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Protease B of *Saccharomyces cerevisiae*: Isolation and Regulation of the *PRB1* Structural Gene

Charles M. Moehle, Martha W. Aynardi, Michael R. Kolodny, Frances J. Park and Elizabeth W. Jones

Department of Biological Sciences, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213

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ABSTRACT

We have isolated the structural gene, *PRB1*, for the vacuolar protease B of *Saccharomyces cerevisiae* from a genomic library by complementation of the *prb1-1122* mutation. Deletion analysis localized the complementing activity to a 3.2-kilobase pair *XhoI-HindIII* restriction enzyme fragment. The fragment was used to identify a 2.3-kilobase mRNA. S1 endonuclease mapping indicated that the mRNA and the gene were colinear. No introns were detected. The mRNA is of sufficient size to encode a protein of about 69,000 molecular weight, a number much larger than either the mature enzyme (≃30,000 protein molecular weight) or the sole reported precursor (≃39,000 protein molecular weight). These results suggest that proteolytic processing steps beyond that thought to be catalyzed by protease A may be required to convert the initial glycosylated translation product into mature protease B. The *PRB1* mRNA is made in substantial amounts only when the cells have exhausted the glucose supply and enter the diauxic plateau. There is an extended time lag between *PRB1* transcription and expression of protease B activity. A deletion that removes about 83% of the coding region was constructed as a diploid heterozygote. Spores bearing the deletion germinate, grow at normal rates into colonies, and have no obvious phenotype beyond protease B deficiency.

THE vacuole of the yeast *Saccharomyces cerevisiae* contains a number of the major hydrolases of the cell including several proteases, a repressible alkaline phosphatase and most of the cellular RNase activity (WIEMKEN, SCHELLENBERG and URECH 1979). The vacuole is an acidic compartment (SALHANY *et al.* 1975; NICOLAY *et al.* 1982) that is thought to share features with lysosomes of animal cells. The levels of the vacuolar hydrolases vary with the carbon and nitrogen sources supplied to cells (DISTEL *et al.* 1983; HANSEN *et al.* 1977; SAHEKI and HOLZER 1975). They also vary with growth stage, reaching maximum levels when the cells approach stationary phase (FREY and ROHM 1978; SAHEKI and HOLZER 1975; TRUMBLY and BRADLEY 1983), a phenomenon thought to reflect a release from carbon catabolite repression.

Most, if not all, of the vacuolar hydrolases are glycoproteins and are synthesized first as inactive precursors [see ACHSTETTER and WOLF (1985) and JONES (1984) for reviews]. Like externally secreted proteins, precursors to vacuolar hydrolases pass through the endoplasmic reticulum and the Golgi membranes. The vacuolar hydrolase precursors are sorted from secreted proteins within the Golgi and pass along a route that leads to the vacuole (STEVENS, ESMON and SCHEKMAN 1982).

One of the vacuolar hydrolases is the endoproteinase protease B (LENNEY *et al.* 1974; MATERN, BETZ and HOLZER 1974). Protease B activity is known to participate in the nitrogen starvation-induced protein degradation seen in vegetative cells, in cells in sporulation medium and during ascus maturation (WOLF and EHMANN 1979; ZUBENKO and JONES 1981). Protease B is a glycoprotein of about 33,000 molecular weight that contains one glycosidic side chain (KOMINAMI, HOFFSCHULTE and HOLZER 1981; MECHLER *et al.* 1982a). One kinetic precursor to protease B of 42,000 molecular weight has been detected (MECHLER *et al.* 1982a). The expression of protease B activity is known to depend upon the levels and function of protease A, the product of the *PEP4* gene (AMMERER *et al.* 1986; JONES, ZUBENKO and PARKER 1982; JONES *et al.* 1981, 1986; WOOLFORD *et al.* 1986), apparently because protease A activity is required for proper activation of the protease B zymogen (AMMERER *et al.* 1986; MECHLER *et al.* 1982b; WOOLFORD *et al.* 1986). In the absence of protease A activity in the *pep4-3* mutant, the 42,000 molecular weight precursor accumulates (MECHLER *et al.* 1982b).

Activity of protease B increases as the cells approach the end of the growth phase (SAHEKI and HOLZER 1975; JONES *et al.* 1986). Levels rise at least 300 fold during the entire derepression (JONES *et al.* 1986). To facilitate investigation of the mechanism(s) of regulation of this protease and of the pathway of processing and localization of this enzyme, we have isolated its structural gene. We report here the cloning of *PRB1*, the structural gene for protease B (ZUBENKO, MITCH-

TABLE 1

***S. cerevisiae* strains used in this study**

Strain	Genotype
BJ1308	α *leu2 prb1-1122 prc1-407*
BJ1825	α *leu2 ura3-52 trp1 prb1-1122 prc1-407*
BJ2341	α *leu2 prb1-1122*
BJ2378	BJ1825 into which plasmid FP8 was integrated
BJ2555	BJ1825 into which plasmid FP8ΔS was integrated
BJ2665	**a** *leu2 ura3-52*
BJ3040	**a** *ade2-101 his4-539 lys2-801 ura3-52*
BJ3041	α *trp1 leu2 ura3-52 can1*
BJ3042	BJ3040 × BJ3041
BJ3043	BJ3042 transformed with YEp24 and the linear PRB1-Δ1.6R containing fragment. This diploid is *PRB1 CAN1/prb1-Δ1.6R can1*

ELL and JONES 1979, 1980), and provide evidence that at least part of the regulation of this enzyme is at the level of transcription. The data also indicate that there is a very substantial time lag between increased transcription of the gene and the detection of increased protease B activity.

