).

We made use of the conditional *GAL1::CLN3* gene to recover *cln3 bck2 GAL1::CLN3* strains on galactose medium. In short-term assays these strains required galactose for proliferation, arresting as large, round, unbudded G_1 cells in the first cell cycle following shift to glucose. When these strains were incubated for a prolonged period on galactose medium, some of the cells escaped the arrest, forming small, slowly growing colonies (data not shown). Thus, *bck2::ARG4* spores absolutely require *CLN3*, while *bck2::ARG4* vegetative cells are dramatically impaired by the lack of *CLN3*. *CLN1 cln2 cln3 bck2 GAL1::CLN3* strains are completely inviable on glucose medium, indicating that Bck2 is required for solo functioning of *CLN1*. These strains exhibit first-cycle arrest as large, unbudded, mononucleate cells upon transfer to glucose medium to turn off the *GAL1::CLN3* gene (Fig. 1 and data not shown). The arrested cells retain high viability for at least 8 h after transfer to glucose medium (data not shown). Isogenic *BCK2* strains exhibit a delay as unbudded cells upon transfer to glucose medium, but eventually resume proliferation (Fig. 1). Substitution of raffinose medium for galactose medium in this protocol gives qualitatively similar results, but the *CLN1 cln2 cln3 GAL1::CLN3 bck2::ARG4* strain may show limited escape from unbudded arrest (data not shown). This may be due to incomplete repression of *GAL1::CLN3* by raffinose compared with that by glucose (15, 20).

The lethality of *CLN1 cln2 cln3 bck2::ARG4* strains was specifically rescued by a low-copy-number plasmid containing *BCK2* but not the same plasmid containing deletions in *BCK2* or containing *bck2::ARG4* (data not shown), demonstrating that the mutant phenotype was specifically due to *BCK2* inactivation.

Function of *CLN1* and *CLN2* in *bck2::ARG4* strains. The inviability of *cln3 bck2::ARG4* spores and the inviability of *cln2 cln3 bck2::ARG4* vegetative cells suggested that the synthesis or function of *CLN1* and *CLN2* was defective in the *bck2::ARG4* background. We constructed *GAL1::CLN1 cln⁻ bck2* strains by tetrad analysis. These strains were viable on

FIG. 1. *bck2* inactivation blocks *CLN1* function. (A) Cells of genotype *CLN1 cln2 cln3 GAL1::CLN3* that were *bck2::ARG4* or *BCK2* were grown to log phase in galactose medium. The cells were shifted to glucose medium, and at intervals aliquots were removed for determination of the percentage of unbudded cells. *BCK2* strain, 1492-21D; *bck2::ARG4* strain, 1371-7A. (B) *CLN1 cln2 cln3 bck2::ARG4 GAL1::CLN3* cells (1052-2D) were grown in galactose medium, plated on solid glucose (YEPD) or galactose (YEPGal) medium, and then photographed after 24 h of incubation. The cells on glucose demonstrate a permanent cell cycle arrest. The doublets observed in the YEPD panel probably derive from initially budded cells, since the proportions of doublets on the plate and budded cells in the population plated were similar (data not shown).

galactose but not on glucose, with approximately normal doubling times (data not shown). Hence, there is no absolute requirement for Bck2 for Cln1 function.

We constructed *cln⁻ bck2* strains containing a construct in which the *CLN3* (cell-cycle-constitutive) promoter drives the *CLN2* coding sequence (by using the *CLN2^{3P}* construct [44]), by mating and tetrad analysis. The ability of *CLN2^{3P}* to rescue *cln⁻ bck2::ARG4* spores was limited. Rescue of *cln⁻ BCK2*

spores by *CLN2*^{3P} was estimated to be 98% (42 of 43); rescue of *cln*⁻ *bck2::ARG4* spores by *CLN2*^{3P} was estimated to be 57% (24 of 42). However, the wild-type *CLN2* gene was completely unable to rescue *bck2::ARG4 cln3* spores (since *bck2 cln3 CLN1 CLN2* spores were inviable [see above]). These results demonstrate that Cln1 and Cln2 have at least partial function in *bck2* strains when expressed from heterologous promoters.

