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Xbp1-Mediated Repression of *CLB* Gene Expression Contributes to the Modifications of Yeast Cell Morphology and Cell Cycle Seen during Nitrogen-Limited Growth

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Yeast cells undergo morphological transformations in response to diverse environmental signals. One such event, called pseudohyphal differentiation, occurs when diploid yeast cells are partially starved for nitrogen on a solid agar medium. The nitrogen-starved cells elongate, and a small fraction form filaments that penetrate the agar surface. The molecular basis for the changes in cell morphology and cell cycle in response to nitrogen limitation are poorly defined, in part because the heterogeneous growth states of partially starved cells on agar media are not amenable to biochemical analysis. In this work, we used chemostat cultures to study the role of cell cycle regulators with respect to yeast differentiation in response to nitrogen limitation under controlled, homogeneous culture conditions. We found that Clb1, Clb2, and Clb5 cyclin levels are reduced in nitrogen-limited chemostat cultures compared to levels in rich-medium cultures, whereas the Xbp1 transcriptional repressor is highly induced under these conditions. Furthermore, the deletion of *XBP1* prevents the drop in Clb2 levels and inhibits cellular elongation in nitrogen-limited chemostat cultures as well as inhibiting pseudohyphal growth on nitrogen-limited agar media. Deletion of the *CLB2* gene restores an elongated morphology and filamentation to the *xbp1*Δ mutant in response to nitrogen limitation. Transcriptional activation of the *XBP1* gene and the subsequent repression of *CLB* gene expression are thus key responses of yeast cells to nitrogen limitation.

Many yeast cells in the wild undergo morphological transitions in response to diverse environmental signals. Transitions between yeast and filamentous forms have been implicated in foraging for nutrients, in the avoidance of toxins, and in the infection of plants and animals by fungal pathogens (33, 38). Most laboratory strains of *Saccharomyces cerevisiae* respond poorly to such environmental stimuli, apparently because early yeast geneticists selected mutants that maintained stable yeast-form growth that were easier to cultivate in the laboratory (26). Recent work suggests that more feral yeast strains show morphological differentiation in response to a rich variety of signals. Diploid yeast cells undergo pseudohyphal differentiation in response to limited nitrogen starvation (16), in the presence of alcohols (9, 31), or in the presence of some types of sugars (14, 23, 46). Haploid yeast cells can show invasive growth on a nitrogen-rich agar medium in response to a depletion of fermentable carbon sources (8, 41). The best studied of these morphological transitions is that of diploid yeast cells subjected to a partial nitrogen starvation, in which case the starved cells elongate and are inhibited for entry into anaphase in mitosis (4, 22). On agar media containing limiting nitrogen, a fraction of the cells form pseudohyphal filaments that penetrate the agar surface.

Two major signal transduction pathways involving a mitogen-activated protein (MAP) kinase cascade and the cyclic AMP (cAMP)-dependent protein kinase pathway have been implicated in pseudohyphal differentiation (33, 37, 39). These pathways are thought to activate key transcription factors, Ste12-Tec1 (32) and Flo8 (26, 39, 42, 43), that control the expression of genes required for pseudohyphal differentiation. Both transcription factors contribute to the expression of *FLO11*, a gene encoding a cell surface protein implicated in the adhesion of cells that form pseudohyphal filaments (18, 23, 27, 28). Several other transcription factors, including Phd1 (15), Ash1 (5), and Sok2 (47), also regulate pseudohyphal growth and contribute to the expression of *FLO11* (38). In addition, the related transcription factors Fkh1 and Fkh2 may repress some aspects of pseudohyphal growth by promoting the expression of a set of genes in S phase (the *CLB2* cluster) that includes the mitotic cyclin gene *CLB2* (19, 40, 48).

Cellular elongation is one of the most evident aspects of nitrogen-limited growth of yeast cells. This elongation is due to a prolonged period of polarized growth to the bud apex (24). Polarized bud growth is initiated at the Start of the cell cycle, when Cdc28 is activated by the G_1 cyclins Cln1 and Cln2 (25), and inactivation of Cln1 and Cln2 inhibits pseudohyphal growth (29). Apical growth in yeast is blocked by the appearance of the Cdc28-Clb1,2 mitotic kinases (25), and it was suggested that an inhibition of this kinase activity explains the hyperpolarized growth and a delay at the metaphase-to-anaphase transition, seen in wild-type cells overexpressing the *PHD1* gene when they were spread on the surface of synthetic low-ammonium dextrose (SLAD) agar plates (22). The mechanism of this inhibition has not yet been elucidated. In this

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TABLE 1. Yeast strains[a]

Strain	Genotype	Source
CSY1000	*MAT***a** *leu2*::*hisG ura3-52*	G. Fink
CSY1004	*MAT***a** *leu2*::*hisG ura3-52 ras2*::*KANMX4*	This study
CSY2003	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 civ1-4*/*civ1-4*	This study
CSY2027	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 gpa2*::*KANMX4*/*gpa2*::*KANMX4*	This study
CSY2029	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 ash1*::*KANMX4*/*ash1*::*KANMX4*	This study
CSY2032	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 ash1*::*KANMX4*/*ash1*::*KANMX4 civ1-4*/*civ1-4*	This study
CSY2034	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 gpa2*::*KANMX4*/*gpa2*::*KANMX4 civ1-4*/*civ1-4*	This study
CSY2068	*MAT***a**/α *ste20*Δ/*ste20*Δ *ura3-52*/*ura3-52 civ1-4*/*civ1-4*	This study
CSY2069	*MAT***a**/α *ste12*Δ/*ste12*Δ *ura3-52*/*ura3-52 civ1-4*/*civ1-4*	This study
CSY2124	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 xbp1*::*KANMX4*/*xbp1*::*KANMX4*	This study
CSY2030	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 phd1*::*KANMX4*/*phd1*::*KANMX4*	This study
CSY2033	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 phd1*::*KANMX4*/*phd1*::*KANMX4 civ1-4*/*civ1-4*	This study
CSY2125	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 tec1*::*KANMX4*/*tec1*::*KANMX4*	This study
CSY2028	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 ras2*::*KANMX4*/*ras2*::*KANMX4*	This study
CSY2031	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 ras2*::*KANMX4*/*ras2*::*KANMX4 civ1-4civ1-4*	This study
CSY2123	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52*	This study
CSY2126	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 cdc28-6*/*cdc28-6*	This study
CSY2127	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 clb2*::*KANMX4*/*clb2*::*KANMX4*	This study
CSY2128	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 STE11-4-(URA3)*/*STE11-4-(URA3)*	This study
CSY2222	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 xbp1*::*KANMX4*/*xbp1*::*KANMX4 clb2*::*KANMX4*/*clb2*::*KANMX4*	This study
CSY2223	*MAT***a**/α *leu2*::*hisG*/*LEU2 trp1*::*hisG*/*TRP1 ura3-52*/*ura3-52 xbp1*::*KANMX4*/*xbp1*::*KANMX4 STE11-4-(URA3)*/*STE11-4-(URA3)*	This study
HLY952	*MAT***a**/α *ste12*Δ/*ste12*Δ *ura3-52*/*ura3-52*	G. Fink
HLY492	*MAT***a**/α *ste20*Δ/*ste20*Δ *ura3-52*/*ura3-52*	G. Fink

[a] All strains are congenic with Σ1278b.

work, we used chemostats to study the regulation of Cdc28-Clb kinases in wild-type cells during nitrogen-limited growth.

