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Proline Utilization in *Saccharomyces cerevisiae*: Analysis of the Cloned *PUT2* Gene

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The *PUT2* gene was isolated on a 6.5-kilobase insert of a recombinant DNA plasmid by functional complementation of a *put2* (Δ^1-pyrroline-5-carboxylate dehydrogenase-deficient) mutation in *Saccharomyces cerevisiae*. Its identity was confirmed by a gene disruption technique in which the chromosomal *PUT2*$^+$ gene was replaced by plasmid DNA carrying the *put2* gene into which the *S. cerevisiae HIS3*$^+$ gene had been inserted. The cloned *PUT2* gene was used to probe specific mRNA levels: full induction of the *PUT2* gene resulted in a 15-fold increase over the uninduced level. The *PUT2*-specific mRNA was approximately 2 kilobases in length and was used in S1 nuclease protection experiments to locate the gene to a 3-kilobase *Hin*dIII fragment. When Δ^1-pyrroline-5-carboxylate dehydrogenase activity levels were measured in strains carrying the original plasmid, as well as in subclones, similar induction ratios were found as compared with enzyme levels in haploid yeast strains. Effects due to increased copy number or position were also seen. The cloned gene on a 2μ-containing vector was used to map the *PUT2* gene to chromosome VIII.

The yeast *Saccharomyces cerevisiae* can grow on proline as the sole source of nitrogen. Proline utilization occurs inside the mitochondrion, where two nuclear-encoded enzymes convert proline into glutamate (8, 9, 11). Both these enzymes, proline oxidase and Δ^1-pyrroline-5-carboxylate (P5C) dehydrogenase, are induced by proline (6, 7). Two systems transport proline across the cell membrane; these are regulated by nitrogen catabolite repression and not by proline induction (8, 9, 24). Mutations resulting in deficiencies in proline oxidase (*put1*), P5C dehydrogenase (*put2*), or proline transport (*put4* and *gap*) activities have been described previously (8, 24).

Since proline utilization involves at least three compartments within the yeast cell, its regulation is expected to be complex. Almost nothing is known about events controlling *PUT1* and *PUT2* transcription; this report presents the first indication of regulation at the level of RNA synthesis or stability. The *PUT3* gene appears to be involved in regulation of the pathway, since a mutation in this gene results in constitutive expression of both proline oxidase and P5C dehydrogenase (9). Once the messages are made and exported to the cytoplasm, the proteins are synthesized on cytoplasmic ribosomes and shipped across the mitochondrial membranes to the appropriate mitochondrial compartment. Proline must be transported across the mitochondrial membrane to serve as substrate. In contrast, proline biosynthesis involves a different set of enzymes, and the last step is known to occur in the cytoplasm (7).

A molecular analysis of proline utilization was begun in order to understand more about the regulation of this multicompartment system. This report describes the isolation and identification of the *PUT2* gene on a recombinant DNA plasmid by functional complementation of a *put2* mutation in *S. cerevisiae*. The location of the gene on the plasmid was determined by S1 nuclease mapping (4), and the cloned gene was used as a probe for mRNA levels under inducing and noninducing conditions. The effect of gene dosage on enzyme level was determined by comparison of yeast strains carrying a single chromosomal gene with those carrying the gene on single-copy and multicopy plasmids. In addition, the *PUT2* gene was mapped to chromosome VIII by using the cloned gene on a recombinant 2μ plasmid.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* and bacterial strains used in this work are described in Table 1. Strains MB1000, MB211-3B, and MB214-18B are isogenic and were derived from Σ1278b (8, 9). Strains DBY785 and DBY939 are congenic to S288C and were obtained from C. Falco and M. Carlson, respectively. MB331-17A was derived from a cross between MB211-3B and