Since *cln1 cln2 CLN3* cells are significantly larger than *CLN1 CLN2 CLN3* cells (18), *CLN1* and *CLN2* activity can be monitored by assaying cell volume. The modal electronic cell volume of log-phase cells of various genotypes was determined as previously described (7) (data not shown). *CLN1 CLN2 CLN3 bck2::ARG4* cells have volumes 30% greater than that of the wild type, but deletion of both *CLN1* and *CLN2* increased volumes of *BCK2* and *bck2::ARG4* cells by similar factors (1.5- and 1.7-fold, respectively). Therefore, at least by the assay of cell size, *CLN1* and *CLN2* appear to function similarly in *bck2::ARG4* and *BCK2* strains, provided *CLN3* is present.

Transcriptional regulation of *CLN2* in *bck2::ARG4* strains. *CLN1* and *CLN2* RNAs are expressed at essentially normal levels in *bck2* mutant strains (data not shown). However, this result is hard to interpret fully: first, Cln function activates transcription of *CLN1* and *CLN2* (9, 11), and second, *CLN1* and *CLN2* expression is periodic in the cell cycle (45), making a determination of average asynchronous levels relatively uninformative. Therefore, we examined the expression of *CLN2* nonfunctional reporter gene constructs in *bck2* or *BCK2* cells lacking *cln* function (i.e., *cln1 cln2 cln3 GAL1::CLN3* or *GAL1::CLN1* strains incubated in raffinose or glucose medium for 2.5 h to induce G₁ arrest [9]). *bck2* strains show lower expression of *cln2* RNA than do the *BCK2* strains at the block (Fig. 2). In one experiment examining four *BCK2* and four *bck2::ARG4 cln*⁻ *GAL1::CLN3* strains incubated in glucose for 3 h, we quantitated this effect roughly by comparison to intensities of serial dilutions. (Note: this method of quantitation is accurate only to within a factor of about 2 [data not shown].) *cln2* transcript levels in *cln*-blocked *BCK2* strains were approximately 1.2 ± 0.1 arbitrary units (mean ± standard error of the mean), whereas in the same experiment the levels in *cln*-blocked *bck2::ARG4* strains were approximately 0.14 ± 0.02 arbitrary units (data not shown). Upon reactivation of the *GAL1::CLN3* construct, *cln1* and *cln2* expression promptly rose, with similar kinetics and to nearly the same level as in the *BCK2* strain (Fig. 2 and data not shown). When this experiment was performed by using *GAL1::CLN1* instead of *GAL1::CLN3*, we observed a lessened ability of *GAL1::CLN1* to transactivate reporter *cln2* transcription in the *bck2::ARG4* compared with that in the *BCK2* strain (Fig. 2). These defects in transcriptional regulation of *CLN2* (and *CLN1* [data not shown]) may contribute to synthetic lethality of *cln3* and *bck2*.

Disruption of *bck2*, *swi4*, or *swi6* suppresses *BYC1-8* activity. *bck2::ARG4*, *swi4::LEU2*, and *swi6::LEU2* efficiently suppressed *BYC1-8 cln* bypass activity. *BYC1-8/+ cln*⁻ diploids yielded approximately 50% segregants with the Byc phenotype (ability to grow without *GAL1::CLN* expression). In contrast, when the diploids were also heterozygous for a marked *swi4*, *swi6*, or *bck2* disruption, all segregants with a Byc phenotype were *SWI*⁺ *BCK2*⁺, while all segregants scored as *swi4*, *swi6*, or *bck2* required *GAL1::CLN* expression for viability. From four such crosses, 53 of 129 *SWI*⁺ *BCK2*⁺ spores had the Byc phenotype, whereas 0 of 35 *swi4*, 0 of 21 *swi6*, and 0 of 68 *bck2* spores had the Byc phenotype (data not shown). The Byc activity of *BCK2* on a low-copy-number centromere-containing plasmid was eliminated in a *swi4 cln*⁻ strain and partially