MATERIALS AND METHODS

Yeast strains and low-ammonium agar media. The yeast strains used in this work are listed in Table 1. PCR-based deletion of coding sequences with the *KANMX4* cassette was performed as previously described (30) for *MAT*a and *MAT*α haploid strains of the Σ1278b background. Homozygous diploid strains were then produced by mating. The *civ1-4* (45) and *cdc28-6* mutant genes were introduced into the Σ1278b background by cloning the mutant genes into the pRS306 (*URA3*) integrative vector and targeting the mutant genes to their corresponding chromosomal loci by digesting with a restriction enzyme that cuts once within the promoter region of the gene and transforming *MAT*a *ura3* and *MAT*α *ura3* haploid strains of the Σ1278b background. Ura^+ transformants were then streaked on 5-fluoro-orotic acid (5-FOA) plates at 24°C in order to select for excision of the integrated plasmid (2). Ura^- colonies growing on the 5-FOA plates were then replica plated on yeast extract-peptone-dextrose (YPD) plates at 37°C to screen for those excision events that retained the *civ1-4* and *cdc28-6* thermosensitive mutations. The resulting haploid strains were then mated to generate homozygous diploid strains. Homozygous diploid *civ1-4 ste20*Δ and *civ1-4 ste12*Δ strains were constructed by integrating one copy of pRS306-*civ1-4* into CSY2067 and CSY2066, followed by selection for plasmid excision on 5-FOA plates and, finally, retransformation with pRS306-*civ1-4* and a second round of plasmid excision at 24°C on 5-FOA plates. The doubly transformed strains were then replica plated at 37°C to test for the replacement of both copies of the wild-type gene by the *civ1-4* mutation.

SLAD agar medium was prepared as previously described (16). A low-ammonium glycerol medium (SLAYP) supported pseudohyphal growth of wild-type cells but not *xbp1* mutants (see Fig. 6). SLAYP was composed of 1.7 g of yeast nitrogen base (YNB) without amino acids and without ammonium sulfate (Difco) per liter, 50 mM sodium phthalate (pH 5), 25 mg of ammonium sulfate per liter, 3% glycerol, and 2% agar.

Plasmids. A *Sac*I-*Hin*dIII *XBP1* fragment (−1995 bp 5′ of the ATG start codon and 187 bp downstream from the TAA stop codon) was prepared by PCR and inserted into the corresponding sites of the pRS416 (*CEN-URA3*) vector. A *Sac*I-*Hin*dIII fragment beginning with the ATG start codon of *XBP1* and ending 187 bp downstream of the TAA stop codon was prepared by PCR and cloned into the corresponding sites of the pYES2 (2μm *URA3-pGAL*) vector in order to place *XBP1* under the control of the *GAL* promoter in a multicopy vector. The *CDC28-43244* gene was isolated from pSF19-*CDC28-43244* (7) by partial digestion with *Xho*I and *Xba*I and inserted into the YEplac195 (2μm *URA3*) vector. The pFG(TyA)::*lacZ-LEU2* reporter construct was a gift from Gerry Fink, the *STE11-4* gene was a gift from George Sprague, and pGR103 (2μm *URA3-PDE2*) was a gift from Georges Renault and Michel Jacquet.

Chemostat cultures. Chemostat cultures were performed at 25°C in a simple, custom-made 1-liter glass vessel (see Fig. 3B) and using general conditions that were outlined previously (4). Fresh medium was delivered from the reservoir to the culture vessel with a peristaltic pump at a flow rate of 100 ml/h. For nitrogen limitation studies, cells were cultured in a filter-sterilized, synthetic, low-ammonium phthalate medium (SLAP) composed of 1.7 g of YNB per liter without ammonium sulfate and without amino acids (Difco), 50 mM sodium phthalate (pH 5.0), 100 mg of ammonium sulfate per liter, and 30 g of dextrose per liter. Sodium phthalate is a nonmetabolizable pH buffer. Cells grown in rich-medium chemostats were cultivated in SLAP containing 5 g of ammonium sulfate per liter. Cells grown in glucose-limited chemostats were cultivated in medium containing 6.7 g of YNB without amino acids (Difco) per liter, 50 mM sodium phthalate (pH 5), and 0.5% glucose. In order to ensure equilibrium conditions in the chemostat, cells were cultivated for 40 to 45 h before harvesting for biochemical analyses, although similar results were obtained when cells were cultivated for as little as 20 h.

Electrophoretic separation of nonphosphorylated Cdc28 from phospo-Thr-169 Cdc28. Conditions allowing the electrophoretic separation of Cdc28 phosphorylated on Thr-169 from unphosphorylated Cdc28 were adopted from those of Espinoza et al. (13). Cell extract (30 μg) was electrophoresed in thin (0.75-mm) 24-cm-long Laemmli 12.5% polyacrylamide gels (acryl-bis, 30:0.8 or 29:1) for at least 15 h at 15 mA and 200 V with constant amperage. Acrylamide and bis-acrylamide were from Sigma, and ammonium persulfate and TEMED (N,N,N′,N′-tetramethylethylenediamine) were from Bio-Rad. After electrophoretic transfer to 0.22-mm nitrocellulose membranes, Cdc28 was detected with rabbit polyclonal antibodies or, in the case of Cdc28-hemagglutinin, with mouse 12CA5 antihemagglutinin ascites fluid as the primary antibody and alkaline phosphatase-coupled anti-rabbit or anti-mouse antibodies as the secondary antibody. Bands were then revealed with 5-bromo-4-chloro-3-indolyl-1-phosphate–nitroblue tetrazolium colorimetric reagents, leading to a purple precipitate directly on the transfer membrane. Colorimetric detection yields bands that are finer than those obtained by chemiluminescence, although the colorimetric detection is less sensitive. The Cdc28 signal can be increased by immunoprecipitating from larger quantities of yeast protein extract, although we did not need to do so for the Western blot shown in Fig. 4.

