

Regulation of the Proteinase B Structural Gene *PRB1* in *Saccharomyces cerevisiae*

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The expression of *PRB1*, the gene that encodes the precursor to the soluble vacuolar proteinase B (PrB) in *Saccharomyces cerevisiae*, is regulated by carbon and nitrogen sources and by growth phase. Little or no *PRB1* mRNA is detectable during exponential growth on glucose as the carbon source; it begins to accumulate as cells exhaust the glucose. Previous work has shown that glucose repression of *PRB1* transcription is not mediated by *HXX2* or by the *SNF1*, *SNF4*, and *SNF6* genes (C. M. Moehle and E. W. Jones, *Genetics* 124:39–55, 1990). We analyzed the effects of mutations in the *MIG1*, *TUP1*, and *GRR1* genes on glucose repression of *PRB1* and found that mutations in each partially alleviate glucose repression. *tup1* and *mig1* mutants fail to translocate all of the Prb1p into the lumen of the endoplasmic reticulum. A screen for new mutants revealed mutations in *MIG1* and *REG1*, genes already known to regulate glucose repression, as well as in three new genes that we have named *PBD1* to *PBD3*; all cause derepressed expression. Mutations that result in failure to completely derepress *PRB1* were also identified in two new genes, named *PND1* and *PND2*. Good nitrogen sources, like ammonia, repress *PRB1* transcription; mutations in *URE2* do not affect this response. Derepression upon transfer to a poor nitrogen source is dependent upon *GLN3*.

The lysosome-like vacuole of the yeast *Saccharomyces cerevisiae* is an acidic compartment that contains a number of proteases, including proteinase A, proteinase B (PrB), and carboxypeptidase Y. These proteases participate in protein degradation and turnover. PrB accounts for a large fraction of the protein degradation under limiting nutrient and stressful conditions (17, 40, 49).

The *PRB1* gene in *S. cerevisiae* encodes the serine endoproteinase B precursor. The 69-kDa precursor is posttranslationally processed to a 31-kDa active PrB protease as it traverses the secretory pathway. Proteolytic cleavages are effected autocatalytically and by proteinase A (26, 32). Levels of PrB activity increase as the cells progress from the log phase to the stationary phase of growth; they are also responsive to the nitrogen and carbon sources present in the growth medium (12, 25, 27). *PRB1* mRNA is barely detectable during the early log phase of growth (Fig. 1). As the cells begin to deplete the glucose supply, a transcriptional derepression of *PRB1* occurs, causing a dramatic increase in *PRB1* mRNA levels. This increase in mRNA levels does not immediately result in a substantial increase in PrB activity, however, in part due to a deficit in posttranslational processing activity (27). When the cells enter the diauxic phase of growth, the *PRB1* mRNA levels fall; however, mRNA levels remain higher in diauxic-phase cells than in log-phase cells. It is during the diauxic stage that one begins to detect PrB activity. A second transcriptional derepression of *PRB1* occurs as the cells approach the stationary phase of growth, leading to an eventual 100-fold increase in PrB activity (25).

Glucose is the preferred carbon source for *S. cerevisiae*. Extensive investigations of glucose regulation of the *SUC2* and *GAL* genes have led to the identification of a number of reg-

ulatory genes, including *HXX2*, *SSN6*, *TUP1*, *MIG1*, *GRR1*, *REG1*, *SNF1*, *SNF4*, and *SNF6* (15, 34, 43). These genes effect global regulation of a number of glucose-repressible genes, mainly at the level of transcription. *HXX2*, *SNF1*, *SNF4*, and *SNF6* are known not to participate in glucose repression of *PRB1* (27). Because we turned up *mig1* and *reg1* mutants in a recent mutant screen, we examined the effects of the *mig1*, *tup1*, *reg1*, and *grr1* mutations on *PRB1* expression.

The preferred nitrogen sources for yeast are glutamine, asparagine, and ammonia, while the nonpreferred are proline and glutamate. Glutamine, asparagine, and ammonia repress synthesis of a constellation of enzymes involved in nitrogen metabolism, including PrB (12, 20). Although *GLN3*, *DAL80*, and *URE2* are considered the three main global regulators in nitrogen catabolite regulation, other genes have also been shown to play a role (3, 5, 6, 8, 23, 37, 38). *DAL80* and *URE2* act negatively, while *GLN3* is required for derepression of nitrogen catabolite-responsive genes brought about by transfer to a poor nitrogen source. All three genes act at the level of transcription (2, 5, 6, 8, 23).