FIG. 2. Altered transcriptional regulation of *CLN2* in *bck2::ARG4* strains. All strains tested were *cln1-del cln2-delx3 cln3-del*. (A) Strains contained a *GAL1::CLN3* cassette (8) integrated at *leu2*; (B) strains contained a *GAL1::CLN1* cassette (36) on a CEN plasmid. The strains were *BCK2* or *bck2::ARG4* as indicated. The *cln2-delx3* gene produces an appropriately regulated nonfunctional transcript (9). Cells were synchronized in G₁ by incubation in raffinose for 2.5 h (turning off *GAL1::CLN1* or *GAL1::CLN3*), and then cell cycle progression was restarted by addition of galactose (9). RNA was extracted at 12-min intervals. RNA blots (4) were probed with *CLN2* probe or with a probe detecting both Protein 1 RNA, a non-cell-cycle-regulated transcript, or histone H2A RNA, which is expressed in S phase (34). The *CLN2* blots and Pr1/H2A blots for the pairs of strains tested were probed and washed on the same membrane.

suppressed in a *swi6 cln*⁻ strain (data not shown), suggesting a common element(s) in the *BYC1-8* and *BCK2* pathways.

The Byc activity observed in *cln*⁻ *BYC1-8/+* heterozygous diploids was suppressed in diploids also heterozygous for *bck2::ARG4*, *swi4::LEU2*, or *swi6::LEU2* (Table 3), so *BYC1-8* activity is highly sensitive to dosage of these START regulators.

Swi4 functions together with *Swi6* to make the SBF transcription factor involved in regulation of *HO* as well as other genes (probably including *CLN1* and *CLN2*) (2, 3, 30–33). *Swi6* is also a component of the DSC-1/MBF transcription factor involved in the regulation of genes required for DNA synthesis (10, 29). The DNA-binding component of DSC-1/MBF is encoded by the *MBP1* gene (10, 31a). *BYC1-8* is not suppressed by deletion of *MBP1* (data not shown), so suppression of

TABLE 3. Suppression of *BYC1-8 cln* bypass activity in heterozygous diploids by heterozygosity of *swi4*, *swi6*, or *bck2*^a

Strain	Genotype	Plating efficiency		
		Expt 1	Expt 2	Expt 3
1446-8A	<i>cln⁻ byc⁺</i>	$<1 \times 10^{-5}$	$<2 \times 10^{-5}$	ND
1000-3B	<i>cln⁻ BYC1-8</i>	0.6	0.6	ND
x1460	<i>cln⁻ BYC1-8/+ swi4/+</i>	6×10^{-4}	7×10^{-4}	ND
x1461	<i>cln⁻ BYC1-8/+ swi6/+</i>	3×10^{-2}	ND	3×10^{-2}
x1464	<i>cln⁻ BYC1-8/+ bck2/+</i>	4×10^{-4}	4×10^{-3}	ND
x1463	<i>cln⁻ BYC1-8/+</i>	0.5	0.3	0.9

^a 1446-8A is a *cln⁻ GAL::CLN3* control haploid strain. 1000-3B is isogenic except for the presence of *BYC1-8*. x1460, x1461, x1463, and x1464 are diploid strains derived by mating 1000-3B to isogenic *cln⁻* haploids carrying *swi4::LEU2*, *swi6::LEU2*, no additional mutation, or *bck2::ARG4*, respectively. Strains were grown to stationary phase on yeast extract-peptone (YEP)-galactose plates and inoculated into water at a similar density. The suspensions were sonicated, and then serial dilutions were made in water. A total of 10 μ l of the dilutions was spotted on YEP-galactose or on YEP-glucose. After 2 days of incubation at 30°C, colonies were counted in the spots, and the number of CFU per unit volume was determined. The plating efficiency was defined as the number of CFU on glucose divided by the number on galactose per unit volume. The significantly higher plating efficiency of x1461 (*swi6::LEU2/+*) compared with those of the *swi4* and *bck2* heterozygotes may be due to accumulation of *SWI6⁺* homozygotes before plating on glucose, since in both experiments six of six glucose-viable clones tested from x1461 were *Leu⁻*. ND, not determined.

BYC1-8 by *swi4::LEU2* and *swi6::LEU2* is probably due to lack of SBF activity.