MATERIALS AND METHODS

Materials. Restriction enzymes, T4 DNA ligase, T4 DNA polymerase, the Klenow enzyme, DNase I and calf alkaline phosphatase were purchased from New England BioLabs, Inc., Bethesda Research Laboratories, Inc., or Boehringer Mannheim Biochemicals and were used according to the directions of the manufacturers. Polynucleotide kinase and deoxynucleoside triphosphates were purchased from Pharmacia P-L Biochemicals; *E. coli* DNA polymerase I was a kind gift from WILLIAM E. BROWN, Carnegie Mellon University. [α-^{32}P]dATP (3000 Ci/mmol), [α-^{32}P]dCTP (600 Ci/mmol) and NENSorb were purchased from New England Nuclear Corporation; [γ-^{32}P]ATP (7000 Ci/mmol) was obtained from ICN Biomedicals Inc. Glusulase was purchased from Endo Labs or Sigma Chemical Company; S1 nuclease, *p*-aminosalicylic acid, *m*-cresol, *N*-acetyl-DL-phenylalanine β-naphthyl ester, Fast Garnet GBC, *N*-benzoyl-L-tyrosine *p*-nitroanilide and ampicillin from Sigma Chemical Company; Hide Powder Azure (HPA) and Azocoll from Calbiochem; polyethylene glycol 4000 and 6000 from BDH Chemicals, Ltd.; triisopropylnaphthalene sulfonic acid from Scientific Products; NACS columns and penicillin-streptomycin solution from Gibco-BRL Laboratories; Bio-Rad protein dye reagent from Bio-Rad Laboratories; and γ-hydroxylquinoline from Fisher Scientific. Nitrocellulose type HAHY paper was obtained from Millipore Corporation.

Media. YEPG (ZUBENKO, MITCHELL and JONES 1980) YEPD and synthetic media (ZUBENKO, PARK and JONES 1982) for yeast cultures, LB medium and LB medium supplemented with ampicillin (DAVIS, BOTSTEIN and ROTH 1980) were prepared and used as described previously.

Strains. Relevant yeast strains and their genotypes are presented in Table 1. All yeast strains were derived in our laboratory from strain X2180-1B (α *gal2 SUC2*) or from crosses between the strains in our isogenic series and strains congenic to strain X2180-1B obtained from D. BOTSTEIN and M. CARLSON. Bacterial strains JA226 hsdR$^-$ hsdM$^+$ (DEVENISH and NEWLON 1982), HB101 and RR1 (MANIATIS, FRITSCH and SAMBROOK 1982) were used to propagate plasmids.

Plasmids. The parent plasmids were YEp24 (BOTSTEIN *et al.* 1979), YEp13 (BROACH, STRATHERN and HICKS 1979) and YCp50 (KUO and CAMPBELL 1983). The original complementing plasmids FP8 and FP34 are derivatives of YEp13; MK4 and MK16 are derivatives of YEp24. Deletion derivatives of MK4, MK4G1 and MK4G2, were made by partial digestion of MK4 with *Bgl*II followed by ligation. The procedures have been described previously (MANIATIS, FRITSCH and SAMBROOK 1982). The *Kpn*I deletion of MK4G1, MK4G1ΔK, was made by cleaving MK4G1 with KpnI, destroying the *Kpn*I site with T4 DNA polymerase plus excess dNTPs, and ligating the product. FP8ΔH is a derivative of FP8 deleted for sequences between the *Hin*dIII site in the insert and the *Hin*dIII site within the pBR322 sequence to the right. FP8ΔS, a derivative of FP8 deleted for the sequences between the *Sac*I site in the insert rightward through the 1998-bp *Eco*RI-*Pst*I fragment of 2 μ to the *Sac*I site in the *Pst*I fragment that carries *LEU2*, was constructed by cleaving with *Sac*I, destroying the *Sac*I site with T4 DNA polymerase plus excess dNTPs, and ligating the product. FP8ΔS lacks all save 245 bp of the 2 μ sequences, including the 2 μ ori and the 599-bp sequence that comprises half of the inverted repeat in the intact 2 μ molecule. The 3.2-kb *Xho*I-*Hin*dIII fragment of MK4G1 was subcloned into *Sal*I-*Hin*dIII cut YCp50 to give CM5XH. CM5XHΔC is a derivative of CM5XH deleted for the sequences between the leftmost *Cla*I site of the insert and the *Cla*I site in the YCp50 vector. CM5XHΔ1.6R was constructed by partial digestion of CM5XH with *Eco*RI followed by ligation. In this plasmid the 1.6-kb *Eco*RI fragment has been deleted but the 100-bp *Eco*RI fragment has been retained. The 4.9-kb *Hin*dIII fragment from MK4G1, extending from a *Hin*dIII site within the YEp24 vector to the left of the insert in Figure 1 to the leftmost *Hin*dIII site within the insert was subcloned into the *Hin*dIII site in YCp50 to give CM5HH. The fragment in CM5HH was so oriented that cleavage of CM5HH with *Cla*I at the internal *Cla*I site of the insert and the *Cla*I site of the vector YCp50 followed by ligation yielded CM5HHΔC as depicted in Figure 1. CM7 is a derivative of pBR325 (BOLIVAR 1978) containing the 1.6-kb *Eco*RI fragment from CM5HX subcloned into the *Eco*RI site.

Nucleic acid preparation. Bacterial plasmid DNA was purified from Brij-deoxycholate-treated bacterial spheroplasts by cesium chloride-ethidium bromide density gradient centrifugation (PETES *et al.* 1978). DNA minipreparations were made by the alkaline lysis method of BIRNBOIM and DOLY (1979). Yeast genomic DNA was prepared from spheroplasts (DAVIS *et al.* 1980) as described by LAST, STAVENHAGEN and WOOLFORD (1984). Yeast RNA was prepared from cells harvested after growth at 30° in YEPD medium as described previously (HEREFORD and ROSBASH 1977; KIRBY 1965; LARKIN 1985). DNA was labeled *in vitro* to 10^8 cpm/μg with [α-^{32}P]dCTP by nick translation (RIGBY *et al.* 1977). The techniques used for preparation and analysis of DNA fragments on agarose gels and most of the general procedures used have been described previously (MANIATIS, FRITSCH and SAMBROOK 1982). Gel slices containing DNA fragments were excised from agarose gels after electrophoresis, staining in ethidium bromide (1 μg/ml), and visualization with long-wave UV light. DNA was extracted by using an IBI analytical electroeluter according to the instructions of the manufacturer.