The control circuits for regulation of vacuolar hydrolases have not been investigated. We have chosen to investigate the regulation of the *PRB1* gene, since PrB plays such a major role in the life of the cell. In order to completely understand the regulation of *PRB1*, we must account for the regulatory circuitry that effects its regulation and identify the genes involved. In this study we examined the effects of mutations in *MIG1*, *TUP1*, and *GRR1* (glucose regulatory genes) and in *GLN3* and *URE2* (nitrogen regulators) on *PRB1* expression. The results we present here indicate that the regulatory circuit governing glucose repression of *PRB1* shares some components with that governing glucose repression of the *SUC2* and *GAL* genes. Similarly, *GLN3*, but not *URE2*, is involved in nitrogen regulation of *PRB1*. In addition, we isolated mutants that show elevated or reduced expression of *PRB1*. Two of the mutations were found to be allelic to the previously identified *mig1* and *reg1* mutations.

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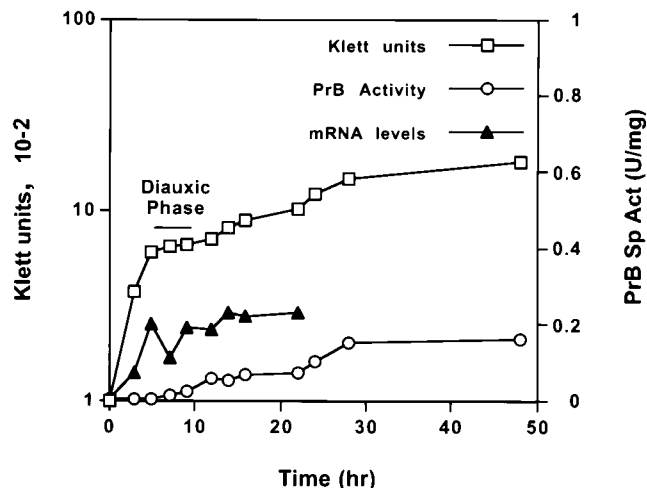


FIG. 1. Levels of *PRB1* transcription and PrB specific activity in a wild-type strain as a function of growth stage. *PRB1* mRNA is barely detectable during the early growth stage. Transcriptional activation of *PRB1* occurs in the pre-diauxic phase. When the cells enter the diauxic phase of growth, mRNA levels fall. Exit from the diauxic phase leads to reactivation of *PRB1* transcription. PrB activity is detectable only during the post-diauxic phase. This figure is adapted from reference 27. One OD₆₀₀ unit equals 40 to 45 Klett units.

MATERIALS AND METHODS

Strains and culture. The strains used in this work and their genotypes are listed in Table 1. Growth media used were yeast extract-peptone-dextrose (YEPD), synthetic complete medium, and omission medium as previously described (50). For analysis of nitrogen-mediated regulation, the derepressing medium contained 0.1% proline as the nitrogen source; the repressing medium contained 0.1% proline plus 0.5% ammonium sulfate. *Escherichia coli* strains were grown in Luria-Bertani medium; for plasmid selection, ampicillin (100 µg/ml) was added (22).

Genetic analysis. Genetic crosses, sporulation, tetrad analysis, and complementation analysis were performed by standard procedures as previously described (13).

DNA manipulations. Standard procedures for plasmid DNA manipulation, isolation, and transformation into bacteria were performed as previously described (21). Yeast transformation was performed by the lithium acetate method (14).

***PRB1-lacZ* reporter plasmid.** A DNA fragment spanning from position -1281 to +4 (the ATG codon starts at +1) upstream of *PRB1* was cloned into YEp356R and YIp356R (30). A *Bam*HI site was introduced by site-directed mutagenesis at position +4 of the *PRB1* open reading frame (ORF) (ATG GATCCA [*Bam*HI site underlined]) in plasmid pF8 (24). The resulting plasmid was then cleaved at the *Hind*III site and filled in with Klenow (Boehringer Mannheim Biochemicals, Indianapolis, Ind.), followed by cleavage with *Bam*HI to give a fragment spanning from -1281 to +4. This fragment was then cloned between the *Sma*I and *Bam*HI sites of YEp356R. The multiple-cloning sequence between the *Bam*HI site and the *lacZ* ORF was deleted to generate an in-frame fusion protein in plasmids pBJ5923 (in YEp356R) and pBJ5893 (in YIp356R). pBJ5893 was integrated at the genomic *ura3* locus after linearization at the *Stu*I site within the plasmid-borne *URA3* gene to create BJ6134 and BJ6239. The integration events were confirmed by DNA blot analysis.

