Consequences of Growth Media, Gene Copy Number, and Regulatory Mutations on the Expression of the \textit{PRB1} Gene of \textit{Saccharomyces cerevisiae}

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\textbf{ABSTRACT}

Glucose represses \textit{PRB1} expression at the level of transcription. However, release from glucose repression initially does not result in accumulation of protease B (PrB) activity despite transcriptional derepression. PrB activity accumulates only upon a second transcriptional derepression as the cells approach stationary phase. Increasing the \textit{PRB1} gene dosage on \textit{2\mu}-based plasmids does not overcome glucose repression. Glucose-mediated repression of \textit{PRB1} is not subject to the same genetic controls as \textit{SUC2}. Mutation of the \textit{HXK2} gene, which confers glucose-insensitive expression of secreted invertase, had no effect on \textit{PRB1} expression at the level of PrB activity. Strains bearing a mutation in any of the \textit{SNF1-SNF6} genes cannot derepress secreted invertase synthesis, but did derepress PrB synthesis when grown in the absence of glucose. Mutation of the \textit{SNF2} or \textit{SNF5} gene led to accumulation of PrB activity to levels ten times that of wild type. Polymorphism for a suppressor gene was observed: in \textit{snf5}-bearing strains, one allele of this suppressor gene resulted in elevated levels of PrB and the other allele resulted in wild-type levels of PrB; neither allele suppressed the Suc− phenotype of the \textit{snf5} mutant. Re-examination of published data on \textit{SUC2} expression in \textit{snf2} and \textit{snf5} mutants and examination of \textit{PRB1} expression in these mutants paradoxically suggest that the \textit{SNF2} and \textit{SNF5} gene products might act as negative regulators of gene expression.

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\textit{Saccharomyces cerevisiae}, except that cAMP does not reverse the repression in yeast (\textit{Matsumoto} \textit{et al.}, 1990).
et al. 1982, 1983; Eraso and Gancedo 1984). Not all prokaryotes use cAMP to regulate carbon catabolite repression, however. Neither cAMP nor adenylate cyclase has been demonstrated in various species of Bacillus, yet these bacteria are subject to carbon catabolite repression (cited in Fisher and Magasanik 1984).

Much effort has been spent trying to determine the mechanism(s) of glucose repression in yeast. Most, but not all, of this work has used either the sucrose or galactose utilization pathways as model systems. A number of yeast mutants have been identified that are defective in aspects of catabolite repression [reviewed in Carlson (1987) and Johnston (1987), and references contained therein]. These studies have demonstrated that the straightforward molecular model of cAMP-mediated catabolite repression in E. coli does not apply to yeast (Matsumoto et al. 1982, 1983; Eraso and Gancedo 1984).

An important part of glucose signal transduction in yeast occurs at the level of glucose uptake and the phosphorylation of hexoses (reviewed in Carlson 1987). Wild-type yeast has low affinity and high affinity glucose (and fructose) uptake systems; the functioning of the high affinity system is itself subject to carbon catabolite repression (Bisson and Fraenkel 1984; Bisson 1988) and influenced by the hexose and glucose kinases (Bisson and Fraenkel 1983). The SNF3 gene product has been implicated in high affinity glucose uptake (Neigeborn et al. 1986; Bisson et al. 1987; Celenza, Marshall-Carlson and Carlson 1988). Function of the high affinity glucose/fructose transporter requires a functional kinase for the cognate hexose (Bisson and Fraenkel 1983). In yeast there are two hexokinases (PI and PII isozymes) and one glucokinase; they are encoded by the HXX1, HXX2 and GLK1 genes, respectively (Lobo and Maitra 1977; Maitra and Lobo 1983). Of these three kinases, loss of the hexokinase PII isozyme causes the cell to become resistant to glucose-mediated repression of SUC2, SUC3, CYC1, GAL10, MAL2, and presumably other repressible genes (Entian 1980; Entian and Mecke 1982; Ma and Botstein 1986).

The products of at least six genes, SNFI-SNFIN6, are required for the derepression of secreted invertebrate synthesis (Carlson, Osmond and Botstein 1981; Neigeborn and Carlson 1984). Suppressors of these snf mutations have also been isolated (Carlson et al. 1984; Neigeborn, Rubin and Carlson 1986). Based on similarities of subtle phenotypes and interactions with suppressor mutations, the SNF genes and their mutations are generally placed in three groups: snfl and snf4 mutants are similar; snf2, snf5 and snf6 mutants are similar; and, the snf3 mutant is by itself. The SNF1 gene encodes a protein kinase (Celenza and Carlson 1986); mutations at this locus are epistatic to the hxx2 mutations mentioned above (Neigeborn and Carlson 1984). The SNF3 gene encodes part of the high affinity glucose transporter (Bisson et al. 1987; Celenza, Marshall-Carlson and Carlson 1988). The functions of the other SNF gene products are not known. The snf2, snf5 and snf6 mutants show a limited derepression of invertase activity and fail to show high level derepression of acid phosphatase, which itself is not subject to glucose repression. Because of this, it has been suggested that the SNF2, SNF3 and SNF6 gene products are involved in high level gene expression and not intimately involved in glucose repression (Abrams, Neigeborn and Carlson 1986).

All the same, the SNFI-HXX2 mediated pathway of glucose repression and derepression is neither monolithic nor even entirely proven. Although most mutations of SNF genes confer a Gal+ phenotype, no suppressor of these snf mutations has been identified that can restore galactose utilization (Johnston 1987). Furthermore, it appears that derepression of SUC2 can occur in the absence of the SNFI gene product. A snfl snn1 double mutant undergoes glucose-mediated repression and derepression (Carlson et al. 1984). There are also a few indications that, in some genes that are repressed by glucose, this regulation is not mediated through the SNF gene products. While expression of PET494, a nuclear gene that is required for mitochondrial translation of cytochrome c oxidase subunit III (Costanzo and Fox 1986), is repressed by glucose, mutations in HXX2, SNI, SNF2 or SNF6 (a suppressor of snfl) have little effect on expression of PET494 (Marykwas and Fox 1989). Further evidence of multiple pathways of glucose repression has come from J. Flick and M. Johnston (personal communication and summarized in] Johnston 1987), who have reported that glucose repression of the GAL1 promoter can be separated into two independently acting components, one that acts upon the GAL4 binding site (UAS), and another that acts upon DNA sequences between the UAS and the TATA box.

In this communication, we present the results of studies on regulation of the PRB1 gene, as measured by accumulation of the transcript and of protease B activity, in response to growth conditions, gene copy number, and mutations in unlinked loci. These results show that synthesis of this transcript is tightly repressed by glucose, but is not subject to the same genetic regulation that has been reported for SUC2, another glucose-repressible gene. Derepression of PRB1 occurs in the snf1-snf6 mutants, and glucose-mediated repression of PRB1 occurs in an hxx2 mutant. In addition to repression at the level of transcript accumulation, at least two forms of posttranscriptional regulation of protease B expression occur: one that is
mediated through the PEP4 locus, and one that is mediated through a separate, albeit undefined, path.