DISCUSSION

Genetics of *cln* bypass. Table 4 summarizes the genetic interactions described in this paper.

The selection for *cln* bypass mutations is quite specific: 12 of 12 independent mutations were dominant mutations most likely in the same gene, *BYC1*. *BYC1-8* does not suppress the requirement for *CDC28* for cell cycle START; this result suggests that either *CDC28* activation is cyclin independent in *BYC1-8* strains or else that *BYC1* allows expression or utilization of cyclin homologs normally not adequate for *CDC28* activation at START.

Inactivation of potential regulators of *CLN1* and *CLN2* transcription (*SWI4* and *SWI6* [32, 33]) suppresses *BYC1-8*. This suggests the possibility that *BYC1-8* works through affecting transcription of some other gene (for example, another cyclin gene) that allows *CLN1,2,3* independence. *CLB1*, *CLB2*, *CLB3*, *CLB4*, *CLB5*, *CLB6*, and *HCS26* are other cyclin genes in *S. cerevisiae*; these cyclins probably have diverse biological roles, and the degree of overlap of their functions has not been fully determined (13, 14, 33, 36, 38, 42). We have tested knockouts of these cyclin genes (except for *CLB6* [38]) for suppression of *BYC1-8*. *HCS26* could be an attractive candidate, since it is likely to be under the transcriptional control of *Swi4/Swi6* (33). None of these knockouts clearly inactivates *BYC1-8 cln* bypass activity, although the *cln⁻ BYC1-8 GAL1::CLN3* strains that were also either *clb5*, *clb2*, or *hcs26* were significantly less vigorous on glucose medium than *CLB⁺*

HCS26⁺ controls (data not shown). These cyclin knockouts might need to be combined in some way to effectively suppress *BYC1-8*, or another cyclin might be the critical target. The genetic data are also consistent with other models; for example, *SWI4* and *SWI6* might be required for transcription of *BYC1*. It is unlikely that *Swi4/Swi6* controls *BCK2* transcription, because *BCK2* transcription is not cell cycle regulated (32a).

A model for α -factor induction of arrest before START in which α -factor results in functional inactivation of all three *CLN* genes was proposed (6). However, if α -factor resulted only in inactivation of *CLN* gene function, then a *cln* bypass mutation should also be α -factor resistant, which is not the case for *BYC1-8* (see above). Various cyclin genes from other organisms can rescue *cln*-deficient *S. cerevisiae* when expressed from strong promoters (21, 22, 25, 28, 46); the rescued strains are sensitive to α -factor in all cases tested. It has been argued (43) that the foreign cyclins may activate expression of additional endogenous yeast cyclin genes and that these genes are the targets of α -factor regulation in the rescued yeast.

***BCK2* and *CLN* function.** *bck2* strains have a strong requirement for *CLN3* even in the presence of *CLN1* and *CLN2*. We observed effects of *bck2* inactivation on *CLN1* and *CLN2* transcriptional control. However, substitution of the *CLN3* promoter for the *CLN2* promoter in the *CLN2* gene completely rescues *cln1,2,3 bck2* strains but only partially rescues *cln1,2,3 bck2::ARG4* strains in tetrad analysis (see above). Thus, *bck2* inactivation may affect *CLN1* and *CLN2* function at both transcriptional and posttranscriptional levels. In contrast, *bck2* inactivation has only minor effects on *CLN3* function as measured by the cell volume assay (see above); in the presence of *CLN3*, *bck2* inactivation has little or no effect on *CLN1* and *CLN2* transcriptional control (Fig. 2 and data not shown) or function (by cell volume assay [see above]). This suggests that *Cln3*-dependent transcriptional activation of *CLN1* and *CLN2* overcomes the defect in *CLN1* and *CLN2* transcription due to *bck2* inactivation.

