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Proline Utilization in *Saccharomyces cerevisiae*: Analysis of the Cloned *PUT1* Gene

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The *PUT1* gene was isolated by functional complementation of a *put1* (proline oxidase-deficient) mutation in *Saccharomyces cerevisiae*. Three independent clones with overlapping inserts of 6.8, 10.5, and 11 kilobases (kb) were isolated from *S. cerevisiae* genomic libraries in YEp24 (2μm) and YCp50 (*CEN*) plasmids. The identity of the *PUT1* gene was determined by a gene disruption technique, and Southern hybridization and genetic analyses confirmed that the bona fide gene had been cloned. Plasmids containing the *PUT1* gene restored regulated levels of proline oxidase activity to *put1* recipient strains. The *PUT1* DNA was present in a single copy in the yeast genome and encoded a transcript of ca. 1.5 kb. S1 nuclease protection experiments were used to determine the direction of transcription of the *PUT1* message and to localize its 5′ and 3′ termini within a subcloned 3-kb DNA fragment. Approximately 50-fold more *PUT1*-specific mRNA was detected in induced (proline-grown) cells than in uninduced (ammonia-grown) cells. A yeast strain carrying the previously identified *put3* regulatory mutation that caused constitutive levels of proline oxidase activity was found to have sevenfold elevated *PUT1* mRNA levels under noninducing conditions. The absence of a functional electron transport system in vegetative petite (*rho*$^-$) strains interfered with their ability to use proline as a nitrogen source. Although these strains were Put$^-$ and made no detectable proline oxidase activity, *PUT1* message was detected under inducing conditions. The *PUT1* gene was mapped distal to the *GAL2* gene on chromosome XII by tetrad analysis.

Metabolic conversion of proline to glutamate in *Saccharomyces cerevisiae* cells permits them to grow on a medium containing proline as the sole source of nitrogen. This two-step pathway occurs inside mitochondria and is catalyzed by the nuclearly encoded, cytoplasmically synthesized, and mitochondrially imported enzymes, proline oxidase and Δ^1-pyrroline-5-carboxylate (P5C) dehydrogenase (7, 8, 10). The activity levels of both enzymes are induced by proline, while the two transport systems that bring proline into the cell, the proline-specific (*PUT4*) permease and the general amino acid permease, are regulated not by proline induction but by nitrogen catabolite repression (18).

Several mutations in nuclear genes that affect proline utilization have been identified and characterized. Mutations in the *PUT1* gene result in a deficiency in proline oxidase activity, and those in the *PUT2* gene cause a reduction in P5C dehydrogenase activity (7). The *put3* mutation results in inducer-independent expression of both of these enzymes (8).

The *PUT2* gene has been cloned and characterized (6), sequenced (17), and recently identified as the structural gene for P5C dehydrogenase (J. Kaput and M. C. Brandriss, unpublished results). The gene is regulated at the level of RNA by proline, and the increase in enzyme activity seen in the *put3* mutant is correlated with an increase in *PUT2*-specific mRNA in that strain (6).

This report describes the isolation and identification of the *PUT1* gene from *S. cerevisiae* genomic libraries by complementation of a *put1* (proline oxidase-deficient) mutation. The cloned gene was used to analyze mRNA levels in the wild-type strain under noninducing and inducing conditions and in a respiratory-deficient strain. The size and direction of the *PUT1*-specific mRNA was determined by S1 mapping on a 3-kilobase (kb) *Kpn*I fragment. Proline oxidase levels in a yeast strain bearing a single copy of *PUT1* on a centromere-containing plasmid were compared with those in a strain carrying *PUT1* on a multicopy plasmid. The *PUT1* gene was mapped to chromosome XII by virtue of its linkage to *GAL2* and *PEP3*.

MATERIALS AND METHODS

Strains and media. The *S. cerevisiae* strains used in this work are described in Table 1. Strains C15-1A and C15-1B are meiotic products of a cross between strains DBY785 and MB379-1C. The *put1-207* mutation in strains C67 and C68 was isolated after ethyl methanesulfonate mutagenesis in a derivative of strain MB1000 and does not complement the *put1-54* allele. The media used for growth of *S. cerevisiae* strains have been described previously (7), except that 2% glucose was supplied instead of galactose. Proline and ammonium sulfate concentrations were 0.1 and 0.2%, respectively. EBgal medium, used for scoring the Gal$^-$ phenotype, was described by Matsumoto et al. (22).

Genetic analysis. Mating, sporulation, and tetrad analysis were carried out by standard procedures (31).

Plasmids and yeast genomic libraries. Plasmid pJH-U1 was kindly provided by John Hill. This plasmid was constructed by insertion of the *Hin*dIII fragment of the yeast *URA3* gene into the *Hin*dIII site of the multiple cloning site of the plasmid pUC18.

Shuttle vectors YEp24 (5) and YCp50 (28; kindly provided by J. Thomas) contain the bacterial Amp^r gene and the *S. cerevisiae URA3* gene for selection and sequences allowing autonomous replication (2 μm or *ARS1*). Plasmid YEp24

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source or reference
MB1000	*MAT*α wild type	7
MB1001	*MAT*α *rho*$^-$	9
MB1433	*MAT*α *ura3-52 trp1 PUT1*	6
MB379-1C	*MAT*α *his4-42 put1-54*	M. C. Brandriss
MB379-4C	*MAT***a** *his4-42 put1-54*	M. C. Brandriss
MB214-18B	*MAT*α *put3*	8
C15-1A	*MAT***a** *ura3-52 trp1 put1-54*	This work
C15-1B	*MAT***a** *ura3-52 his4-42 gal2*	This work
C1000	*MAT*α *ura3-52 trp1 put1::URA3*	This work
C1001	*MAT*α *ura3-52 trp1 put1::URA3*	This work
DBY785	*MAT***a** *ura3-52 trp1 gal2*	D. Botstein
BJ2785	*MAT*α *ura3 trp1 pep 3-12*	E. W. Jones
K381-10A	*MAT*α *ura3-1 ade6 arg4 aro7-1 asp5 met14 lys2-1 pet17 trp1*	16
C55	*MAT*α *ura3-52 trp1* + + *put1::URA3*	This work
	*MAT***a** *ura3-52* + *gal2 his 4-42* +	
C60	*MAT*α *ura3-52 trp1* + *put1::URA3*	This work
	*MAT***a** + + *his4-42 put1-54*	
C67	*MAT*α + + + *pep3-12 trp1 ura3*	This work
	*MAT***a** *his4-42 gal2 put1-207* + + +	
C68	*MAT*α + *asp5 gal2 put1-207 trp1 lys2-1*	This work
	*MAT***a** *ura3-52* + + + + +	