MATERIALS and METHODS

Chemicals, media and supplies: Azocoll and Hide Powder Azure (HPA) were purchased from Calbiochem, San Diego, California, glass beads, 0.44-0.46 mm diameter, from Thomas Scientific, Swedesboro, New Jersey, protein dye reagent from BioRad, Richmond, California, and deoxyribonucleoside triphosphates from Pharmacia, Piscataway, New Jersey. Deoxyribonuclease I and β-glucuronidase (type H-1, from Helix pomatia) were from Sigma Chemical, St. Louis, Missouri, Glusulase from Endo Labs, Garden City, New Jersey, and Zymolyane through Miles Laboratories, Elkhart, Indiana. Nitrocellulose HAHY was from Millipore, Bedford, Massachusetts, most media supplies from Difco, Detroit, Michigan, and [α-32P]dCTP (>600 Ci/mmol) from New England Nuclear, Boston, Massachusetts. Klenow fragment of DNA polymerase I was the kind gift of William Brown, Carnegie Mellon University, Pittsburgh, Pennsylvania.

Media and growth conditions: All rich media for yeast contained, per liter, 10 g yeast extract and 20 g peptone (YPE) supplemented with one of the following: 20 g dextrose (YPD), 20 g potassium acetate (YPEA), 20 g raffinose (YPE-Raf), or 50 g glycerol (YPEG). All synthetic media were standard (Jones, Benko and Parker 1982). In most experiments using liquid media, cell growth was monitored as turbidity with a Klett-Summerton colorimeter (Arthur H. Thomas, Philadelphia, Pennsylvania). By empirical observation, our Klett meter had a linear response from approximately 20-120 units. A detailed correction curve was plotted by measuring the turbidity of one saturated culture and multiple dilutions of that culture. Turbidity values for all samples outside of the linear range were adjusted according to the correction curve, and the corrected values were plotted in each figure. Occasionally, cell growth was monitored as turbidity at 600 nm (A600) in a Zeiss PMQ3 spectrophotometer. An A600 of 1.0 corresponded to 40-45 Klett units.

For growth curve experiments with plasmid-bearing strains, cells were precultured at 25-30°C in standard synthetic media with the appropriate nutritional supplements. For the other growth curve experiments, cells were precultured at 25-30°C in YEPD medium. The precultures were transferred to 1 liter of YEPD or YEPDA in 2.8-liter Fernbach flasks and cultured at 30°C with vigorous aeration in a rotary air shaker (New Brunswick Scientific, New Brunswick, New Jersey). Samples were removed aseptically at the indicated times. For some experiments multiple cultures of 50 ml of YEPD in 300 ml side-arm flasks were used instead of the Fernbach flasks to allow turbidity measurements to be made without removing any of the culture.

For media-shift experiments (Celenza and Carlson 1984), the cells were precultured in 4 ml YEPD, transferred to 60 ml YEPD in 300-ml side-arm flasks, and cultured at 30°C in the air shaker. When the cultures reached mid-exponential phase (approximately 100 Klett units), they were split into two parts: 12 ml were added to 50 ml of YEPD in another flask; the other 48 ml were collected by centrifugation, resuspended in 10 ml of YEP-LD, and transferred to a fresh flask that contained 50 ml of YEP-LD. The YEPD cultures were harvested when they reached 100 Klett units; the YEP-LD cultures were harvested after 3 hr.

For an "end point" assay, 4 ml of YEPD in an 18 x 150 mm test tube was inoculated with a small colony of cells and cultured on a tube roller at 25-30°C. After 2.5 days, cells from 1.5 to 2.0 ml of the culture were harvested by centrifugation and washed once with 2 ml of deionized water. Unlike the other culturing conditions described above, the end point assay allowed all of the samples in a given exper-

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</tr>
<tr>
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<td>MATa his4-539, lys2-801, snf2-50 ura3-52 SU2/C7 Gal* [MCY637]</td>
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<td>MATa snf3-39 ura3-52 SU2/C7 Gal* [MCY600]</td>
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<td>MATa his4-539, snf4-319, SU2/C7 Gal* [MCY681]</td>
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<td>MATa leu2 ura3-52</td>
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<td>MATa leu2 [Prb+] [XCMM50-7D]</td>
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All strains were derived in this laboratory except where noted. All strains are congenic to X2180 and S288C.

* Obtained from M. Carlson and colleagues.

Genotypes presented in parentheses are inferred.
imment to be harvested at the same time. For this reason, washed cells from the end point samples were usually extracted and assayed fresh, without freezing, whereas washed cells from samples collected from growth curve and media-shift experiments were frozen at -20°C, and extracts were made and assayed later after thawing.

Protein extraction for assays: Each cell pellet (derived from about 50 O.D. units of cells) was resuspended in approximately 1.5 ml of 4°C extract buffer (0.1 M Tris, pH 7.6), and added to a 12 x 35 mm tube that contained 0.45 mm diameter glass beads. Care was taken to fill each tube to the rim so as to minimize foaming during the homogenization step. The cells were disrupted in a Braun homogenizer (Braun-Melsungen, West Germany) for 3 min at room temperature. Extracts were clarified by centrifugation at 27,000 x g for 20 min at 4°C. Supernatants were transferred to fresh tubes and kept on ice.

Enzyme and protein assays: Most assays were performed on duplicate samples to ensure consistent results. If the duplicate values varied by more than 25%, a third assay was performed. Protein concentrations were determined by the method of BRADFORD (1976) using the Bio-Rad protein dye reagent with BSA as a standard and absorbance at 595 nm. Protease B was assayed with Azocoll as a substrate as described (JUNI and HEYM 1968; ZUBenko, MITCHell and JONES 1979), with slight modification. The reaction mixture to be harvested at the same time. For this reason, washed cells from the end point samples were usually extracted and assayed fresh, without freezing, whereas washed cells from samples collected from growth curve and media-shift experiments were frozen at -20°C, and extracts were made and assayed later after thawing.

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plasmid pYACT1 contains the ACT1 gene (NG and ABELSON 1980). The plasmid pRB528 contains DNA from the HXK2 locus that has had the HXK2 gene replaced with the URA3 gene (MA and BOTSTEIN 1986).