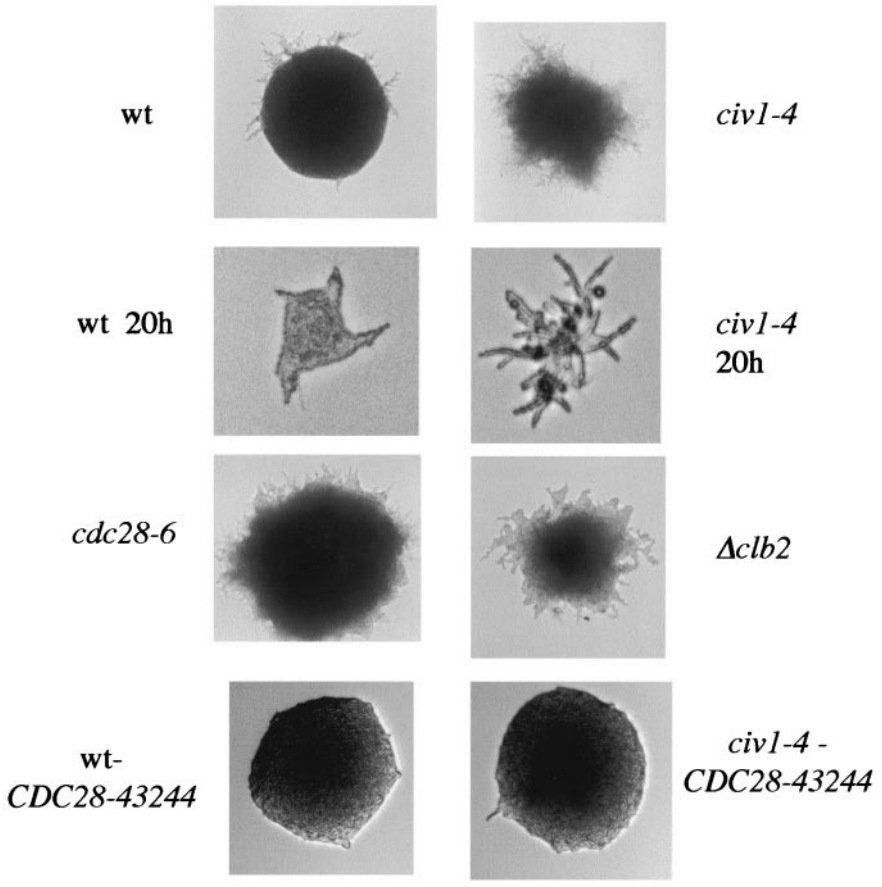

FIG. 1. Partial inactivation of Cak1 stimulates pseudohyphal growth, whereas expression of a Cak1-independent form of Cdc28 represses pseudohyphal growth. Wild-type (CSY2123), *civ1-4* (CSY2003), *cdc28-6* (CSY2126), and *clb2*Δ (CSY2127) strains containing the YEplac195 vector and wild-type and *civ1-4* strains containing YEplac195-*CDC28-43244*, encoding a Cak1-independent form of Cdc28, were cultivated on SLAD plates for 20 h or 3 days at 25°C. The colonies at 20 h are shown at a higher magnification than the colonies photographed after 3 days of growth.

Quantitative RT-PCR. Quantitative reverse transcription PCR (RT-PCR) was performed as described by Godon et al. (17). cDNAs were synthesized from 1 μg of total RNA using primers specific for each mRNA. PCR amplification with [^{32}P]dCTP was performed for 15 cycles for *ACT1* mRNA and 25 cycles for the remaining mRNAs using the following primers: *CLB1* (CCAGTCTAGGACGT TAGCGAAGTT and AGTAATTGGCAAACGGGATA), *CLB2* (CAGTCTC GAACTCTTGCCAAATTC and AGCCCATTGGACGGAAATTATAGA), *CLB3* (GAACGGCTTAGAATTTGAATTG and TAATGCTATCCACTTCG CTACGAT), *CLB5* (CATCGCACAACTATTTACTCGACA and ACATTGC CATTGCGCTTACGGTAG), *XBP1* (AGAGGTGACAGCGTTTCCACTAGC and GTAAGACTGGCAAATAAGGTCCC), and *ACT1* (TTGGATTCCGGT GATGGTGTTACT and TGAAGAAGATTGAGCAGCGGTTTG). ^{32}P-labeled PCR products were then separated by polyacrylamide gel electrophoresis and quantified with a PhosphorImager (Molecular Dynamics).

Microscopy and flow cytometry. Cells were visualized with a Zeiss Axiophot microscope fitted with a charge-coupled device camera for image acquisition. Cells were prepared for flow cytometry as previously described (36) and analyzed on a Becton Dickinson FACSCalibur.

RESULTS

Cak1 mutants show derepressed pseudohyphal growth. The molecular basis for yeast cell elongation and the inhibition of mitosis during pseudohyphal growth on nitrogen-limited media is unknown (22). We noticed that strains expressing mutations of the yeast Cdk-activating kinase (*cak1*/*civ1*) such as *civ1-4* (45) show a derepressed pseudohyphal growth at the permissive temperature of 25°C similar to that seen with a *clb2*Δ mutant or with certain *cdc28* mutants (Fig. 1) (1, 10). This result indicates that partial inhibition of CAK activity can stimulate pseudohyphal growth. Several regulatory pathways are required for pseudohyphal differentiation in the wild-type strain (33). These include a MAP kinase and a cAMP kinase pathway (37, 39, 43) and a transcription factor called Ash1 (5). Inactivation of the MAP kinase pathway with *ste20* or *ste12* mutants or inactivation of the cAMP pathway with a *gpa2* mutant or through the overexpression of the cAMP phosphodiesterase Pde2, or deletion of the *ASH1* gene, all eliminated or severely inhibited pseudohyphal growth in the *civ1-4* mutant (Fig. 2). The derepressed pseudohyphal growth of the *civ1-4* mutant thus depends on the normal regulatory pathways that are required for pseudohyphal growth in the wild-type strain.

Cak1 phosphorylates Cdc28 on Thr-169 (12, 20, 45). This phosphorylation is required for Cdc28 kinase activity. Partial inactivation of Cak1 leads to reduced activating phosphorylation of Cdc28 and a decrease in Cdc28 protein kinase activity. Cross and Levine isolated multiply mutated forms of Cdc28 that no longer require Cak1 phosphorylation for its activity (6, 7). One such mutant, Cdc28-43244, was introduced into the wild type and the *civ1-4* mutant on a multicopy plasmid in order to test its effect on pseudohyphal growth. Strikingly, Cdc28-43244 strongly inhibited pseudohyphal growth of both

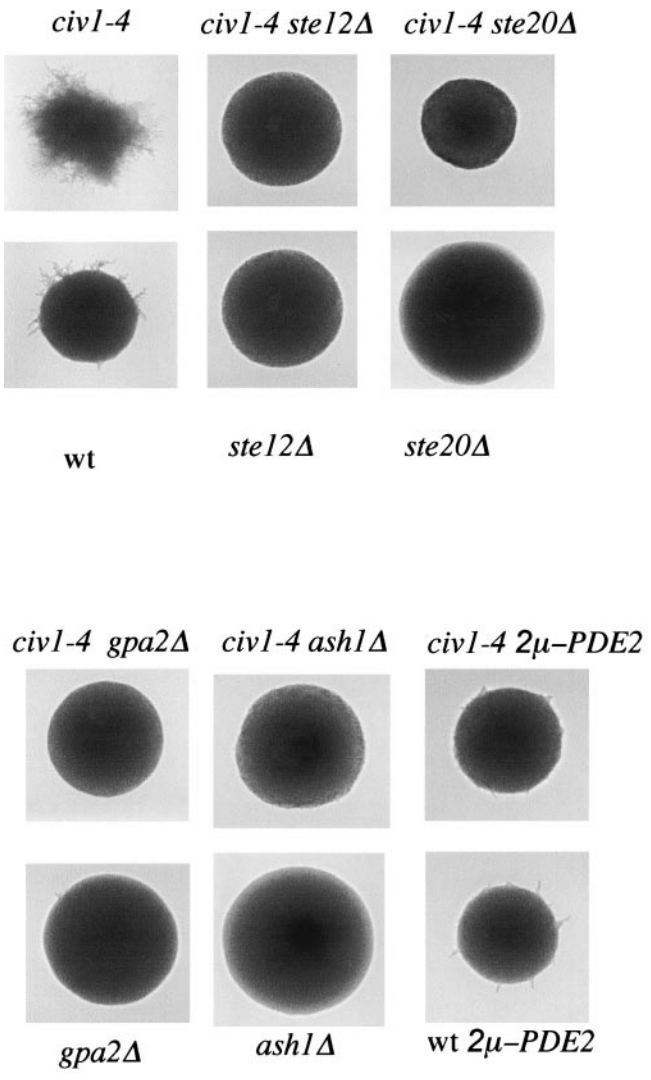

FIG. 2. The derepressed pseudohyphal growth of *civ1-4* (CSY2003) mutants requires the function of the *STE* MAP kinase pathway, the cAMP pathway, and the Ash1 transcription factor, as for the pseudohyphal growth of wild-type cells. Cells with the 2μm *PDE2* construct overexpress the *PDE2* gene on a multicopy plasmid. Colonies are shown after 3 days of growth on SLAD plates at 25°C. Colonies are shown at the same magnification.

the wild type and the *civ1-4* mutant on a low-nitrogen (SLAD) agar medium (Fig. 1). This result suggested that dephosphorylation of Cdc28 might be required for pseudohyphal growth.