Since the null phenotype of *BCK2* appears most closely related to *CLN1* and *CLN2* transcription and/or function, it appears reasonable to attribute the *cln* bypass activity of *BCK2* on a plasmid to effects on transcription and/or function of another cyclin or cyclins, following logic similar to that applied to *BYC1* above. We do not yet have candidates for this cyclin target of Bck2 action. Bck2 was independently isolated as a suppressor of lethality due to protein kinase C inactivation (23). It was concluded (23) that Bck2 functioned on a "branch"

TABLE 4. Summary of genetic interactions among *CLN* genes, *SWI4*, *SWI6*, *BCK2*, and *BYC1-8*

<i>CLN^a</i>	<i>BYC1</i>	Growth ^b			
		wt ^c	<i>bck2</i>	<i>swi4</i>	<i>swi6</i>
1- 2-	wt	++	++	—	—
1- 3-	wt	++	+/-	—	—
2- 3-	wt	++	—	—	—
1- 2- 3-	wt	—	—	—	—
2- 3- 3-	<i>BYC1-8</i>	+	—	—	—

^a Abbreviations for *CLN* background: 1-, *cln1*; 2-, *cln2*; 3-, *cln3*.

^b ++, vigorous growth; +, weaker growth; +/-, marginal growth; —, no growth. Data for first three rows for *swi4* and *swi6* are from reference 32.

^c wt, wild-type.

of the protein kinase C pathway (26, 27), conceivably bringing input from other regulatory systems. Perhaps these observations suggest some link between *CLN/CDC28* function and the protein kinase C pathway.

ACKNOWLEDGMENTS

We thank M. Hoek for excellent technical assistance; B. Futcher, D. Levin, K. Nasmyth, S. Reed, and J. Thorner for communicating results prior to publication; M. Goebel for computer analysis of sequences; J. McKinney for strain constructions; B. J. Andrews for *swi4::URA3*, *swi6::LEU2*, and *swi4::LEU2* plasmids; C. Koch for the *mbp1::URA3* plasmid; and D. Levin for the YEP24-BCK2 plasmid.

F.R.C. is a Scholar of the Lucille P. Markey Charitable Trust. This work was supported by the Markey Trust, by the Arnold and Marie Beckman Foundation, and by PHS grant GM47238.