**Nucleic acid manipulations:** DNA probes were labeled in vitro to $2 \times 10^6$ cpm/µg with [α-32P]dCTP by nick-translation using DNase I and the Klenow fragment of DNA polymerase I (RIGBY et al. 1977). For yeast RNA preparation, extreme care was taken to avoid ribonuclease contamination (MANIATIS, FRITSCH and SAMBROOK 1982). Total yeast RNA was prepared by the method of (KIRBY 1965) as modified (HEREFORD and ROUSH 1977; LARKIN 1985), subjected to electrophoresis on 1% agarose-MOPS-formaldehyde gels (LARKIN and WOOLFORD 1983), and transferred to nitrocellulose (THOMAS 1980; LAST, STAVENHAGEN and WOOLFORD 1984). The blots were hybridized overnight at 42°C in 2 ml (per 100 cm² of nitrocellulose) of 50% formamide, 5 × SSPE, 0.2% SDS, and 100 ng/ml denatured salmon sperm DNA. The blots were hybridized overnight at 42°C in 2 ml (per 100 cm² of nitrocellulose) of 50% formamide, 5 × SSPE, 1 × Denhardt’s solution (50 × is 1% BSA, 1% polyvinyl pyrrolidone, 1% Ficoll), 1% glycine, 0.1% SDS, and 100 µg/ml denatured salmon sperm DNA, and labeled probe (DAYS, BOTSTEIN and ROETH 1980). After hybridization, the blots were washed at 45°C: three times with 2 × SSPE, 0.2% SDS, and, one time with 0.2 × SSPE, 0.2% SDS.

** Autoradiography and densitometry:** Autoradiographs of RNA and DNA blots were made at −70°C using Kodak XAR or XRP film and intensifying screens. Densitometer tracings of autoradiographs were made using a Zeineh densitometer on multiple exposures of the same filter.

**RESULTS**

**Protease B activity as a function of growth stage and gene dosage in YEPD medium:** When we first obtained putative PRBl clones, we sought to confirm that the cloned DNA contained the PRBl gene by several independent experiments (MOEHLE et al. 1987a). For one of these experiments we measured protease B levels in transformed and untransformed strains. While PRBl-containing plasmids did confer an increase in protease B activity, this increase was not commensurate with the increased gene dosage. Because protease B activity levels are also lower in pep4/PEP4 strain than in a PEP4 homozygote (JONES, ZUBENKO and PARKER 1982), we tested whether co-introduction of the cloned PEP4 gene (WOOLFORD et al. 1986) on a high copy vector would allow protease B activity levels to be commensurate with PRBl gene dosage. As can be seen from the following experiment, protease B activity levels are dependent on PRBl gene dosage, PEP4 gene dosage, and growth conditions.

For this experiment, four isogenic strains that differed in the number of copies of the PEP4 and PRBl genes were constructed to study the effect of gene dosage on protease B expression (Figure 1). Varying the copy number of PRBl and PEP4 had a negligible effect on doubling time, final cell density, and the general shape of the growth curve. Regardless of plasmid content, the cultures grew exponentially with a doubling time of approximately 110 min until the glucose was exhausted (time = 20 hr), when growth abruptly was suspended. (At the plateau, the medium contained 0.1% glucose or less; data not shown, see also LILLIE and PRINGLE 1980). After a pause of 3–5 hr, exponential growth resumed, albeit at less than one-tenth the former rate. The experiment was stopped just as the cultures reached stationary phase (time = 64 hr, cf. Figure 6 in MOEHLE et al. 1987a). Varying the copy number of PRBl and PEP4 did have a significant effect on the levels of protease B.

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**TABLE 2**

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* Percentage of cells retaining this phenotype (plasmid).
activity, however. The lower set of curves in Figure 1 correspond to the levels of protease B activity that were measured for each time point. Consistent with our earlier work (Moehle et al. 1987a), protease B was undetectable in the untransformed cells growing exponentially on YEPD medium. Once the glucose was exhausted (at 20 hr), protease B activity derepressed slightly. As the culture approached stationary phase, a second and major derepression was observed (at about 40 hr).

For the pPEP4-transformed strain the only difference observed was an earlier onset of the second derepression (at about 30 hr). Therefore, under some conditions the PEP4 gene product, protease A, is rate-limiting for protease B expression. Higher PEP4 gene dosage had no effect on glucose-mediated repression or derepression, and had no effect on the level of protease B activity in stationary-phase cells.

Protease B activity in the pPRB1-transformed strain was repressed by glucose equally as well as in the untransformed strain, but the extent of derepression (at 20 hr) and the level of activity in stationary-phase cells was much greater.

The strain transformed with both plasmids showed normal glucose-mediated repression and derepression of protease B activity except that derepression (at 20 hr) was of a greater magnitude than for any of the other strains. For the double-transformant the second derepression started at an earlier time (at about 30 hr), and the final level of protease B activity was higher than for any of the other strains.

As would be expected for 2µ-based plasmids, significant plasmid loss was encountered in this experiment (Table 2), so the true effect of increased gene dosage would be underreported in Figure 1. For each time point from the transformed cultures, we calculated a protease B specific activity, correcting for plasmid loss (not shown). For the last time point, the pPRB1-transformed and the doubly transformed cultures would have exhibited PrB activities of about 1.0 units/mg and 5.4 units/mg, or approximately three and 16 times that of the untransformed culture, respectively. At this particular time point, the pPEP4 plasmid alone had a negligible effect on PrB activity.

**Protease B activity as a function of growth stage and gene dosage in YEPAc medium**: In the previous section, it might be argued that protease B expression was regulated by growth stage rather than by glucose repression. In order to address this point, the same strains were grown in rich medium containing acetate, rather than glucose, as the carbon source (Figure 2). In this experiment the transformed strains had a slightly faster doubling time than the untransformed strain (4.5 hr vs. 5.5 hr, some data not shown), but the final cell densities of the four cultures were essentially the same. The growth curve in Figure 2 is roughly analogous to the second leg of the growth curve in Figure 1. In both cases, the cultures were growing exponentially in a nonfermentable carbon source until some unspecified nutrient became rate-limiting and the cultures entered stationary phase. Surprisingly, all four cultures had comparable levels of protease B activity throughout this experiment, even though a significant fraction of the cells retained the plasmids, i.e., there appeared to be no gene dosage effect for the PRB1 structural gene in this experiment (not shown). The plot of protease B activity resembled that for the second leg of growth (post-diauxic) of the untransformed strain in Figure 1: a moderate level of protease activity was observed during exponential growth, followed by a derepression of activity as the cells approached stationary phase (at about 35 hr).

**Effect of snf mutations on protease B expression in media-shift experiments**: This and other work (Hansen et al. 1977; Moehle et al. 1987a) suggested that PRB1, like SUC2 (Carlson and Botstein 1982), was subject to glucose repression in rich medium. We
tested the expression of PRB1 in mutants that are
defective in regulation of other glucose repressible
genes. The snf1-snf6 mutants have been identified by
their sucrose non-fermenting phenotype. When
grown in low glucose medium, these mutants fail to
derepress expression of secreted invertase (CARLSON,
OSMOND AND BOTSTEIN 1981; NEIGEBORN AND CARL-
SON 1984).