was maintained in high copy, whereas plasmid YCp50 contained the centromere of chromosome IV (*CEN4*) and was maintained in one to two copies per cell. Yeast genomic libraries constructed in YEp24 (11) and YCp50 (J. Thomas and M. Rose, personal communication) vectors were kindly provided by the laboratory of David Botstein. These libraries were constructed by ligation of yeast genomic DNA partially digested with *Sau*3A into the single *Bam*HI site of the YEp24 or YCp50 vector.

Reagents. Restriction endonucleases were obtained from New England Biolabs or Boehringer Mannheim Biochemicals and used as recommended by the manufacturer. Bovine intestinal alkaline phosphatase was obtained from Sigma Chemical Co. S1 nuclease was purchased from Bethesda Research Laboratories.

DNA preparation and transformation. Isolation of plasmid DNA from spheroplasts of *S. cerevisiae* was performed by the method of Birnboim and Doly (4). Preparation of plasmid DNA from *Escherichia coli* was carried out by the alkaline denaturation method (4) or by the CsCl gradient method (21).

E. coli transformation was done by the $CaCl_2$ method described by Cohen et al. (13). *S. cerevisiae* transformation was performed by the spheroplasting method as described previously (15). Yeast genomic DNA was isolated by the method of Davis et al. (14).

Electrophoresis of DNA, transfer to nitrocellulose, and hybridization. The methods used for electrophoresis, transfer to nitrocellulose, and hybridization have been described previously (6).

RNA preparation. Total RNA from *S. cerevisiae* was prepared as described by Needleman et al. (27). Poly (A)-containing RNA was purified by chromatography on oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.) as described previously (6).

Electrophoresis of RNA, transfer to nitrocellulose and hybridization. Total or poly (A)-containing RNA was subjected to 1.0% agarose gel electrophoresis and transferred to nitrocellulose membranes by the method described previously (6). Hybridization was carried out with nick-translated DNA probes as described by Thomas (34). The amount of total or poly (A)-containing RNA loaded in each lane was adjusted so that the *PUT1*-specific message was visible on the autoradiograms. The induction ratios of the *PUT1*-specific message were determined by measuring the intensity of the bands with respect to the amount of RNA loaded. A Zeineh scanning densitometer (Model SL-DNA) with a tungsten lamp was used for this purpose.

S1 mapping. For 3′ end labeling of the DNA fragment, plasmid pWB8 (Fig. 1) was first digested with the restriction enzyme *Pvu*II and then treated with T4 DNA polymerase as described by Challberg and Englund (12). End-labeled DNA was further digested with restriction enzyme *Kpn*I to generate the 2-kb *Kpn*I-*Pvu*II and 1.1-kb *Pvu*II-*Kpn*I single end-labeled DNA fragments.

For 5′ end labeling, the plasmid pWB8 was digested with restriction enzyme *Kpn*I and subjected to electrophoresis. The 3-kb *Kpn*I DNA fragment was electroeluted (21), digested with *Pvu*II endonuclease, dephosphorylated with bovine intestinal phosphatase as described previously (21), and then phosphorylated with T4 polynucleotide kinase by the method of Maxam and Gilbert (23). The labeled DNA was then digested with both *Cla*I and *Bgl*II enzymes to generate the 1.6-kb *Cla*I-*Pvu*II and 0.7-kb *Pvu*II-*Bgl*II single end-labeled DNA fragments.

S1 mapping was performed as described by Berk and Sharp (3), using empirically determined hybridization temperature (45°C) and S1 concentration. The reaction mixture contained 5 μg of poly (A)-containing RNA or 20 μg of total RNA, with bulk *E. coli* B tRNA (Schwartz/Mann) used as carrier. Electrophoresis on a 1.0% alkaline agarose gel was carried out by the method of McDonnell et al. (24).

Proline oxidase assay. Growth of yeast cells for the assay of proline oxidase activity was carried out as described previously (6). Cells were harvested and permeabilized, and the enzyme reactions were carried out as described previously (7). The values of proline oxidase in this report were approximately fivefold lower than previously reported (7) due to the substitution of glucose for galactose in the growth medium.

Protein determination. Whole-cell protein values were determined by a modification (33) of the method of Lowry et al. (19), with bovine serum albumin as the standard.

