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POG1, a Novel Yeast Gene, Promotes Recovery From Pheromone Arrest via the G1 Cyclin *CLN2*

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Manuscript received September 9, 1998
Accepted for publication November 3, 1998

ABSTRACT

In the absence of a successful mating, pheromone-arrested *Saccharomyces cerevisiae* cells reenter the mitotic cycle through a recovery process that involves downregulation of the mating mitogen-activated protein kinase (MAPK) cascade. We have isolated a novel gene, *POG1*, whose promotion of recovery parallels that of the MAPK phosphatase Msg5. *POG1* confers α-factor resistance when overexpressed and enhances α-factor sensitivity when deleted in the background of an *msg5* mutant. Overexpression of *POG1* inhibits α-factor-induced G1 arrest and transcriptional repression of the *CLN1* and *CLN2* genes. The block in transcriptional repression occurs at SCB/MCB promoter elements by a mechanism that requires Bck1 but not Cln3. Genetic tests strongly argue that *POG1* promotes recovery through upregulation of the *CLN2* gene and that the resulting Cln2 protein promotes recovery primarily through an effect on Ste20, an activator of the mating MAPK cascade. A *pog1 cln3* double mutant displays synthetic mutant phenotypes shared by cell-wall integrity and actin cytoskeleton mutants, with no synthetic defect in the expression of *CLN1* or *CLN2.* These and other results suggest that *POG1* may regulate additional genes during vegetative growth and recovery.

THE yeast *Saccharomyces cerevisiae* has **a** and α haploid cells that mate to produce **a**/α diploids (Sprague and Thorner 1992). The haploids secrete pheromones, **a**-factor and α-factor, that act on haploid cells of the opposite mating type. The pheromone binds to and activates the Ste2 receptor in **a** cells and the Ste3 receptor in α cells. Receptor activation turns on a signal transduction cascade that induces the transcription of a number of genes involved in mating and causes cells to undergo cell-cycle arrest in G1 phase and morphological changes (termed shmoo formation).

The receptors transduce the pheromone signal to a heterotrimeric G-protein consisting of the Gpa1 (Gα), Ste4 (Gβ), and Ste18 (Gγ) subunits (Dietzel and Kurjan 1987; Miyajima *et al.* 1987; Whiteway *et al.* 1989). In the absence of pheromone, Gpa1 binds to and maintains Ste4/Ste18 (Gβγ) in an inactive state. Upon activation of the receptor, Gpa1 is released from Ste4/Ste18, allowing Ste4 to transduce the signal to a highly conserved mitogen-activated protein kinase (MAPK) cascade. Ste4 activates the MAPK cascade by binding to Ste20 (Leeuw *et al.* 1998), a Cdc42-activated kinase (Simon *et al.* 1995; Peter *et al.* 1996; Leberer *et al.* 1997), and to Ste5, a LIM/Ring-H2 domain protein (Inouye *et al.* 1997; Feng *et al.* 1998). Ste5 acts as a scaffold (Elion 1995) for Ste11 (a MAPK kinase kinase), Ste7 (a MAPK kinase), and Fus3/Kss1 (MAPKs, of which Fus3 is most critical for mating; Elion *et al.* 1991; Madhani *et al.* 1997). Ste20 activates Ste11 through an unknown mechanism that requires the presence of Ste5 (Leberer *et al.* 1992; Wu *et al.* 1995; Feng *et al.* 1998).

Once activated, the MAPKs act on a transcription factor, Ste12 (Elion *et al.* 1991, 1993), which induces the expression of numerous genes required for signal transduction, G1 arrest, and mating (Song *et al.* 1991). In addition, Fus3 activates a cyclin-dependent kinase inhibitor, Far1, which inhibits the activity of the G1 cyclin-dependent kinase, Cdc28 (Chang and Herskowitz 1990; Peter and Herskowitz 1994; Jeoung *et al.* 1998). Fus3 and Kss1 also repress the transcription of the G1 cyclin genes, *CLN1* and *CLN2* (Cherkasova *et al.* 1999). The combination of transcriptional repression of cyclin genes and inactivation of the Cln/Cdc28 complexes leads to arrest in the G1 phase of the cell cycle (Valdivieso *et al.* 1993; Cherkasova *et al.* 1999).

The response elicited by pheromone is transient. In the absence of mating, cells reenter the cell cycle through a process of recovery or desensitization (Sprague and Thorner 1992). Regulators of recovery include Gpa1 and Sst2, which inhibit the activity of Ste4/Ste18 (Dietzel and Kurjan 1987; Dohlman and Thorner 1997) and phosphatases such as Msg5, Ptp2, and Ptp3, which inactivate Fus3 (Doi *et al.* 1994; Zhan *et al.* 1997). In addition to driving the G1- to S-phase transition (Hadwiger *et al.* 1989), the G1 cyclin Cln2 may also have a function in recovery because overexpression of *CLN2* blocks the ability of cells to arrest in the presence of

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α-factor (Oehlen and Cross 1994). Cln2 overproduction inhibits the mating MAPK pathway between the Ste4 and Ste11 steps, suggesting that Ste5, Ste20, or Ste11 may be direct targets of Cln2/Cdc28 (Wassmann and Ammerer 1997).

To identify other components involved in the recovery process, we isolated genes that block pheromone-induced G1 arrest when overexpressed. Among the genes isolated, we found a novel gene, *POG1.* Double mutant analysis suggests that *POG1*'s promotion of recovery parallels that of *MSG5. POG1* requires *CLN2* but not the other G1 cyclins to promote recovery. Consistent with this, *POG1* overexpression leads to elevated levels of *CLN1* and *CLN2* mRNAs in the presence of α-factor. This loss of transcriptional repression occurs through SCB/MCB promoter elements and requires Bck1, a MAPK kinase kinase known to upregulate Swi4-dependent cell-cycle box (SCB)/*Mlu*I cell-cycle box (MCB) promoter elements during vegetative growth (Madden *et al.* 1997). Additional genetic evidence suggests that *STE20* may be a key target of control for the promotion of recovery of *POG1* and *CLN2.* Finally, *POG1* has a vegetative function that may be redundant with *CLN3* and distinct from its ability to regulate the *CLN1* and *CLN2* genes.

MATERIALS AND METHODS

Yeast strains, media, and genetic manipulation: Yeast strains and plasmids are listed in Table 1. Standard methods were used for microbial and molecular manipulations (Guthrie and Fink 1991). *msg5::Leu2* from pSPdel was introduced as described (Doi *et al.* 1994). *hml*α*::LEU2* from pCW9-1 (provided by C. White, Frederick Cancer Institute, Frederick, Maryland) was introduced as a *Bam*HI fragment. All strain constructions by gene replacement were confirmed by Southern analysis (Sambrook *et al.* 1989).

cDNA library screen: EY1118 cells were transformed with a yeast cDNA plasmid library that expresses cDNA inserts from the *GAL1* promoter (Liu *et al.* 1992). Ura^+ transformants were first selected on glucose-uracil plates. A total of 80,000 colonies were then screened for α-factor resistance by replica plating them onto galactose-uracil plates spread with 4.2 μg of α-factor. In a secondary screen, α-factor-resistant transformants were retested for dependence on galactose for growth in the presence of α-factor. In a tertiary screen, transformants were passaged over 5-fluoroorotic acid + uracil plates to select for loss of the plasmid DNA to confirm the dependence of α-factor resistance on the presence of the plasmid. Positive plasmids were then rescued from yeast (Hoffmann and Winston 1987) and retransformed into EY1118 to confirm galactose-dependent growth in the presence of α-factor.

Recombinant DNA techniques: Standard methods were used for all recombinant DNA techniques (Sambrook *et al.* 1989). Plasmids were transformed into *Escherichia coli* HB101. DNA sequencing was performed by the dideoxy chain termination method (Sambrook *et al.* 1989) with Sequenase (United States Biochemical Corp., Cleveland). Terminal sequences of the isolated cDNAs were determined using a 3′ T7 primer and a 5′ primer (5′-TCGAGGTCGACCCACGC) synthesized to match polylinker sequences in the vector.