As a preliminary experiment to determine whether
expression of PRB1 was governed by these genes,
protease B activity was measured in six snf mutants
and a SNF control strain that had been grown in a
media-shift experiment (described in MATERIALS AND
METHODS). Unfortunately, in this experimental para-
digm the SNF control showed a rather unimpressive
derepression of protease B activity (2–3-fold, data not
shown); most likely 3 hr was not a sufficient amount
of time for full derepression of protease B activity.
Since such a small derepression occurred in the SNF
control, it was not possible to determine if any of the
snf mutants failed to derepress protease B. Unexpect-
edly, under both conditions the snf2 and snf3 mutants
expressed 5–10-fold higher levels of protease B than
did the SNF control strain. The derepression ratio
was 2–3 times greater for the snf2 and snf3 mutants
also (data not shown).

Protease B activity as a function of growth stage
in snf mutants grown in YE PD medium: Since the
medium-shift paradigm was inadequate for measuring
protease B derepression, a different approach was
tried. A set of growth curve experiments, similar to
those depicted in Figures 1 and 2, were performed
with six snf mutants and a control SNF strain (BJ2502–
2507 and BJ2665, respectively) in order to assess the
kinetics of protease B derepression in these strains.
The six mutants could be placed in two groups based
on the kinetics of growth and accumulation of pro-
tease B activity.

The snf1Δ3, snf4–319 and snf6–719 mutants were
similar in their growth characteristics: they grew ex-
ponentially until they reached 350–450 Klett units,
entered a plateau, and reached a final turbidity of
500–600 Klett units. The kinetics of accumulation of
protease B activity were also similar for each of these
mutants: during exponential growth a constant low
level of activity was observed, followed by a sev-
fold derepression of activity as the cultures stopped
growing. The snf6 mutant is representative of this
group (Figure 3A). (It is difficult to see in Figure 3A,
but the protease B activity in the snf6 strain at 23 hr
and 43 hr is, respectively, two and three times that at
19 hr.)

We also tested a mutant bearing the snf1–28 point
mutation in this same experimental paradigm (Figure
4). The snf1–28 mutation is less deleterious to cells,
and presumably leakier, than snf1 deletion mutations
(L. NEIGEBORN AND M. CARLSON, personal communi-
cation). In our experiments, the snf1–28 mutant did
grow better than the snf1Δ3 mutant. The kinetics of
protease B expression of the two strains bearing dif-
ferent snf1 mutations were similar: there was a con-
stant low level of protease B activity observed during
exponential growth, followed by a wild type derepres-
sion as growth slowed down. Instead of ceasing growth
around 500 Klett units, however, the snf1–28 mutant
culture reached 800 Klett units before the experiment
was stopped. In keeping with this extended growth,
the snf1–28 mutant accumulated a higher level of
protease B than did the snf1Δ3 mutant. In summary,
accumulation of protease B activity in the snf1–28
mutant resembled that of an SNF strain.

The snf2, snf3 and snf5 mutants shared similarities
with one another, but were more heterogeneous than
the snf1-snf4-snf6 group. The growth curves for the
snf2 and snf3 mutants were similar to wild type. The
snf5 mutant grew less well. The kinetics of protease B
expression in the snf2 and snf3 mutants were different
from those of the SNF strain in that the mutants were
incompletely repressed in the presence of glucose and
derepressed much more rapidly (Figures 3B and 5).
The snf2 and snf5 mutants were alike in that they
expressed protease B activity on a grand scale: in both
of these mutants protease B activity reached ten times
the level observed in the SNF strain (Figure 3B). The
elevated protease B activity phenotype (Prbf) of the
snf2 and snf5 mutants was almost exactly the opposite
of that predicted from their Suc− phenotype.

Segregation analysis of Prb phenotypes in snf
mutants: To test for co-segregation of the Prb and
Snf phenotypes, each of the six snf mutants was
crossed to an SNF strain. Tetrads were dissected
and scored for all of the segregating genetic markers,
except for gal2. Because the growth curve experi-
ments showed that the difference between each snf
mutant and wild type was most noticeable in stationary
phase, all segregants and parents were tested in the
"end point" assay (described in the MATERIALS AND
METHODS).

The results of the Prb and Snf phenotype co-seg-
regation analyses for the snf1, snf2, snf3, snf4 and snf6
mutants are presented in Table 3. The snf1 and snf4
mutations had no discernible effect on protease B
activity. Various snf1 and snf4 segregants were lower
than, equal to, or higher than the SNF segregants with
respect to protease B activity in the end point assay.
The snf2 mutation cosegregated with a threefold in-
crease in protease B activity in 19 tetrads. The snf3
mutation cosegregated with a 50% increase in pro-
tease B activity in six tetrads. The snf6 mutation
cosegregated with a 50% reduction in protease B
activity in seven tetrads. While the Prb phenotypes of
the snf3 and snf6 mutants were repeatable, the mag-
FIGURE 3.—Expression of protease B activity as a function of growth stage and SNF genotype in YEPD-grown cells. Exponentially growing cultures were used to inoculate 1 liter of YEPD medium each. Growth was monitored as turbidity (open symbols) and cell-free extracts were assayed for protease B activity (closed symbols). Strains used were: (A) BJ2507 (snf6−719) and BJ2665 (SNF6); (B) BJ2503 (snf2−50), BJ2506 (snf5−18), and BJ2665 (SNF).

An unexpected result was obtained when the segregants from the snf5/SNF5 diploid were assayed for protease B activity. Three classes of tetrads were seen (Table 4): one class segregated 2 Snf+ Prb+:2 Snf− Prbf (parental ditype), a second gave 2 Snf+ Prb+:2 Snf− Prbf (nonparental ditype), and the third gave 2 Snf+ Prb+:1 Snf− Prb+:1 Snf− Prbf (tetratype). The ratio of these three classes (4:2:8, respectively) suggested that two genes were segregating that affected protease B levels: the first gene, snf5, conferred elevated levels of PrB expression; the second gene, called sup-s, suppressed the elevated protease B levels caused by the snf5 mutation.

Two diploids were constructed to test the suppressor hypothesis (Table 5). A diploid of the genotype snf5/snf5 sup-n/sup-s would be expected to give rise to tetrads that are all 0 Snf+4 Snf−, and 2 Prb+:2 Prbf+. Unfortunately, snf5 homozygotes do not sporulate (Abrams, Neigeborn and Carlson 1986), a result not changed by the heterozygous suppressor. A diploid heterozygous for snf5 and homozygous for the non-suppressing allele (sup-n, we have not tried to establish the dominance relationship of the two forms of the suppressor gene) should give rise to tetrads that all segregate 2 Snf+ Prb+:2 Snf− Prbf+, since the suppressor has no effect on levels of protease B activity in the SNF5 background. The SNF5 parent of this diploid came from one of the NPD tetrads in Table 4. All 12 tetrads assayed from this snf5 heterozygote, sup-n homozygote gave the expected ratio of 2 Snf+:2 Snf− Prbf+.