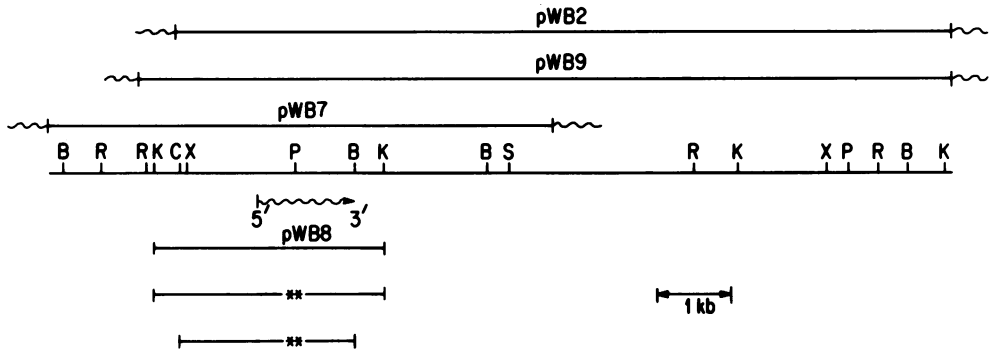

FIG. 1. Restriction map of the *PUT1* region and strategy for mapping the *PUT1* transcript. Plasmids pWB2 and pWB9 contain 10.5- and 11-kb inserts, respectively, in the vector YCp50. Plasmid pWB7 contains a 6.8-kb DNA insert in the vector YEp24. The 3-kb *Kpn*I fragment from plasmid pWB7 was inserted into the *Kpn*I site of plasmid YCp50 to form plasmid pWB8. The fragments of DNA shown under plasmid pWB8 were end labeled (fragments *Kpn*I-*Pvu*II and *Pvu*II-*Kpn*I were 3' end labeled; fragments *Cla*I-*Pvu*II and *Pvu*II-*Bgl*II were 5' end labeled) as indicated by the astericks. Wavy line represents the approximate position of the *PUT1* mRNA; arrow indicates the direction of its transcription. Abbreviations: B, *Bgl*II; C, *Cla*I; K, *Kpn*I; P, *Pvu*II; R, *Eco*RI; S, *Sac*I; X, *Xho*I.

RESULTS

Molecular cloning of the *PUT1* gene. The *PUT1* gene was isolated on recombinant DNA plasmids by functional complementation of a *put1* mutation in *S. cerevisiae*.

Two yeast genomic DNA libraries (one in plasmid YCp50 and the other in plasmid YEp24; see Materials and Methods) were used to transform *S. cerevisiae* C15-1A (*ura3 put1*) to uracil prototrophy. Approximately 10,000 independent Ura$^+$ transformants from each library were pooled and screened for their ability to grow on a medium containing proline as the sole nitrogen source (Put$^+$ phenotype). Those that were Put$^+$ were purified. Ura$^+$ Put$^+$ transformants that carried 2 μm plasmids showed the simultaneous loss of the two plasmid-borne markers after growth on a permissive medium at a frequency expected for genes carried by such a vector. The Ura$^+$ Put$^+$ transformants containing *CEN* sequences were very stable and showed the loss of these two genes at a frequency of 1 to 5%. Both types of plasmid DNAs were isolated from these cells and used to retransform the *put1* strain C15-1A to Put$^+$. This second transformation step eliminated the presence of extra plasmids and further purified the plasmid containing the *PUT1*-complementing DNA sequences. Plasmid DNAs were then isolated from these transformants and used to transform *E. coli* to ampicillin resistance. The plasmid DNA made from these *E. coli* transformants was used to transform the *put1* yeast strain C15-1A. All of the Ura$^+$ transformants obtained were Put$^+$.

Three independent clones that could complement the *put1-54* mutation were identified. Restriction maps of the yeast DNA inserts in plasmids pWB2, pWB7, and pWB9 are shown in Fig. 1. Plasmid pWB8 (Fig. 1), constructed by inserting a 3-kb *Kpn*I fragment from the region of overlap into the *Kpn*I site of the YCp50 vector, still contained the *PUT1*-complementing activity. Southern hybridization analysis of yeast genomic DNAs prepared from the wild-type yeast strain MB1000 and the *put1* mutant strain, with this 3-kb *Kpn*I fragment as the probe, revealed that this *PUT1*-complementing DNA was present in a single copy in the yeast genome (data not shown).

Identification of the cloned DNA. To prove that the cloned DNA insert was the bona fide *PUT1* gene and not a suppressor of the *put1* mutation, several experiments were carried out.

The *PUT1*-complementing DNA insert was disrupted on a plasmid and reintroduced into the yeast genome to replace the resident, wild-type copy. Two plasmids, pWB11 and pWB12, were constructed by inserting the *URA3* marker within the *PUT1*-complementing DNA (Fig. 2). Plasmid pWB11 contained a simple insertion of a 1.1-kb *Sma*I

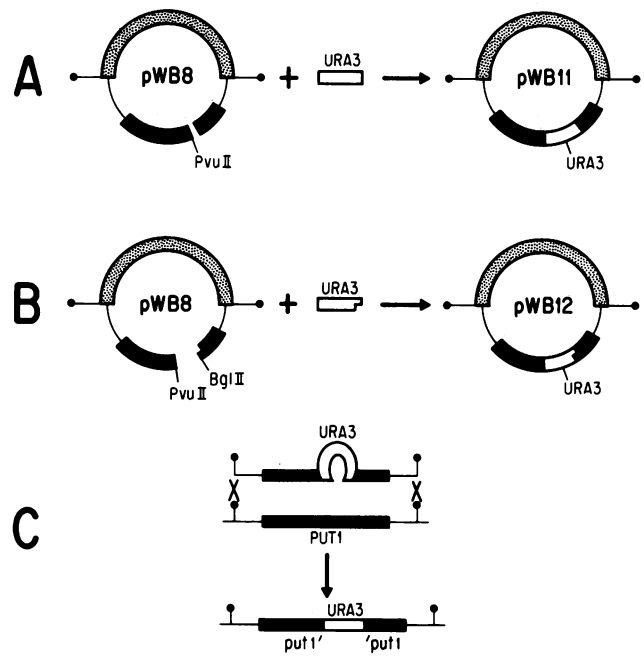

FIG. 2. Formation of the *put1* gene disruptions. (A) Plasmid pWB8 was digested with restriction enzyme *Pvu*II. A 1.1-kb *Sma*I fragment from plasmid pJH-U1 carrying the *URA3* gene was ligated into this site to form plasmid pWB11. (B) Plasmid pWB8 was digested with endonucleases *Pvu*II and *Bgl*II, and the 0.8-kb piece of DNA was removed. A 1.1-kb *Sma*I-*Bam*HI *URA3* fragment isolated from the plasmid pJH-U1 was ligated to the remainder of plasmid pWB8 to form plasmid pWB12. (C) DNAs from plasmids pWB11 and pWB12 were each digested with endonuclease *Kpn*I, and the linear DNA fragments were used to transform strain MB1433 (*ura3 PUT1*) to Ura$^+$. Resulting transformants had replaced the chromosomal *PUT1* region with the *put1::URA3* disruption, as shown, to become Ura$^+$ Put$^-$. Symbols: ▬, *PUT1* gene; ▨, YCp50 vector DNA; thin line, *S. cerevisiae* DNA; ↟, *Kpn*I restriction site; X, sites of cross-overs.