Chemostat cultures provide homogeneous nitrogen-limited growth for biochemical investigations. Cells grown on nitrogen-limited agar media are in heterogeneous physiological states; although most cells are elongated relative to cells grown in rich media, only a small percentage of cells form pseudohyphal filaments that penetrate the agar surface (Fig. 1). Furthermore, cells that are at the interior of colonies will be more starved than cells that are at the edge of the colonies or that are in filaments projecting from the colonies. We examined the DNA content of wild-type diploid yeast cells growing on nitrogen-limited SLAD agar medium (Fig. 3A). Approximately 1,000 cells were spread on the surfaces of SLAD plates and incubated for 19, 36, or 72 h at 25°C. Cells were then scraped from the surface, and their DNA content was analyzed by flow cytometry. Cells grown for 19 h on SLAD plates were mainly budded with a 4N DNA content, but by 36 h of culture most cells accumulated in the unbudded state with a 2N DNA content. Infrequent filament formation became visible on these plates after about 2 days of culture. The accumulation of unbudded 2N cells after 36 h of growth on the SLAD plates can be accounted for by the nitrogen starvation experienced by most of the cells. The cycling cells that generate the pseudohyphal filaments represent only a small percentage of the total number of cells on the SLAD plates.

In order to overcome the problems inherent in the analysis of heterogeneous cell populations on agar media, we chose to examine chemostat cultures (Fig. 3B) as a source of nitrogen-limited cells (4). Cells growing in liquid chemostat cultures are in a homogeneous environment in which the degree of nitrogen starvation is precisely controlled, and it is easy to prepare large quantities of biomass for biochemical characterization. As observed for cells grown on nitrogen-limited agar media, yeast cells grown in nitrogen-limited chemostat cultures are elongated compared to cells grown in rich media (Fig. 3C). Extended filaments of cells were not found in the chemostats, but occasional clusters of three or four cells were observed. Cell elongation and pseudohyphal filamentation on nitrogen-limited SLAD plates requires the diploid cell state (16), and we found that haploid cells were not highly elongated during growth in nitrogen-limited chemostats (Fig. 3C). Expression of the Cak1-independent Cdc28-43244 mutant prevented cellular elongation of wild-type diploid cells in nitrogen-limited chemostats (Fig. 3C), as it did for cells on the surfaces of SLAD plates (Fig. 1 and data not shown). Finally, expression of a Ty1-*lacZ* reporter construct by the Ste12-Tec1 transcription factor is increased during pseudohyphal growth (32), and we found that there was a strong 12-fold induction of Ty1-*lacZ* expression for cells grown in nitrogen-limited chemostat cultures compared to rich media (Fig. 3D). These results show that nitrogen-limited growth in a chemostat shares many characteristics of nitrogen-limited growth on agar media.

Flow cytometry and microscopic analysis showed that diploid cells grown in nitrogen-limited chemostats had an increased proportion of unbudded cells with a 2N DNA content compared to cells grown in rich media (Fig. 3E and F). This G_1-phase accumulation may reflect an inhibition of cell growth and the passage of Start due to the partial nitrogen starvation. We also compared the fraction of budded cells containing a single nucleus (pre-anaphase cells) versus those containing two nuclei (post-anaphase cells). Cells grown in nitrogen-limited chemostats had an increased proportion of pre-anaphase cells compared to those grown in rich media (Fig. 3F). This result suggests that cells in nitrogen-limited chemostats are delayed at the metaphase-to-anaphase transition after having accumulated sufficient mass to pass Start and enter a new cell cycle.

Cdc28 is not dephosphorylated during nitrogen-limited growth in chemostats or on agar plates, but mitotic cyclin levels are reduced. Genetic results suggest that inhibition of Cdc28-cyclin B activity is responsible for the cell elongation and the pre-anaphase delay observed in cells growing in nitrogen-limited conditions (21, 22). Given our genetic results suggesting that partial dephosphorylation of Cdc28 might be re-

