MAPPING OF THE PROTEINASE B STRUCTURAL GENE PRBl, IN SACCHAROMYCES CEREVISIAE AND IDENTIFICATION OF NONSENSE ALLELES WITHIN THE LOCUS

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ABSTRACT

We report the mapping of the structural gene for proteinase B, PRBl. It is located 1.1 cM proximal to CAN1 on the left arm of chromosome V of Saccharomyces cerevisiae. We have identified 34 amber and 12 ochre mutations among the 126 prbl mutations in our collection.

YEAST cells contain a prominent organelle, the vacuole, which contains three proteinases (CABIB, ULANE and BOWERS 1973; HASILIK, MÜLLER and HOLZER 1974; LENNEY et al. 1974), as well as other hydrolases. Proteinase A (EC 3.4.23.8) is an endoproteinase active at acid pH, proteinase B (EC 3.4.22.8) is an endoproteinase active near neutrality, and proteinase C (EC 3.4.23.—) is a carboxypeptidase active near neutrality (LENNEY and DALBEC 1967; HATA, HAYASHI and DOI 1967). In an attempt to determine the physiological role of proteinase B, we isolated 147 strains that were deficient in proteinase B activity. Some 82,400 mutagenized clones were grown at 23° and screened for proteinase B activity at 36° to allow isolation of conditional lethal mutations, were proteinase B essential for viability. All of the mutations in these strains were recessive and have been assigned to five complementation groups: 126 were assigned to prbl, two to prb2, 17 to prb3, one to prb4 and one to pep16. Of these 147 prb- mutations, only two, prbl-29 and prbl-38, caused the production of a thermolabile proteinase B activity; the remainder resulted in a proteinase B deficiency at both 23° and 36°. None of these 147 mutations produced lethality at any temperature. We have presented evidence that PRBl is the structural gene for proteinase B. (MITCHELL and JONES 1977; ZUBENKO, MITCHELL and JONES 1978, 1979a,b).

We report the mapping of PRBl on the left arm of chromosome V, between MAK10 and CAN1, and the identification of 34 amber and 12 ochre mutations among the 126 prbl mutations in our collection.

MATERIALS AND METHODS

Yeast strains: Mutants were isolated in 2 genetic backgrounds: M16-14C (a leu1-1 ser1-171) and a trpl derivative of X2180-1B (a gal2). The parent strains were s26 (X2180-1B background), which carries a lysis mutation resulting in lysis of a fraction of the cells in a colony.
and which was isolated by the procedure of Cabib and Duran (1975), and ape23 (a \textit{leu1-1 ser1-171 pep15-1}), which also carries a lysis mutation. Outcross parents for each isogenic series were derived from M16-14C (the parent of ape23) and X2180-1B by mutation and crosses within a series and had the genotype \textit{a met- ser1-171 thr1} and \textit{a ade6 his5}, respectively (Zubenko, Mitchell and Jones 1979b; Jones 1977).

The amber mutations, \textit{trp1-1} and \textit{tyr7-1}, as well as the ochre mutations, \textit{his5-1} and \textit{lys1-1}, were obtained from G. Fink. The \textit{mak10} mutation was obtained from R. Wickner. Mutations at \textit{CAN1} were obtained by selection for growth on canavanine-containing medium. Gene symbols are denoted as follows: \textit{a} and \textit{a} mating-type alleles; \textit{ade6}, \textit{his5}, \textit{leu1}, \textit{met-}, \textit{ser1}, \textit{thr1} and \textit{trp1}, requirements for adenine, histidine, leucine, methionine, serine, threonine and tryptophan, respectively; \textit{mak10}, maintenance of killer; \textit{can1}, resistance to canavanine; \textit{gal2}, inability to ferment galactose; \textit{pep15}, decreased ability to cleave acetylphenylalanine \textit{β}-naphthyl ester; \textit{prb1}, decreased ability to cleave Hide Powder Azure (HPA) (Calbiochem).

\textbf{Media}: YEPD, KAc, SC and omission media were as described by Jones and Lam (1973). For YEPG agar, 5% glycerol replaced the glucose in YEPD. Canavanine medium was arginine omission medium to which canavanine sulfate (Sigma) was added to a final concentration of 60 mg/l.

\textbf{Genetic methods}: Procedures for sporulation, dissection and scoring of nutritional markers were as described by Mortimer and Hawthorne (1969). The \textit{mak10-1} mutation was scored as described by Somers and Bevan (1969).