Plasmids: ZM43 and ZM44 are *GAL1* promoter derivatives of YCplac33 (*URA3 CEN*) and YCplac111 (*LEU2 CEN*), respectively, (provided by Z. Moqtaderi, Harvard Medical School, Boston; Gietz and Sugino 1988). pGALSTE20, a *Bam*HI-*Pvu*-III fragment containing the *STE20* coding sequence (CDS) except for amino acid residues 1–69, was isolated from pSTE20-5 (Leberer *et al.* 1992) and cloned into the *Bam*HI-*Hin*dIII (blunt-ended) sites of ZM44. A *Bst*YI-*Bam*HI fragment containing *STE20* amino acid residues 1–69 then was cloned into the *Bam*HI site to generate pML52. YEpMSG5, a *Xba*I-*PST*I fragment from YCpMSG5 (Doi *et al.* 1994), was transferred to *Xba*I-*Pst*I-cleaved YEplac195, generating pML31.

Cloning of *POG1* and gene deletion: The *POG1* cDNA in pML2 was isolated from a pRS316-based cDNA library (Liu *et al.* 1992). The genomic copy of *POG1* was cloned as follows: pML32, containing a genomic copy of *POG1*, was isolated from a Yep24-based genomic yeast library (Carlson and Botstein 1982) probed with *POG1* cDNA sequences. A 4-kb *Sal*I-*Hin*dIII fragment from pML32 containing the *POG1* CDS was then cloned into the *Sal*I-*Hin*dIII sites of YEplac195 and YEplac181 to generate pML33 and pML34.

pog1::HIS3 deletion mutation was as follows: A fragment containing 1100 bp of *POG1* 5′ flanking sequences (−171/−1278 from the ATG) was isolated from pML58 (pBlueScript containing a *Sal*I-*Eco*RV fragment from YEpPOG1) as a *Sph*I-*Bam*HI fragment and transferred to YIplac211. A fragment containing 1155 bp of *POG1* 3′ flanking sequences (+153/+1308 from the stop codon) was amplified by polymerase chain reaction (PCR) using primers: (A) 5′-CCGTCAGGATCCACTCCTTATCTCATTTCA-3′ (a *Bam*HI site that is added is underlined) and (B) 5′-CCGTCGAATTCGTTCCTCTTTGTTTCTGG-3′ (an *Eco*RI site that is added is underlined). The *Bam*HI-*Eco*RI PCR product was cloned into YIplac211 containing the −171/−1278 piece to generate pML59. A *Bam*HI *HIS3* gene fragment from pUC18-HIS3 (provided by D. Kodosh) was then introduced into pML59 to generate pML60. For gene replacement, pML60 was digested with *Sph*I and *Eco*RI and the resulting *pog1::HIS3* fragment was used for transformation. Replacement of the genomic *POG1* locus was confirmed by Southern analysis (Sambrook *et al.* 1989).

Epitope tagging of *POG1*: To place the green fluorescent protein (GFP) tag on the N terminus of Pog1, an *Acc*I fragment from pML33 containing amino acid residues 48–351 of Pog1 was blunt-ended and cloned into the *Bam*HI (blunt-ended) site of pCGF-1A (Lee *et al.* 1996), creating pML61. To HA-tag Pog1 at the C terminus the *POG1* CDS was amplified by PCR using primers: (C) 5′-CCGTCAGAATTCATGAAGCAGGAGCCACAT-3′ (an added *Eco*RI site is underlined) and (D) 5′-CGCTCAGTCGACGAATGAAGGTTAGGAAGG-3′ (an added *Sal*I site is underlined). The *Eco*RI-*Sal*I PCR fragment was cloned into pBluescript to generate pML62. A HA triple tag from pGTE1 (Tyers *et al.* 1992) was cloned into the *Bse*RI site within the *POG1* CDS in pML62, introducing the HA tag between amino acids 345–347 of the *POG1* CDS, generating pML68. Then the *Eco*RI-*Sal*I fragments from pML62 and pML68 were transferred to pDAD2 (*URA3 2*μ), placing the untagged and HA-tagged copies of *POG1* under the control of the *GAL1* promoter and resulting in plasmids pML67 and pML72. None of the plasmids containing tagged copies of Pog1 conferred α-factor resistance in halo assays.

Halo and spotting assays: α-Factor sensitivity was measured by halo assay as described (Elion *et al.* 1990) using 50 μl of an overnight culture or of cultures equalized for cell density. α-Factor peptide (synthesized by C. Dahl, Harvard Medical School, Boston) was dissolved in 90% methanol and stored at −20°. Unless indicated otherwise, 420 ng of α-factor was used for all *sst1* strains. All halo assays were done at least twice using independent transformants. For spotting assays, cells were diluted to the same A_{600} (0.4–0.5) and then diluted serially 100×

TABLE 1

Yeast strains and plasmids

	Genotype or descriptions	Source or reference
Strains[a]		
EY698	*MAT*α	R. Rothstein
EY699	*MAT***a**	R. Rothstein
EY957	EY699 *sst1::hisG*	Elion *et al.* (1993)
EY1027	EY957 *cln2::LEU2*	E. A. Elion
EY1028	EY957 *cln1::TRP1*	E. A. Elion
EY1118	EY957 *his3Δ200 lys2::FUS1-HIS3*	Lyons *et al.* (1996)
EY1298	*MAT***a** *sst1::hisG STE11-4 far1Δ his3Δ200 lys2::FUS1-HIS3*	Lyons *et al.* (1996)
EY2022	EY1298 *ste20::TRP1*	Feng *et al.* (1998)
CY326	EY1118 *CLN2::CLN2-HA LEU2*	V. Cherkasova
ML201	*MAT***a** *cln3::URA3*	B. Futcher
C699-59	*MAT***a** *sst1::hisG bck1::HIS3 ade3::hisG*	B. Errede
MLY4	EY1118 *hml*α*::LEU2*	This study
MLY16	EY698/EY699	This study
MLY17	MLY16 *pog1::HIS3*	This study
MLY28	*MAT***a** *pog1::HIS3* progeny of MLY17	This study
MLY30	*MAT*α *pog1::HIS3* progeny of MLY17	This study
MLY18	MLY16 *msg5::LEU2*	This study
MLY81	*MAT***a** *msg5::LEU2* progeny of MLY18	This study
CY3557	*MAT***a** *ssd1-d ura3-52 leu2Δ1 his3Δ200 ade2-101 lys2-801 plus SCB-lacZ at HIS3 locus*	B. Andrews
Plasmids		
pGALCLN2	pML1-*GALCLN2 URA3 CEN*	This study
pGALPOG1	pML2-*GALPOG1 URA3 CEN*	This study
YEpMSG5	pML31-*MSG5* in YEplac195	This study
YEpPOG1-1	pML33-*POG1* in YEplac195	This study
YEpPOG1-2	pML34-*POG1* in YEplac181	This study
pGALSTE20	pML52-*GALSTE20* in ZM44	This study
pCGF-POG1	pML61-GFP-tagged *POG1 (GAL URA3 2*μ*)*	This study
pPOG1-HAc	pML72- HA-tagged *POG1 (GAL URA3 2*μ*)*	This study
ppog1::HIS3	pML60-*pog1::HIS3*	This study
pGALGPA1m	*GALGPA1val50 URA3 CEN*	Miyajima *et al.* (1989)
pGALSTE4	pYEE116-*GALSTE4 HIS3 CEN*	Elion *et al.* (1991)
pGALMSG5	*GALMSG5 URA3 CEN*	Doi *et al.* (1994)
ADH-CLN2	*ADH-CLN2 URA3 2*μ	M. Peter
pYBS45	*FUS1-lacZ LYS2 CEN*	Lyons *et al.* (1996)
pSPdel	*msg5::LEU2*	Doi *et al.* (1994)
pCW9-1	*hml*α*::LEU2*	C. White
YEplac195	*URA3 2*μ	Gietz and Sugino (1988)
YEplac181	*LEU2 2*μ	Gietz and Sugino (1988)
ZM43	*URA3 CEN GAL1*	Z. Moqtaderi
ZM44	*LEU2 CEN GAL1*	Z. Moqtaderi

[a] All strains are isogenic derivatives of W303 (*ura3-1 his3-11,15 leu2-3, 112 trp 1-1 ade2-1 can1-100 Gal*+) except for CY3557.

over a 10,000-fold range before spotting 5 μl of each dilution on solid medium. For α-factor resistance tests, yeast cells were spotted onto plates spread with 4.2 μg of α-factor.

Growth conditions: Yeast strains were grown in selective synthetic complete (SC) medium containing either 2% dextrose or 2% galactose. All strains were grown at 30° except for C699-59 and its control strain, which were grown at 25°. For α-factor inductions, logarithmically growing cells were adjusted to the same A_{600} (0.4–0.6) and divided into two with one-half receiving α-factor. Cultures were incubated with shaking for 2 hr and then harvested. For galactose induction of genes under the *GAL1* promoter, the cells were grown in 2% galactose for 2–2.5 hr prior to the addition of α-factor. All *sst1* cultures were treated with 50 nm α-factor unless otherwise stated. *SST1* cultures were treated with 1 mm α-factor.