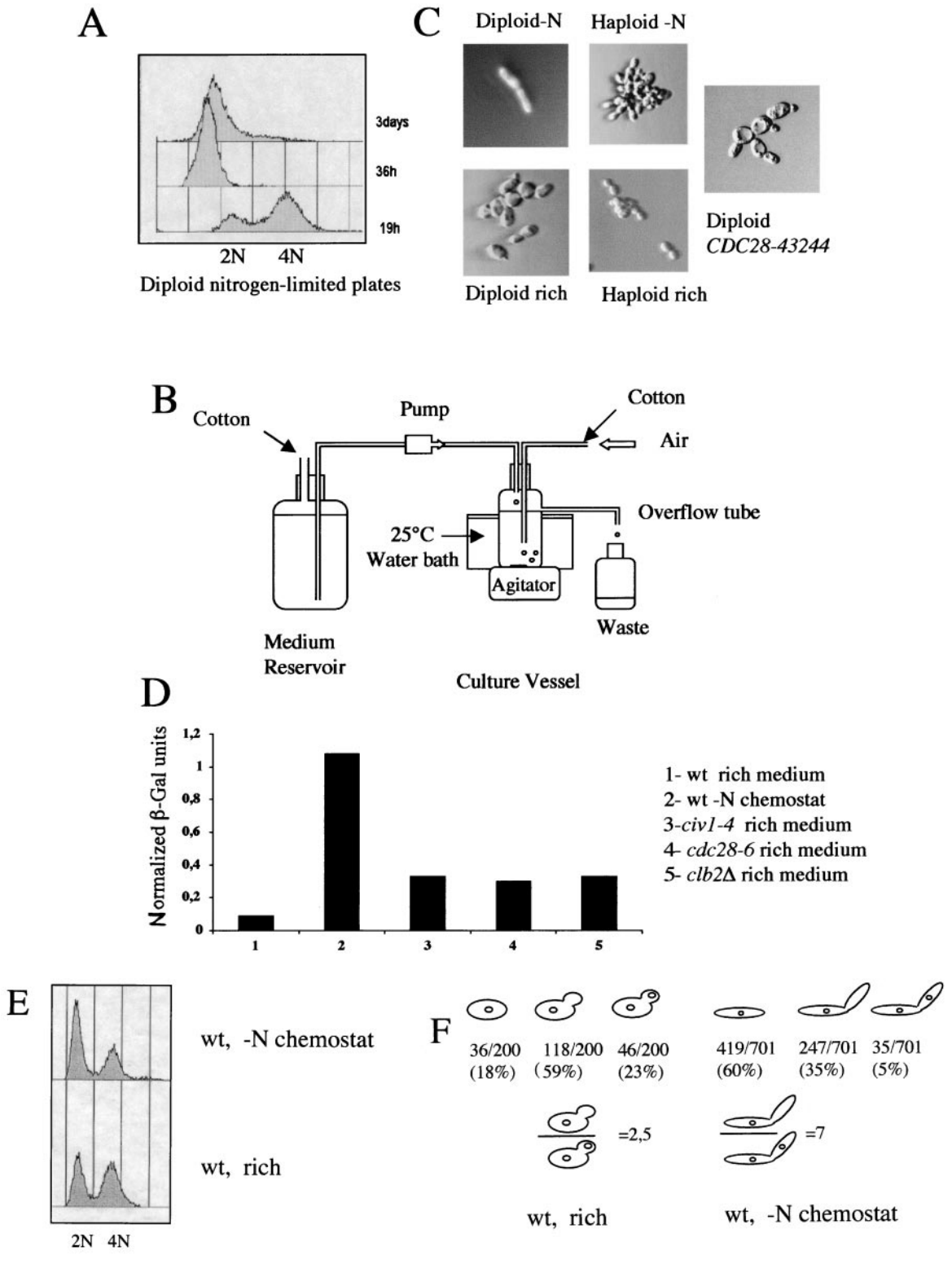

FIG. 3. (A) The vast majority of wild-type cells accumulate with a G_1-phase 2N DNA content after 36 h of growth on SLAD plates. (B) Schematic diagram of the chemostat used in this work. (C) Morphology of cells grown in chemostats. Wild-type diploid cells (CSY2123) in nitrogen-limited chemostats (−N) are elongated compared to those in rich medium. Wild-type haploid (CSY1000) cells do not elongate in response to the nitrogen limitation, and cellular elongation is blocked in diploid cells by the expression of Cdc28-43244, a Cak1-independent form of Cdc28. (D) Transcription of the Ste12-Tec1 Ty1-*lacZ* reporter construct [pFG(TyA)::*lacZ-LEU2*] is highly induced in wild-type (wt) cells grown in nitrogen-limited chemostats compared to that in rich medium. Ty1-*lacZ* transcription is also induced at lower levels in *civ1-4* (CSY2003), *cdc28-6* (CSY2126), and *clb2*Δ (CSY2127) mutants grown in a rich medium at 25°C. (E) Wild-type diploid cells (CSY2123) grown in nitrogen-limited chemostats contain more cells with a 2N DNA content (G_1 phase) than do the same cells grown in a rich medium. (F) Wild-type diploid cells (CSY2123) grown in nitrogen-limited chemostats contain a higher proportion of pre-anaphase to post-anaphase mitotic cells than do the same cells grown in a rich-medium chemostat.

quired for filamentous growth, we examined whether phosphorylation of Cdc28 is altered during nitrogen-limited growth of wild-type cells. Under appropriate electrophoretic conditions, Cdc28 phosphorylated by Cak1 on Thr-169 migrates slightly faster than unmodified Cdc28 (13). Cdc28 was mainly phosphorylated on Thr-169 in wild-type cells growing in rich media, whereas it was mainly dephosphorylated in the *civ1-4* mutant at the permissive temperature of 24°C and totally dephosphorylated in this mutant at the restrictive temperature of 37°C (Fig. 4A). We then examined the level of Cdc28 phosphorylation in cells growing at equilibrium in chemostats under nitrogen-limited or glucose-limited growth conditions, in cells on the surfaces of SLAD plates, and in cells in stationary phase in rich medium (YPD) batch cultures. A slight dephosphorylation of Cdc28 was observed in glucose-limited chemostat cultures, but Cdc28 was mainly in the Thr-169-phosphorylated form in cells grown in nitrogen-limited chemostats or on the surfaces of nitrogen-limited SLAD plates after 2 days of growth or in stationary-phase cells in batch cultures (Fig. 4A). Thus, growth in nitrogen-limited media and growth to stationary phase in rich media do not induce dephosphorylation of Cdc28 on Thr-169.

Since activating phosphorylation of Cdc28 was not reduced during nitrogen limitation, we decided to examine the levels of Clb2 protein in wild-type diploid cells grown in nitrogen-limited chemostats and rich media. Clb2 protein levels were greatly reduced in extracts from diploid cells growing in nitrogen-limited chemostats, as determined by immunoblotting with anti-Clb2 antibodies (Fig. 4B). In striking contrast, Clb2 protein levels were not decreased in wild-type haploid cells growing in nitrogen-limited chemostats (Fig. 4C) or in diploid wild-type cells growing in glucose-limited chemostats (data not shown). These results show that the decrease in Clb2 levels is a diploid-specific developmental response to the partial nitrogen starvation and does not represent a nonspecific starvation response.

It was previously reported that Clb2 levels are not diminished in *STE11-4* mutant cells grown in a rich medium compared to levels in wild-type cells (1). Ste11-4 is a constitutively active form of the MEK kinase that is thought to be turned on during pseudohyphal growth (41, 44). *STE11-4* cells show a pseudohyphal phenotype even when they are grown in rich media, and these cells have been proposed as a model for studying filamentous growth in wild-type cells (1). We examined Clb2 levels in a *STE11-4* mutant grown under nitrogen limitation in a chemostat. In striking contrast to the wild-type diploid cells, the *STE11-4* mutant did not show reduced levels of Clb2 during nitrogen-limited growth (Fig. 4B). The *STE11-4* mutation thus prevents a reduction of Clb2 levels that is observed in the wild-type diploid strain during continuous nitrogen-limited growth in a chemostat. We thus feel that the *STE11-4* mutant does not accurately reflect the response of wild-type diploid cells to nitrogen limitation, although it may be a valid model for other types of filamentous growth (8, 9, 23, 31, 46).

Modification of gene expression during nitrogen-limited growth in chemostats. We used quantitative RT-PCR to determine whether the drop in Clb2 protein levels during nitrogen-limited growth was correlated with a drop in *CLB2* mRNA levels, and to monitor the transcriptional response of a series