RNA extractions and hybridizations. Total RNA was extracted from log-phase cells (optical density at 600 nm [OD₆₀₀], 0.8 to 1.2) by the hot acidic phenol method and quantitated spectrophotometrically (4). Five-microgram samples of RNA were blotted onto a nylon membrane (Schleicher & Schuell, Keene, N.H.) through a slot blotter. The dried membrane was prehybridized for 2 to 6 h in a solution containing 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 50% formamide, 5× Denhardt's solution, 200 µg of salmon sperm DNA/ml, and 7% formaldehyde. ³²P-labeled DNA probes were added to fresh hybridization solution (2× SSC, 50% formamide, 5× Denhardt's solution, and 3% formaldehyde) and hybridized for 16 h at 46°C. The membrane was washed twice with 2× SSC-0.1% sodium dodecyl sulfate (SDS) at 46°C for 20 min and twice in 2× SSC-1% SDS at 60°C for 20 min. Two final washes were carried out at 60°C in 2× SSC-0.1% SDS for 20 min. The membrane was then exposed to X-ray film (Biomax; Kodak) for 24, 48, and 72 h. Northern analysis probes included a 0.7-kb PCR-generated fragment encoding the *pro* region of Prb1p and an internal fragment of *ACT1*, which served to normalize the amount of RNA loaded into each slot. Probes were labeled in vitro with [α-³²P]dCTP by random primer extension with a kit from Amersham, Arlington Heights, Ill.

β-Galactosidase assays. Cells transformed with the *PRB1-lacZ* reporter plasmid were grown to an OD₆₀₀ of 0.8 to 1.2 in 5 ml of synthetic medium supplemented to ensure growth and plasmid retention. β-Galactosidase activity was assayed by the permeabilized-cell method or with cell extracts, as previously described (36). Activities are presented in Miller units (22). Values are averages of at least three independent experiments performed in duplicate. The standard deviation was generally <25%. Values from the β-galactosidase assays were always compared to those for the isogenic wild-type parent.

Mutagenesis. For isolating *PRB1* derepressed (*pbd*) mutants, patches of yeast strains BJ6134 and BJ6239 were grown on YEPD plates overnight. Patches were then replica plated onto YEP-sucrose containing 0.2% 2-deoxyglucose and 20 µg of antimycin A/ml (YEPS-DG). Plates were exposed to UV light for various lengths of time and then incubated anaerobically for 3 to 5 days at 30°C to select for mutants. Colonies that grew on YEPS-DG were then replica plated onto YEPD plates containing 0.2% X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). Colonies were visually screened for an "early blue" phenotype (blue color within 2 days compared to 3 to 4 days for the wild type). The primary selection was performed because we initially thought that both strains carried a *PRB1-SUC2* reporter construct integrated at the *SUC2* locus. However, DNA blot analysis confirmed that there was a single integration of *PRB1-lacZ* at the *ura3* locus and that the strains had an intact wild-type *SUC2* gene. For isolating *PRB1* nonderepressible (*pbn*) mutants, BJ6239 was mutagenized with 3% ethyl methanesulfonate and cells were plated directly onto YEPD plates (36). After 2 days of incubation at 30°C the plates were replica plated onto YEPD containing 0.2% X-Gal. Plates were incubated at 30°C and visually screened after 2 days for white or pale-blue colonies; control colonies were dark blue. All putative mutants were then assayed for β-galactosidase activity.

Preparation of yeast whole-cell extracts. Cell protein extracts were prepared from cells grown to mid-log phase in appropriate medium as described previously (48). The protein concentration was determined by protein assay (Bio-Rad, Hercules, Calif.) with bovine serum albumin as the standard. Samples were either used immediately or stored at -80°C.

Immunoblotting. Immunoblotting was performed as described by Woolford et al. (48). Affinity-purified rabbit anti-PrB antibody (26) was used as the primary antibody. Immune complexes were visualized according to the manufacturer's instructions by use of a secondary antibody, horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad). Routinely, 5-µg samples of protein extract from stationary-phase cells were used for immunoblotting. For mid-log-phase cells, a 50-µg sample of protein extract was used for detecting PrB antigen. Most of the nonspecific background bands were a consequence of overloading.