Preparation of yeast protein extracts: Cells were harvested at 4°, washed twice with cold sterile water, and frozen in dry ice. Whole cell extracts were prepared by lysis with glass beads as described (Elion *et al.* 1993) using modified H buffer adjusted to 125 mm NaCl, 2.5 mm benzamidine, and 1 μg/ml aprotinin. Protein concentration was determined with the Bio-Rad (Richmond, CA) protein assay.

β-Galactosidase assays: Yeast strains transformed with pYBS45 (Lyons *et al.* 1996) were either left untreated or treated with 50 nm (*sst1*) or 2 mm (*SST1*) α-factor for 2 hr. Protein extracts were prepared and assayed for β-galactosidase (*lacZ*) activity as described (Elion *et al.* 1995).

Western blots: Yeast protein extract (50–100 μg) was loaded in 8% SDS-PAGE gels. 12CA5 mouse monoclonal antibody (ascites fluid from Harvard University Antibody Facility) at a 1/10,000 dilution was used to detect Cln2HA (Tyers *et al.* 1993). Western blots were done as described (Harlow and

Lane 1988). Blots were developed with the Amersham (Arlington Heights, IL) ECL kit according to the manufacturer's instructions using Fuji RX X-ray film.

Northern blots: Total RNA was prepared and 10–20 μg of RNA was loaded in duplicate 1% formaldehyde-agarose gels, transferred to nitrocellulose (Schleisser and Schuller) and probed by Southern analysis (Sambrook *et al.* 1989). DNA probes were labeled by the random-primed method. The probes used were a 1.8-kb *Sal*I-*Not*I *CLN2* fragment from pGALCLN2 (pML1), a 1.6-kb *Bam*HI *CLN1* fragment from EB608 (Elion *et al.* 1991), a 1.4-kb *Hin*dIII-*Spe*I *CLN3* fragment from pBF30 (provided by B. Futcher), and *Xho*I-*Hin*dIII *ACT1* from pYEE15 (Elion *et al.* 1991). Blots were probed simultaneously with *ACT1* as a control for loading and *CLN1*, *CLN2*, or *CLN3*. Relative amounts of mRNA were quantified using a Fuji Imaging plate.

RNase protection: *lacZ* and A*CTI* probes were synthesized from pSPCTV and pSACTall (Stuart and Wittenberg 1994) using the Promega (Madison, WI) Riboprobe kit. RNase protection was performed as described in Stuart and Wittenberg (1994) using a protocol generously provided by D. Stuart.

Cell morphology and indirect immunofluorescence: All microscopy was performed using an Axioscope fluorescence microscope (Carl Zeiss, Thornwood, NY). To determine the percentage of budded cells, cells were fixed in 3.7% formaldehyde and briefly sonicated before quantitation as described (Elion *et al.* 1990). Fluorescence signal from GFP-tagged Pog1 was examined in the FITC channel after cells were grown in 2% galactose for 1 hr. Detection of C-terminal HA-tagged Pog1 through the use of 12CA5 monoclonal antibody and DTGF-labeled goat anti-mouse antibody was done as described (Lee *et al.* 1996). Images shown in Figure 6C were captured with a Toshiba 3CCD camera and Phase 3 Imaging System by Media Cybernetics.

Protein sequence analysis: The Pog1 amino acid sequence was analyzed using BLASTP/X (Altschul *et al.* 1990), BEAUTY (Worley *et al.* 1995), TFASTA/FASTP (Pearson and Lipman 1988), MOTIFS, and BLOCKS (Henikoff and Henikoff 1994) database search programs.

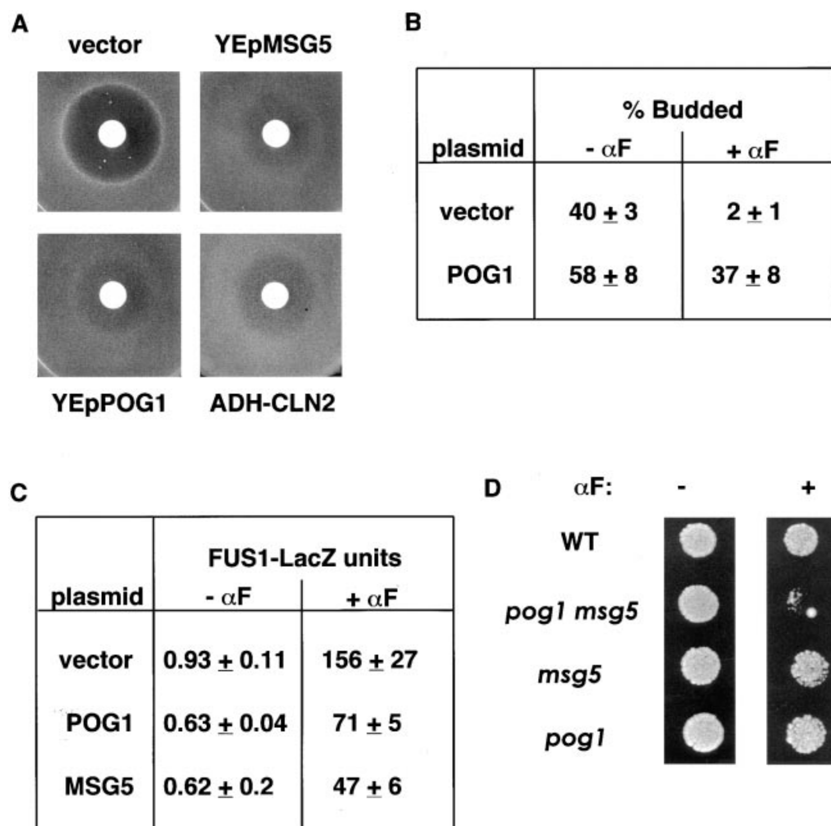
RESULTS

Isolation of cDNAs that block pheromone-induced G1 arrest: To isolate genes that promote recovery, we transformed a *MAT***a** *sst1*Δ strain (EY1118) with a yeast cDNA library under the control of the *GAL1* promoter (Liu *et al.* 1992) and screened for α-factor-resistant colonies on selective medium containing galactose. *sst1*Δ strains are supersensitive to α-factor (Chan and Otte 1982) due to the loss of the Sst1 protease that degrades α-factor (Ciejek and Thorner 1979; Sprague and Herskowitz 1981). We identified seven plasmids that conferred galactose-dependent resistance to α-factor. Partial sequencing of the cDNA inserts revealed that they encoded ∼278 amino acids of the C terminus of *SIR4* and the entire coding sequences of *CLN2*, *GPA1*, and a novel gene we named *POG1* (for *P*romoter *o*f *G*rowth; GenEMBL AC Z46833, ORF YI8277.07, and SwissProt. AC P40473). *CLN2* and *GPA1* encode positive regulators of recovery (Dietzel and Kurjan 1987; Miyajima *et al.* 1987; Oehlen and Cross 1994), confirming the validity of the screen. In contrast, the Sir4 C-terminal fragment confers α-factor resistance by an indirect mechanism that is linked to derepression of silent *MAT*α information at *HML*α (data not shown; Marshall *et al.* 1987). The *POG1* cDNA does not promote α-factor resistance through derepression of *HML*α as it confers α-factor resistance in a strain that is deleted for *HML*α (MLY4; data not shown).

To rule out the possibility that the α-factor resistance of the *POG1* cDNA was the result of cDNA library construction and/or growth of cells on galactose, we isolated the native *POG1* gene from a 2μ-based yeast library (YEpPOG1; see materials and methods). *MAT***a** *sst1*Δ cells overexpressing *POG1* (YEpPOG1) displayed obvious resistance to α-factor compared to cells transformed with a vector control (Figure 1A; compare turbid halo to clear halo). The ability of the YEpPOG1 plasmid to confer α-factor resistance is similar to that of the ADH-CLN2 and YEpMSG5 plasmids (Figure 1A). Thus, in this assay, the *POG1* gene promotes α-factor resistance as well as two other known promoters of recovery.