Proteinase B-deficient mutants were isolated in ape23 and sl26 after ethyl methanesulfonate mutagenesis by the procedure described by Zubenko, Mitchell and Jones (1979b). To follow the segregation of \textit{prb1} mutations in meiotic tetrads, streaks of cells growing on YEPD or YEPG plates were overlaid with 4 ml of 0.5% agar containing 50 to 100 mg HPA, 20 mg of sodium dodecyl sulfate and 1 mg of cycloheximide. After incubation at 36°C, streaks containing proteinase B activity solubilize the HPA particles on top of and surrounding the streaks (Zubenko, Mitchell and Jones 1979b). Growth on YEPD allows visualization of activity in the absence of a lysis mutation. Maximal sensitivity for detection of activity by plate assay is achieved by growing cells bearing the lysis mutation on YEPG. An example of the assay is shown in Figure 1.

\textbf{Figure 1}.—Plate assay showing the segregation of \textit{prb1-2} in tetrads derived from a \textit{prb1-2/+} diploid. Each horizontal set of four stripes is one tetrad. The segregation is ++ -- ++ (upper) and -- ++ --.
Preparation of extracts and proteinase B assay: Cell-free extracts were prepared by the homogenization of cell suspensions with glass beads as described by ZUBENKO, MITCHELL and JONES (1979b). Proteinase B activity was assayed according to ZUBENKO, MITCHELL and JONES (1979b). One unit of proteinase B activity is defined as the change of one absorbance unit at 520 nm per min for the 0.74 ml reaction mixture at 37°. Protein concentrations were determined by the method of Lowry et al. (1951). Specific activity is given in units/mg of extract protein.

RESULTS

Mapping of PRB1: In routine outcrosses involving prb1-8, tight linkage of this mutation to a can1 mutation was detected. In order to map PRB1 with respect to MAK10 and CAN1 on the left arm of chromosome V, we sporulated a diploid heterozygous for mak10-1, prb1-8, can1 and leu1-1. The data in Table 1 show frequencies of ditype and tetratype segregations with respect to leu1 for mak10, prb1-8 and can1. The distances of these markers from their centromeres were calculated (Perkins 1949) assuming a second-division segregation frequency of 4.9% for leu1 (Mortimer and Hawthorne 1966). These data are consistent with the established order of MAK10 and CAN1 with respect to their centromere (Wickner and Liebowitz 1976) and suggest that PRB1 lies between these two loci. Table 2 includes the number of tetrads that represented parental ditypes and tetratype segregations for the three gene pairs. These data establish the following order: centromere-MAK10-PRB1-CAN1. The four tetrads in which can1 and prb1 were tetratype showed prb1 and mak10 in parental ditype configuration. Likewise, the 20 tetrads in which mak10 and prb1 were tetratype showed prb1 and can1 in parental ditype configuration. Thus, the order given is confirmed.

PRB1 nonsense alleles: In order to allow definitive statements about the physiological role of proteinase B, we sought mutants totally deficient in proteinase B activity. Since we had no way of identifying deletions, we screened the collection for nonsense mutations. Nonsense suppressors were isolated by selecting for co-reversion of established amber (trp1-1, tyr7-1) or ochre (his5-1, lys1-1) mutations in strains that carried either prb1-6 or prb1-8. The prb1 mutations were chosen at random. The genotypes of the strains used are shown in Table 3. The presence of the amber or ochre suppressors was verified by insuring that the suppressors were dominant, were separable from the mutations that they suppressed and acted only on amber or ochre alleles, respectively. None of the suppressors obtained suppressed either prb1-6 or prb1-8, for all suppressor-bearing strains were Hpa-.

| TABLE 1 |
| Tetrad data indicating linkage to centromere of mak10, prb1 and can1 |
|---|---|---|
|   | leu-mak10 | leu-prb1 | leu-can1 |
| Ditypes: tetratype | 75:105 | 68:112 | 64:116 |
| cM from centromere | 28.8 | 30.9 | 32.1 |
Four amber suppressor-bearing and four ochre suppressor-bearing strains derived from strain 1 were crossed to the prbl mutants obtained from the ape23 parent strain. The diploids were tested for HPA cleavage after growth on YEPG, since the lysis mutation was heterozygous in the diploids. A positive HPA test for such a diploid implied that the prbl allele introduced from the ape23 collection was a nonsense mutation. By this method, one amber mutation, prbl-9, and no ochre mutations were detected among the 10 prbl mutations isolated in the M16-14C genetic background.