Biochemical assays. PrB activity in late-log-phase cells was determined with Azocoll (Calbiochem, San Diego, Calif.) as a substrate as described by Jones (16); specific activity is defined as (1.91)(ΔOD₅₂₀)/(milligrams of protein/minute)

TABLE 1. Strains and plasmids used in this study and their genotypes

Strain or plasmid	Genotype
BJ5991	<i>MATα prb1-Δ1.6R ura3-52 trp1 can1 suc2-Δ9</i>
BJ6134	<i>MATα his3Δ200 leu2-3,112 trp1 URA3 ura3-52::PRB1_{UAS}-lacZ SUC2</i>
BJ6239	<i>MATα leu2-3,112 trp1 URA3 ura3-52::PRB1_{UAS}-lacZ lys2 SUC2</i>
BJ6977	<i>MATα his3Δ200 leu2::HIS3 lys2-801 ura3-52 mig1Δ::LEU2</i>
BJ6978	<i>MATα his3Δ200 leu2::HIS3 lys2-801 ura3-52</i>
BJ7140 ^a	<i>MATα leu2-3,112 ura3-52</i>
BJ7141 ^a	<i>MATα leu2-3,112 ura3-52 gln3Δ::LEU2</i>
BJ8727 ^a	<i>MATα leu2-3,112 ura3-52 ure2Δ::LEU2</i>
BJ8316 ^b	<i>MATα his3Δ200 leu2-3,112 ura3-52 lys2-801 ade2-101 grr1Δ::LEU2</i>
BJ8317 ^b	<i>MATα his3Δ200 leu2::HIS3 ura3-52 lys2-801 ade2-101 trp1-901 tyr1-801</i>
BJ8319 ^b	<i>MATα his3Δ200 lys2-801 ura3-5 ade2-101 trp1-901 tyr1-801 tup1Δ::TRP1</i>
pPHU1	<i>PRB1::his4::URA3^c</i>
pBJ5923	<i>PRB1_{UAS}-lacZ</i> in YEp356R
pBJ5893	<i>PRB1_{UAS}-lacZ</i> in YIp356R

^a Obtained from B. Magasanik. PH5 (*ure2Δ::LEU2*), PM71 (*gln3Δ::LEU2*), and PM38 (wild-type parent).

^b Obtained from M. Johnston. YM2955 (*grr1Δ::LEU2*), YM4127 (wild-type parent), and YM4513 (*tup1Δ::TRP1*).

^c YCp50 derivative in which *LYS2* replaces *URA3* as the selectable marker.

as previously described (16). Invertase assays were performed on the *pbd* mutants under glucose-repressed and -derepressed conditions (mid-log phase) as described previously (11).

Prb1p-Ura3p translocation assay. The translocation assay is a modification of the selection scheme used by Deshaies and Schekman (9) for isolating translocation-defective mutants. The full-length *PRB1* gene was fused to a fragment of *HIS4* onto which the reporter gene *URA3* had been fused to create an in-frame fusion in plasmid pPHU1; the plasmid backbone is YCp50, in which *LYS2* replaces *URA3* as the selectable marker (28).

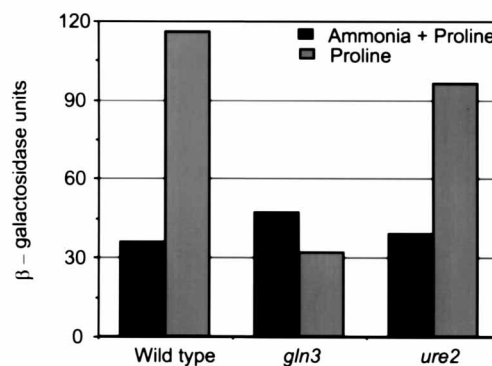
RESULTS

Expression of *PRB1* is regulated by Gln3p. *GLN3* and *URE2* had been proposed to be the main regulators of nitrogen catabolite-responsive (NCR) genes. Under nitrogen-limiting conditions, Gln3p activates transcription by binding to GATAA sequences in the upstream promoter elements of target genes (1, 2, 20, 24). Ure2p, the negative regulator, antagonizes Gln3p in the presence of readily utilizable nitrogen sources (20). Because the DNA sequence upstream of the *PRB1* ORF contained GATAA sequences at positions -138, -413, and -691 (relative to the ATG codon), we examined the role of *GLN3* in the regulation of *PRB1*. We determined the levels of PrB activity in wild-type and *gln3* cells grown to late log phase (OD_{600} , 4 to 6) in a medium containing proline (derepressed) or proline and ammonia (repressed) as nitrogen sources by the azocoll method. For the wild-type strain, a threefold increase in PrB activity was observed in cells grown with proline compared to cells grown with proline plus ammonia as the nitrogen source (specific activities of 2.40 and 0.75 U/mg, respectively); similar observations were made by Hansen et al. (12) for cells grown on a poor nitrogen source. For the *gln3* mutant grown in proline plus ammonia, the levels of PrB activity were comparable to those for the wild type grown in the same medium. However, with proline as the sole nitrogen source, we observed no increase in PrB activity in the *gln3* mutant. Rather, there was a decrease of about twofold in PrB activity in *gln3* cells grown on proline alone compared to the PrB activity in *gln3* cells grown on proline plus ammonia (specific activities of 0.40 and 0.81 U/mg, respectively).