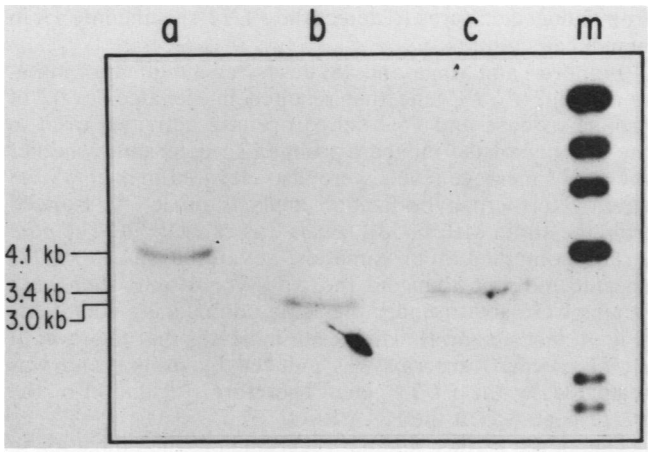

FIG. 3. Southern analysis of wild-type and *put1*-disrupted *S. cerevisiae* strains. 10 μg of yeast genomic DNA from each strain were digested with the restriction enzyme *Kpn*I. The DNAs were probed with the nick-translated 3-kb *Kpn*I fragment from plasmid pWB8. Lanes: a, genomic DNA from strain C1000; b, genomic DNA from parent strain MB1433; c, genomic DNA from strain C1001; m, *Hin*dIII-digested λ DNA as marker. The insertion of the *URA3* gene into the *PUT1* region caused the *Kpn*I fragment to increase in size by ca. 1.1 kb in strain C1000 and by 0.4 kb in strain C1001.

fragment carrying the *URA3* gene from plasmid pJH-U1 into the unique *Pvu*II site in the insert of plasmid pWB8. Plasmid pWB12 was constructed by deleting an 0.8-kb *Pvu*II-*Bgl*II fragment in the *PUT1*-complementing DNA and inserting a 1.1-kb *Sma*I-*Bam*HI *URA3* fragment isolated from plasmid pJH-U1 into the same region. These two plasmids were introduced into the *put1* mutant strain C15-1A and now failed to complement the *put1-54* mutation. This indicated that the *PUT1*-complementing DNA in the insert on plasmid pWB8 had been disrupted by the insertion of the *URA3* marker in plasmid pWB11 or pWB12. These two plasmids were then digested with restriction enzyme *Kpn*I. A 4.1-kb DNA fragment from plasmid pWB11 and a 3.4-kb DNA fragment from plasmid pWB12 were isolated. These DNA fragments carrying the *URA3* marker were introduced by transformation into the *ura3-52 PUT1* yeast strain, MB1433 (Fig. 2). Ura^+ transformants were selected. If the cloned gene were the bona fide *PUT1* gene, homologous recombination (30) between the incoming DNA fragment and the genomic *PUT1* locus would result in the replacement of the resident *PUT1* gene by the mutated copy, causing the transformants to become *put1*. Of the 32 Ura^+ transformants obtained, 19 were also Put^-. One Ura^+ Put^- transformant of each type was analyzed further.

Genomic DNAs digested with *Kpn*I from strains C1000 (carrying the simple *URA3* insertion), C1001 (carrying the insertion of *URA3* and an 0.8-kb deletion), and MB1433 (the parent strain) were subjected to Southern analysis (32) as described in Methods. Blots were probed with the 3-kb *Kpn*I insert from plasmid pWB8. The 3-kb *Kpn*I DNA fragment in the genome of the parental strain MB1433 (Fig. 3, lane b) was replaced by a 4.1-kb fragment in strain C1000 (lane a) or a 3.4-kb fragment in strain C1001 (lane c), which corresponded to the sizes of the *Kpn*I fragments from plasmids pWB11 and pWB12, respectively. Therefore, in strains C1000 and C1001, the homologous genomic region was replaced by the plasmid-borne *URA3*-disrupted fragment by homologous recombination.

The following genetic experiments confirmed that the *PUT1*-complementing DNA was the bona fide *PUT1* gene and that the native *PUT1* region had been disrupted. First, a genetic complementation test was carried out by crossing strain C1000 (*put1::URA3*) to strain MB379-4C containing the *put1-54* mutation. The *put* mutation carried in strain C1000 was unable to complement the *put1-54* mutation. Tetrad analysis was performed on the diploid strain (C60) isolated from the above cross. All 18 tetrads analyzed showed a 0 *PUT1*:4 *put1* genotype, as determined by complementation tests. This result indicated that the disrupted gene and the *PUT1* gene were allelic and were not two noncomplementing unlinked genes (2).

Second, cosegregation of the *URA3* marker and the disrupted *put1* gene was studied by tetrad analysis of a diploid (C55) made by crossing strains C1000 and C15-1B (*MATa ura3-52 his4-42 gal2*). The *URA3* and *put1* markers cosegregated in every tetrad (18 PD:0 NPD:0 T). This result indicated that the *URA3* marker and the *put1* locus in strain C1000 were tightly linked. Identical results were obtained in analogous experiments with strain C1001 (data not shown).

These results prove that the cloned DNA carrying the *PUT1*-complementing activity contained the bona fide *PUT1* gene and not a suppressor of the *put1* mutation.

Chromosomal mapping of the *PUT1* gene. The *PUT1* gene was located on chromosome XII by its linkage to the *gal2*, *asp5*, and *pep3* markers. Mapping data for two diploid strains, C67 and C68, are shown in Table 2. The *pep3-12* mutation in strain C67 was derived from strain BJ2785, and the *asp5* mutation in strain C68 was derived from strain K381-10A. The *PUT1* gene is approximately 36 centimorgans from *GAL2* and 22 centimorgans from *PEP3*.