There are four possible explanations for the nature of this suppressor: (1) it is an allele of SSN20, a known suppressor of snf5 alleles (Neigeborn, Rubin and Carlson 1986); (2) it is an otherwise silent allele of PRBI that is not responsive to the loss of the SNF5 gene product; (3) it is an informational suppressor of the snf5 nonsense allele; or, (4) it is a novel snn mutation. We feel that this suppressor is not likely to be an allele of SSN20 because it does not confer either of two phenotypes common to all other mutant alleles of ssn20: there is no suppression of the raffinose growth defect of the snf5 mutant by sup-s, and the strains carrying the suppressor do not have a Tsm− phenotype (Neigeborn, Rubin and Carlson 1986). In order to establish whether the suppressor was an allele of PRBI, the original diploid in Table 4 was reconstructed using a spontaneous can1 derivative of the SNF5 parent. The can1 and PRBI genes are tightly linked (1 cM, Zubenko, Mitchell and Jones 1980); therefore, the two parental PRBI genes can be identified in the segregants by their growth response on arginine omission medium containing canavanine. If the suppressor of the Prbf+ phenotype were a PRBI allele, the Prbf+ phenotype should be tightly linked to the Can+ phenotype in this cross. This was not the case; the Prb and Can phenotypes segregated independently in six tetrads assayed (not shown), thereby
proving that the Prb† suppressor was not an allele of PRBI. It is not likely that this is a simple informational suppressor, because an amber allele of SNF5 was used in these experiments and two other amber mutations segregating in these crosses were not suppressed (his4-539, lys2-801). Even if this were an amber suppressor, a snf5 sup-s combination most likely would be analogous to having a missense allele of SNF5 that is showing aberrant regulation of its targets. This suppressor has not been tested for suppression of other snf5 alleles, nor for suppression of any snf2 alleles. We do not know the nature of this suppressor yet. The suppressor failed to show linkage to MAT, CAN1, LEU2, LYS2, SNF5 or URA3.

Requirement of an intact PRBI gene for the Prb† phenotype of snf2 and snf5 mutants: An snf2 and an snf5 mutant were each crossed to a prb1 mutant in order to verify that the Prb† phenotype of the snf mutants was due to increased protease B activity and not due to an increase in some minor and heretofore unknown yeast endoproteinase. For both crosses, all

FIGURE 4.—Expression of protease B activity as a function of growth stage and SNF1 genotype in YEPD-grown cells. Exponentially growing cultures of BJ5155 (snf1-28) and BJ2665 (SNF1) were used to inoculate multiple 500 ml flasks containing 50 ml of YEPD each. Growth was monitored as turbidity (upper curves). Samples were withdrawn at intervals, and cell-free extracts were assayed for protease B activity (lower curves). The growth and protease B activity curves presented are composites of three to five overlapping cultures. In this experiment, a slightly modified protease B assay was used. Instead of incubating the assay for 15 min and shaking the tubes once a minute, the assay was incubated for 1 hr and the tubes were shaken every 5 min. The protease B activity is expressed as the quotient of the absorbance from the Azocoll assay divided by the absorbance from the protein concentration assay.

four possible combinations of Prb and Snf phenotypes were observed (not shown). The recovery of segregants with the Snf Prb phenotype proved that the PRBl gene was necessary for protease B activity in the snf2 and snf5 mutants.

Transcriptional component of the snf2 and snf5 Prb† phenotype: Because expression of protease B potentially is subject to post-translational controls, e.g., processing of the Prb zymogen by protease A, it was important to determine whether the Prb† phenotype of the snf2 and snf5 mutants was correlated with increased steady state levels of the PRBl transcript. Cultures of SNF, snf2-50, and snf5-18 strains were grown in YEPD medium. Culture samples were taken at early and middle-to-late exponential phase. Total RNA from these culture samples was analyzed on Northern blots probed with labeled DNA from the PRBl and ACT1 genes. Figure 6 shows representative autoradiographic exposures of these probed RNA blots and densitometric tracings of the autoradiographs. The data show that the snf2 mutant had a significant level of PRBl mRNA during exponential growth on glucose, whereas the SNF strain had none. The snf5 mutant appeared to be intermediate between the SNF and snf2 strains.

Figure 7 presents the results from another, albeit nonoverlapping, attempt to address the issue of PRBl transcript levels in these same three strains. Essentially, this is the same experiment as the one just described, except that here all of the samples were
TABLE 3

Co-segregation analyses of the Prb phenotypes of snf1, snf2, snf3, snf4 and snf6 mutants

<table>
<thead>
<tr>
<th>Diploid</th>
<th>Tetrads Assayed</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>α snf1-Δ3</td>
<td>5</td>
<td>No discernible differencea</td>
</tr>
<tr>
<td>a SNF1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α snf2-250</td>
<td>19</td>
<td>2 Snf+ Prb+ : 2 Snf+ Prb+a</td>
</tr>
<tr>
<td>a SNF2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α snf3-39</td>
<td>6</td>
<td>2 Snf+ Prb+ : 2 Snf- Prb+b (50% increase)</td>
</tr>
<tr>
<td>a SNF3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α snf4-319</td>
<td>5</td>
<td>No discernible differencec</td>
</tr>
<tr>
<td>a SNF4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>α snf5-719</td>
<td>7</td>
<td>2 Snf+ Prb+ : 2 Snf- Prb-</td>
</tr>
<tr>
<td>a SNF6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The snf mutants used were BJ2502, BJ2503, BJ2504, BJ2505 and BJ2507, respectively. The SNF strain was BJ2665 in each case.

a Values ranged from 50% to 150% of the wild type control.

b In each experiment, the mean value for the snf2 segregants ranged from 190% to 280% of the mean value for the SNF2 segregants; the individual values for each genotype were within 25% of the mean.

c The mean value for the snf3 segregants was 160% of the mean value for the SNF3 segregants; the individual values for each genotype were within 25% of the mean.

d All values save one ranged from 75% to 200% of SNF4 parent.

e For most SNF6 segregants the URNA/Aano (protease B activity) ranged from 0.25 to 0.70; all snf6 segregants ranged from 0.07 to 0.25.

high through the rest of the growth curve and into stationary phase. For the snf2 mutant, protease B activity levels were mostly consistent with PRBl RNA levels.