POG1 encodes a protein of 351 amino acids with a predicted mass of ∼40 kD and no significant homologies to known proteins or protein motifs on the basis of protein-sequence comparison searches (see materials and methods). Pog1 has an acidic N-terminal half and a basic C-terminal half rich in proline residues. Localization studies on hemagglutinin (HA)- and GFP-tagged versions of Pog1 suggest that the protein localizes in the nucleus (data not shown). However, all tagged derivatives of Pog1 examined to date are nonfunctional, so these data must be viewed with caution. On the basis of Northern blot analysis, *POG1* mRNA levels are similar in **a** and α haploid and **a**/α diploid cells and are not affected by pheromone exposure (data not shown), suggesting that *POG1* has a function that is not restricted to regulation of the pheromone response pathway.

***POG1* overexpression blocks G1 arrest and inhibits expression of *FUS1*:** We determined whether pGAL-POG1 promotes α-factor resistance by interfering with G1 arrest and shmoo formation. Cells overexpressing *POG1* were treated with a slightly subsaturating concentration of α-factor (F. Farley and E. A. Elion, unpublished results) and the percentage of budded cells was determined before and after exposure to α-factor. Cells overexpressing *POG1* had a slightly greater percentage of budded cells than the control cells prior to exposure to α-factor, with essentially identical cell morphology (Figure 1B). After a 2-hr exposure to α-factor, the *POG1* overexpressing cells maintained a high percentage of budded cells while nearly all of the control cells underwent G1 arrest. Cells overexpressing *POG1* in the presence of α-factor were also blocked for shmoo formation. Only a small fraction (∼10%) of the unbudded *POG1*-transformed cells displayed the projections typical of cells responding to pheromone (compared to ∼70% of the unbudded control cells). Thus, overexpression



plasmid	% Budded - αF	% Budded + αF
vector	40 ± 3	2 ± 1
POG1	58 ± 8	37 ± 8

plasmid	FUS1-LacZ units - αF	FUS1-LacZ units + αF
vector	0.93 ± 0.11	156 ± 27
POG1	0.63 ± 0.04	71 ± 5
MSG5	0.62 ± 0.2	47 ± 6

Figure 1.—Overexpression of *POG1* inhibits G1 arrest, shmoo formation, and activation of the *FUS1* gene. (A) Halo assays of EY1118 cells transformed with YEpac195 (vector), YEp-POG1-1, YEpMSG5, or ADH-CLN2. Filters contain 420 ng of synthesized α-factor. Plates were incubated at 30° for 2 days. (B) EY1118 cells containing ZM43 (vector), pGALPOG1, or pGALMSG5 were treated with 25-nm of α-factor for 2 hr and the percentage of budded cells were quantitated. Shown is the average of three experiments. (C) EY1118 cells containing the *FUS1-lacZ* reporter plasmid pYBS45 and either ZM43 (vector) or pGALPOG1 were treated with 50 nm of α-factor for 2 hr and β-galactosidase activity quantitated. The β-galactosidase units shown are the average of six independent transformants. (D) *pog1 msg5* double mutant is hypersensitive to α-factor. Spotting assays of cultures of *MAT***a** spore clones from a *pog1* × *msg5* cross. Cultures were spotted onto a YPD plate spread with 17 μg of α-factor (+) or with no α-factor (−). Plates were incubated at 25°.

of *POG1* blocks pheromone-induced G1 arrest and cell morphological changes.

We determined whether the *POG1*-induced block in G1 arrest and projection formation correlated with inhibition of the MAPK cascade, by assaying the effect of *POG1* overexpression on α-factor-induced transcription of the *FUS1* gene. The *FUS1* gene is strongly induced by pheromone and is dependent upon an active MAPK pathway (McCaffrey *et al.* 1987; Trueheart *et al.* 1987; Elion *et al.* 1991). For comparison, we monitored the effect of pGALMSG5, a known inhibitor of Fus3 (Doi *et al.* 1994). *MAT***a** *sst1*Δ cells were cotransformed with vector pGALPOG1 or pGALMSG5, and a second plasmid containing a *FUS1-lacZ* reporter gene (pYBS45). Strains were first grown in medium containing galactose to induce the expression of *POG1* and *MSG5* and then incubated with pheromone and assayed for β-galactosidase levels. Overexpression of *POG1* causes a reproducible twofold decrease in the levels of *FUS1* expression (Figure 1C). This level of inhibition is similar to that caused by overexpression of *MSG5* (Figure 1C; Doi *et al.* 1994).

Deletion of *POG1* increases the α-factor sensitivity of an *msg5* mutant: We next examined the effect of a *POG1* deletion on pheromone response and growth. A *pog1* null strain was created by replacing one chromosomal copy of the *POG1* gene in a wild-type diploid strain (MLY16) with a *pog1::HIS3* allele lacking the *POG1* coding sequence (MLY17; see materials and methods). Upon sporulation and tetrad dissection of the heterozygous diploid, all four spores were equally viable. Compared with isogenic wild-type spore clones, the *pog1::HIS3* spore clones exhibited no obvious growth defects, no heightened α-factor sensitivity in halo assays, and no differences in levels of *FUS1-lacZ* expression in either the absence or presence of α-factor (data not shown).

It was possible that the absence of a phenotype for the *pog1* null was due to the fact that recovery is regulated at multiple levels, any of which might operate in parallel with *POG1.* For example, deletion of the *MSG5* gene causes only a slight increase in α-factor sensitivity (Doi *et al.* 1994), most likely because it is only one of three phosphatases that regulates Fus3 (Zhan *et al.* 1997). We tested whether *POG1* regulates recovery parallel to *MSG5* by constructing a *msg5 pog1* double mutant. An isogenic *msg5::LEU2* disruption strain (MLY81) was mated to a *pog1*Δ mutant (MLY30). *MAT***a** wild-type, single, and double-mutant spore clones were tested for α-factor sensitivity using a spotting assay. As shown in Figure 1D, the *pog1 msg5* double mutant is more sensitive to α-factor than either single mutant, suggesting that *POG1* promotes recovery in parallel with *MSG5.*

***POG1* requires *CLN2* to promote recovery:** We investigated whether *POG1* requires any of the genes known to regulate the recovery response, as further evidence for a physiological role in regulating recovery. The ability of pGALPOG1 to promote α-factor resistance was tested in a variety of strains harboring deletions in regu-

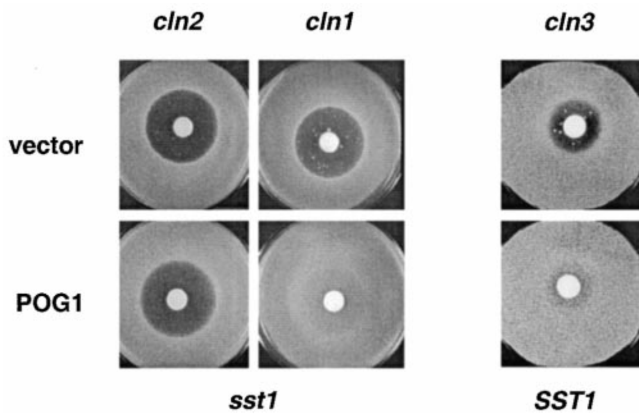


Figure 2.—*POG1* requires *CLN2* to promote recovery. Halo assays of *cln2* (EY1027) and *cln1* (EY1028) cells transformed with ZM43 (vector) or pGALPOG1, and *cln3* (ML201) cells transformed with YEplac181 (vector) or YEpPOG1-2. α-Factor in the amount of 420 ng was used for the *sst1* strains and 17 μg of α-factor was used for the *SST1* strains.

lators of recovery, using halo assays as a monitor. pGAL-POG1 conferred α-factor resistance to both *sst2*Δ and *msg5*Δ deletion strains (data not shown), suggesting that *POG1* functions in parallel to both *SST2* and *MSG5*. By contrast, pGALPOG1 was unable to confer α-factor resistance to a *cln2* deletion mutant (EY1027; Figure 2). The requirement for the *CLN2* G1 cyclin is remarkably specific, as *POG1* overexpression confers significant α-factor resistance to isogenic *cln1* (EY1028) and *cln3* (ML201) mutants. Thus, *POG1* requires *CLN2*, but not *CLN1* or *CLN3*, to promote growth in the presence of pheromone.