In diploid strain C67, an unexplained interaction between *pep3* and *gal2* prevented us from scoring the *gal2* marker. We could still score the *put1* and *pep3* mutations. The *gal2-put1* and *put1-pep3* distances determined here, when added together, agreed well with the *gal2-pep3* distance determined by Zamb and Petes (35). *PUT1* showed no linkage to *ASP5*. The *CEN12-asp5* and *asp5-gal2* distances agreed well with previously published values (25, 26). The order and distances (in centimorgans) between these genes on chromosome XII are as follows: *CEN12*-22-*ASP5*-36-*GAL2*-36-*PUT1*-22-*PEP3*.

Regulation of the *PUT1* gene at the RNA level. The cloned *PUT1* gene was used to study the regulation of *PUT1* gene expression. Total RNA and poly (A)-containing RNA were

TABLE 2. Chromosomal mapping of *PUT1*

Strain, marker[a]	No. of asci					Map distance (cM)[d]
	Segregation[b]		Ascus type[c]			
	FD	SD	PD	NPD	T	
Strain C68						
CEN12-asp5	16	13				22 (18)
asp5-gal2			8	0	21	36 (32)
gal2-put1			8	0	21	36
asp5-put1			8	3	18	Unlinked
Strain C67						
put1-pep3			17	0	14	22

[a] See Table 1 for genotypes of strains C67 and C68. The *CEN12-asp5* interval was measured with respect to the *trp1* marker. All other markers segregated unlinked to chromosome XII markers.

[b] FD, first-division segregation; SD, second-division segregation.

[c] PD, parental ditype; NPD, nonparental ditype; T, tetratype.

[d] Distance in centimorgans equals [100 × (T + 6NPD)]/[2 × (PD + NPD + T)], using the equation derived by Perkins (29) and the abbreviations given in footnotes *b* and *c*. Numbers in parenthesis are published values (25).

prepared from the wild-type strain MB1000 grown in either the uninduced condition (minimal ammonia medium) or the induced condition (minimal proline medium). The RNAs were separated by electrophoresis on an agarose gel and transferred to nitrocellulose paper as described in Materials and Methods. The radioactively labeled 3-kb *Kpn*I *PUT1* DNA fragment was used as a probe to detect the RNAs homologous to the *PUT1* DNA. A single band corresponding to an RNA ca. 1.5 kb in length was detected when the RNAs were isolated from yeast cells grown in either ammonia medium or proline medium (Fig. 4, lanes d and e [total RNA] and lanes g and h [poly(A) RNA]). The level of *PUT1*-specific mRNA in proline-grown cultures was approximately 50-fold greater than that found in ammonia-grown cultures. Under partial induction (ammonia plus proline), the cultures had levels of *PUT1*-specific RNA approximately sevenfold higher than that found under uninduced conditions (Fig. 4, lanes c and e). The basal level of expression of the *PUT1* gene is so low that it was necessary to load an amount of RNA from uninduced cultures about 10 times that required from induced cultures to detect the *PUT1*-specific mRNA in uninduced cultures.

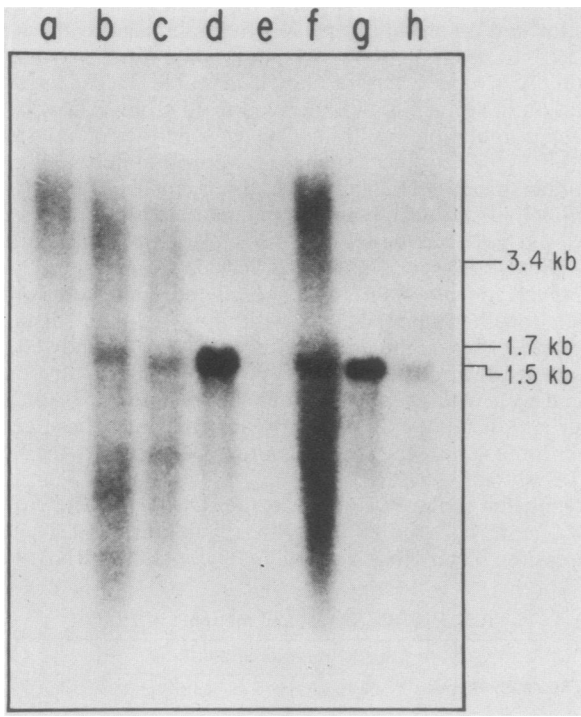

FIG. 4. Northern analysis of the *PUT1* gene. The nick-translated 3-kb *Kpn*I *PUT1* DNA fragment from plasmid pWB8 was used as the probe. Lane a, 48 μg of total RNA from *rho*⁻ strain MB1001 grown in ammonia medium; lane b, 47 μg of total RNA from MB1001 grown in proline-ammonia medium; lane c, 33 μg of total RNA from wild-type strain MB1000 grown in proline-ammonia medium; lane d, 4.4 μg of total RNA from MB1000 grown in proline medium; lane e, 43 μg of total RNA from MB1000 grown in ammonia medium; lane f, 66 μg of total RNA from *put3* strain MB214-18B grown in ammonia medium; lane g, 0.6 μg of poly (A)-containing RNA from MB1000 grown in proline medium; lane h, 7 μg of poly (A)-containing RNA from MB1000 grown in ammonia medium. The *PUT1* DNA probe detected a *PUT1* transcript of ca. 1.5 kb. The labels 3.4 and 1.7 kb refer, respectively, to the sizes of the large and small subunits of ribosomal RNAs from *S. cerevisiae*. The faint band in lane e is plainly visible on darker exposures of the X-ray film.

Brandriss and Magasanik (8) described a regulatory mutation in the *PUT3* gene that resulted in elevated levels of proline oxidase and P5C dehydrogenase activities even in the absence of the inducer, proline. To determine whether the *PUT1* message levels were also elevated in such a *put3* strain, Northern hybridization analysis on RNAs isolated from the strain MB214-18B (*put3*) was carried out. The *put3* strain contained approximately sevenfold more *PUT1*-specific message than did the wild-type strain when both strains were grown under the same noninducing conditions (Fig. 4, lanes e and f). This result indicates that the level of *PUT1*-specific transcript was induced by proline and was regulated by the *PUT3* gene. Therefore, regulation of the *PUT1* gene was at the RNA level.