The wild-type, *gln3*, and *ure2* strains were transformed with pBJ5923 bearing the *PRB1-lacZ* reporter. The resultant strains were examined for the roles of *GLN3* and *URE2* in regulating expression of the fusion construct, thus eliminating any requirement for posttranslational processing in expression. Cells were grown to logarithmic phase in a proline-based medium, with or without ammonia, and then assayed for β -galactosidase activity. The levels of β -galactosidase activity increased approximately threefold under derepressing conditions in the wild type (Fig. 2A), results quite comparable to those seen for expression of PrB activity. In contrast, there was no increase in β -galactosidase activity in the *gln3* mutant grown in proline alone, confirming the results seen for PrB activity. In the *ure2* mutant, β -galactosidase levels still responded to repression by ammonia; the levels seen under repressing and nonrepressing conditions were roughly comparable to those seen for the wild type (Fig. 2A).

In order to determine whether the results obtained with the *PRB1-lacZ* reporter truly reflect differences in the transcription of *PRB1*, we performed Northern analysis for *PRB1* expression. We observed transcriptional activation of *PRB1* in wild-type cells grown to log phase in the absence of ammonia (Fig. 2B). In the *gln3* mutant, *PRB1* mRNA levels did not increase under nitrogen-limiting conditions. In the *ure2* mutant, the level of *PRB1* mRNA was still responsive to the presence of ammonia and regulation seemed to be unperurbed. The levels of *ACT1* mRNA were comparable across conditions and strains, ensuring that equivalent amounts of

A).



B).

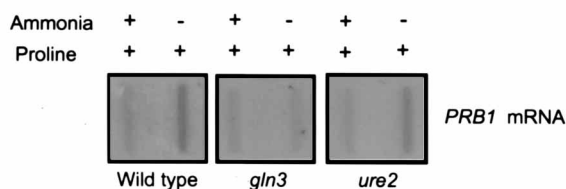


FIG. 2. Effect of nitrogen-regulatory mutations on *PRB1* expression. (A) Transcriptional activity of the *PRB1-lacZ* reporter construct was measured by β -galactosidase assay in BJ7140 (wild type), BJ7141 (*gln3*), and BJ8727 (*ure2*) grown to log phase (OD_{600} , 0.8 to 1.2). (B) Northern slot blot showing *PRB1* mRNA levels in the wild-type (*GLN3 URE2*), *gln3*, and *ure2* strains. Synthetic medium containing either 0.1% proline plus 0.5% ammonia (repressing) or 0.1% proline (derepressing) as the nitrogen source with 2% glucose as the carbon source was used for growing the strains.

RNA were loaded (data not shown). The results obtained by our Northern analysis agree with the results obtained with the reporter construct. Thus, the results in Fig. 2 show that Gln3p is required for the activation of *PRB1* transcription in cells grown on a poor nitrogen source and that Ure2p is not involved in the negative regulation of *PRB1*. Because *PRB1* transcription is repressed by good nitrogen sources, there must be a negative regulator. Two obvious candidates are Dal80p, which is proposed to compete with Gln3p for binding at the GATAA sequences of some NCR genes in the presence of a good nitrogen source (7), and Nil2p, which negatively regulates some NCR genes in the presence of glutamine and glutamate (37).

Glucose regulation of *PRB1*. Earlier studies on the regulation of *PRB1* suggested that glucose-mediated repression of *PRB1* did not seem to involve *HXK2* or the *SNF1*, *SNF4*, and *SNF6* genes (27). To extend these observations we tested the effects of mutations in the glucose regulators *MIG1* and *TUP1* (encoding nuclear proteins) and *GRR1* (encoding a cytoplasmic protein) on *PRB1* expression.