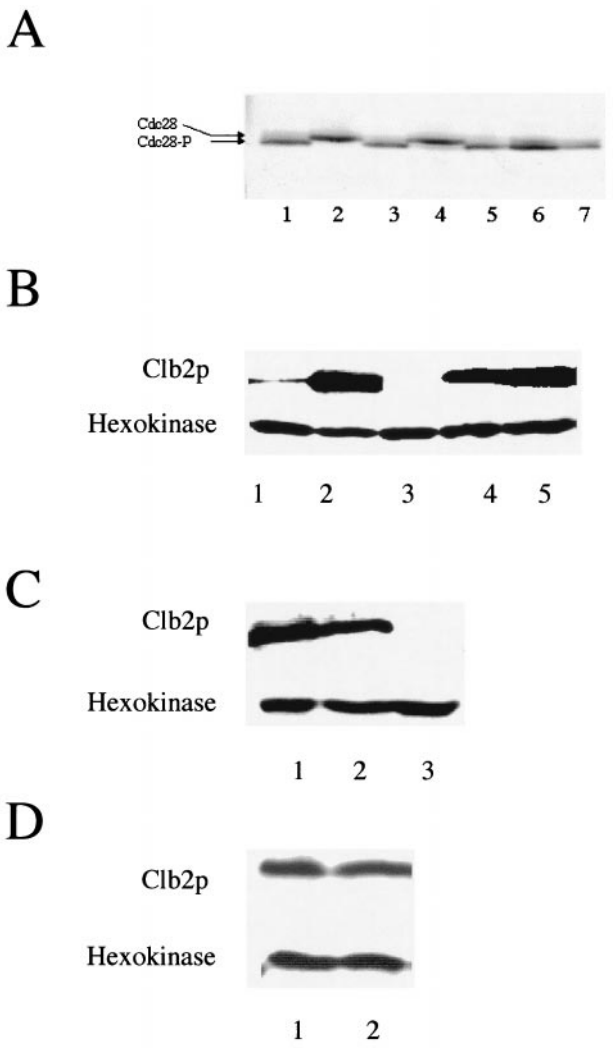

FIG. 4. (A) Cdc28 is mainly phosphorylated on Thr-169 in cells grown under nitrogen limitation. Thr-169-phosphorylated and nonphosphorylated forms of Cdc28 were electrophoretically separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Protein extracts were prepared from the following strains: the wild type (CSY2123) in rich medium at 25°C (lane 1), the *civ1-4* mutant (CSY2003) in rich medium at the restrictive temperature of 37°C (lane 2), the wild type in a nitrogen-limited chemostat at 25°C (lane 3), the *civ1-4* mutant (CSY2003) in rich medium at 25°C (lane 4), the wild type (CSY2123) scraped from the surface of nitrogen-limited SLAD plates after 2 days of incubation at 25°C (lane 5), the wild type (CSY2123) grown in a glucose-limited chemostat at 25°C (lane 6), and the wild type (CSY2123) in stationary phase in YPD (lane 7). (B) Clb2 protein levels are significantly reduced in wild-type diploid cells (CSY2123), but not in *STE11-4* diploid cells (CSY2128), grown in nitrogen-limited chemostats. Clb2 and hexokinase protein levels in whole-cell protein extracts were determined by immunoblotting for the following strains: the wild-type diploid (CSY2123) grown in a nitrogen-limited chemostat (lane 1), the wild-type diploid (CSY2123) grown in a rich medium (lane 2), a *clb2*Δ strain (CSY2127) grown in a rich medium (lane 3), a *STE11-4* diploid (CSY2128) grown in a nitrogen-limited chemostat (lane 4), and a *STE11-4* diploid (CSY2128) grown in a rich medium (lane 5). (C) The wild-type haploid strain (CSY1000) grown in a nitrogen-limited chemostat (lane 2) does not have reduced levels of Clb2p compared to the same strain grown in a rich medium (lane 1). Lane 3 contains a protein extract from a *clb2*Δ mutant (CSY2127) grown in a rich medium. (D) A homozygous diploid *xbp1*Δ mutant (CSY2124) has no less Clb2p when grown in a nitrogen-limited chemostat (lane 2) than when grown in a rich medium (lane 1).

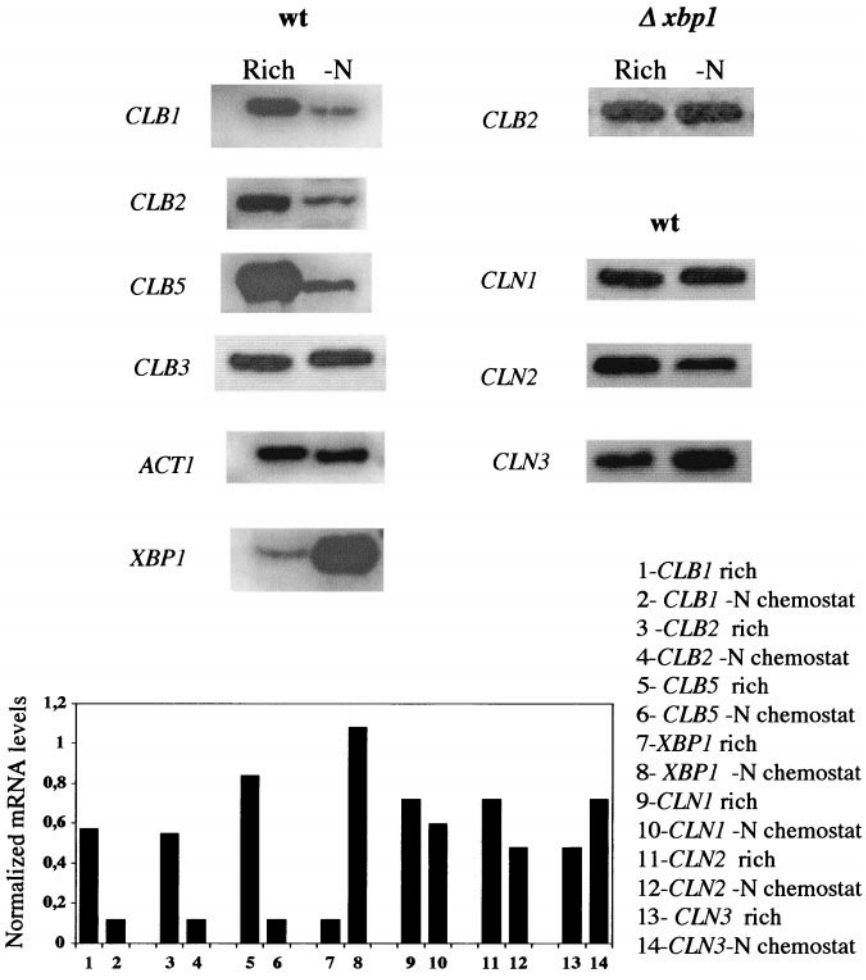

FIG. 5. Quantitative RT-PCR of *CLB, CLN, XBP1*, and *ACT1* mRNA levels in wild-type (wt) diploid cells (CSY2123) grown in rich-medium and nitrogen-limited chemostats. *CLB1, CLB2*, and *CLB5* mRNA levels are decreased, *CLB3* and *ACT1* mRNA levels are constant, *CLN1* and *CLN2* mRNA levels are slightly decreased, *CLN3* mRNA levels are slightly increased, and *XBP1* mRNA levels are increased eightfold in nitrogen-limited chemostats compared to rich-medium chemostats. The decrease in *CLB2* mRNA levels is blocked in a homozygous diploid *xbp1* mutant (CSY2124) grown in a nitrogen-limited chemostat.

of cyclin genes (Fig. 5). *ACT1* mRNA levels were unchanged in nitrogen-limited and rich-medium cultures, and they were thus used as a normalization standard. *CLB1, CLB2*, and *CLB5* mRNA levels were reduced five- to sevenfold in wild-type cells grown in nitrogen-limited chemostats compared to rich-medium chemostats, whereas *CLB3* levels were unchanged. We also examined the mRNA levels for the *CLN1, CLN2*, and *CLN3* G_1 cyclin genes. The *CLN1* and *CLN2* mRNA levels showed a modest decline during growth in nitrogen-limited chemostats, whereas the *CLN3* mRNA was slightly increased (Fig. 5).