One of the earliest findings regarding proline utilization in *S. cerevisiae* was the observation that vegetative petite (*rho*⁻) strains failed to utilize proline as the sole source of nitrogen (7, 20). *rho*⁻ strains were shown to have the second Put enzyme, P5C dehydrogenase, but were lacking detectable levels of proline oxidase (9). Since proline oxidase in a variety of organisms is linked to the respiratory chain (1), the inability to measure active proline oxidase in *rho*⁻ strains could have been due to the limitations of the enzyme assay, in which the natural electron acceptor missing in petite strains was used. Like the *rho*⁻ strains, *put1* mutants are also deficient in proline oxidase activity, but they are not respiratory deficient. To determine whether the *PUT1* gene was expressed in *rho*⁻ strains, the cloned DNA was used to identify the presence of the specific mRNA. Northern hybridization analysis on the total RNAs isolated from the *rho*⁻ strain MB1001 grown in minimal ammonia-proline medium revealed a single band 1.5 kb in length when the cloned 3-kb *Kpn*I *PUT1* DNA fragment was used as a probe (Fig. 4, lane b). The level of the *PUT1*-specific message in the *rho*⁻ strain was approximately equivalent to that found in the wild-type *rho*⁺ strain MB1000 grown under the same conditions (Fig. 4, cf. lanes b and c). However, in contrast to the results obtained with the wild type, no band was detected in the *rho*⁻ strain grown in noninducing conditions (Fig. 4, lane a). Further experiments will be required to determine whether the regulation of the *PUT1* gene is altered in ammonia-grown *rho*⁻ strains.

RNA mapping. To determine the location of the *PUT1* transcript within the 3-kb *Kpn*I DNA fragment on plasmid pWB8, S1 mapping was carried out by the method of Berk and Sharp (3).

Since insertion of a 1.1-kb *URA3* fragment into the *Pvu*II site within this 3-kb *Kpn*I DNA fragment on plasmid pWB8 abolished the *PUT1*-complementing activity, it was expected that the *PUT1* gene would be located in this region. The *Pvu*II site was therefore chosen for the end-labeling reactions. Plasmid pWB8 was digested with various restriction endonucleases and end labeled as described in Materials and Methods. The 2- and 1.1-kb 3′-labeled DNA fragments from *Pvu*II and *Kpn*I digestions and the 1.6- and 0.7-kb 5′-labeled DNA fragments from the *Pvu*II, *Cla*I, and *Bgl*II digestions were denatured and allowed to hybridize with the poly (A)-containing RNAs isolated from proline-grown or ammonia-grown yeast cultures. After S1 nuclease treatment, RNA protected a 0.8-kb DNA fragment from the 1.1-kb 3′-labeled *Pvu*II-*Kpn*I fragment (Fig. 5, lanes e and g) and a 0.5-kb DNA fragment from the 5′-labeled 1.6-kb *Cla*I-*Pvu*II fragment (lanes c and d). No protected DNA was detected when the 3′-labeled 2.0-kb *Kpn*I-*Pvu*II fragment (Fig. 5, lanes f