The *PRB1-lacZ* reporter was transformed into the wild type and the *mig1*, *tup1*, and *grr1* mutants. The transformants were grown under glucose-repressing conditions. Increases of about 3- and 3.3-fold in levels of β -galactosidase were seen in *mig1* and *tup1* mutants, respectively (Fig. 3A). The *grr1* mutant showed a twofold increase. The effect of *tup1* was not unexpected since Tup1p, along with Ssn6p, is recruited by Mig1p into a binding complex that causes repression in the presence

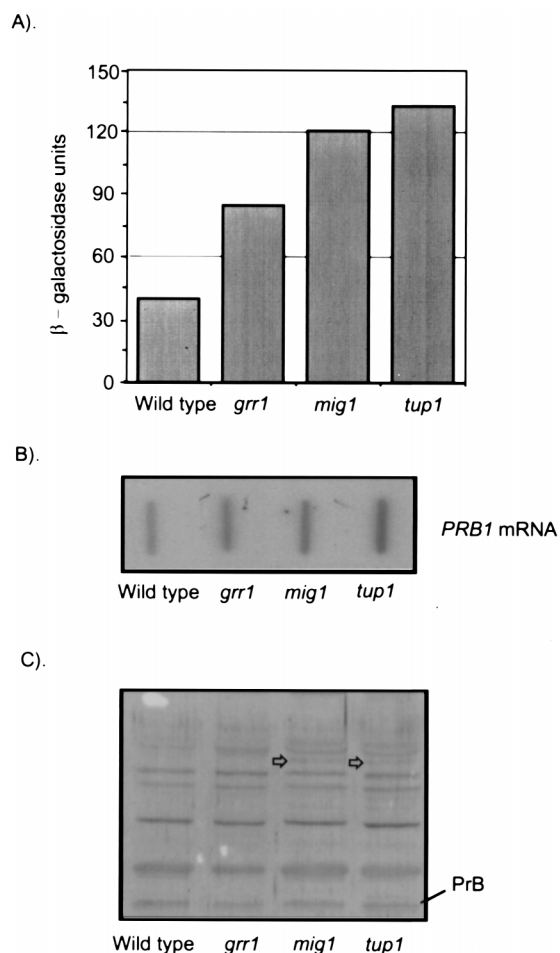


FIG. 3. Effects of glucose-regulatory mutations on *PRB1* expression. (A) Transcriptional activity of the *PRB1-lacZ* reporter construct was measured by β -galactosidase assay in strains BJ8317 (wild type), BJ8319 (*tup1*), BJ8316 (*grr1*), and BJ6977 (*mig1*) grown to log phase (OD_{600} , 0.8 to 1.2). (B) Northern slot blot showing *PRB1* mRNA levels in the wild-type, *tup1*, and *grr1* strains. (C) Immunoblot of cell extracts from strains with the indicated genotype to detect levels of PrB antigen by using anti-PrB. Cells were grown under glucose-repressing conditions (2% glucose in YEP). The 69-kDa precursor form of PrB (arrows) and the mature-size 31-kDa form (bar) are indicated.

of glucose (42). Recently, another zinc finger protein, Mig2p, was shown to be involved in the glucose repression of *SUC2* expression (19). The levels of β -galactosidase in the wild type and the *mig2* mutant were comparable (data not shown).

In agreement with the results for β -galactosidase, the *tup1*, *mig1*, and *grr1* mutants had increased levels of *PRB1* transcript as determined by Northern analysis (Fig. 3B). The *ACT1* mRNA levels confirmed that equivalent amounts of RNA were loaded (data not shown). These results suggest that these mutations cause a partial release from glucose repression, leading to increases in mRNA levels during log phase. It has been observed that increases in mRNA levels do not cause an immediate increase in PrB levels (25) (Fig. 1). For this reason, we decided to check the levels of PrB antigen in these mutants by immunoblot analysis (Fig. 3C). We observed little effect of the mutations on the levels of mature-size PrB antigen in the mutants. However, we did observe a larger form of PrB antigen, approximately 69 kDa, in the *mig1* and *tup1* mutants. The size of this species corresponds to that of the untranslocated form of the PrB precursor (Prb1p) (26). In wild-type cells, the