XBP1 codes for a repressor of *CLN1* and *CLB2* expression during sporulation, and *XBP1* expression is induced by diverse stresses (34, 35). We found that *XBP1* mRNA levels were increased eightfold in nitrogen-limited chemostats compared to rich-medium chemostats. Xbp1 is required for some of the transcriptional modifications observed during nitrogen-limited growth in chemostats, since the decreases in *CLB2* mRNA (Fig. 5) and Clb2 protein (Fig. 4D) were abolished in an *xbp1*Δ mutant grown in a nitrogen-limited chemostat. The absence of significant *CLN1* repression in the presence of eightfold-elevated *XBP1* mRNA is not exceptional; a similar result was reported for cells treated with diamide (35). Reduced *CLB1,2* expression will limit Cdc28-Clb1,2 kinase activity, which in turn could explain the elongated cell morphology of cells grown in nitrogen-limited media. Furthermore, we found that Ty1-*lacZ* activity was increased threefold in *clb2*Δ*, civ1-4*, and *cdc28-6* mutants grown in rich medium compared to the wild-type strain (Fig. 3D). This stimulation of the expression of a Ste12-Tec1 reporter gene correlates well with the enhanced pseudohyphal growth shown by these mutants (Fig. 1) and suggests that Cdc28-Clb2 can inhibit the Ste12-Tec1 transcriptional activation complex.

***XBP1* is required for pseudohyphal growth.** We made an *xbp1*Δ homozygous diploid strain and found that it was inhibited for pseudohyphal filament formation on low-ammonium dextrose (SLAD) and glycerol (SLAYP) agar media (Fig. 6C through F). Moreover, individual *xbp1*Δ cells did not show the characteristic elongated cell shape exhibited by wild-type diploid cells on nitrogen-limited media (Fig. 6A through D). Fi-

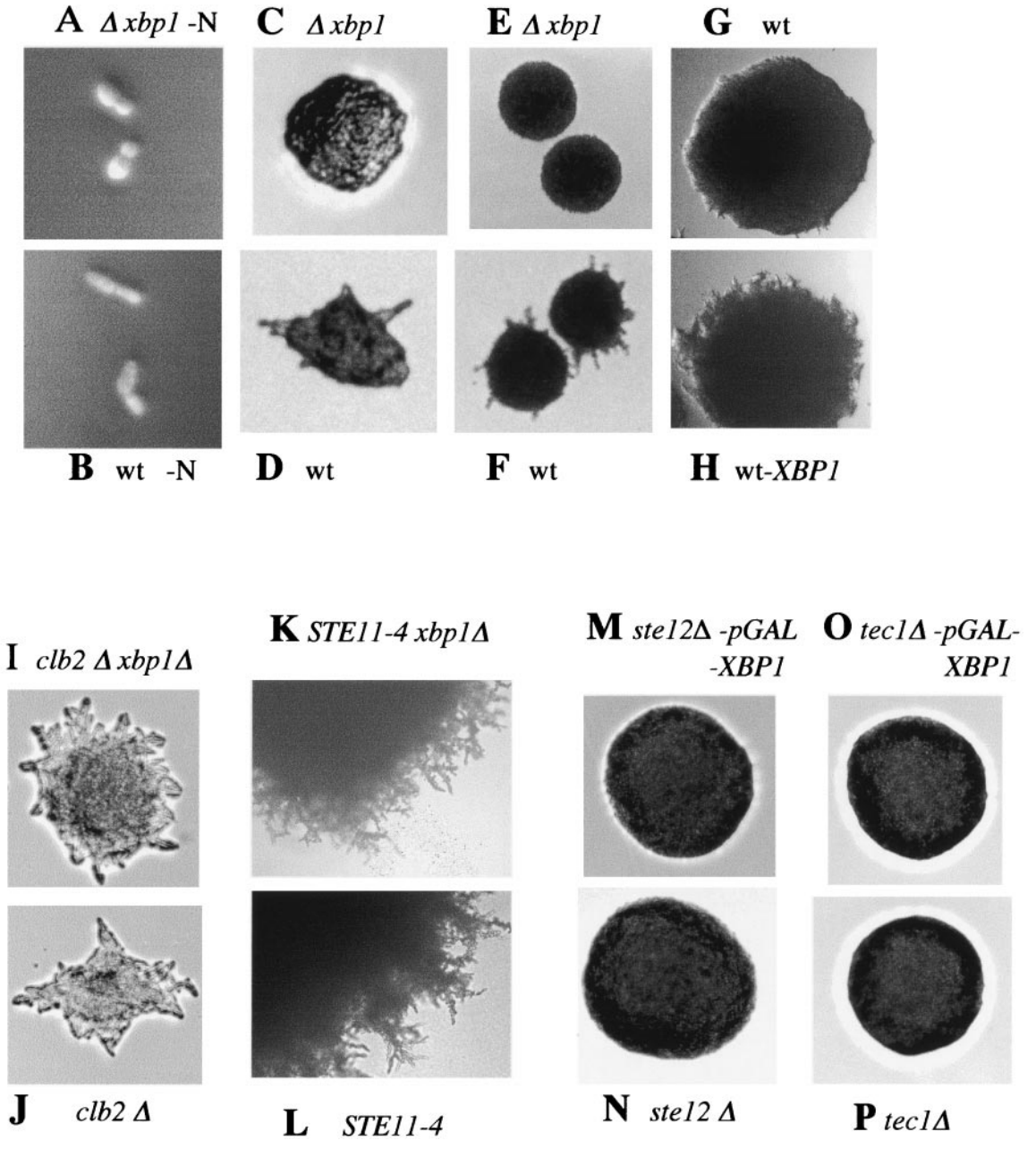

FIG. 6. Homozygous diploid *xbp1* mutant cells (CSY2124) are inhibited for cellular elongation in nitrogen-limited chemostats (A and B) or after 20 h of growth on SLAD plates (C and D). Deletion of *XBP1* completely blocks filament formation of the wild-type strain (CSY2123) on SLAYP plates, even after 5 days of growth at 30°C (E and F). Overexpression of *XBP1* from the *GAL* promoter stimulates filamentation on plates containing synthetic low-ammonium medium with 2% galactose compared to the same strain expressing *XBP1* from its normal promoter on a centromeric plasmid (G and H). Cells in panels A and B are shown at a higher magnification than cells in panels C through H. (I through P) Colonies of cells of the indicated genotypes after growth for 3 days (I, J, M, N, O, and P) or 7 days (K and L) on SLAD plates at 30°C.

nally, overexpression of *XBP1* from the *GAL* promoter stimulated pseudohyphal growth (Fig. 6G and H). Thus, *XBP1* is required for normal pseudohyphal differentiation. We next deleted the *CLB2* gene in an *xbp1*Δ mutant in order to test the importance of *CLB2* as a target of Xbp1-mediated repression during nitrogen-limited growth. Deletion of *CLB2* largely restored elongated cell growth and filamentation to an *xbp1*Δ mutant on a nitrogen-limited SLAD agar medium (Fig. 6I, compare with Fig. 6C and J). *clb2*Δ was thus largely epistatic to *xbp1*Δ. These results suggest that *CLB2* is an important repression target of Xbp1 during nitrogen-limited growth.

We also attempted to place *XBP1* action with regard to the MAP kinase pathway regulating pseudohyphal growth by genetic epistasis experiments. Constitutive activation of the MAP kinase pathway through the expression of *STE11-4* restored filamentous growth to the *xbp1*Δ mutant on nitrogen-limited SLAD plates (Fig. 6K and L). In contrast, *XBP1* overexpression from the *GAL* promoter did not suppress the filamenta-

tion defect of *ste12*Δ and *tec1*Δ mutants (Fig. 6M through P). These results show that constitutive activation of the MAP kinase cascade by the *STE11-4* mutation can bypass the requirement for Xbp1 in pseudohyphal growth, but overexpression of *XBP1* cannot bypass the requirement for the Ste12 and Tec1 transcription factors. These results thus place *XBP1* function upstream of or in parallel to the MAP kinase cascade and Ste12-Tec1 transcription factor function.