Electrophoresis, transfer and hybridization of DNA. Restriction fragments of genomic DNA were subjected to electrophoresis on 0.8% agarose gels (MANIATIS, FRITSCH and SAMBROOK 1982). DNA was transferred from gels to nitrocellulose filters by using the method of SOUTHERN

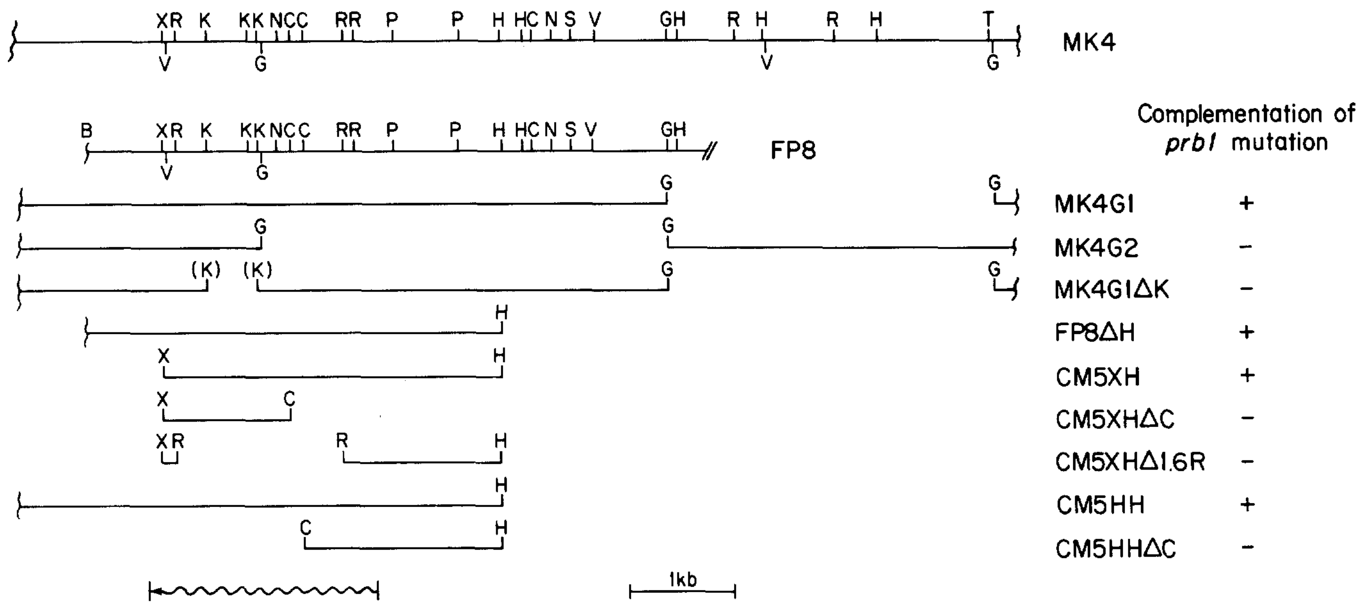


FIGURE 1.—Restriction maps and complementation results for plasmids MK4 and FP8 and their derivatives. Endonuclease cleavage sites are indicated (B, *Bam*HI; G, *Bgl*II; T, *Bst*EII; C, *Cla*I; R, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; N, *Nco*I; P, *Pst*I; V, *Pvu*II; S, *Sac*I and X, *Xho*I). Solid lines indicate insert DNA sequences that are present. The wavy line represents the *PRB1* mRNA. See MATERIALS AND METHODS for details of construction.

(1975) with the following modifications: the gels were rinsed twice in 0.25 M HCl for 15 min. prior to denaturation and the transfers were done in 20 × SSC (1 × SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (LARKIN and WOOLFORD 1983). Hybridizations were done in 50% formamide as described previously (DAVIS, BOTSTEIN and ROTH 1980) by using gel-purified probes.

Electrophoresis, transfer and hybridization of RNA. A 20-μg portion of total RNA was subjected to electrophoresis on 1% agarose-formaldehyde gels (LARKIN and WOOLFORD 1983) and was transferred to nitrocellulose as described previously (LAST, STAVENHAGEN and WOOLFORD 1984). Hybridizations were done in 50% formamide by using gel-purified DNA probes (DAVIS, BOTSTEIN and ROTH 1980). Approximate RNA sizes were determined by using DNA restriction fragments as standards.

S1 Nuclease Mapping. The *PRB1* mRNA was mapped relative to the cloned gene by the S1 nuclease method of BERK and SHARP (1977). DNA probes were labeled at the 5′ end with polynucleotide kinase and [α-^{32}P]ATP (7000 Ci/mmol). DNA probes were labeled at the 3′ end with Klenow fragment of *E. coli* DNA polymerase I, [α-^{32}P] dATP (3000 Ci/mmol), and dTTP. After labeling, the labeled DNA was separated from unincorporated label on either a NENSorb or a NACS column. The DNA was then digested with a second restriction enzyme and probes were gel purified (MANIATIS, FRITSCH and SAMBROOK 1982). Strand specific probes (1–5 × 10^5 cpm) were hybridized to total yeast RNA (50 μg). The hybrids were then digested with S1 nuclease. After S1 nuclease treatment, the samples were ethanol precipitated and analyzed on acrylamide-urea gels (MAXAM and GILBERT 1977).