POG1* blocks α-factor-induced repression of *CLN1* and *CLN2* mRNAs:** The requirement for *CLN2* for *POG1*-dependent α-factor resistance suggested that *POG1* might upregulate the levels of Cln2 in the presence of α-factor. We first examined Cln2 levels in a *MATa** *sst1*Δ strain containing an integrated copy of a HA-tagged *CLN2* gene (CY326). Normally, the level of Cln2-HA is significantly reduced by α-factor (Figure 3A, vector control) as a result of transcriptional repression of the *CLN2* gene (Valdivieso *et al.* 1993; Cherkasova *et al.* 1999). However, the abundance of Cln2-HA is only slightly reduced when *POG1* is overexpressed. *POG1* overexpression overcomes the α-factor inhibition of Cln2-HA to almost the same degree as *GPA1*^{val50}, a gene known to cause hyperadaptation (Miyajima *et al.* 1989). This loss of negative control is at the level of *CLN2* mRNA. Overexpression of *POG1* and *GPA1*^{val50} prevents α-factor-induced inhibition of both *CLN1* and *CLN2* mRNAs (Figure 3B). *POG1* overexpression has no obvious effect on the level of *CLN3* mRNA, in contrast to *GPA1*^{val50}, which clearly affects *CLN3* mRNA levels.

***POG1* stimulates transcription via SCB/MCB elements:** We determined whether *POG1* was regulating the expression of *CLN1* and *CLN2* through a common promoter element. Cell-cycle-dependent transcription of *CLN1* and *CLN2* is primarily regulated by the Swi4 and Swi6 transcription factors that act as a complex on SCB and MCB elements upstream of the transcription start of both genes (Nasmyth and Dirick 1991; Ogas *et al.* 1991). In the *CLN2* promoter, SCB and MCB elements are present in UAS1, with a second UAS, UAS2, contributing only slightly to cell-cycle transcription (Fig-

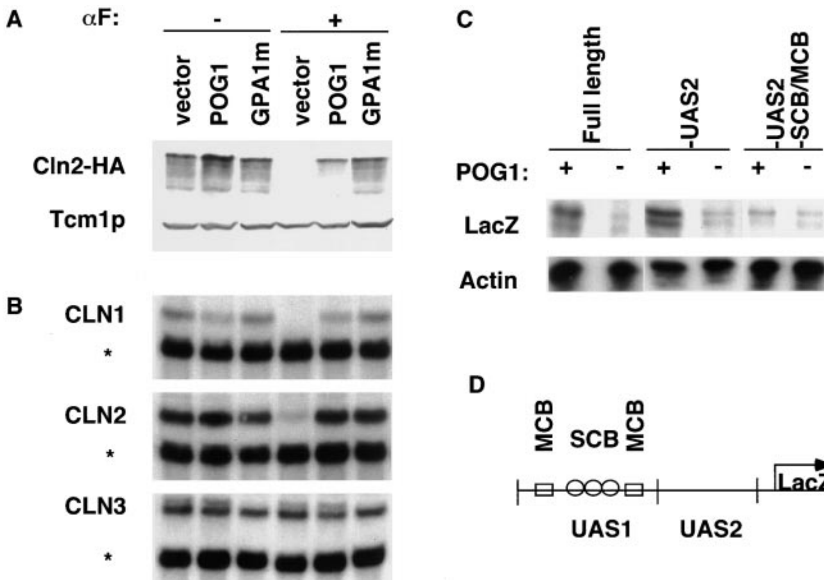


Figure 3.—Effect of *POG1* overexpression on cyclin expression. (A) Western blot of Cln2-HA protein levels in CY326 cells (EY1118 with an integrated copy of CLN2-HA). A ribosomal protein, Tcm1p, was used as a loading control. (B) Northern blots of G1 cyclin mRNA levels in EY1118. An asterisk indicates ACT1 mRNA used as a loading control. For (A) and (B) yeast cells containing ZM43 (vector), pGALPOG1 or pGALGPA1^{m} (GPA1^{val50}) were grown as in materials and methods, with cultures induced with 50 nm α-factor for 2 hr where indicated. (C) Requirement for SCB/MCB elements. Strain EY1118 containing one of three deleted versions of the *CLN2* promoter fused to a *CYC1-lacZ* reporter gene (Stuart and Wittenberg 1994) was transformed with YEplac181 (vector) or YEpPOG1-2. Logarithmically growing cultures were adjusted to a similar A_{600} (0.4–0.6), treated with 50 nm α-factor for 2 hr, harvested, and total RNA isolated. *lacZ* and *ACT1* RNAs were analyzed by quantitative RNase protection. The *CLN2* promoter sequences tested were full length (pΔ5′728), which includes sequences from −728/−256 (sufficient for wild-type levels of CLN2 expression), −UAS2 (pΔ5′728se) containing a deletion of −505/−409 from the −728/−256 promoter, and −UAS2 −SCB/MCB (pΔSCB/MCBse), which is pΔ5′728se with the SCB/MCB elements mutated. (D) Schematic of the *CLN2* promoter showing the relative positions of UAS1 and UAS2 and of the SCB and MCB elements.

ure 3D; Stuart and Wittenberg 1994). We tested whether *POG1* acts through the SCB/MCB elements using a *CLN2-lacZ* promoter fusion gene containing either the full-length *CLN2* promoter (−729 − 256) or two mutant derivatives, one lacking UAS2 and the other lacking both UAS2 and functional SCB/MCB elements within UAS1 (Stuart and Wittenberg 1994). RNase protection analysis showed that *POG1* is able to increase expression of the *lacZ* gene when it is controlled by either the full-length *CLN2* promoter or the derivative lacking UAS2. Mutation of the SCB and MCB elements blocked the effect (Figure 3C), suggesting that *POG1* acts through SCB or MCB elements.

This result was confirmed independently using a chromosomally integrated *lacZ* reporter gene under the control of synthetic SCB elements (CY3557; Table 2). *POG1* overexpression induced the levels of β-galactosidase activity ~2- to 2.5-fold in the presence of α-factor compared to control. A parallel experiment was conducted in an isogenic strain harboring a multicopy plasmid containing the *lacZ* reporter gene under the control of synthetic MCB elements (Di Como *et al.* 1995). Overexpression of *POG1* had no obvious effect on the expression of the *MCB-lacZ* reporter gene (data not shown). However, the absence of an effect could be due to the high dosage of the reporter gene coupled with the stability of β-galactosidase or to the absence of proper control by the MCB elements when they are out of the context of the *CLN2* promoter. From these results we conclude that overexpression of *POG1* either indirectly or directly stimulates SCB-dependent transcription in the presence of α-factor. Further work is needed to demonstrate whether *POG1* acts through MCB elements.

***POG1* and *CLN2* both require *STE20* to promote efficient recovery:** We performed genetic epistasis tests to determine the step(s) at which *POG1* might regulate the mating signal transduction pathway. Overexpression of *POG1* blocked the ability of *pGALSTE4* (Gβ) to induce G1 arrest, as demonstrated by the restoration of growth

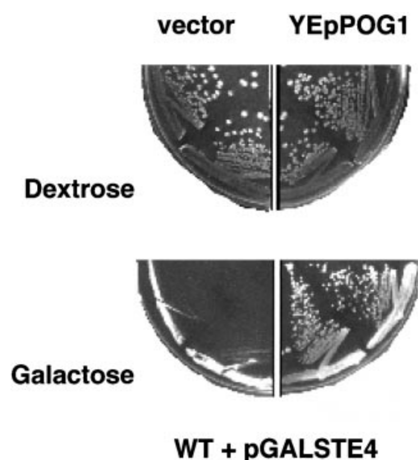


Figure 4.—*POG1* blocks G1 arrest induced by overexpression of *STE4.* Wild-type (EY957) cells containing pGALSTE4 and either YEplac195 (vector) or YEpPOG1-1 were streaked on selective SC solid media containing either 2% dextrose or 2% galactose to induce expression of *STE4.* Plates were incubated for 2–3 days at 30°. Streak outs are from the same plate and were placed side by side for presentation purposes.

on galactose plates for the *pGALSTE4* strain harboring YEpPOG1 (Figure 4). *POG1* also efficiently promotes α-factor resistance in the presence of *STE11-4* (Figure 5A), a gain-of-function allele of *STE11* that causes high constitutive and induced signaling (Stevenson *et al.* 1992). Note that in the *STE11-4* strains shown in Figure 5, A and B, *FAR1* is deleted in order to reduce *STE11-4*-induced growth inhibition in the absence of pheromone. In these strains, α-factor mediates G1 arrest through enhanced transcriptional repression of *CLN1* and *CLN2* (Cherkasova *et al.* 1999). Thus, *POG1* acts a step(s) below Gβ in the pathway that may be distinct from Ste11.