The low frequency of nonsense mutations in the ape23 collection and a similar result among the 116 members of the sl26 collection prompted us to increase the sensitivity of our screen by introducing the lysis mutation into suppressor-bearing parents derived from strain 2 (amber suppressors) or strain 3 (ochre suppressors). As the suppressor-bearing strains proved to be unstable, as was previously reported (LIEBMAN and SHERMAN 1976; LIEBMAN, SHERMAN and STEWART 1976), we maintained selection for the suppressors throughout the procedure. Using these new strains and procedures, we crossed the tester strains to the 116 prbl mutants isolated in the X2180-1B genetic background. We identified 33 amber mutations and 12 ochre mutations among these 116 prbl mutations.

**Analysis of nonsense mutations:** Table 4 provides conclusive evidence that prbl-9 is an amber mutation, as inferred from the screen. The first entry shows the result of an outcross of the suppressor-bearing strain used to identify prbl-9 as an amber mutation. The Hpa phenotype segregated 2:2 in all 18 tetrads, reflecting the fact that the amber suppressor does not suppress prbl-6. Two-gene segregation for the tryptophan and tyrosine auxotrophies was observed as expected, since trpZ-l and tyr7-1 are amber mutations. Of the Hpa+ spore clones obtained, the vast majority were Can+ because of the coupling of PRBl and CAN1 in the diploid. The frequency of clones that did not require tryptophan or tyrosine among those that were Hpa+ was not different from the overall frequency of Trp+ or Tyr+ spore clones obtained in the cross. The inability to utilize glycerol (Gut+) as a source of carbon was recessive and co-segregated with the

**TABLE 2**

Tetrad data indicating linkage of mak10, prbl and can1

<table>
<thead>
<tr>
<th></th>
<th>mak10-prbl</th>
<th>prbl-can1</th>
<th>mak10-can1</th>
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</thead>
<tbody>
<tr>
<td>PD:T:NPD</td>
<td>160:20:0</td>
<td>176:4:0</td>
<td>156:24:0</td>
</tr>
<tr>
<td>cM</td>
<td>5.6</td>
<td>1.1</td>
<td>6.7</td>
</tr>
</tbody>
</table>

**TABLE 3**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotypes of tester strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ade6 trp1-l tyr7-l his5-l lys1-l prbl-6 can1</td>
</tr>
<tr>
<td>2</td>
<td>met+ trp1-l tyr7-l his5-l lys1-l prbl-8 can1</td>
</tr>
<tr>
<td>3</td>
<td>met- tyr7-l his5-l lys1-l prbl-8 can1</td>
</tr>
</tbody>
</table>
### TABLE 4

*Tetrad data indicating that prb1-9 is a nonsense mutation*

<table>
<thead>
<tr>
<th>Relevant genotype</th>
<th>Hpa* Hpa*</th>
<th>Trp* Trp*</th>
<th>Tyr* Tyr*</th>
<th>Gut* Gut*</th>
<th>Phenotype of Hpa* spore clones</th>
<th>Phenotype of Gut* spore clones</th>
</tr>
</thead>
<tbody>
<tr>
<td>SUP-a trp1-1 tyr7-1 prb1-6 cam1 + +</td>
<td>3 2 1 0</td>
<td>3 2 1 0</td>
<td>3 2 1 0</td>
<td>3 2 1 0</td>
<td>+: -</td>
<td>+: -</td>
</tr>
<tr>
<td>+ - + -</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
<td>0 1 2 3 4</td>
<td>+: -</td>
<td>S: R</td>
</tr>
<tr>
<td>SUP-a trp1-1 tyr7-1 prb1-6 cam1 + + prb1-9 +</td>
<td>0 0 1 5 4</td>
<td>1 9 0 0 0</td>
<td>0 10 0 0 0</td>
<td>0 10 0 0 0</td>
<td>0 7 7 0 7 0 6 1 7 13 20 0 20 0</td>
<td>16 0 16 0 16 0</td>
</tr>
<tr>
<td>+ + + +</td>
<td>2 5 1 0 0</td>
<td>3 2 3 0 0</td>
<td>3 4 1 0 0</td>
<td>0 0 8 0 0</td>
<td>9 16 19 6 20 5 25 0</td>
<td>16 0 16 0 16 0</td>
</tr>
<tr>
<td>SUP-a trp1-1 tyr7-1 prb1-9 + + +</td>
<td>0 0 10 0 0</td>
<td>0 10 0 0 0</td>
<td>0 10 0 0 0</td>
<td>0 10 0 0 0</td>
<td>0 20 20 0 20 0 20 0</td>
<td>20 0 20 0 20 0</td>
</tr>
<tr>
<td>+ trp1-1 tyr7-1 prb1-9 - + +</td>
<td>0 0 10 0 0</td>
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<td>0 10 0 0 0</td>
<td>0 10 0 0 0</td>
<td>0 20 20 0 20 0 20 0</td>
<td>20 0 20 0 20 0</td>
</tr>
</tbody>
</table>
amber suppressor (LIEBMAN, SHERMAN and STEWART 1976). These observations contrast with the dominant effects of certain amber suppressors reported by CLAISSE and HAWTHORNE (1973) where sporulation and glycerol utilization were prevented by the suppressors.