TABLE 1. Strains used in this work

Strain	Genotype	Source or reference
S. cerevisiae		
MB1000	*MAT*α wild type	(8)
MB211-3B	*MAT*α *his4-42 put2-57*	(10)
MB214-18B	*put3*	(9)
DBY785	*MAT*α *ura3-52 trp1-1*	D. Botstein
DBY939	*MAT*α *suc2-215 ade2-101*	(12)
MB331-17A	*MAT*α *ura3-52 trp1-1 put2-57*	This work
MB1433	*MAT*α *ura3-52 trp1-1 PUT2*	This work
W301-18A	*MAT*α *ade2-1 his3-11,15 ura3-1 trp1-1 leu2-3,112*	R. Rothstein
MB1426	*MAT*α *ade2-1 his3-11,15 ura3-1 trp1-1 leu2-3,112 put2::HIS3*	This work
K381-15C	*MAT***a** *ura3-1 ade6 arg4 aro7-1 asp5 met14 lys2-1 pet17 trp1*	(23)
K393-27C	*MAT***a** *ura3-1 his2 leu1-12 lys1-1 met4 pet8*	(23)
K396-27B	*MAT***a** *ura3-1 ade1 his1 leu2 lys7 met3 trp5-d*	(23)
SP2-3C	*MAT***a** *leu1 leu2 pet3*	S. Klapholz
JW913-4D	*MAT*α *arg4 thr1 cup1*	(20)
X3127-2A	*MAT***a** *lys7 leu1 leu2 trp1 pet17 ura3 thr1 arg4 his5 ade5 gal2*	S. Klapholz
MB638	*MAT***a** *arg4 thr1* + *cup1* + / *MAT*α + + *put2-57* + *pet3*	This work
E. coli		
HB101	*hsdR hsdM proA2 lacZ24 leuB thi-1 rpsL20 supE44 recA13*	(26)

DBY785 and carries the *put2-57* region of MB211-3B. W301-18A is related to X2180 and was obtained from R. Rothstein. S. Klapholz graciously provided the *ura3* mapping strains (23), and J. Welch provided a copper-sensitive strain (20).

Plasmids. Two *S. cerevisiae-Escherichia coli* shuttle vectors carrying a single *Hin*dIII site were kindly provided by F. Winston. Both were derived from plasmid YIp5 (6). Plasmid pCGS42 carries the *S. cerevisiae URA3* gene and 2μ DNA for selection and autonomous replication in yeasts and displays the inherent instability characteristic of 2μ-containing plasmids. Plasmid pFW14 contains, in addition to the *URA3* gene, the centromere of chromosome III (*CEN3*) and a sequence allowing autonomous replication (*ars1*), which together cause the plasmid to be maintained stably and in low copy number (one to two copies per cell) in *S. cerevisiae* (13).

Genetic analysis. The methods used have been previously described (8).

Media. The media used for growth of *S. cerevisiae* strains have been described (8), except that 2% glucose was supplied instead of galactose. Proline and ammonium sulfate concentrations were 0.1 and 0.2%, respectively.

E. coli was grown in LB (26) broth or agar supplemented with ampicillin (Bristol Laboratories; 100 μg/ml) as required. M9 medium (29) supplemented with Casamino Acids (1 g/liter) was used for large-scale plasmid preparation.

Restriction enzymes and chemicals. Restriction endonucleases were obtained from New England Biolabs or Boehringer Mannheim Corp. Avian myeloblastosis virus reverse transcriptase was purchased from Life Sciences, Inc. NAD and Meldola Blue were purchased from Boehringer Mannheim Corp. Agarose HGT was purchased from Seakem. DL-(+)-Allo-δ-hydroxylysine hydrochloride was purchased from Calbiochem, and *p*-iodonitrotetrazolium violet (INT), *o*-aminobenzaldehyde, and *N*-tris(hydroxymethyl)methylaminopropane-sulfonic acid were purchased from Sigma Chemical Co. S1 nuclease was purchased from Miles Laboratories.

DL-Δ^1-Pyrroline-5-carboxylate was synthesized by the method of Williams and Frank (39) and used as described previously (8).

DNA preparation. Large-scale plasmid DNA was prepared from *E. coli* by the method of Clewell and Helinski (14). Rapid isolation of plasmid DNA from either *E. coli* or spheroplasts of *S. cerevisiae* was performed essentially as described by Birnboim and Doly (5). *S. cerevisiae* genomic DNA was isolated by the method of Davis et al. (17).