Transformation. Bacteria were transformed by using the $CaCl_2$ protocol (MANIATIS, FRITSCH and SAMBROOK 1982) with selection for resistance to ampicillin. Yeast cells were transformed by the spheroplast method (HSIAO and CARBON 1979) or by a modification of the lithium acetate method of ITO *et al.* (1983) as described by WOOLFORD *et al.* (1986).

Preparation of extracts and protease B activity assays. Extracts were prepared and assayed for protease B activity as described previously (ZUBENKO, MITCHELL and JONES 1979). Protein concentration was estimated using Coomassie Brilliant Blue G-250 as described previously (BRADFORD 1976).

Genetic methods. The procedures used for routine sporulation, dissection and scoring of nutritional markers have been described previously (HAWTHORNE and MORTIMER 1960). The *prb1* marker was scored on the basis of an inability of colonies to catalyze solubilization of Hide Powder Azure. Streaks or replica plates of cells grown for 2 days on YEPG agar were overlaid with 4 ml of 0.5% agar containing 50–100 mg HPA, 20 mg of SDS, 1 mg of cycloheximide, 1 mg of streptomycin and 1000 units of penicillin G. The use of YEPG agar allows detection of protease B activity in colonies in the absence of lysis mutations. Some lysis takes place during growth of cells on YEPG agar. The HPA was prepared as described previously (ZUBENKO, MITCHELL and JONES 1979). The *prc1* marker was scored on the basis of an inability to cleave acetylphenylalanine β-naphthyl ester in agar overlay (JONES 1977) or benzoyltyrosine-*p*-nitroanilide in a well test (WOOLFORD *et al.* 1986).

RESULTS

Cloning of the *PRB1* gene. Plasmids capable of complementing the *prb1-1122* mutation were recovered from the YEp24 bank (CARLSON and BOTSTEIN 1982). After transformation of BJ1825 by the modified lithium acetate procedure and selection for Ura$^+$, approximately 13,000 Ura$^+$ transformants were replica plated onto YEPG and tested for the ability to solubilize HPA. Three Prb$^+$ transformants were identified. Plasmids capable of effecting complementation of the *prb1-1122* mutation after being passaged through *E. coli* were recovered from two of the transformants. The restriction map for the smaller of the two, MK4, is shown in Figure 1. MK16 shows the identical map and contains an additional 2.7 kb of

TABLE 2

FP8 integrates into and destabilizes chromosome *V*

III: $\frac{leu2}{leu2}$ *V*: O— *ura3 prb1 can1* [*LEU2 PRB1*][a] / + *prb1* +

Phenotypic ratio[b]	No. of the following tetrad types *Ura*+ : *Ura*− 2:2	3:1	4:0
2 Leu+ Prb+ Can^r:2 Leu− Prb− Can^s	41[c]	0	0
0 Leu+ Prb+ Can^r:4 Leu− Prb− Can^s	24	0	8

[a] The diploid is a cross of BJ2378, a stable Leu+ Prb+ transformant of a Can^r derivative of BJ1825, *α leu2 ura3-52 trp1 prb1-1122 prc1-407 can1*, to BJ2341, **a** *leu2 prb1-1122.* By hypothesis the *LEU2* and *PRB1* markers of the plasmid FP8 (in brackets) have integrated into chromosome *V*. The order of the plasmid markers with respect to *prb1* and *can1* is unknown.

[b] Two additional aberrant tetrads were obtained: one gave 2:2 segregation for Prb and Ura but all four spores were Leu− Can^s; another gave 2:2 segregation for Ura but all four spores were Leu− Prb+ Can^s.

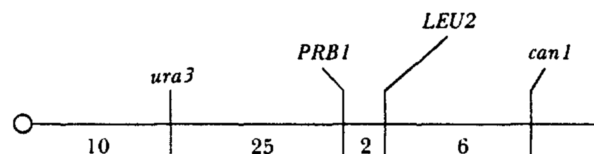
[c] Of these 41, 22 gave tetratype segregation of *URA3* with *LEU2*, *PRB1* and *can1*, the remaining 19 were parental ditype yielding a map distance of 28 cM (PERKINS 1949).

TABLE 3

FP8ΔS integrates at the *PRB1* locus

III: $\frac{leu2}{leu2}$ *V*: O— *ura3 prb1* + [*LEU2 PRB1*][a] / + *prb1 can1*

Marker pair	No. of the following tetrad types: Parental ditype	Nonparental ditype	Tetra type	cM
ura3-cen5[b]	6	15	5	9.6
can1-ura3	10	0	16	30.8
can1-leu2	23	0	3	5.8
can1-prb1	22	0	4	7.7
ura3-leu2	12	0	14	26.9
ura3-prb1	13	0	13	25.0
prb1-leu2	25	0	1	1.9



[a] The cross is between BJ2555, a Leu+ Prb+ transformant of BJ1825, *α leu2 trp1 ura3-52 prb1-1122 prc1-407* and a Can^r derivative of BJ2341, **a** *leu2 can1.*

[b] Parental ditype and nonparental ditype refer to segregation of *ura3* with respect to *trp1.* Together the ditypes mark first division segregation for *ura3.*

sequence at the left end of the insert. By methods similar to those described above, except that transformants were selected for Leu+ instead of Ura+, two plasmids able to complement the *prb1-1122* mutation were recovered from the YEp13 bank (NASMYTH and REED 1980; NASMYTH and TATCHELL 1980). The restriction map for the smaller of these, FP8, is also shown in Figure 1. The larger, FP34, is identical to FP8 except that an additional 5 kb of DNA are present at the right end of the insert. The portion of overlapping sequence for the four plasmids is about 6 kb. The *Bam*HI sites at the left insert junction of plasmids FP8 and FP34 apparently were created by ligation of a Sau3A tail to the *Bam*HI tail of the plasmid, for no *Bam*HI site is present in the MK4 and MK16 plasmids. The 4.6-kb *Bam*HI-*Sac*I fragment from FP8 hybridized to the DNA inserts of MK4 and MK16, indicating that the three plasmids do indeed contain homologous sequences (data not shown).