Two experiments suggest that *POG1* may act at the *STE20* step of the pathway. First, overexpression of *STE20* in a *MAT***a** *sst1*Δ strain greatly reduces the ability

TABLE 2

Effect of *POG1* on expression of a *SCB-lacZ* reporter gene

	β-Galactosidase units			
	Experiment 1		Experiment 2	
Strain	− α F	+ α F	− α F	+ α F
CY3557 *SCB-lacZ::HIS3*				
+ vector	13 ± 0.5	7	33	16
+ POG1	19 ± 2	14 ± 0.5	60	41

Strain CY3557 harboring an integrated *SCB-lacZ* reporter gene was transformed with vector (YEplac181) or YEpPOG1-2. In experiment 1, two independant transformants were grown and induced with 5-μM α-factor for 2 hr and, in experiment 2, a pool of four transformants was assayed. The standard error is shown for experiment 1. β-Galactosidase activity was assayed as described (Elion *et al.* 1995) and is presented as Miller units. The relative values within each experiment are very similar; differences in absolute β-galactosidase values between experiment 1 and experiment 2 may be due to the use of different α-factor stock solutions.

of *POG1* to promote α-factor resistance, although *MSG5* is still able to efficiently promote recovery (Figure 5D). Second, *POG1* loses most of its ability to promote α-factor resistance in the absence of *STE20*, as assessed in a pheromone responsive *STE11-4 ste20*Δ strain (compare Figure 5, A and B). *STE11-4* partially bypasses the requirement for *STE20* for α-factor-dependent activation of the MAPK cascade and G1 arrest, providing a means to assess α-factor sensitivity in the absence of *STE20* (Lyons *et al.* 1996; Feng *et al.* 1998). These results suggest that *POG1* requires *STE20* to promote cell division in the presence of α-factor.

We tested the possibility that *POG1* requires *STE20* to upregulate *CLN2* transcription in the presence of α-factor, by determining the ability of *POG1* to block repression of *CLN2* transcription in the *STE11-4 ste20*Δ strain. Northern blots show that *STE20* is not required for the enhanced levels of *CLN2* mRNA produced by *POG1* (Figure 5C). Thus, the inability of *POG1* to induce recovery is not due to a loss in its ability to induce *CLN2* expression, raising the possibility that *CLN2* requires *STE20* to promote recovery. Consistent with this, *ADH-CLN2* also has a reduced ability to confer α-factor resistance in a *STE11-4 ste20*Δ (EY2022) strain compared with a *STE11-4* strain (EY1298; data not shown). Furthermore, we find that in the presence of excess *STE20*, *CLN2*-overexpressing cells display a reduced ability to recover (Figure 5D). However, *CLN2* has a greater ability than *POG1* to promote recovery in the presence of excess *STE20*, as shown by the slightly greater turbidity of the pGALCLN2 halo compared with the pGALPOG1 halo. This difference could be due to quantitative differences in the levels of *CLN2* mRNA produced in the two strains. For example, the level of *CLN2* mRNA produced from the *ADH-CLN2* gene is approximately fivefold greater than the level of *CLN2* mRNA generated by YEpPOG1 in the presence of α-factor (data not shown). Taken together, the results suggest that *STE20* is an important target of control for recovery events that are mediated by *CLN2.*

POG1* has a vegetative function that is redundant with *CLN3*:** Deletion of *POG1* does not affect the rate of appearance or levels of *CLN1* and *CLN2* mRNAs during recovery (data not shown), raising the possibility that *POG1* regulates G1 cyclin transcription through a redundant mechanism. *CLN3* is a positive regulator of *CLN1* and *CLN2* transcription (Tyers and Futcher 1993; Nasmyth 1996) and functions in parallel with other genes to control expression of the G1 cyclins (Di Como *et al.* 1995). We therefore compared the ability of *MATa** wild-type, single, and double-mutant spore clones from a *pog1*Δ (MLY30) × *cln3*Δ (ML201) cross to undergo α-factor-induced G1 arrest and regulate *CLN1* and *CLN2* transcription. *pog1 cln3* double mutants were more sensitive than the wild-type strain, but as sensitive to α-factor as the *cln3* single mutants. Northern blot analysis of *cln3* and *pog1 cln3* strains did not reveal an obvious difference in the rate of appearance or absolute levels of *CLN1* and *CLN2* mRNAs during recovery (data not shown).

In contrast, *pog1 cln3* double mutants were found to exhibit two vegetative growth defects. First, a *pog1 cln3* double mutant has a more pronounced temperature-sensitive growth defect than a *cln3* single mutant, as shown by spotting equal numbers of cells on YPD plates and incubating them at 37° (Figure 6A). The *pog1* single mutant has no obvious temperature sensitivity. The temperature sensitivity of the *pog1 cln3* double mutant is remedied by the inclusion of either 12 mm Mg^{2+}, 1 m sorbitol (Figure 6A), or 25 mm Ca^{2+} (data not shown) in the medium. Sorbitol functions as an osmotic stabilizer and remedies cell lysis (Cid *et al.* 1995). Ca^{2+} and Mg^{2+} may also stabilize the cell membrane and enhance cell wall biosynthesis (Lin and Macey 1978; Levin *et al.* 1990; Cyert *et al.* 1991; Marini *et al.* 1996). Microscopic examination of cells grown on solid medium at 37° overnight and then resuspended in 1 m sorbitol for osmotic support show that the *pog1 cln3* cells are, on

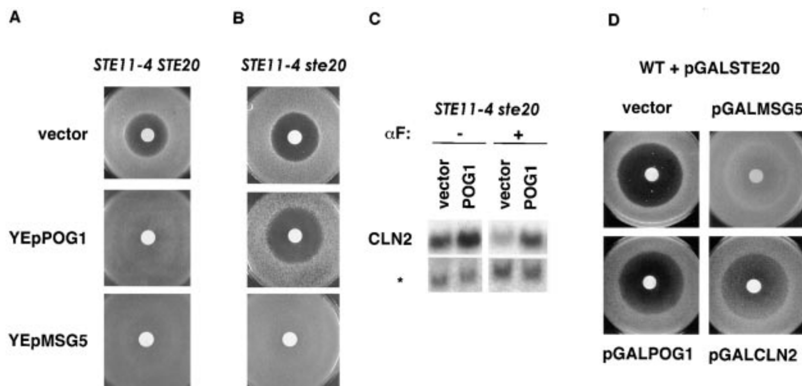


Figure 5.—*POG1* requires *STE20* to promote recovery. (A and B) *STE20* is required for *POG1* to promote recovery in a *STE11-4* strain. Halo assays of *STE11-4 far1* (EY1298) and *STE11-4 ste20*Δ *far1*Δ (EY2022) cells containing YEplac195 (vector), YEpPOG1-1, ADH-CLN2, or YEpMSG5. α-Factor in the amount of 1.3 μg was used in A and 2.5 μg of α-factor was used in B. EY1298 and EY2022 are more resistant to α-factor than an isogenic *sst1* strain, necessitating a greater amount of α-factor in order to form an easily discernible halo. At least two transformants were tested for each strain. (C) Northern blot of *CLN2* mRNA levels in the *STE11-4 far1*Δ *ste20*Δ strain (EY2022) containing YEplac195 (vector) or YEpPOG1-1. Cells were induced with 100 nm a-factor. The filter was probed sequentially using *ACT1* as the last probe. An asterisk indicates *ACT1* mRNA used as a loading control. (D) Overexpression of *STE20* inhibits the ability of *POG1* and *CLN2* to promote recovery. Halo assays of EY1118 cells containing GALSTE20 and either vector (ZM43), pGAL-POG1, pGALCLN2, or pGALMSG5. α-Factor in the amount of 420 ng was applied to the filters. Media contains 2% galactose to induce expression of the exogenous genes.

average, larger sized than the single mutants or wild type (Figure 6C).

Second, a *pog1 cln3* double mutant has greatly reduced viability when it is starved for a carbon source (Figure 6B). The effect of starvation for a carbon source was examined by patching wild-type, single-, and double-mutant strains onto solid SC medium lacking dextrose. After several days at 25°, the patches were transferred to fresh YPD plates for further incubation. A loss of viability after carbon source starvation suggests that the *pog1 cln3* double mutant is unable to enter G_o (Werner-Washburne *et al.* 1993). Collectively, these results suggest that, parallel to *CLN3*, *POG1* promotes vegetative growth.

The *pog1 cln3* mutant shares some characteristics of Pkc1 pathway mutants: the temperature sensitivity of the double mutant is rescued by sorbitol, Mg^{2+}, and Ca^{2+}, and the morphological defects are accentuated in solid medium (Lee and Levin 1992; Paravicini *et al.* 1992; Marini *et al.* 1996). In contrast to Pkc1 pathway mutants (Costigan *et al.* 1992), the *pog1 cln3* double mutant is not sensitive to nitrogen starvation (Figure 5B) or caffeine and can grow in the presence of nonfermentable carbon sources such as ethanol and glycerol (data not shown). In addition, overexpression of *POG1* does not rescue growth defects of Pkc1 pathway mutants (*e.g.*, *bck1*Δ and *mpk1*Δ, data not shown). For these reasons, *POG1* is unlikely to be directly involved in the Pkc1 pathway.