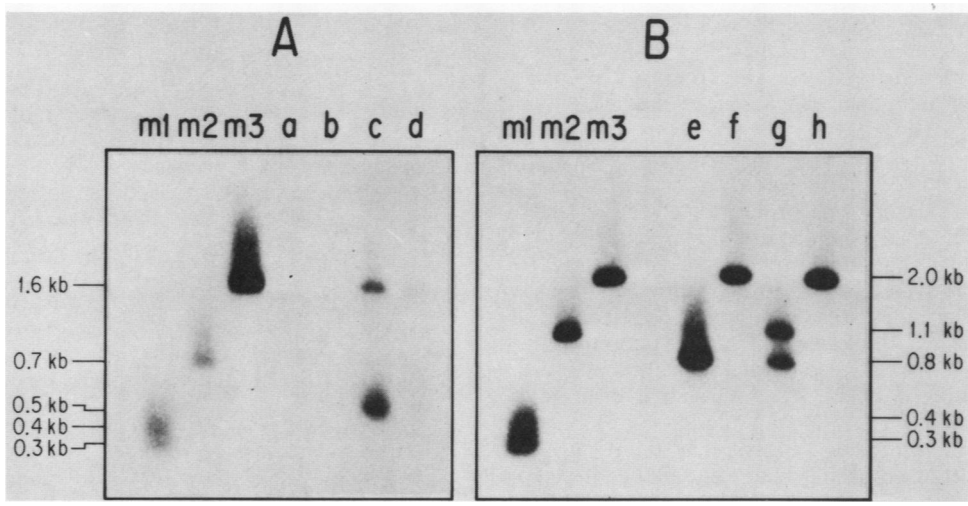

FIG. 5. S1 mapping of the *PUT1* gene. Total RNA (20 μg) or poly (A)-containing RNA (5 μg) from strain MB1000 grown in ammonia or proline medium was used for each reaction. (A) Blot hybridizations with 5'-labeled probes. The probes (see the legend to Fig. 1) were also used as markers. Lane m1, 0.3-kb *Kpn*I-*Cla*I fragment and 0.4-kb *Kpn*I-*Bgl*II fragment; lane m2, 0.7-kb *Pvu*II-*Bgl*II fragment; lane m3, 1.6-kb *Cla*I-*Pvu*II fragment. Lanes a and b, poly(A)-containing RNAs from proline-grown and ammonia-grown cells, respectively, hybridized with the 5'-labeled 0.7-kb *Pvu*II-*Bgl*II fragment. No protected band was detected. Lanes c and d, poly(A)-containing RNAs from proline-grown and ammonia-grown cells, respectively, hybridized with the 5'-labeled 1.6-kb *Cla*I-*Pvu*II fragment. A 0.5-kb piece of DNA was protected by these RNAs. (B) Blot hybridizations with 3'-labeled probes. Markers: lane m1, 0.3-kb *Kpn*I-*Cla*I fragment and 0.4-kb *Kpn*I-*Bgl*II fragment; lane m2, 1.1-kb *Pvu*II-*Kpn*I fragment; lane m3, 2.0-kb *Kpn*I-*Pvu*II fragment. Lanes e and g, total and poly (A)-containing RNA, respectively, from proline grown cells, hybridized with the 1.1-kb 3'-labeled *Pvu*II-*Kpn*I fragment. An 0.8-kb fragment of DNA was protected by these RNAs. Lanes f and h, total RNA and poly (A)-containing RNA, respectively, from proline-grown cells, hybridized with the 2.0-kb 3'-labeled *Kpn*I-*Pvu*II DNA fragment. No protected band was seen. This result determined the orientation and approximate position of the *PUT1* transcript as shown in Fig. 1.

and h) or the 5'-labeled 0.7-kb *Pvu*II-*Bgl*II fragment (lanes a and b) was used as a probe. This result placed the 5' end of the *PUT1* transcript 0.5 kb from the *Pvu*II site towards *Cla*I and the 3' end 0.8 kb from the *Pvu*II site towards the *Bgl*II site (Fig. 1). The length of the *PUT1* mRNA as found by S1 mapping is in good agreement with the size determined from the Northern analysis.

Induction of proline oxidase in strains bearing *PUT1*-containing plasmids. Proline oxidase levels were measured under noninducing (ammonia as the sole source of nitrogen) and inducing (ammonia and proline as the nitrogen sources) conditions in strains carrying the *PUT1* plasmids pWB2 and pWB7 or the parent plasmids YCp50 and YEp24. There was a two- to threefold induction of proline oxidase activity in strains carrying plasmids pWB2 (Table 3, experiment 2) or pWB7 (experiment 4) when the cells were grown in medium containing ammonia and proline. The low proline oxidase activity of the mutant *put1* strain did not increase upon induction in the strains bearing the plasmids lacking the *PUT1* insert (YCp50 or YEp24; Table 3, experiments 3 and 5). This result indicated that the cloned *PUT1* gene restored inducible proline oxidase activity to the *put1-54* mutant strain C15-1A. The induction ratio was comparable to that obtained for a wild-type (but not isogenic) strain, MB1000 (Table 3, experiment 1) (7).

DISCUSSION

The *PUT1* gene was isolated on three independent clones from yeast genomic libraries by its ability to complement a *put1* mutation in *S. cerevisiae*. When the plasmid-borne gene was interrupted by the *URA3* gene, it lost its *PUT1*-complementing activity. Integration of the disrupted gene into the yeast genome resulted in replacement of the genomic copy and formation of a *put1* mutation that mapped to the correct locus. This provided evidence that the bona fide *PUT1* gene was isolated and eliminated the possibility of having cloned a suppressor gene or a gene that was

TABLE 3. Proline oxidase levels in plasmid-bearing and wild-type strains

Expt no.	Strain[a]	Location of *PUT1* gene	Proline oxidase sp act on the following nitrogen source[b]	
			Ammonium	Ammonium plus proline
1	MB1000	Chromosome	0.29	1.19
2	C15-1A (pWB2)	Single-copy plasmid	0.31 (99–100)	1.07 (95–99)
3	C15-1A (YCp50)	Chromosome (defective)	0.22 (95–99)	0.29 (95–99)
4	C15-1A (pWB7)	Multicopy plasmid	0.42 (45–71)	0.90 (49–69)
5	C15-1A (YEp24)	Chromosome (defective)	0.20 (86)	0.32 (80)

[a] Strain MB1000 is wild type. Strain C15-1A is *put1*. Plasmid pWB2 is plasmid YCp50 with a 10.5-kb *PUT1* insert. Plasmid pWB7 is plasmid YEp24 with a 6.8-kb *PUT1* insert.

[b] Units of specific activity are nanomoles of P5C formed per minute per milligram of protein. Ammonium sulfate and proline were supplied at 0.2 and 0.1%, respectively. Numbers in parenthesis refer to the percent Ura^+ (plasmid-bearing) cells in each culture.

noncomplementing and nonallelic to the *PUT1* gene, as recently reported by Atkinson (2). The *PUT1* gene is found in single copy in the yeast genome, and encodes a proline-inducible mRNA of ca. 1.5 kb that increases about 50-fold upon full induction. The *PUT1* gene was mapped to chromosome XII by virtue of its linkage to *GAL2* and *PEP3*.

The work reported in this paper provides evidence that is consistent with *PUT1* being the structural gene for proline oxidase. The *PUT1* DNA complements the genetic lesion, restores measurable and appropriately regulated proline oxidase activity, and encodes a proline-inducible mRNA that responds, like proline oxidase activity (8), to the effects of the *put3* mutation. The extent of RNA induction correlated well with the 20-fold induction in proline oxidase activity reported previously (7). The difference in maximum expression between RNA and enzyme activity may reflect the limitations of the whole-cell enzyme assay used rather than a form of posttranscriptional or translational control. In addition, a previous study (8) showed a gene dosage effect on proline oxidase activity in *PUT1/put1* heterozygous diploids. We acknowledge that the above do not constitute proof and are merely suggestive that the *PUT1* gene encodes proline oxidase. Definitive proof awaits purification and analysis of this enzyme.

The cloned *PUT1* gene carried by the single-copy plasmid pWB2 or the multicopy plasmid pWB7 restored the inducibility for proline oxidase in the recipient *put1-54* mutant strain C15-1A (Table 3). The strain bearing the multicopy *PUT1* plasmid pWB7 did not show the elevated levels of proline oxidase expected with increased gene dosage. This plasmid displayed greater instability than its parent plasmid, YEp24, which would lead to a lower apparent enzyme activity in the population. This finding suggests that the yeast strain cannot tolerate additional copies of the insert DNA found in pWB7.