To determine whether we had cloned *PRB1*, we tested whether an integrated copy of the cloned gene mapped to the *PRB1* locus, which is known to map one cM proximal to *can1* on chromosome *V* (ZUBENKO, MITCHELL and JONES 1980). The plasmid FP8 was transformed into a canavanine resistant derivative of BJ1825 and Leu+ transformants were selected. As expected, the transformants were Leu+ and Prb+. After growth of the transformants in the nonselective medium YEPD, most cells lost the plasmid and became Leu− and Prb−. A minority remained Leu+ and Prb+ and maintained the phenotype stably, indicating that the plasmid had integrated into a chromosome. One of these, BJ2378, was crossed to BJ2341 and the diploid was sporulated and dissected. The expected disposition of the markers is indicated in Table 2 as are the results. In 41 of the tetrads, the genes conferring the Leu+, Prb+ and Can^r phenotypes showed 2:2 segregation and were completely linked. These data indicate that the plasmid had integrated adjacent to *can1* on the left arm of chromosome V. The linkage of the three markers to *ura3* (27 cM) is in agreement with this conclusion. In 32 of the tetrads, the plasmid-borne *LEU2* and *PRB1* markers were not found in the spores and all of the spores bore the input *CAN1* allele derived from the homologous chromosome. We assume that this class arose from destabilization of the chromosome arm by the 2 μ sequences present in the FP8 plasmid that integrated (FALCO *et al.* 1982).

To test the conclusion, free of interference from the destabilization phenomenon, that we had indeed cloned *PRB1*, a derivative of FP8, FP8ΔS, was prepared. FP8ΔS lacks most of the 2 μ sequences present in YEp13 and is therefore an integrating plasmid. FP8ΔS was transformed into BJ1825 and Leu+ transformants were selected. One such, BJ2555, was crossed to a canavanine resistant derivative of BJ2341. Segregation data for the diploid are presented in Table 3. The genes conferring the Prb+ and Leu+ phenotypes segregated 2:2, indicating that the sequences are in the chromosome. The two genes are tightly linked to one another as well as to *CAN1* as expected if FP8ΔS carries the *PRB1* gene. From the two sets of data, we infer that we have indeed cloned *PRB1*, the structural gene for protease B.

In order to determine which portion of the insert

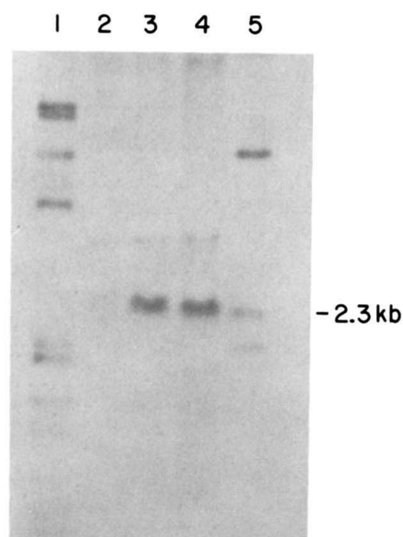


FIGURE 2.—Northern blot of total yeast RNA from strain BJ2665. RNA was prepared from YEPD cultures at densities of approximately 40, 660 and 1200 Klett units (lanes 2–4; 12–18 μg per lane). Size standards in lanes 1 and 5 are *E. coli* phage λ DNA digested with *Eco*RI plus *Hin*dIII (lane 1) and with *Hin*dIII (lane 5). The blot was hybridized with nick-translated λ DNA and the 2.3-kb *Ava*I-*Ava*I *PRB1* fragment (see Figure 3, *Xho*I restriction sites are a subset of *Ava*I restriction sites). One fragment in lane 5 is 2.3 kb in size.

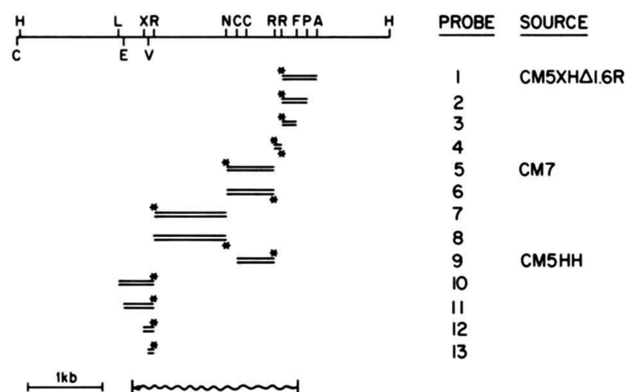


FIGURE 3.—S1 nuclease mapping. The *PRB1* message was mapped with probes 1 and 4–10; probes 2, 3, 11, 12 and 13 were used in conjunction with DNA sequencing ladders to determine the size of partially protected hybrids. The S1 nuclease-protected mRNA is indicated at the bottom of the figure. The probes are presented in the conventional manner; *i.e.*, the top strand runs 5'→ 3', the asterisks denote the position of ^{32}P label. The probes were constructed as follows. Probes 1–4: plasmid CM5XHΔ1.6R was incubated with *Eco*RI followed by phosphatase, then polynucleotide kinase and finally with *Ava*I. The 0.1-kb *Eco*RI-*Eco*RI fragment (probe 4) and the 0.48-kb *Eco*RI-*Ava*I fragment (probe 1) were gel purified. Portions of probe 1 were cut with *Pst*I or *Hin*fI to make probes 2 and 3. Probes 5 and 8: plasmid CM7 was incubated with *Nco*I followed by phosphatase, then polynucleotide kinase and finally with *Eco*RI and the indicated fragments were gel purified. Probes 6 and 7: plasmid CM7 was incubated with *Eco*RI, followed by phosphatase, then polynucleotide kinase and finally with *Nco*I and the indicated fragments were gel purified. Probes 9–13: plasmid CM5HH was treated with *Eco*RI, then the Klenow fragment of DNA polymerase plus nucleotides and finally with *Cla*I. Probe 9 was gel purified and its identity was verified. With probe 9, a second *Eco*RI-*Cla*I fragment that contained probes 10–13 was purified. Portions of this fragment were cut with *Bal*I, *Hae*III, *Xho*I and *Pvu*II to make probes 10–13. As with probes 2 and 3, probes 10–13 were used without further purification. Abbreviations: A, *Ava*I; C, *Cla*I; E, *Hae*III; F, *Hin*fI; H, *Hin*dIII; L, *Bal*I; N, *Nco*I; P, *Pvu*II; R, *Eco*RI and X, *Xho*I. Additional *Hae*III and *Hin*fI sites are present in the DNA fragment beyond those shown.