The second entry in Table 4 shows the analysis of the Hpa+ diploid from which we inferred that prbl-9 is an amber mutation. The 2:2, 1:3 and 0:4 segregation for Hpa+:Hpa- are inferred to be nonparental ditype, tetratype and parental ditype segregations, respectively, of SUP-a and prbl-9. Since the diploid lacks a wild-type PRBl allele, all Hpa+ spore clones derived from this diploid must bear SUP-a and prbl-9 and do not require tryptophan or tyrosine, as expected. The excess of Can+ clones among those that are Hpa+ supports the inference that prbl-9 is suppressed by SUP-a. An Hpa+ clone from one of the tetrads was inferred from the segregations to be of genotype SUP-a trpl-1 prbl-9 and possibly tyr7-1. This Hpa+ clone was outcrossed to a wild-type strain to ensure that Hpa- meiotic segregants could be obtained. The third entry of Table 4 shows the results of this cross and confirms the genotype SUP-a trpl-2 prbl-9 tyr7-1. This same Hpa' segregant was crossed to a strain bearing trpl-1, tyr7-1 and prbl-9. For all 10 tetrads obtained from this diploid, the abilities to cleave HPA and to synthesize tryptophan and tyrosine co-segregated with the inability to utilize glycerol. These results prove that prbl-9 is an amber mutation.

An abbreviated analysis of four prbl nonsense mutations isolated in the sl26 parent strain was performed. Diploids that were heterozygous for SUP-a, tyr7-1 and can1, and which carried two defective prbl heteroalleles, were constructed. The prbl heteroalleles were either prbl-8 (a nonsuppressible allele) and prbl-1511 or prbl-8 and prbl-1517. The can1 allele was coupled to prbl-8 in each case. For both crosses, tetrads showed 2:2, 1:3 and 0:4 segregations for the Hpa phenotype, representing nonparental ditype, tetratype and parental ditype segregations for the SUP-a prbl-1511 or SUP-a prbl-1517 gene pairs. All Hpa+ spore clones were Tyr+ and Can+. An analogous cross was performed for the ochre mutations, prbl-1122 and prbl-1411, by employing SUP-o, his5-1 and lys1-1, and analogous results were obtained. In addition, an Hpa+ spore was outcrossed to a wild-type strain to ensure that Hpa- segregants could be recovered. Such Hpa- segregants were found.

Efficiency of suppression: Since prbl-9 was one of only a few prbl mutations to be identified in our initial screen for nonsense mutations, which utilized diploids heterozygous for the lysis mutation, we suspected that the suppressor used to identify prbl-9 was very efficient. To test this, we repeatedly crossed a strain bearing prbl-9 and this amber suppressor to M16-14C to obtain a strain bearing both of these mutations in a background that approximated the M16-14C genetic background. Once obtained, a strain of genotype, a leul-1 ser1-171 prbl-9 SUP-a, was grown in liquid YEPD culture medium, the cells were harvested, and the level of proteinase B in extracts made from these cells was compared to those obtained for strains of genotype a leul-1 ser1-171 prbl-9 and a leul-1 ser1-171 (M16-14C). The results are shown in Table 5. The suppressor-bearing strain was inoculated heavily into three separate vessels. Aliquots were
withdrawn from each culture before harvesting and streaked out onto YEPD agar, since we knew that this amber suppressor was lost from growing populations of cells when there was no selective pressure applied to maintain it. The frequency of HPA+ cells in three populations was determined at the time the cells were harvested; these results are also shown in Table 5.