Whether the *PUT1* gene is the structural gene for proline oxidase or some other effector of its activity, its transcription occurred in respiratory-deficient cells and was, therefore, not the direct cause of the Put^- phenotype of rho^- strains. Whether proline oxidase polypeptide is made under these conditions or is enzymatically silent remains to be determined.

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LITERATURE CITED

1. **Adams, E., and L. Frank.** 1980. Metabolism of proline and the hydroxyprolines. Annu. Rev. Biochem. **49**:1005–1061.
2. **Atkinson, D. K.** 1985. Two recessive suppressors of *Saccharomyces cerevisiae CHO1* that are unlinked but fall in the same complementation group. Genetics **111**:1–6.
3. **Berk, A. J., and P. A. Sharp.** 1977. Sizing and mapping of early adenovirus mRNAs by gel electrophoresis of S1 endonuclease-digested hybrids. Cell **12**:721–732.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7**:1513–1523.
5. **Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeast (SHY): a eucaryotic system of biological containment for recombinant DNA experiments. Gene **8**:17–24.
6. **Brandriss, M. C.** 1983. Proline utilization in *Saccharomyces cerevisiae*: analysis of the cloned *PUT2* gene. Mol. Cell. Biol. **3**:1846–1856.
7. **Brandriss, M. C., and B. Magasanik.** 1979. Genetics and physiology of proline utilization in *Saccharomyces cerevisiae*: enzyme induction by proline. J. Bacteriol. **140**:498–503.
8. **Brandriss, M. C., and B. Magasanik.** 1979. Genetics and physiology of proline utilization in *Saccharomyces cerevisiae*: mutation causing constitutive enzyme expression. J. Bacteriol. **140**:504–507.
9. **Brandriss, M. C., and B. Magasanik.** 1980. Proline: an essential intermediate in arginine degradation in *Saccharomyces cerevisiae*. J. Bacteriol. **143**:1403–1410.
10. **Brandriss, M. C., and B. Magasanik.** 1981. Subcellular compartmentation in control of converging pathways for proline and arginine metabolism in *Saccharomyces cerevisiae*. J. Bacteriol. **145**:1359–1364.
11. **Carlson, M., and D. Botstein.** 1982. Two differentially regulated mRNAs with different 5′ ends encode secreted and intracellular forms of yeast invertase. Cell **28**:145–154.
12. **Challberg, M. D., and P. T. Englund.** 1980. Specific labeling of 3′ termini with T4 DNA polymerase. Methods Enzymol. **65**:39–43.
13. **Cohen, S. N., A. C. Y. Chang, and L. Hsu.** 1973. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. USA **69**:2110–2114.
14. **Davis, R. W., M. Thomas, J. Cameron, T. P. St. John, S. Scherer, and R. A. Padgett.** 1980. Rapid DNA isolations for enzymatic and hybridization analysis. Methods Enzymol. **65**:404–411.
15. **Hinnen, A., J. B. Hicks, and G. R. Fink.** 1978. Transformation of yeast. Proc. Natl. Acad. Sci. USA **75**:1929–1933.
16. **Klapholz, S., and R. E. Esposito.** 1982. A new mapping method employing a meiotic Rec^- mutant of yeast. Genetics **100**:387–412.
17. **Krzywicki, K. A., and M. C. Brandriss.** 1984. Primary structure of the nuclear *PUT2* gene involved in the mitochondrial pathway for proline utilization in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4**:2837–2842.
18. **Lasko, P. F., and M. C. Brandriss.** 1981. Proline transport in *Saccharomyces cerevisiae*. J. Bacteriol. **148**:241–247.
19. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
20. **Lundgren, D. W., M. Ogur, and S. Yuen.** 1972. The isolation and characterization of a *Saccharomyces* mutant deficient in Δ^1-pyrroline-5-carboxylate dehydrogenase activity. Biochem. Biophys. Acta **286**:360–362.
21. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
22. **Matsumoto, K., A. Toh-e, and Y. Oshima.** 1978. Genetic control of galactokinase synthesis in *Saccharomyces cerevisiae*: evidence for constitutive expression of the positive regulatory gene *gal4*. J. Bacteriol. **134**:446–457.
23. **Maxam, A. M., and W. Gilbert.** 1980. Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. **65**:499–560.
24. **McDonnell, M. W., M. N. Simon, and F. W. Studier.** 1977. Analysis of restriction fragments of T7 DNA and determination of molecular weights by electrophoresis in neutral and alkaline gels. J. Mol. Biol. **110**:119–146.
25. **Mortimer, R. K., and D. Schild.** 1980. Genetic map of *Saccharomyces cerevisiae*. Microbiol. Rev. **44**:519–571.
26. **Mortimer, R. K., and D. Schild.** 1985. Genetic map of *Saccha-*

romyces cerevisiae, edition 9. Microbiol. Rev. **49**:181–212.
27. **Needleman, R. B., D. B. Kaback, R. A. Dubin, E. L. Perkins, N. G. Rosenberg, K. A. Sutherland, D. B. Forrest, and C. A. Michels.** 1984. *MAL6* of *Saccharomyces*: a complex genetic locus containing three genes required for maltose fermentation. Proc. Natl. Acad. Sci. USA **81**:2811–2815.
28. **Parent, S. A., C. M. Fenimore, and K. A. Bostian.** 1985. Vector systems for the expression, analysis and cloning of DNA sequences in *S. cerevisiae*. Yeast **1**:83–138.
29. **Perkins, D. D.** 1949. Biochemical mutants in the smut fungus *Ustilago maydis*. Genetics **34**:607–626.
30. **Rothstein, R. J.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101**:202–211.
31. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1978. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
32. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
33. **Stewart, P. R.** 1975. Analytical methods for yeasts. Methods Cell Biol. **12**:111–147.
34. **Thomas, P.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
35. **Zamb, T. J., and T. D. Petes.** 1982. Analysis of the junction between ribosomal RNA genes and single-copy chromosomal sequences in the yeast *Saccharomyces cerevisiae*. Cell **28**: 355–364.