DISCUSSION

Xbp1-mediated repression of *CLB2* expression can explain the elongated phenotype of wild-type yeast cells under nitrogen-limited growth conditions. Cellular elongation is one of the most evident phenotypes associated with the growth of wild-type diploid yeast cells in nitrogen-limited media (4, 16, 21, 22). This phenotype can be explained by a delayed or inefficient repression of polarized growth to the bud tip by Cdc28-Clb1,2 protein kinases (24, 25). There are four known mechanisms that could potentially account for Cdc28-Clb1,2 protein kinase inhibition: Swe1 inhibitory phosphorylation of Cdc28 Tyr-19 (3), inhibition of Cdk-Clb1,2 activity by a Cdk inhibitor such as Sic1 (1), a decrease in Clb1,2 protein levels, and a decrease in Cak1 activating phosphorylation of Cdc28. Although Swe1 inhibition of Cdc28 may contribute to filamentous growth in certain circumstances (10), it is not required for cellular elongation during nitrogen-limited growth (1, 22), and no clear evidence has yet been found for the role of a Cdk inhibitor in filamentous growth. In contrast, overexpression of *CLB2* (1, 10, 22) and expression of a Cak1-independent form of Cdc28 (this paper) both block cellular elongation and pseudohyphal growth in response to a nitrogen starvation. Moreover, deletion of *CLB2* or partial inactivation of Cak1 stimulates cellular elongation and pseudohyphal growth. Thus, the genetic analyses suggest that reduced Clb2 levels or reduced activating phosphorylation of Cdc28 could be responsible for inhibition of Cdc28-Clb2 kinase activity during nitrogen-limited growth. Unfortunately, the genetic analyses do not indicate which of these pathways are actually used by wild-type diploid yeast cells during pseudohyphal growth. Thus, direct biochemical analysis of wild-type cells during pseudohyphal growth is required to determine which regulatory pathways are really employed during this growth state. However, the heterogeneous physiological states of wild-type cells undergoing pseudohyphal differentiation on nitrogen-limited agar media are an obstacle to their biochemical analysis. Filament formation is observed for only a small fraction of wild-type cells growing on nitrogen-limited agar media. Furthermore, cells within colonies are likely to be highly starved for nitrogen, whereas cells on the outer edges of colonies and in penetrating filaments will experience different degrees of starvation. It is thus impossible to prepare physiologically homogeneous populations of wild-type cells from nitrogen-limited agar plates on which pseudohyphal growth is typically studied. We therefore used chemostat cultures to determine whether either of these two regulatory pathways could be implicated in cellular elongation during nitrogen-limited growth of wild-type diploid yeast cells. Chemostats provide a simple means of preparing large quantities of homogeneous cultures in which cells are grown under continuous, precisely defined conditions of nutrient limitation for biochemical analysis. It was previously shown that wild-type diploid cells are elongated when they are grown in nitrogen-limited, but not glucose-limited, chemostats (4). We show here that as for growth on nitrogen-limited agar media, this elongation response is specific for diploid cells (Fig. 3), is partially dependent on Ste20 and Ste12 (data not shown), and is accompanied by the transcriptional induction of a Ste12-Tec1 reporter construct (Fig. 3). On the other hand, we saw little or no indication of filament formation in our nitrogen-limited chemostats, and we observed low levels of expression of *FLO11* (data not shown), which encodes a cell surface adhesion protein implicated in filament formation on agar plates (23, 27, 39, 43). The weak *FLO11* expression in our nitrogen-limited glucose-rich chemostats may be due to glucose repression of *FLO11* transcription (14). We observed frequent chains of cells and increased expression of *FLO11* in nitrogen-limited chemostat cultures containing 3% galactose instead of 3% glucose, although the individual cells in the chains were less elongated then when cells were cultivated in glucose (data not shown). Altogether, these results suggest that our nitrogen-limited chemostat cultures reproduce many, but not all, aspects of nitrogen-limited pseudohyphal growth on solid agar media.

We found no evidence for a decrease in the level of Cdc28-activating phosphorylation during nitrogen-limited growth (Fig. 4) despite strong genetic data suggesting that a decrease in activating phosphorylation was required for pseudohyphal growth. How can this apparent contradiction be resolved? It is possible that partial inactivation of Cak1 mimics an event, such as inhibition of Cdc28-Clb1,2 activity, that normally occurs by a distinct mechanism in wild-type cells during nitrogen-limited growth, and it remains possible that Cdc28-activating phosphorylation is regulated during other conditions that lead to filamentous growth (8, 9, 23, 31, 46). However, it is less clear how the Cak1-independent Cdc28-43244 mutant is so effective in blocking pseudohyphal growth (Fig. 1) and cellular elongation (Fig. 3C) in response to nitrogen starvation. Possibly, this inhibition may be related to the weak kinase activity associated with Cdc28-43244–Cln2 in vitro (7). The Cln1 and Cln2 G_1 cyclins are required for pseudohyphal growth (29), so the weak in vitro kinase activity of Cdc28-43244–Cln2 could mean that this mutant, although active in the absence of Cak1, may not sustain sufficient G_1 cyclin kinase activity in vivo to support pseudohyphal growth.

Although we did not observe decreased activating phosphorylation of Cdc28, we did observe significant decreases in *CLB1, CLB2*, and *CLB5* gene expression during nitrogen-limited growth. Xbp1 is synthesized in response to diverse types of stress and it is required for repression of *CLN1* and *CLB2* transcription during sporulation (34, 35). We showed that *XBP1* is highly expressed during growth of wild-type yeast cells in nitrogen-limited chemostats (Fig. 5). Deletion of *XBP1* prevents the fall in Clb2 levels normally observed in wild-type cells grown under nitrogen limitation, as well as inhibiting cellular elongation in nitrogen-limited chemostats and cellular elongation and pseudohyphal filament formation on nitrogen-limited agar media (Fig. 6). *CLB2* overexpression also inhibits cellular elongation and filament formation (1, 10). Furthermore, *CLB2* deletion restored cellular elongation and pseudohyphal growth to an *xbp1*Δ mutant (Fig. 6). These combined results strongly suggest that transcriptional induction of the *XBP1* gene and

subsequent repression of *CLB2* gene expression is a key response to nitrogen limitation leading to modifications of the yeast cell cycle and cell morphology. Further work is necessary to determine the functional significance of the *CLB5* transcriptional repression, to specify the mechanism of action of Xbp1 with regard to *CLB* gene repression, and to order the action of Xbp1 and Clb1,2 with regard to the different signal transduction pathways implicated in pseudohyphal growth. Finally, many genes involved in filamentation in *S. cerevisiae* have apparent orthologs in *Candida albicans* that have been implicated in morphogenetic pathways contributing to the virulence of this pathogenic yeast (11). A sequence coding for a protein with low but significant similarity to Xbp1 is found in the *C. albicans* genome (http://www-sequence.stanford.edu/group/candida), and it will be interesting to determine whether it also contributes to morphogenesis and virulence in *Candida*.

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