Protease B expression in an hxk2 mutant: The HXK2 gene product is both a hexose kinase and a regulator of glucose repression in yeast (Entian 1980; Ma and Botstein 1986). Loss of this gene product results in constitutive synthesis of secreted invertase. The HXK2 gene was replaced by the URA3 gene in a plasmid containing HXK2 and contiguous DNA by Ma and Botstein (1986). We used this cloned disruption allele to replace the wild type HXK2 allele of the strain BJ3341. Disruption of the HXK2 locus was verified by probing a nitrocellulose blot of an electro-phoretogram of restriction enzyme digests of DNA from Ura4 transformants with a 32P-labeled HXK2 DNA fragment (not shown).

Figure 8 represents a growth curve experiment in which protease B expression of an hxk2 mutant (B3404) was compared to that of an HXK2 strain (B3541). Protease B expression was clearly repressed in the presence of glucose. After glucose exhaustion, the hxk2 mutant derepressed PrB activity with kinetics similar to that of the HXK2 strain. Therefore, the HXK2 gene product had little or no effect on protease B expression.

The hxk2 mutation had an interesting effect on the growth curve. The hxk2 strain grew exponentially on glucose at the same rate as wild type, but was able to shift from exponential growth on glucose to exponential growth on ethanol without a perceptible pause. This is perfectly in keeping with the constitutive expression of glucose repressible genes during growth on glucose by this mutant. The mutant grew more slowly on ethanol than did the HXK2 strain.

**DISCUSSION**

Summary of regulation of PRBl expression at the level of transcription and at the level of protease B activity: PRBl gene expression is repressed by glucose, but growth in rich medium without glucose is
not sufficient for full expression of this gene. Some condition associated with entry into stationary phase is required for full expression. Increasing the PRB1 gene dosage cannot overcome glucose repression, but under some circumstances can result in increased protease B activity in cells grown in the absence of glucose. Glucose-mediated repression and derepression of PRB1 is not subject to the same sort of control by regulatory genes (SNF1-SNF6, HXK2) that affect regulation of the expression of SUC2, the hallmark glucose-repressible gene of S. cerevisiae (Figure 9).

There appear to be at least two components of posttranscriptional regulation of PrB production. One component involves the PEP4 gene product protease A and is presumed to be the protease A-catalyzed processing of the PrB zymogen. In addition to protease A, there must be other, albeit unidentified, factors that exert posttranscriptional control of protease B expression, for it appears that transcription of PRB1 is uncoupled from production of active protease B as the cells enter the diauxic plateau: although mRNA levels rise upon glucose exhaustion, the levels of protease activity do not rise commensurately.

**Transcriptional regulation of PRB1 as a function of growth stage:** Expression of the PRB1 transcript varies greatly as cells progress from exponential to stationary phase on YEPD medium. The PRB1 transcript is expressed at negligible levels in yeast cells during exponential growth on glucose (Figures 6 and 7, see also MOEHLE et al. 1987a). As the cells deplete the glucose, a dramatic increase in the transcript level is observed (Figure 7 and MOEHLE et al. 1987a). Once the cells have entered the diauxic plateau, the level of the PRB1 transcript falls somewhat, but is still higher than during exponential growth (Figure 7 and other data not shown). As the cultures approach stationary phase, another dramatic increase in the transcript is observed (Figure 7).

**Regulation of protease B expression as a function of growth stage and gene copy number:** Consistent with the PRB1 transcript levels, protease B activity is repressed in cells growing in YEPD. Yeast cells that are growing exponentially in YEPD contain low-to-undetectable levels of protease B activity (e.g., Figure 1, also SAHEKI and HOLZER 1975; HANSEN et al. 1977; MOEHLE et al. 1987a). This lack of protease B activity is due to glucose repression and not some other aspect of exponential growth, because protease B activity is expressed during exponential growth on ethanol (the second stage of growth in Figure 1) or acetate as the carbon source (Figure 2, also HANSEN et al. 1977), but is not expressed during exponential growth on acetate plus glucose (HANSEN et al. 1977). Glucose repression of PRB1 is mediated at the level of transcription (Figures 6 and 7, also, MOEHLE et al. 1987a). The repression of protease B by glucose is a tight control, because increasing the PRB1 gene dosage with 2µ-
based plasmids did not overcome this repression (Figure 1).

Cells undergoing a diauxic shift from growth on glucose to growth on ethanol undergo a small derepression of protease B activity. After yeast cells that are growing exponentially on YEPD medium deplete the glucose, growth halts as the cells undergo a diauxic shift. During this quiescent period, protease B activity increases to a low but measurable level. Derepression of mRNA levels seems much greater than the increases in protease B activity, however (Figure 7). Increasing the PRB1 gene dosage with 2µ-based plasmids results in a 10- to 20-fold increase in this derepression of protease activity (Figure 1).

Full derepression of protease B activity does not occur until cells approach the stationary phase of growth. After cells have completed the diauxic shift from growth on glucose to growth on ethanol, the level of protease B activity stays constant for a while. As these cells near stationary phase, protease B activity increases another 3- to 10-fold (Figure 1, also SAHEKI and HOLZER 1975; MOEHLE et al. 1987a). The post-diauxic growth of YEPD-grown cells is analogous to the growth of cells in YEPAc. During exponential growth in YEPAc, protease B levels appear fairly constant. As these cells enter stationary phase, protease B levels increase by approximately threefold (Figure 2).

Cells with a higher PRB1 gene dosage undergo a similar but slightly different pattern of postdiauxic protease B expression. After these cells complete the diauxic shift, protease B levels actually go down by a third. This decrease in activity is too great to be accounted for by cytoplasmic dilution by cell division. This observation, together with the transcription pattern described above, implies that there is some active
mechanism for protease B translational control, inactivation, or turnover in postdiauxic cells, because the level of protease B activity does not adequately reflect the level of transcript accumulation. As the cells carrying extra copies of PRB1 enter stationary phase, protease B activity increases at least twofold, which is similar to the increase observed with one copy of PRB1 (Figure 1 and MOEHLE et al. 1987a).

**Protease B expression in regulatory mutants:** Control of expression of protease B activity differs from that of secreted invertase in the regulatory mutants snf1–snf6 and hxx2. The most important point to make about these mutants is that once the glucose was exhausted, derepression of PRB1 expression occurred in all six of the snf mutants. For snf1, snf4 and snf6, the final level of protease B activity appeared to be less than wild type, but this can be explained as a consequence of trying to grow cells too long under non-permissive conditions (i.e., no glucose for much longer than 3 hr). Curiously, strains that had mutations at five of the six snf loci (snf5 being the exception) express higher levels of protease B during growth on glucose than do our laboratory SNF strains; in the snf1, snf4 and snf6 mutants this was still a very low level of expression. We have not rigorously tested whether this is conferred by the snf mutations, but we do not believe that this is simply a difference in strain background, as all of these strains were derived from a common genetic background (S288C/X2180) and the snf5 mutant behaved like wild type during exponential growth.