69-kDa PrB precursor is translocated into the lumen of the endoplasmic reticulum, where it is processed to yield a 40-kDa species. The 69-kDa form of Prb1p is a transient species that is never seen in immunoblots with wild-type-protein extracts. In order to determine whether an untranslocated form of PrB was accumulating, we assayed for translocation of a Prb1p-His4p-Ura3p fusion protein in the *tup1* and *mig1* mutants, using the *PRB1::his4::URA3* fusion reporter-bearing plasmid, pPHU1. The reporter enzyme Ura3p is involved in the biosynthesis of UMP and is required for growth in the absence of exogenously supplied uracil (35). Translocation of the fusion across the endoplasmic reticulum in wild-type cells sequesters Ura3p from the cytoplasm and renders *ura3* cells unable to grow on a medium lacking uracil. When translocation of Prb1p-His4p-Ura3p is blocked or slowed, the fusion remains in the cytoplasm, thereby allowing the cells to grow in the absence of uracil (28, 29). Both the *tup1 ura3* and *mig1 ura3* mutants carrying the fusion plasmid were able to grow on a medium lacking uracil (Table 2), suggesting that the precursor was accumulating in the cytoplasm of these mutants at 30°C; similar results were also obtained at 37°C. The wild-type *ura3* parent grew little at either temperature. Hence, increased transcription of *PRB1* in these mutants does not result in increased levels of PrB activity or mature-size antigen possibly because of a limitation in the cells' translocation capacity. As there is no evidence for a deficit in translocation capacity in the wild type at later growth stages when the flux through the pathway must be great (not just increased Prb1p but also Suc2p, etc.), these observations imply that synthesis of components of the translocation apparatus must be regulated vis-à-vis the growth stage and that the needed translocation components will comprise a larger fraction of total protein at late growth stages than at earlier growth stages.

Isolation of regulatory mutants. Our present study of the various regulatory mutants suggests that additional genes may be required for the regulation of *PRB1*. In order to identify these additional factors, we screened for early blue mutants that showed increased levels of β -galactosidase activity in cells bearing the *PRB1-lacZ* reporter (Table 3). Outcrosses of the mutants to the wild type indicated that all of the mutants contained single gene mutations, since altered β -galactosidase expression segregated 2:2 in all tetrads. Pairwise complementation tests indicated that the mutants fell into five complementation groups. Two *mig1* alleles and one *reg1* allele were obtained, as were alleles in three new genes: *PBD1* (1), *PBD2* (1), and *PBD3* (2) (*PRB1* derepressed). The *mig1* alleles resulted in increases of about 3.5- to 5-fold in β -galactosidase activity compared to the wild-type parent, and the *reg1* allele resulted in an increase of about 3-fold. We also transformed the *PRB1-lacZ* reporter into a strain carrying an allele of *reg1* (*reg1-501*) obtained from M. Johnston. Levels of β -galactosi-

TABLE 2. Growth properties of various strains carrying a *PRB1::his4::URA3* fusion plasmid

Strain (relevant genotype)	Growth on synthetic complete medium without uracil ^a	
	30°C	37°C
BJ8317 (wild type)	+	±
BJ6977 (<i>mig1</i>)	+++	+++
BJ8319 (<i>tup1</i>)	+++	++

^a Growth was scored on a scale from ± (little or no growth) to +++ (abundant growth). Results showed that some Prb1p-His4p-Ura3p fusion protein remains untranslocated in the *mig1* and *tup1* mutants.

TABLE 3. β -Galactosidase and invertase activities in *pbd* and *pnd* mutants

Mutation ^a	β -Galactosidase activity ^b	Invertase sp act ^c	
		Repressed	Derepressed
None (wild type)	13	<2	423
<i>pbd1</i>	58	<2	414
<i>pbd2</i>	48	<2	522
<i>pbd3</i>	73	64	1,171
<i>reg1</i>	41	96	1,507
<i>mig1</i>	64	60	1,496
<i>mig1</i> Δ :: <i>LEU2</i>	55	62	1,369
None (wild type) ^d	66	ND ^e	ND
<i>pnd1</i>	18	ND	ND
<i>pnd2</i>	38	ND	ND

^a All strains carrying these mutations also carry *PRB1*_{UAS}-*lacZ* integrated at the *ura3-52* locus. Strain background is either BJ6134 or BJ6239.

^b Activity expressed in Miller units.

^c Micromoles of glucose released/minute/OD of cells.

^d Mid-log phase.

^e ND, not determined.

dase were elevated about fourfold in the *reg1-501* mutant compared to those in its wild type (data not shown).

Because *MIG1* and *REG1* are known to be involved in the glucose repression of *SUC2* (15), we assayed the new mutants for alterations in invertase expression. As expected, the *mig1* and *reg1* mutants showed increased invertase activity under repressing conditions; the *pbd3* mutant did also (Table 3). However, the *pbd1* and *pbd2* mutants did not; *SUC2* was apparently regulated normally in these mutants.