Electrophoresis of DNA, transfer to nitrocellulose, and hybridization. Agarose gel electrophoresis of DNA was carried out in 50 mM Tris base–67 mM boric acid–10 mM EDTA (pH 8.0) or 40 mM Tris base–5 mM sodium acetate–1 mM EDTA (pH 7.9). The DNA fragments were transferred from the gels to nitrocellulose filters by the method of Southern (35). Hybridizations were carried out at 65°C in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) plus 1% Sarkosyl overnight with probes nick translated by the method of Rigby et al. (33).

The filters were exposed to Kodak XRP-5 film at −70°C with DuPont Cronex Lightning-Plus screens.

DNA transformation. Plasmid DNA transformation of *E. coli* was carried out by the method of Cohen et al. (15). DNA transformation of *S. cerevisiae* was done by the method of Hinnen et al. (21).

RNA preparation. Total RNA from *S. cerevisiae* was prepared by the guanidinium-thiocyanate method of Hirsh and Davidson (22). This method was modified for yeasts by L. Feldberg and D. Kaback, using a Braun homogenizer to open the cells. Polyadenylic acid [poly(A)]-containing RNA was purified by chromatography on oligodeoxythymidylic acid-cellulose (Collaborative Research, Inc.). RNA was loaded on the column in binding buffer containing 0.5 M NaCl–0.01 M Tris (pH 7.5)–0.5% sodium dodecyl sulfate–1

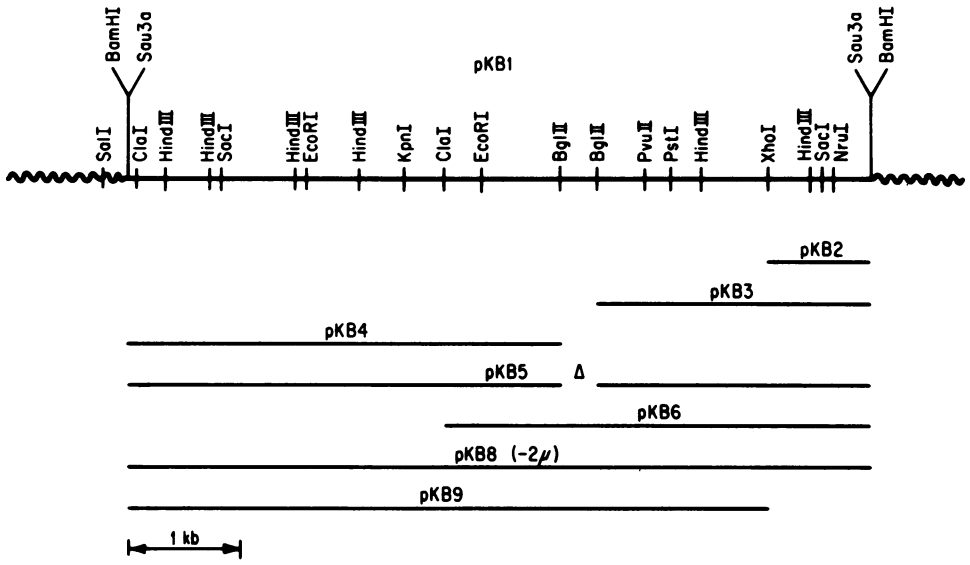

FIG. 1. Restriction map of the *PUT2* region in plasmid pKB1 and its subclones.

mM EDTA. After the column was washed with binding buffer, poly(A)-containing RNA was eluted with 0.01 M Tris (pH 7.5)–0.05% sodium dodecyl sulfate–1 mM EDTA. The RNA was concentrated by ethanol precipitation and suspended in 0.1 ml of 10 mM Tris (pH 7.5)–1 mM EDTA. A 0.9-ml amount of dimethyl sulfoxide and 0.1 ml of lithium chloride buffer (1 M LiCl, 10 mM Tris [pH 7.5], 50 mM EDTA) were added, and the mixture was heated at 50 to 55°C for 5 min. Immediately, 10 ml of binding buffer was added, and the oligodeoxythymidylic acid-cellulose chromatography was repeated as above. The poly(A)-containing RNA was concentrated by ethanol precipitation.

Electrophoresis of RNA, transfer to nitrocellulose, and hybridization. Total or poly(A)-containing RNA was subjected to agarose gel electrophoresis in 50 mM boric acid–5 mM sodium borate–1 mM EDTA–5 mM methylmercuric hydroxide (3). The RNA was transferred to nitrocellulose membranes, and hybridization was carried out with nick-translated DNA probes as described by Thomas (37). A Zeineh soft-laser scanning densitometer was used to compare gel bands on the autoradiograms.