The SNF3 gene product has been implicated in high affinity glucose transport in S. cerevisiae, both biochemically and by its sequence similarity to a human glucose transporter (Bisson et al. 1987; Celenza, Marshall-Carlson and Carlson 1988). The level of protease B activity of snf3–39 cells growing under repressing conditions was about equal to that of SNF3 cells at the end of the diauxic plateau (Figure 5). This mutant experienced a significant derepression of protease B activity as the culture reached the end of the exponential phase of growth. Stationary phase protease B activity levels in the mutant were approximately 50% higher than in the SNF3 strain.

Protease B expression in the snf3 mutant is exactly as would be predicted for a high affinity glucose transporter mutant: as the glucose concentration becomes low and the cell must rely on the high affinity uptake system (late exponential phase), the cell perceives a lack of glucose, rather than low glucose, and derepression occurs sooner. What is not clear is why this mutant should fail to regulate secreted invertase. It was reported that snf3 null mutants have a different Suc phenotype than do the point mutants, that is, the null mutants show the predicted regulation of the SUC2 gene (Neieborn et al. 1986). It has been suggested that the SNF3 gene product plays a regulatory role in glucose repression (Neieborn et al. 1986), but recently the snf3–39 bearing strain has been found to carry a second mutation, tightly linked to the snf3–39 allele, that interacts with it to produce an altered repression response. Thus the discrepancy may now be resolved (M. Carlson, personal communication).

Protease B expression in snf2 and snf5 mutants was very different from that expected. Both SNF2 and SNF5 appear to be negative regulators of PRB1, whereas they are positive regulators of SUC2. Rather than failing to derepress protease B activity, as might have been predicted, stationary phase cells of these two mutants have ten times as much protease B activity as do SNF strains (Figure 3B). During exponential growth, protease B levels in the snf2 mutant are comparable to those of a SNF2 strain in stationary phase. Once this mutant runs out of glucose, protease B levels undergo a dramatic derepression and the levels continue to increase until the culture reaches

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**FIGURE 8.—Expression of protease B activity as a function of growth stage and HXX2 genotype in YEPD-grown cells.** Exponentially growing cultures of BJ3404 (hxx2 Δ:URA3) and BJ3341 (HXX2) were used to inoculate multiple 300 ml flasks containing 50 ml of YEPD each. Growth was monitored as turbidity (open symbols). Samples were withdrawn at intervals, and cell-free extracts were assayed for protease B activity (closed symbols). The growth and protease B activity curves presented are composites of three to five overlapping cultures. In this experiment, a slightly modified protease B assay was used. Instead of incubating the assay for 15 min and shaking the tubes once a minute, the assay was incubated for 1 hr and the tubes were shaken every 5 min. The protease B activity is expressed as the quotient of the absorbance from the Azocoll assay divided by the absorbance from the protein concentration assay.
FIGURE 9.—Transduction of glucose concentration into gene expression in yeast. External glucose (GLC) enters the cell via a low affinity, high $K_m$ transporter (open box) or a high affinity, low $K_m$ transporter (encoded in part by the $SNF3$ gene). Once the glucose is inside the cell, either the $GLK1$, $HXK1$ or $HXK2$ gene products phosphorylate it to glucose-6-phosphate (GLC-P). Entry of glucose via the high affinity transporter requires that at least one of these three kinases be present. During growth on high concentrations of glucose, the $HXK2$ gene product causes efficient repression of secreted invertase but not protease B. The $HXK1$ gene product causes an inefficient repression, and the $GLK1$ gene product has no effect on secreted invertase. The effect of the last two kinases on $PRB1$ is unknown, but $PRB1$ expression is repressed during growth on high concentrations of glucose. During growth on low concentrations of glucose, both secreted invertase and protease B expression are derepressed at the level of transcription. During growth on high concentrations of glucose, partial repression of both cytoplasmic invertase and protease B requires the $SNF2$ gene product. During growth on low concentrations of glucose, partial repression of both cytoplasmic invertase and protease B requires the $SNF2$ and $SNF3$ gene products.

stationary phase. In the $snf5$ mutant, expression of protease B activity was similar to that of the $SNF5$ strain during the exponential and diauxic plateau growth stages (Figure 3B). Once this mutant resumed growth however, protease B activity accreted unabated through the rest of the experiment, even after the cells reached stationary phase, in contrast to the wild-type strain, in which stationary phase cells normally maintain a constant protease B specific activity. The effects of the $snf2$ and $snf5$ lesions do not appear to be additive because stationary phase cultures of $snf2$ $snf5$ double mutants have levels of protease B activity comparable to the single mutants (data not shown). This is consistent with the hypothesis that these two genes act on a common point in the regulatory circuit (Neigeborn and Carlson 1984; Neigeborn, Rubin and Carlson 1986). It is interesting that Bisson (1988) recently found that levels of the glucose-repressible, high affinity glucose transporter are substantially higher in a $snf5$ mutant than in the $SNF$ strain. The $snf2$ mutation had little effect on expression of the transporter, positive or negative. Thus, there already may be three different response patterns for the $SNF2$ $SNF5$ gene pair.

Transcriptional control of $PRB1$ in $snf2$ and $snf5$ mutants: Because the $snf2$ and $snf5$ mutations had such dramatic effects on the regulation of protease B expression, we also investigated their effects on $PRB1$ transcription. Substantially more $PRB1$ transcript was expressed in the $snf2$ strain than the $SNF$ strain during exponential growth on glucose (Figures 6 and 7). As the glucose was exhausted, this mutant showed a sharp increase in $PRB1$ transcript level. The level of transcript stayed fairly constant throughout the rest of the experiment with no further derepression as the culture entered stationary phase. (This mutant does not appear to undergo a pause in growth when the glucose is depleted.) Similar to the $SNF$ strain, the $snf5$ mutant had negligible and low levels of $PRB1$ transcript during the early and late exponential growth stages, respectively (Figures 6 and 7). Unlike the $SNF$ strain, the $snf5$ mutant underwent only a mild derepression of $PRB1$ transcription at the end of exponential growth. After this mutant left the diauxic plateau stage, a sharp increase in $PRB1$ transcript level was observed.

In order to explain the combined data on $PRB1$ and protease B expression represented in Figures 3B, 6 and 7, it would be helpful to first recall and distill these results into the simple summary shown in Table 6. In more descriptive terms: during exponential growth on glucose, $PRB1$ transcription is repressed by
the SNF2 gene product in combination with other factors that do not include the SNF5 gene product. This reasoning comes from the observations that PRBI is expressed in the presence of glucose in the snf2 mutant, but not in the snf5 mutant or SNF strain. As the glucose becomes depleted, PRBI expression is not fully derepressed regardless of SNF2 or SNF5 genotype, because the other unknown factors still effect some contribution to PRBI repression in the absence of the SNF2 or SNF5 gene products. After the diauxic shift is over and the cells have resumed exponential growth on ethanol, repression of PRBI begins again (Figure 7), this time mediated by both the SNF2 and SNF5 gene products, since neither the snf2 nor snf5 mutants fully undergo this second repression. In this model, the SNF2 gene product could act with, on, or independently of the SNF5 gene product. Consistent with this model, it has been reported that transcription of SNF2 and SNF5 are not affected by glucose (ABRAMS, NEICEBORN and CARLSON 1986), so their respective gene products are probably present in the cell at all stages of growth.