carried the *PRB1* gene, a number of deletion derivatives of MK4 and FP8 were constructed and tested for retention of the ability to complement the *prb1-1122* mutation. As shown in Figure 1, Fp8ΔH, a derivative of FP8 that lacks insert DNA to the right of the *Hin*dIII site, retains the ability to complement. A number of fragments were subcloned into YCp50 and tested for the ability to complement. Plasmids CM5XH and CM5HH complement the *prb1-1122* mutation and they do so in single copy. *Cla*I deletions that remove the left segment or the right segment of the *Xho*I-*Hin*dIII fragment (CM5HHΔC and CM5XHΔC, respectively) eliminate complementation. From the data obtained, we inferred that the *PRB1* gene lay to the right of the *Xho*I site, overlapping at least one *Kpn*I site and one *Cla*I site, but to the left of the leftmost *Hin*dIII site.

Mapping the transcript of the *PRB1* gene. An RNA blot analysis indicated that the *PRB1* gene encodes a 2.3-kb RNA (Figure 2). In Figure 2, two RNAs are visible, one of about 3 kb in size and one of about 2.3 kb in size. Only the 2.3-kb transcript is seen if the 1.6-kb *Eco*RI-*Eco*RI internal fragment is used as a probe (data not shown). Use of the *Ava*I-*Ava*I fragment either has identified an adjacent transcript or, more likely, the probe was contaminated with a small amount of another probe. To determine where the mRNA begins and ends, whether it contains introns, and the direction of transcription, S1 protection experiments were carried out. The probes used for this determination and their sources are presented in Figure 3. About 0.2 kb of the 0.4-kb, 5' end labeled *Eco*RI-*Ava*I fragment (probe 1) was protected (Figure 4) as was about 0.3 kb of the 0.5-kb, 3' end labeled *Eco*RI-*Bal*I fragment (probe 10) (Figure 5). When hybridized with the mRNA, the 0.1-kb *Eco*RI and 1-kb *Nco*I-*Eco*RI fragments (probes 4 and 7, respectively) were completely resistant to S1 nuclease. The hybrid between the mRNA and the 0.6-kb *Nco*I-*Eco*RI fragment (probe 5) was not completely protected: about half of the hybrid molecules were fully protected and the rest appeared to be 10–30 nucleotides smaller. The heterogeneity could reflect RNA processing or more likely, an S1 artifact. In order to clarify the cause of this heterogeneity, a 3' end labeled fragment was tested (probe 9). The hybrid between the mRNA and this 0.5-kb *Eco*RI-*Cla*I fragment showed a 5–10 nucleotide heterogeneity but was essentially fully protected. When hybridized with the mRNA, the 1-kb and 0.6-kb *Nco*I-*Eco*RI probes (probes 8 and 6, respectively) for detection of opposite

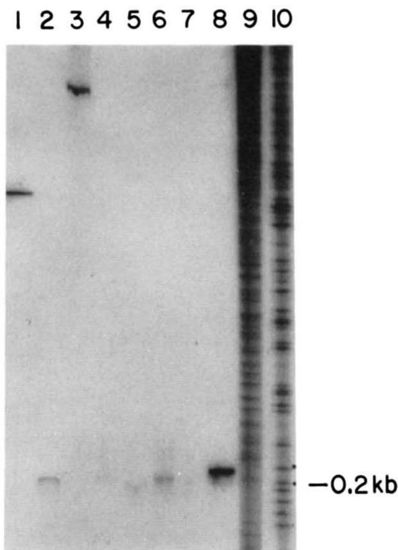


FIGURE 4.—5′ end of *PRB1* message. Total RNA was hybridized to the labeled *Eco*RI-*Ava*I fragment (probe 1 in Figure 3) and digested with S1 nuclease. The size of the protected fragment was 201 nucleotides, as established by comparison to DNA sequencing ladders and probes 2 and 3 in Figure 3. Lane 1: 340 nucleotide probe 2 from Figure 3; lane 3: *Eco*RI-*Ava*I probe with total yeast RNA, no S1; lanes 2, 4, 5, 6, and 7: *Eco*RI-*Ava*I probe with total yeast RNA, with S1; lane 8: 203 nucleotide probe 3 from Figure 3; lanes 9 and 10: DNA sequencing ladders.