***POG1* requires *BCK1* to increase *CLN2* expression in the presence of α-factor:** The partial overlap of phenotypes between the *pog1 cln3* double mutant and Pkc1 pathway mutants suggested that *POG1* may regulate a subset of the same genes that are also regulated by Pkc1. The Pkc1 pathway plays a major role in cell-wall biosynthesis and contributes to the activation of G1 cyclin expression via the Swi4 and Swi6 transcription factors (Madden *et al.* 1997) and G1 cyclins are thought to be activators of the Pkc1 pathway (Zarzov *et al.* 1996; Gray *et al.* 1997). We determined whether *POG1* requires the Pkc1 pathway to regulate the expression of *CLN2* in the presence of α-factor using a *bck1*Δ deletion strain. *BCK1* encodes a MAPK kinase kinase that functions downstream of Pkc1 (Costigan *et al.* 1992) and upstream of the MAPK Mpk1 thought to regulate Swi4 (Madden *et al.* 1997). Northern blots showed that *CLN2* mRNA levels are not elevated to an obvious degree in the *bck1* cells overexpressing *POG1* compared to the wild-type strain (Figure 7A). In halo assays, the *POG1*-

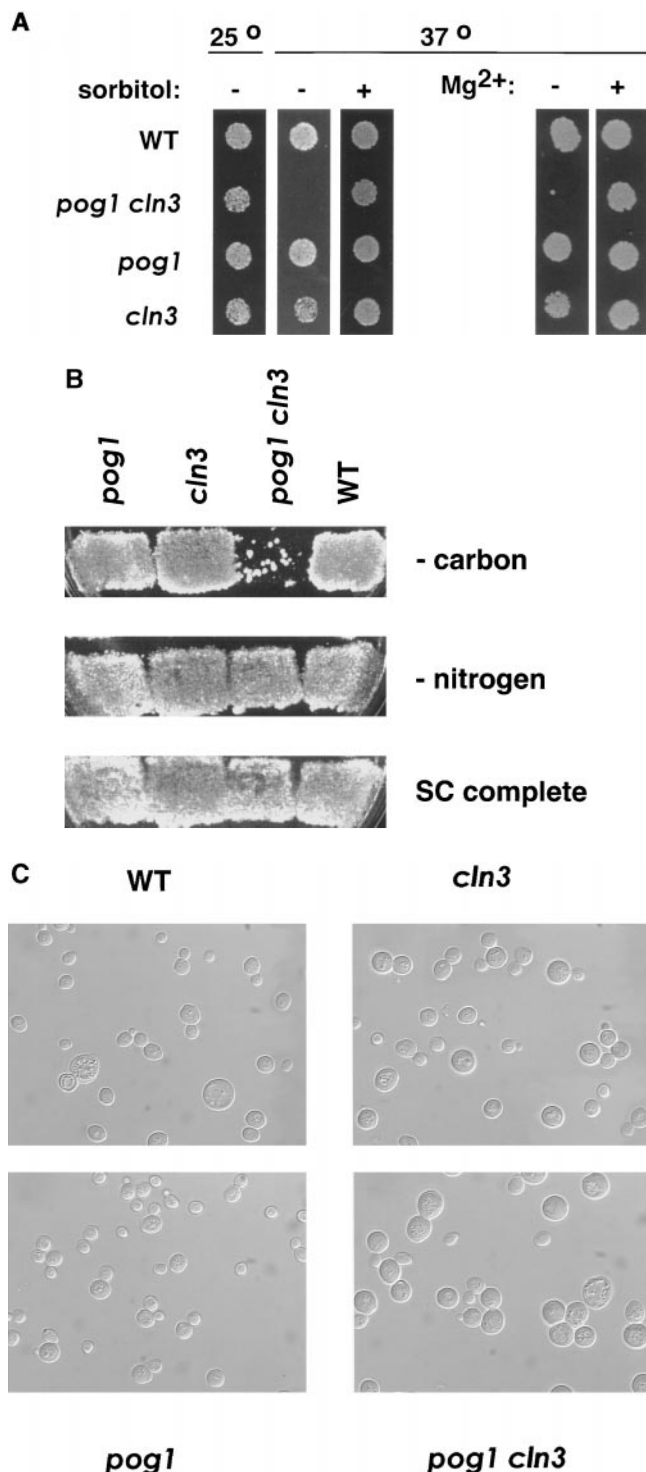


Figure 6.—Phenotypes of a *pog1 cln3* double mutant. (A) Spotting assay of cultures of a *pog1* × *cln3* tetrad. *pog1 cln3* mutant has a temperature-sensitive defect that is remedied by osmotic stabilizers. Cultures were spotted on YPD, YPD + sorbitol, or YPD with 12 mm $Mg2^{+}$. Plates were incubated at the temperatures indicated. At least three tetrads were tested. (B) *pog1 cln3* double mutant is sensitive to carbon source starvation. A tetrad from the *pog1* × *cln3* cross was patched unto SC complete or SC lacking either a carbon or a nitrogen source, incubated at 25° for 7 days, and then replica plated to fresh YPD plates followed by further incubation to determine cell viability. Three tetrads were tested. (C) Morphology of *pog1 cln3* mutant. Cells from the spore clones of a *cln3* × *pog1* tetrad were thinly spread on YPD and incubated at 37° overnight. Cells were then scraped off the dish, resuspended in 1 m sorbitol, and visualized by light microscopy.

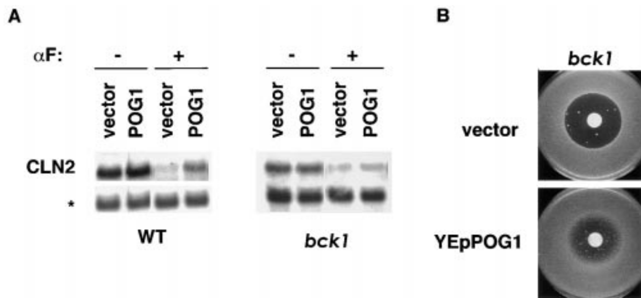


Figure 7.—A *bck1* mutation blocks the ability of *POG1* to stimulate expression of the *CLN2* gene in the presence of pheromone. (A) Northern blots of *CLN2* mRNA levels in *BCK1* (EY1118) and *bck1* (C699-59) cells containing either YEplac195 (vector) or YEpPOG1-1 before and after treatment with 50 nm α-factor for 2 hr. An asterisk indicates *ACT1* as a loading control. (B) Halo assays of *bck1* cells were done as in Figure 1A. Strains were grown at 25°.

overexpressing *bck1* cells exhibited partially filled-in halos indicating that *POG1* can stimulate recovery in the abence of *BCK1* (Figure 7B), although not nearly as well as in its presence (Figure 1A). These results have two implications. First, *POG1* may require *BCK1* to regulate the expression of the *CLN2* gene. Second, *POG1* may promote recovery through additional routes besides upregulation of *CLN2.*

DISCUSSION

***POG1* is a novel regulator of recovery that operates through *CLN2*:** We have isolated and characterized a novel gene, *POG1*, that blocks α-factor-induced G1 arrest when overexpressed. Several lines of evidence argue that *POG1* positively regulates recovery in the presence of α-factor. First, as a multicopy suppressor, *POG1* provides a rate-limiting function that promotes cell division and, in the presence of α-factor, interferes with the ability of cells to arrest in G1 phase and form shmoos (Figure 1). Second, a *pog1* null mutation enhances the α-factor sensitivity of an *msg5* mutant, suggesting that Pog1 promotes recovery in parallel with Msg5, a known regulator of recovery (Figure 1). Third, *POG1* specifically requires the *CLN2* G1 cyclin to promote cell division (Figure 2), and *CLN2* is implicated in having a role in recovery (Oehlen and Cross 1994; Wassmann and Ammerer 1997). Fourth, *POG1* elevates *CLN2* and *CLN1* mRNA levels in the presence of α-factor (Figure 3), providing a molecular explanation for the requirement for *CLN2.* Taken together, these data provide strong support for a model in which *POG1* promotes recovery by overcoming transcriptional repression of *CLN2* and *CLN1.* The resulting increased levels of Cln2 and Cln1 protein may be sufficient to promote recovery. However, the strict requirement for the *CLN2* gene suggests that the Cln2 protein performs essential functions for recovery that are not shared by Cln1, in accordance with previous observations (Oehlen and Cross 1994).