Mutants that express lower levels of β -galactosidase were also identified; two recessive *PRB1* nonderepressible mutants (*pnd*) were recovered. The *pnd1* and *pnd2* mutants produced 27 and 46% as much β -galactosidase, respectively, as the wild type. Because the β -galactosidase assay was performed on cells grown to mid-log phase, the defect in derepression is manifested during the period in which *PRB1* normally undergoes transcriptional derepression. All of these newly isolated regulatory mutants showed differences in *PRB1* mRNA levels in accord with the data on β -galactosidase enzyme levels (data not shown).

DISCUSSION

Many studies through the years have indicated that the levels of vacuolar protease activities respond dramatically to changes in nutritional status. The nature of the carbon and/or nitrogen source, the nature of the medium (rich complex or synthetic), the growth stage, and whether the cells are actually starving all affect the levels of hydrolases (17, 39). It is clear that, at least in part, the protease levels reflect the cells' need for a recycling capability, for these vacuolar proteases play major roles in protein turnover and, in fact, determine the cells' ability to survive under stressful conditions (40). PrB is the key protease for protein degradation for cells under stress (39, 40, 49). Because of its importance to the cell, we elected to study the regulation of PrB production.

Two apparently distinct regulatory circuits that effect glucose repression have been described: the *HAP* complex, which regulates expression of *CYC1* (10), and the *SNF1*, *SNF4*, *SNF6*, *HXX2*, *MIG1*, *SSN6*, *TUP1* circuit, which governs expression of *SUC2* and the *GAL* genes (15, 34). In this circuit, the DNA binding protein Mig1p recruits the general repressor Tup1p and Ssn6p to shut off transcription of target genes in the pres-

ence of glucose (18, 33). The Snf1p-Snf4p protein complex acts to release selected genes from repression by the Mig1p-Tup1p-Ssn6p complex when glucose becomes limiting (46). Whether the Snf1p-Snf4p kinase actually phosphorylates one or more members of the repressor complex to effect release or acts indirectly is unknown, although it is known that Mig1p possesses fewer phosphoryl groups in an *snf1* mutant (41). The action of the Snf1p-Snf4p kinase is antagonized by a protein phosphatase, PP1; the catalytic subunit of this enzyme is encoded by *GLC7*, and the regulatory subunit is encoded by *REG1* (44, 45).

The results presented here suggest that glucose repression of *PRB1* is in part effected by the Mig1p-Tup1p-Ssn6p repressor complex, just as is glucose repression of the *SUC2* and *GAL* genes. We have recently found that the *glc7-T152K* mutant, which was identified as *cid1-226* among mutants selected for derepressed invertase expression (44), is moderately derepressed for *PRB1* expression (31). This result, together with the finding that *reg1* mutants are released from glucose repression of *PRB1*, suggests that the Glc7p-Reg1p protein phosphatase may be antagonizing the action of a protein kinase in regulating *PRB1* expression. If this hypothesis is correct, the identity of the protein kinase remains to be established, since it is not the Snf1p-Snf4p kinase (29); the *PND* genes are obvious candidates to encode the missing kinase.

When cells in a culture exhaust the glucose in the medium, the cells shift to another metabolic mode. The most obvious and immediate need is for enzymes to mobilize and metabolize the new carbon source and to synthesize ATP; hence the observed derepression of invertase, alcohol dehydrogenase, tricarboxylic acid cycle enzymes, cytochromes, and electron transport components (15, 34). Mechanisms to prioritize the genes to be derepressed and to determine the order of their release are required at this growth stage. Should the hypothesis of a shared repressor complex and a shared protein phosphatase with alternative protein kinases prove true, the cell efficiently makes use of common components where possible but can achieve prioritization by varying the protein kinase.

Hxk2p and Grr1p are thought to be part of the signal transduction pathway that senses glucose and transmits a signal somehow to Snf1p-Snf4p (43, 47). We observed a modest derepression of *PRB1* in the *grr1* mutant, but the *hxx2* mutation was reported to be without effect (27), leaving unclear how the signal that results in *PRB1* repression is transmitted. Possibly the *PBD* genes play a role.

We have identified three new genes required for glucose repression of *PRB1* expression. Two of these, *PBD1* and *PBD2*, appear to be specific for *PRB1* expression; *PBD3* affects both *PRB1* and *SUC2*. In addition, we have identified two new genes, *PND1* and *PND2*, that appear to be required for post-diauxic derepression of *PRB1*. Whether the study of these new genes will force alternate views of the regulatory circuitry for glucose repression awaits investigation.

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