REFERENCES

- Aebi, M., G. Kirchner, J.-Y. Chen, U. Vijayraghavan, A. Jacobson, N. C. Martin, and J. N. Abelson. 1991. Isolation of a temperature-sensitive mutant with an altered tRNA nucleotidyltransferase and cloning of the gene encoding tRNA nucleotidyltransferase in the yeast *Saccharomyces cerevisiae*. *J. Biol. Chem.* **265**:16216–16220.
- Andrews, B. J. Personal communication.
- Andrews, B. J., and I. Herskowitz. 1989. The yeast SW14 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription. *Nature (London)* **342**:830–833.
- Andrews, B. J., and L. A. Moore. 1992. Interaction of the yeast Swi4 and Swi6 cell cycle regulatory proteins in vitro. *Proc. Natl. Acad. Sci. USA* **89**:11852–11856.
- Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl. 1987. Current protocols in molecular biology. Wiley Interscience, New York.
- Boeke, J. D., F. LaCrute, and G. R. Fink. 1984. A positive selection for mutants lacking 5' phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance. *Mol. Gen. Genet.* **197**:345–346.
- Chang, F., and I. Herskowitz. 1990. Identification of a gene necessary for cell cycle arrest by a negative growth factor of yeast: FAR1 is an inhibitor of a G1 cyclin, CLN2. *Cell* **63**:999–1011.
- Cross, F. R. 1988. DAF1, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **8**:4675–4684.
- Cross, F. R. 1990. Cell cycle arrest caused by *CLN* gene deficiency in *Saccharomyces cerevisiae* resembles START-I arrest and is independent of the mating-pheromone signalling pathway. *Mol. Cell. Biol.* **10**:6482–6490.
- Cross, F. R., and A. H. Tinkelenberg. 1991. A potential positive feedback loop controlling CLN1 and CLN2 gene expression at the start of the yeast cell cycle. *Cell* **65**:875–883.
- Dirick, L., T. Moll, H. Auer, and K. Nasmyth. 1992. A central role for SWI6 in modulating cell cycle Start-specific transcription in yeast. *Nature (London)* **357**:508–513.
- Dirick, L., and K. Nasmyth. 1991. Positive feedback in the activation of G1 cyclins in yeast. *Nature (London)* **351**:754–757.
- Epstein, C. B. 1992. Ph.D. thesis. Rockefeller University, New York, N.Y.
- Epstein, C. B., and F. R. Cross. 1992. CLB5: a novel B cyclin from budding yeast with a role in S phase. *Genes Dev.* **6**:1695–1706.
- Futcher, B. Personal communication.
- Ghiara, J. B., H. E. Richardson, K. Sugimoto, M. Henze, D. J. Lew, C. Wittenberg, and S. I. Reed. 1991. A cyclin B homolog in *S. cerevisiae*: chronic activation of the Cdc28 protein kinase by cyclin prevents exit from mitosis. *Cell* **65**:163–174.
- Goebel, M. Personal communication.
- Guarente, L., R. R. Yocum, and P. Gifford. 1982. A GAL10-CYC1 hybrid yeast promoter identifies the GAL4 regulatory region as an upstream site. *Proc. Natl. Acad. Sci. USA* **79**:7410–7414.
- Hadwiger, J. A., and S. I. Reed. 1990. Nucleotide sequence of the *Saccharomyces cerevisiae* CLN1 and CLN2 genes. *Nucleic Acids Res.* **18**:4025.
- Hadwiger, J. A., C. Wittenberg, M. D. Mendenhall, and S. I. Reed. 1989. The *Saccharomyces cerevisiae* *CKS1* gene, a homolog of the *Schizosaccharomyces pombe* *suc1+* gene, encodes a subunit of the Cdc28 protein kinase complex. *Mol. Cell. Biol.* **9**:2034–2041.
- Hadwiger, J. A., C. Wittenberg, H. E. Richardson, M. De Barros Lopes, and S. I. Reed. 1989. A family of cyclin homologs that control the G1 phase in yeast. *Proc. Natl. Acad. Sci. USA* **86**:6255–6259.
- Hartwell, L. H. 1991. Twenty-five years of cell cycle genetics. *Genetics* **129**:975–980.
- Johnston, M., and R. W. Davis. 1984. Sequences that regulate the divergent GAL1-10 promoter in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **4**:1440–1448.
- Koff, A., F. Cross, A. Fisher, J. Schumacher, K. Leguellec, M. Philippe, and J. M. Roberts. 1991. Human cyclin E, a new cyclin that interacts with two members of the CDC2 gene family. *Cell* **66**:1217–1228.
- Lahue, E. E., A. V. Smith, and T. L. Orr-Weaver. 1991. A novel cyclin gene from *Drosophila* complements CLN function in yeast. *Genes Dev.* **5**:2166–2175.
- Lee, K. S., L. K. Hines, and D. E. Levin. 1993. A pair of functionally redundant yeast genes (*PPZ1* and *PPZ2*) encoding type 1-related protein phosphatases function within the *PKC1*-mediated pathway. *Mol. Cell. Biol.* **13**:5843–5853.
- Lee, K. S., K. Irie, Y. Gotoh, Y. Watanabe, H. Araki, E. Nishida, K. Matsumoto, and D. E. Levin. 1993. A yeast mitogen-activated protein kinase homolog (Mpk1p) mediates signalling by protein kinase C. *Mol. Cell. Biol.* **13**:3067–3075.
- Levin, D. Personal communication.
- Léopold, P., and P. H. O'Farrell. 1991. An evolutionarily conserved cyclin homolog from *Drosophila* rescues yeast deficient in G1 cyclins. *Cell* **66**:1207–1216.
- Levin, D. E., and E. Bartlett-Heubusch. 1992. Mutants in the *S. cerevisiae* *PKC1* gene display a cell cycle-specific osmotic stability defect. *J. Cell Biol.* **116**:1221–1229.
- Levin, D. E., F. O. Fields, R. Kunisawa, J. M. Bishop, and J. Thorner. 1990. A candidate protein kinase C gene, *PKC1*, is required for the *S. cerevisiae* cell cycle. *Cell* **62**:213–224.
- Lew, D. J., V. Dulic, and S. I. Reed. 1991. Isolation of three novel human cyclins by rescue of G1 cyclin (Cln) function in yeast. *Cell* **66**:1197–1206.
- Lowndes, N. F., A. L. Johnson, L. Breeden, and L. H. Johnston. 1992. SWI6 protein is required for transcription of the periodically expressed DNA synthesis genes in budding yeast. *Nature (London)* **357**:505–513.
- Nasmyth, K. 1993. Control of the yeast cell cycle by the Cdc28 protein kinase. *Curr. Opin. Cell Biol.* **5**:166–179.
- Nasmyth, K. 1993. Regulating the HO endonuclease in yeast. *Curr. Opin. Genet. Dev.* **3**:286–294.
- Nasmyth, K. Personal communication.
- Nasmyth, K., and L. Dirick. 1991. The role of SW14 and SW16 in the activity of G1 cyclins in yeast. *Cell* **66**:995–1013.
- Oehlen, L., and M. Hoek. Personal communication.
- Ogas, J., B. J. Andrews, and I. Herskowitz. 1991. Transcriptional activation of CLN1, CLN2, and a putative new G1 cyclin (HCS26) by SW14, a positive regulator of G1-specific transcription. *Cell* **66**:1015–1026.
- Osley, M. A., J. Gould, S. Kim, M. Y. Kane, and L. Hereford. 1986. Identification of sequences in a yeast histone promoter involved in periodic transcription. *Cell* **45**:537–544.
- Pringle, J. R., and L. H. Hartwell. 1981. The *Saccharomyces cerevisiae* life cycle, p. 97–142. *In* J. Strathern, E. W. Jones, and J. R. Broach (ed.), *The molecular biology of the yeast Saccharomyces cerevisiae*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
- Richardson, H., D. J. Lew, M. Henze, K. Sugimoto, and S. I. Reed. 1992. Cyclin-B homologs in *Saccharomyces cerevisiae* function in S phase and in G2. *Genes Dev.* **6**:2021–2034.
- Richardson, H. E., C. Wittenberg, F. Cross, and S. I. Reed. 1989. An essential G1 function for cyclin-like proteins in yeast. *Cell* **59**:1127–1133.
- Schwob, E., and K. Nasmyth. 1993. CLB5 and CLB6, a new pair of B cyclins involved in DNA replication in *Saccharomyces cerevisiae*. *Genes Dev.* **7**:1160–1175.