Appreciable loss of the suppressor occurred in two of the three cultures that had been heavily seeded with suppressor-bearing cells. When corrected for the loss of the suppressor, proteinase B levels of all three of these cultures were in good agreement. Assuming that the suppressor does not insert an amino acid at the premature termination codon that increases the intrinsic activity of the proteinase B molecule, the efficiency of this amber suppressor is at least 40%. The slow growth of strains bearing this suppressor and the rapid loss of the suppressor in the absence of positive selection for its maintenance are likely the result of a high-efficiency of suppression. However, since we are measuring the efficiency of suppression indirectly, if the suppressor inserts an amino acid that results in a proteinase B molecule with an increased activity toward the artificial substrate, the efficiency of the suppressor could be considerably less than 40%.

DISCUSSION

Proteinase B had been thought to be involved in septum formation during cell division (ULANE and CABIB 1976), in catabolite inactivation of gluconeogenic enzymes following a metabolic shift (MOLANO and GANCEDO 1974; HOLZER 1976; JUSIK, HINZE and HOLZER 1976), in the inactivation of NADP-dependent glutamate dehydrogenase (EC 1.4.1.4) during carbon starvation (HEMMINGS and MAZON 1979) and in the protein degradation that accompanies sporulation (HOPPER et al. 1974; KLAR and HALVORSON 1975; BETZ and WEISER 1976). The evidence for these assertions was derived totally from experiments done in vitro. Our purpose in isolating mutants deficient in proteinase B activity was to determine the physiological role of this proteinase. However, if the phenotypes of mutants that lack a particular enzyme are to be used to infer the physiological function of that enzyme, it is necessary to ensure that the mutations present in those mutants lie in the structural gene for that enzyme. This is especially important if one is to separate pleiotropic effects attending the loss of the enzyme
from pleiotropic effects arising because more than one enzyme activity has been lost as a consequence of the mutation.

Our screen was designed to identify mutations that resulted in a thermolabile proteinase B activity. Of the 147 mutations isolated, 126 were members of the prbl complementation group. One hundred twenty-four of these were not conditional mutations and imparted an HPA- phenotype under both the permissive (23°C) and restrictive conditions (37°C). We inferred that PRBl is the structural gene for proteinase B because alleles at this locus show a dosage effect, because two prbl alleles, prbl-29 and prbl-38, result in the production of thermolabile proteinase B activities that are distinguishable from one another and from that of the wild-type strain in vitro, and finally because extracts made from a tetraploid strain that carries three prbl-38 alleles and one wild-type allele contain a thermolabile component of the total proteinase B activity in a proportion consistent with that expected based on gene dosage considerations (Mitchell and Jones 1977; Zubenko, Mitchell and Jones 1978, 1979a, b).

Since we had no way of identifying prbl deletions, we searched among the prbl mutants for those that carried nonsense mutations in order to be as certain as possible that the strains used in our studies lacked proteinase B activity in vivo. Two lines of evidence suggest that the nonsense mutations that we identified at the prbl locus are not all located at one end of the prbl gene. Following ethyl methanesulfonate mutagenesis, prbl mutants were isolated at a frequency of 0.15%, a frequency comparable to those found for other nonessential loci (0.01 to 1%) (Lindegren et al. 1965). Had the mutations that we recovered been a selected group, we would have expected this frequency to be substantially lower. Second, of the 126 mutations we isolated at the PRBl locus, 46 are amber or ochre mutations. This frequency is comparable to frequencies found for other nonessential structural gene loci (see Singh and Sherman 1975, Table 8; Chatterjee et al. 1979). We may have underestimated the frequency of amber and ochre mutations at the PRBl locus, for our screen relies on detection, in a plate assay, of proteinase B activity itself and may require higher levels of activity as compared to screens based on growth properties.

Experiments that we performed using strains bearing either the prbl-9 or prbl-1122 nonsense mutations have shown that proteinase B activity is not required for the activation of chitin synthetase zymogen during cell division (Zubenko, Mitchell and Jones 1979b), for the inactivation of NADP-dependent glutamate dehydrogenase during carbon starvation (Hemmings, Zubenko and Jones 1980), or for the inactivation of gluconeogenic enzymes when glucose is added to cultures, using acetate as a source of carbon (Zubenko and Jones 1978; Zubenko, Mitchell and Jones 1979a, Zubenko and Jones 1979a). We have found that proteinase B plays an important role in the protein degradation that occurs during sporulation and that in the absence of proteinase B activity, sporulation is abnormal (Zubenko and Jones 1979b; Zubenko and Jones, manuscript in preparation). The integration of all of these findings suggests that proteinase B is not involved in proteolytic processes that require a high degree of specificity, but has an important function in gross protein degradation.
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LITERATURE CITED


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