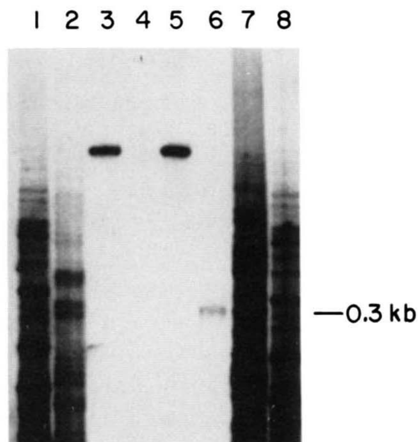
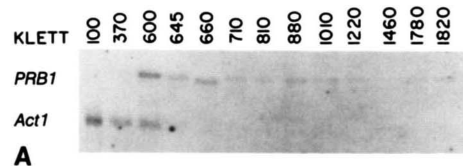


FIGURE 5.—3′ end of *PRB1* message. Total RNA was hybridized to the labeled *Eco*RI-*Bal*I fragment (probe 10 in Figure 3) and digested with S1 nuclease. The size of the protected fragment was 298 nucleotides, as established by comparison to DNA sequencing ladders and probes 9, 11, 12 and 13 in Figure 3. Lanes 1, 2, 7 and 8: DNA sequencing ladder; lane 3: probe plus tRNA, no S1; lane 4: probe plus tRNA, with S1; lane 5: probe plus total yeast RNA, no S1; lane 6: probe plus total yeast RNA, with S1.



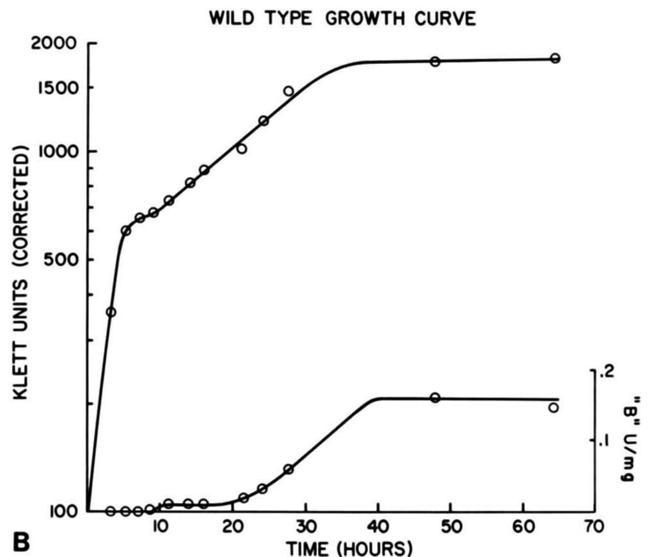


FIGURE 6.—Time course of growth and protease B expression in YEPD medium. Strain BJ2665 was grown to stationary phase and samples were taken at the times indicated. A, Northern blot of total yeast RNA hybridized with ^{32}P-labeled 1.6-kb *Eco*RI-*Eco*RI *PRB1* fragment and the *Act1* plasmid pYACT1. B, Cell density and protease B activity as a function of time.

strand transcripts, were completely digested by S1 nuclease. These results indicate that the mRNA is uninterrupted, transcribed from right to left, and extends for 2.2 kb. These results are summarized in Figures 1 and 3. The summed figure of 2.2 kb is in good agreement with the 2.3 kb estimated by RNA blot analysis. It is interesting to note that the mRNA extends 161 bp to the left of the *Xho*I site and yet the subclone of the *Xho*I-*Hin*dIII fragment in YCp50 (CM5XH in Figure 1) is still able to complement the *prb1-1122* mutation.

Transcription of *PRB1* in relation to growth phase. When yeast cells grow on glucose, very little protease B activity is detected until the cells have exhausted the glucose and have begun to utilize ethanol after emerging from the diauxic plateau (SAHEKI and HOLZER 1975; JONES *et al.* 1986). We have used RNA blot analysis to determine whether any part of this regulation is transcriptional in nature. Cells were grown in YEPD. At intervals samples were removed and cell density was determined. One aliquot for each time point was processed to recover total RNA, a second for assays of protease B specific activity. RNA samples were fractionated on an agarose-formaldehyde gel and transferred to nitrocellulose. The RNA blot was probed with the 1.6-kb *Eco*RI fragment from plasmid CM7 and plasmid YACT1 containing the yeast actin clone (NG and ABELSON 1980) in an attempt to control for loading. Each lane in the gel corresponds to the RNA from 2×10^6 cells. The results are presented in Figure 6. Substantial amounts of *PRB1* mRNA are not present until the supply of glucose is exhausted (around 600 Klett units). Although there is a slight increase in the specific activity of protease B around this time (about 10

hr), the major increase in the specific activity of protease B occurs substantially later, during growth on the ethanol. The actin mRNA proved an inadequate control for RNA loading at late time points, for it appears to show decreased expression at late growth stages. We are unable to identify an appropriate control for this experiment, since we know of no constitutive mRNA that is synthesized throughout growth. However, the major conclusion to be drawn from this experiment, that derepression of *PRB1* mRNA precedes the increase in protease activity by several hours, does not depend upon the mRNA levels that are found at later time points. Since protease B is synthesized via a precursor (MECHLER *et al.* 1982a), whose activation is dependent upon protease A, the product of the *PEP4* gene (AMMERER *et al.* 1986; WOOLFORD *et al.* 1986), a delay in expression of protease B activity could be related to the level or activity of protease A available for processing the protease B precursor. Additional copies of the *PEP4* gene have been shown to alter the timing and level of protease B expression (JONES, ZUBENKO and PARKER 1982; JONES *et al.* 1981, 1986). Whether protease A is the only activity needed during the long delay between derepression of transcription of *PRB1* and the increase in protease B activity (≃10 hr) is unknown.