39. Sherman, F., G. R. Fink, and J. B. Hicks. 1989. Laboratory course manual for methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
40. Sikorski, R. S., and P. Hieter. 1989. A system of shuttle vectors and yeast strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122:19–27.
41. Sun, H., D. Treco, N. P. Schultes, and J. W. Szostak. 1989. Double-strand breaks at an initiation site for meiotic gene conversion. *Nature (London)* 338:87–90.
42. Surana, U., H. Robitsch, C. Price, T. Schuster, I. Fitch, A. B. Futcher, and K. Nasmyth. 1991. The role of CDC28 and cyclins during mitosis in the budding yeast *S. cerevisiae*. *Cell* 65:145–161.
- 42a. Thorner, J. Personal communication.
43. Tyers, M., G. Tokiwa, and B. Futcher. 1993. Comparison of the *Saccharomyces cerevisiae* G1 cyclins: Cln3 may be an upstream activator of Cln1, Cln2 and other cyclins. *EMBO J.* 12:1955–1968.
44. Valdivieso, M. H., K. Sugimoto, K.-Y. Jahng, P. M. B. Fernandes, and C. Wittenberg. 1993. *FAR1* is required for posttranscriptional regulation of *CLN2* gene expression in response to mating pheromone. *Mol. Cell. Biol.* 13:1013–1022.
45. Wittenberg, C., K. Sugimoto, and S. I. Reed. 1990. G1-specific cyclins of *S. cerevisiae*: cell cycle periodicity, regulation by mating pheromone, and association with the p34CDC28 protein kinase. *Cell* 62:225–237.
46. Xiong, Y., T. Connolly, B. Futcher, and D. Beach. 1991. Human D-type cyclin. *Cell* 65:691–699.