S1 mapping. Plasmid pKB1 DNA (Fig. 1) was cut with the restriction enzyme *Bgl*II, extracted with phenol and chloroform, and precipitated with ethanol. For 3′ end labeling, the DNA was suspended in reverse transcriptase buffer and α [^{32}P]dATP was incorporated by the method of Bahl et al. (2). The DNA was extracted with phenol and chloroform, precipitated with ethanol, and digested with endonuclease *Sac*I. The fragments were subjected to agarose gel electrophoresis, and the 3.1-, 2.2-, and 0.34-kilobase (kb) bands were electroeluted.

For 5′ end labeling, the *Bgl*II-cut DNA was first dephosphorylated with calf intestinal phosphatase as described previously (26) and then was 5′ phosphorylated with T4 polynucleotide kinase by the method of Maxam and Gilbert (27). The DNA was then cut with *Sac*I endonuclease, and the fragments were separated by gel electrophoresis and electroeluted.

When plasmid pKB1 was cut with *Cla*I, the 4.8-kb fragment was purified by agarose gel electrophoresis and electroelution. It was then 5′ end labeled as described above.

Strand separation of the 340-base pair (bp) *Bgl*II fragment was carried out by the method of Maxam and Gilbert (27).

The S1 mapping procedure used was that of Berk and Sharp (4), using empirically determined hybridization temperature (45°C) and S1 concentrations. The reaction mixture contained 7.5 μg of poly(A)-containing *S. cerevisiae* RNA, with bulk *E. coli* B tRNA (Schwartz-Mann) used as carrier. Alkaline agarose gel electrophoresis was carried out by the method of McDonnell et al. (28).

Growth of yeast cells for enzyme assays. Twenty-milliliter amounts of the appropriately supplemented minimal medium (glucose-proline, glucose-ammonia-proline, or glucose-ammonia) in 125-ml sidearm flasks were inoculated to a density of 1 to 10 Klett units (blue filter), using a Klett-Summerson colorimeter. Cultures were shaken at 30°C until their densities reached 80 to 100 Klett units.

Preparation of cell extracts. The cells were harvested and washed with an equal volume (20 ml) of water and then with 5 ml of 50 mM potassium phosphate buffer (pH 7.7). Glass beads (0.45 to 0.50 mm; B. Braun; roughly equal in volume to the cell pellet) were added, and the slurry was blended vigorously with a Vortex-Genie mixer for four 30-s intervals interspersed with periods of cooling on ice.

Cell extract was removed from the beads and transferred to a centrifuge tube. The glass-bead slurry was rinsed twice with 1 to 1.5 ml of buffer. The rinses were combined with the extract and centrifuged at 12,100 × *g* for 20 min. The supernatant was used to assay P5C dehydrogenase activity and protein.

P5C dehydrogenase assay. The colorimetric assay for P5C dehydrogenase was suggested by June Bossinger, based on the methods of Dommes and Kunau (18). The assay measures the P5C-dependent production of reduced INT (INTH). Previously, the activity of this enzyme was measured spectrophotometrically by the formation of NADH (8).

The assay mixture contained, per 0.9 ml: 0.1 ml of Triton X-100 (1.5%), 5 μl of Meldola Blue (2.3 mg/ml), 0.1 ml of INT (1.3 mg/ml), 0.05 ml of *N*-tris(hydroxymethyl)methylaminopropane-sulfonic acid (1 M; pH 9.0), 0.05 ml of NAD (20 mg/ml), crude extract up to 0.095 ml, and 0.5 ml of P5C (ca. 5 mM; pH 7.0).

Tubes containing the assay mixture (minus substrate) were kept on ice, and the reaction was started by the addition of P5C or water and transferred to a 30°C water bath. The reaction was stopped by the addition of 0.1 ml of concentrated HCl. The absorbance was read at 492 nm. The millimolar extinction coefficient of INTH is 19.4 (18).

A blank with water substituted for P5C was included for every sample of crude extract, and the absorbance of the blank was subtracted to yield a net absorbance. The assay was linear with time at least to 45 min and with extract concentration. Reactions in which the total absorbance exceeded 1.3 were repeated with diluted extracts.