Deletion of the *PRB1* gene. Protease B has a molecular weight of about 33,000, of which about 3,000 is the single glycosidic side chain. The molecular weight of the precursor is about 42,000, 39,000 of which is protein. A 2.2-kb mRNA could encode a protein of about 633 amino acids or about 69,000 molecular weight, assuming about 300 nucleotides of untranslated 5′ and 3′ RNA. A molecular weight of 69,000 is nearly twice the size of the protein of the one precursor detected. To try to determine whether *PRB1* might encode an additional function and to determine whether it is an essential gene we constructed a deletion that removes the 1.6 kb of DNA between the leftmost and the middle *Eco*RI sites that lie within the transcribed DNA. A fragment bearing the deleted gene was recovered after endonuclease digestion of plasmid CM5XH1.6R (Figure 1) with *Hin*dIII and *Pvu*II and was used, along with plasmid YEp24, to transform the diploid BJ3042 to Ura$^+$. Canavanine resistant mitotic recombinants were selected from individual Ura$^+$ transformants and screened for the Prb$^-$ phenotype. A diploid that yielded Can^r Prb$^-$ mitotic recombinants was sporulated and dissected. Typically, each ascus gave four surviving spores. Of 41 asci, 39 showed 2 Can^r Prb$^-$: 2 Can^s Prb$^+$ (parental ditype) and two showed tetratype segregation, for a map distance of 2.4 cM. The Prb$^-$ clones had no other obvious phenotype. Proof that we had constructed the deletion is provided in Figure 7. Genomic DNA of the parent haploids, the parent diploid, the derivative deletion heterozygote and the four haploid spore clones derived from one tetrad was blotted to nitrocellulose and probed with a 2.3-kb *Ava*I fragment (*Ava*I cleaves at the *Ava*I and *Xho*I sites shown in Figure 3). The wild type haploids and diploids possess a 2.3-kb fragment. Spore clones 8A and 8D possess a 0.7-kb fragment and, as expected, the deletion heterozygote possesses both fragments. These results indicate that we had indeed deleted 1.6 kb from one of the two copies of the *PRB1* gene present in the diploid. It is also clear that *PRB1* is a single copy gene. Because the Prb$^-$ spore clones grew and showed no other obvious phenotype, we infer that *PRB1* function is not essential for growth and that, provisionally at least, *PRB1* encodes only the precursor to protease B. The discrepancy between gene size and protein and precursor size awaits resolution.

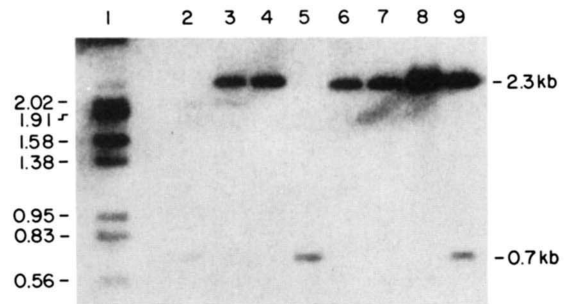


FIGURE 7.—Southern blot of total yeast DNA from *PRB1* and *prb1-Δ1.6R* strains. DNA was prepared for spheroplasts, digested with *Ava*I, fractionated on an agarose gel and transferred to nitrocellulose. The blot was probed with nick-translated 2.3-kb *Ava*I *PRB1* fragment and phage λ DNA. Lane 1: phage λ DNA digested with *Hin*dIII and *Eco*RI. Lanes 2–5: 8A–D, representative tetrad from BJ3043, the *PRB1/prb1-Δ1.6R* heterozygote. Lane 6: BJ3040, **a** *PRB1* parent. Lane 7: BJ3041, α *PRB1* parent. Lane 8: BJ3042, untransformed *PRB1/PRB1* diploid. Lane 9: BJ3043, *PRB1/prb1-Δ1.6R* heterozygote constructed by transformation of BJ3042. The 1.6-kb *Eco*RI deletion reduced the size of the 2.3-kb *Ava*I fragment to 0.7 kb. This can be seen in 8A and 8D (Lanes 2 and 5), and in the heterozygote, which has one wild-type allele (2.3-kb fragment) and one mutant allele (0.7-kb fragment).

DISCUSSION

The results we have presented indicate that little or no *PRB1* mRNA is made during growth on YEPD until the glucose is exhausted and the cells enter the diauxic plateau. The increase in levels of protease B activity lag far behind the transcriptional activation of *PRB1* and indeed appear to occur after the mRNA levels have declined. Since protease B is known to be synthesized via a precursor, it seems likely that a portion of the lag may reflect the time needed for proteolytic processing of the precursor.

Protease B has a protein molecular weight around 30,000 (KOMINAMI, HOFFSCHULTE and HOLZER 1981; MECHLER *et al.* 1982a). One kinetic precursor was detected after a 20-min pulse that had a molecular weight of 42,000 of which about 39,000 presumably

is protein (MECHLER *et al.* 1982a). The mRNA encoded by *PRB1* is 2.3 kb in size. Assuming average sized 5′ and 3′ untranslated regions and a poly(A) tail totaling 400 base pairs one expects about 1.9 kb of coding capacity or 633 amino acids. Assuming an average molecular weight of 110 per amino acid, one expects a glycosylated protein of 72,000 molecular weight. An open reading frame of 635 amino acids has been detected (data not shown). This discrepancy between the observed molecular weight of the precursor (42,000) and the predicted size of the precursor (72,000) raises the possibility that there might be an earlier, larger precursor that might be detected if shorter pulses were employed in kinetic experiments and that additional proteolytic processing steps might be involved in generating active protease B from the initial glycosylated translation product.

The expression of protease B activity is known to be dependent on the activity of the *PEP4* gene product, protease A (AMMERER *et al.* 1986; WOOLFORD *et al.* 1986). Heterozygosity for a *pep4* mutation results in decreased levels of protease B (JONES, ZUBENKO and PARKER 1982; JONES *et al.* 1981) and extra copies of the *PEP4* gene can alter the timing of expression and the levels of protease B activity (JONES *et al.* 1986). If there are other processing events required as well, the timing of expression of protease B activity might well depend upon the expression or activity of the gene product(s) responsible for these other processing events.

The large size of the gene and mRNA relative to the size of protease B raised the possibility that the translation product of the *PRB1* gene might be processed to yield two active gene products. The viability and robust health of the deletion derivative that lacks 83% of the open reading frame (ms in preparation) indicates that if there is a second function beyond protease B activity encoded within the *PRB1* gene, it is not essential for growth and viability nor does its loss lead to an obvious phenotype.

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