***POG1* may regulate transcription of SCB/MCB elements:** *POG1* is likely to operate at the level of transcription of the *CLN1* and *CLN2* genes. Excess *POG1* stimulates the expression of β-galactosidase promoter fusions containing either the *CLN2* SCB/MCB elements or synthetic SCB elements (Figure 3), ruling out a post-transcriptional effect. Two observations suggest that Pog1 may play a direct role in regulating *CLN1* and *CLN2* transcription. First, HA- and GFP-tagged versions of Pog1 localize in the nucleus (data not shown). While these proteins are nonfunctional, their nuclear localization may accurately reflect a nuclear function for Pog1. Second, the predicted Pog1 protein contains two hallmarks of transcription factors, an acidic domain in the N-terminal half and a proline-rich domain in the C-terminal half.

Two interpretations are possible to explain the mechanism by which Pog1 increases the expression of *CLN1* and *CLN2* in the presence of pheromone. Pog1 may be a transcriptional activator that activates by binding directly to the SCB/MCB elements or it may positively regulate the activity of the Swi4/Swi6 complex. Alternatively, Pog1 could be an inhibitor of α-factor-induced repression of the *CLN1* and *CLN2* promoters. Previous work has shown that Fus3 and Kss1 repress transcription of the *CLN1* and *CLN2* genes to promote G1 arrest (Cherkasova *et al.* 1999). Pog1 could interfere with the ability of Fus3 and Kss1 to repress transcription by blocking an α-factor-induced repressor that abrogates transcription through the SCB/MCB elements, or by affecting directly the repressive function of either Fus3 or Kss1.

It seems likely that the ability of Pog1 to upregulate the *CLN1* and *CLN2* genes is dependent upon α-factor, because neither overexpression nor deletion of *POG1* has an obvious effect on *CLN1 CLN2* mRNA levels during vegetative growth, even in the absence of the *CLN3* gene. Potential control by pheromone is not at the level of *POG1* gene expression; the *POG1* gene is not pheromone inducible and it is expressed in diploid cells as well as haploid cells. Perhaps the Pog1 protein or its target(s) are modified in response to α-factor. In this regard, two-hybrid analysis suggests that Pog1 interacts with Kss1 but not Fus3, and Pog1 confers significantly less α-factor resistance when the *KSS1* gene is deleted (data not shown). These observations raise the possibility that Kss1 regulates Pog1 to promote recovery or vice versa. Pog1 also requires Bck1 to stimulate expression of the *CLN2* gene (Figure 7), suggesting that Pog1 or another protein must first be modified (directly or indirectly) by Bck1 in order for Pog1 to function. Swi4 is a possible candidate as it has been implicated as a downstream target of Mpk1 (Madden *et al.* 1997).

***POG1* may regulate additional genes during vegetative growth and recovery:** Our analysis suggests that Pog1 has

additional functions for vegetative growth and recovery that are distinct from transcriptional control of *CLN1* and *CLN2*. *POG1* promotes vegetative growth in parallel with *CLN3*, as shown by the enlarged size, temperature-sensitive, and cellular lysis phenotypes of a *pog1 cln3* double mutant (Figure 6 and data not shown). However, this growth defect does not correlate with reduced rates of appearance or levels of *CLN1* or *CLN2* mRNAs (data not shown). The phenotypes of the *pog1 cln3* double mutant are reminiscent of mutants defective in cell-wall integrity or the actin cytoskeleton (Cid *et al.* 1995; Roemer *et al.* 1998). The Pkc1 pathway regulates cell-wall integrity and nutrient response (Levin and Errede 1995). Mutants with defects in the protein kinases under the control of Pkc1 undergo temperature-sensitive lysis that is remedied by osmotic support, similar to a *pog1 cln3* double mutant (Levin and Errede 1995). Likewise, the actin cytoskeleton mutant *rvs167* is also sensitive to osmotic stress and to starvation for carbon, nitrogen, or sulfur sources (Bauer *et al.* 1993). Thus, *POG1* may regulate genes that control cell-wall integrity or the actin cytoskeleton. Such genes may be controlled by SCB or SCB-like elements.

POG1 may also positively regulate recovery through functions distinct from transcriptional control of the *CLN2* gene. First, although a *bck1* mutation nearly completely blocks the ability of overexpressed *POG1* to upregulate the *CLN2* gene, *POG1* still confers partial α-factor resistance (Figure 7). Second, in the presence of the *STE11-4* mutation, a *POG1* multicopy plasmid confers significantly more α-factor resistance than does an *ADH-CLN2* plasmid, even though the *POG1* plasmid induces less *CLN2* mRNA (data not shown). The *STE11-4* mutation is thought to bypass an inhibitory effect of Cln2/Cdc28 kinase at the Ste11 step of the pathway (Wassmann and Ammerer 1997). Thus, *POG1* overexpression can still promote recovery under conditions that block either the expression or function of the *CLN2* gene, arguing that *POG1* regulates additional genes to promote recovery. Candidate genes include the *PCL2* gene that is regulated by Swi4 and whose expression is highly induced by α-factor (Measday *et al.* 1994, 1997) and the *PCL1,10* genes that are repressed by α-factor (Measday *et al.* 1997). *PCL1,2,10* genes encode 3 of 10 cyclin partners for the Pho85 kinase (Measday *et al.* 1997), which promotes budding in parallel with Cln1/Cdc28 and Cln2/Cdc28 (Measday *et al.* 1994) and regulates cell morphology (Measday *et al.* 1997). *pho85* mutants are more sensitive to α-factor than wild-type cells (Measday *et al.* 1997) and delay recovery from G1 arrest (Cherkasova *et al.* 1999), consistent with the possibility that transcriptional control of Pho85 cyclin partners may play a role in recovery.

Ste20 may be a critical target of control for recovery from G1 arrest: It is noteworthy that disruption or overexpression of *STE20* greatly decreases the ability of both *POG1* and *CLN2* to confer α-factor resistance (Figure 5). Thus, *POG1* and *CLN2* may promote recovery by inhibiting the ability of Ste20 to activate the pheromone response pathway. Consistent with this, overexpression of *POG1* and *CLN2* modestly inhibits the expression of the *FUS1* gene (Figure 1; Oehlen and Cross 1994). Because *POG1* is able to induce the expression of *CLN2* in the absence of a functional *STE20* gene (Figure 5), it seems likely that Pog1 activates the expression of the *CLN2* gene, which in turn promotes recovery through an effect on Ste20. Previous work suggests that Cln2/Cdc28 inhibits the mating signal transduction pathway at or near the Ste11 step (Wassman and Ammerer 1997). Ste20 is essential for the activation of Ste11 (Leberer *et al.* 1992; Feng *et al.* 1998). Furthermore, a *STE11-4* mutation partially bypasses the requirement for Ste20 for signal transduction (Lyons *et al.* 1996), explaining why *STE11-4* can bypass the inhibitory effects of excess *CLN2* (Wassman and Ammerer 1997).

How might Cln2/Cdc28 regulate Ste20 to promote recovery? Ste20 can either promote G1 arrest and shmoo formation by interacting with Gβ (Leeuw *et al.* 1998) or budding and pseudohyphal growth by interacting with Cdc42 (Peter *et al.* 1996; Leberer *et al.* 1997). Cln2/Cdc28 is thought to act upstream of Ste20 and Cla4 to promote budding (Cvrcková *et al.* 1995), suggesting that Cln2/Cdc28 may directly or indirectly alter the specificity of Ste20 from mating to budding. We find that the Cdc42 binding site of Ste20 is not required for *POG1* or *CLN2* overexpression to promote recovery (data not shown), suggesting that the control is not at the level of association with Cdc42. Further studies are required to clarify how Ste20 is regulated to promote recovery.

We thank H. Liu, Z. Moqtaderi, D. Stuart, M. Whiteway, C. Wittenberg and B. Errede for their generous gift of plasmids and strains and Andrew Neuwald for kindly analyzing the Pog1 protein sequence with the Probe program. We thank S. Buratowski, H. Saito, K. Struhl, D. Takemoto, F. Winston, and members of the Elion laboratory for insightful comments on the manuscript. This research was supported by grants to E.A.E. from the March of Dimes (#1-FY96-0925), Council for Tobacco Research, and National Institutes of Health (GM 46962).

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Communicating editor: M. Carlson