After forming this model and then rereading the published reports regarding these mutants, we came to realize that this model is capable of explaining otherwise inexplicable observations from M. CARLSON'S laboratory (ABRAMS, NEICEBORN and CARLSON 1986). This group reported that mutants carrying null alleles of SNF2 or SNF5 failed to undergo high level derepression of the 1.9-kb SUC2 mRNA (encoding the repressible, secreted form of invertase), which is consistent with earlier observations on derepression of secreted invertase activity in EMS-induced mutants (NEICEBORN and CARLSON 1984). The new and here-tofore unaccountable observation they reported was that the 1.8-kb SUC2 mRNA (nonrepressible, intracellular form of invertase) was expressed at elevated levels in either a snf2 or a snf5 mutant grown in derepressing conditions. Under repressing conditions, the snf5 mutant did not contain abnormal levels of the 1.8-kb SUC2 mRNA (ABRAMS, NEICEBORN and CARLSON 1986). (The amount of 1.8-kb mRNA in a repressed snf2 mutant was not reported.) As far as it goes, this description of regulation of the 1.8-kb SUC2 message in snf2 and snf5 mutants exactly parallels our description of regulation of the PRBI message (Figure 9 and Table 6). We propose that the real function of the SNF2 and SNF5 gene products is to be negative regulators or modulators of transcription. The reason that these gene products appear to be positive regulators of secreted invertase is that, in their absence, the 1.8-kb transcript is synthesized preferentially. A corollary of this model is that the SNF2 and SNF5 gene products direct transcription initiation toward the promoter for the 1.9-kb transcript. A similar model for the mechanism of CRP activation of the E. coli lac operon has been reported (MALAN and MCCLURE 1984; MALAN et al. 1984). A promoter competition model has also been proposed to explain the interaction of an insertion element with various his4 alleles in S. cerevisiae (HIRSCHMAN, DURBIN and WINTON 1988).

Most likely the SNF2 and SNF5 gene products affect protease B expression at another level as well, because protease B activity in the snf2 and snf5 mutants accumulates to ten times the level found in the wild type, but the PRBI transcript accumulates to a level that is comparable to wild type. This could easily be a secondary effect of the snf mutations, however. For instance, the mutants may fail to derepress the protease B inactivation system discussed above.

**Expression of protease B in an hxk2 mutant:** Protease B expression was examined in another regulatory mutant, namely a strain bearing a hxk2 null mutation. The HXK2 gene encodes the hexokinase P2 isozyme (LOBO and MAITRA 1977). Mutants that are defective for this isozyme are defective in carbon catabolite repression (ENTIAN 1980). Because these mutants constitutively express α-glucosidase, maltase, invertase, a CYC1-lacZ fusion, and a GAL10-lacZ fusion (ENTIAN 1980; MA and BOTSTEIN 1986), it was suggested that the hxk2 mutant might also constitutively express protease B activity. Because the kinetics of protease B expression in the HXK2 and hxk2-202::URA3 strains were indistinguishable (Figure 8), the HXK2 gene product cannot be required for glucose-mediated repression of PRBI. This does not eliminate the participation of a hexokinase in the regulation of PRBI, however. MA and BOTSTEIN (1986) reported that the HXK1 gene on a 2μ plasmid can partially restore glucose-mediated repression of SUC2 in an hxk1 hxk2 mutant. Initially this particular result was overlooked because we did not critically examine the common knowledge that hexokinase P2 is the regulatory isozyme. Nonetheless, it is apparent from the results reported by MA and BOTSTEIN (1986) that the hexokinase P1 isozyme can inefficiently mediate glucose repression of SUC2. Although it is not generally discussed, the data of MA and BOTSTEIN (1986) indicate that the HXK1 gene plays a minor role in mediating glucose repression of SUC2, CYC1 and

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**TABLE 6**

Summary of PRB expression in snf2 and snf5 mutants

<table>
<thead>
<tr>
<th></th>
<th>expon.</th>
<th>end</th>
<th>diaux.</th>
<th>expon. ethanol</th>
<th>station.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SNF</td>
<td>repr.</td>
<td>derepr.</td>
<td>repr.</td>
<td>repr. → derepr.</td>
<td>derepr.</td>
</tr>
<tr>
<td>snf2</td>
<td>+/-</td>
<td>derepr.</td>
<td>derepr.</td>
<td>derepr.</td>
<td>derepr.</td>
</tr>
<tr>
<td>snf5</td>
<td>repr.</td>
<td>repr.</td>
<td>repr.</td>
<td>derepr. → derepr.</td>
<td>derepr.</td>
</tr>
</tbody>
</table>

Abbreviations and symbols used: expon., exponential; gluc., glucose; diaux., diauxic plateau; station., stationary phase; repr., repressed; derepr., derepressed; →, changes during this growth phase; +/-, intermediate value.
GAL10. Glucose repression of these genes is leakier in an hxk1 strain than it is in the isogenic HXK1 strain, and it is also leakier in an hxk1 hxk2 strain than in the isogenic HXK1 hxk2 strain. Furthermore, HXK1 on a high copy plasmid partially restores glucose repression of SUC2 in an hxk1 hxk2 strain: a 2μm::HXK1 plasmid by itself causes a tenfold reduction in invertase activity during growth on glucose, whereas a 2μm::HXK2 plasmid by itself causes a 100-fold reduction (MA and BOTSTEIN 1986). This opens the possibility that HXK1 and HXK2 represent the first divergent step in overlapping global networks of glucose repression. Both hxk1 single and hxk1 hxk2 double mutants should be examined for regulation of protease B expression.

Analysis of the regulation of protease B production must ultimately account for the regulatory circuit that effects transcriptional regulation, the gene products that govern posttranscriptional regulation and/or processing, and the mechanisms responsible for loss of activity at rates faster than cell division. About the first and last of these three levels we have no information at all. One event of proteolytic processing, the protease A-catalyzed processing at the C terminus of the PrB zymogen (MOEHLE, DIXON and JONES 1989), is presumed to be one component of posttranscriptional regulation. Whether the second component of this regulation will prove to be the proteolytic processing of the PrB zymogen N terminus, catalyzed by an as yet unidentified protease (MOEHLE, DIXON and JONES 1989), and/or regulation of translation should be resolved by future experiments.

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LITERATURE CITED


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