The INT was stored at 4°C in a dark bottle and kept for 2 weeks at most. The solution of Meldola Blue was filtered after it was made up. The assay mixture is light sensitive and was mixed immediately before the assay was carried out. Specific activity was defined as nanomoles of INTH formed per minute per milligram of protein. Each specific activity value represented the average of two or more enzyme assays, in each of which several extract concentrations were measured.

Protein determination. Protein was determined by the method of Lowry et al. (25), using bovine serum albumin as a standard.

RESULTS

Isolation of the *PUT2* gene. The *PUT2* gene was identified on a recombinant DNA plasmid by its functional complementation of a *put2* mutation in *S. cerevisiae*. DNA from *S. cerevisiae* DBY939 (*MATα suc2-215 ade2-101 PUT2*), contained in recombinant plasmid libraries made in plasmid YEp24 (6), was constructed and described by Carlson and Botstein (12) and was kindly provided by Carl Falco. YEp24 is a pBR322-derived plasmid carrying a fragment of 2μ DNA and the *URA3* gene for autonomous replication and selection in *S. cerevisiae*.

Selection for plasmids carrying the *PUT2* gene was carried out in two steps. Library DNA was used to transform *S. cerevisiae* MB331-17A (*MATα ura3-52 trp1-1 put2-57*) to uracil prototrophy. The Ura^+ transformants were pooled and screened for single colonies which were capable of growth on proline as sole nitrogen source (Put^+ phenotype). Three independent Ura^+ Put^+ transformants were studied further.

Growth on a nonselective medium resulted in loss of both markers simultaneously at a frequency expected for genes on 2μ-containing vectors. Plasmid DNA isolated from these transformants was used to transform *E. coli* to ampicillin resistance. Large plasmid DNA preparations were made from *E. coli* for restriction enzyme analysis. The three plasmids were found to have overlapping restriction maps with a common region of 6 kb, suggesting that they represented a single chromosomal region (data not shown). The three plasmids contained yeast DNA inserts of ca. 6.5, 7.5, and 13 kb. When these plasmid preparations were reintroduced into *S. cerevisiae* by transformation, 100% of the Ura^+ transformants were Put^+, and they behaved exactly like the original Ura^+ Put^+ transformants. The plasmid with the smallest insert was called pKB1 and was analyzed further. A map of restriction enzyme sites in the inserted DNA of plasmid pKB1 is shown in Fig. 1.

Subcloning plasmid pKB1. Subclones of plasmid pKB1 were made by cutting the yeast DNA with various restriction enzymes and ligating fragments back into the parental plasmid YEp24 (pKB2, pKB3, pKB4, pKB9) or by partial or complete digestion of plasmid pKB1 with subsequent ligation of the DNA ends (pKB5, pKB6, pKB8). The amount of inserted DNA carried by these subclones is shown in Fig. 1. Plasmid pKB5 is deleted for the 340 bp between the two *Bgl*II sites and contains a single *Bgl*II site. Plasmid pKB8 contains the same insert DNA as plasmid pKB1 but is deleted for the 2μ DNA and is no longer an autonomously replicating plasmid in *S. cerevisiae*.

DNA from each plasmid was isolated and used to transform MB331-17A to Ura^+. The transformants were tested for their ability to use proline as sole nitrogen source, as an indication of the presence of the entire *PUT2* gene. Only transformants containing the stably integrated plasmid pKB8 or the autonomously replicating plasmid pKB9 were able to use proline as the sole nitrogen source. Transformants carrying plasmids pKB2, pKB3, pKB4, pKB5, and pKB6 were all Put^-. These results indicated that the small region of DNA between the *Bgl*II sites was important for *PUT2* function (pKB5) and that the rightmost 1 kb of DNA between the *Xho*I and *Bam*HI-*Sau*3a sites in plasmid pKB1 was dispensable (pKB9). Some DNA to the left of the central *Cla*I site is also important for *PUT2* function, as indicated by the Put^- phenotype of strain MB331-17A(pKB6). The cloned DNA complements the *put2* mutation whether the DNA is on a multicopy plasmid (pKB1 or pKB9) or is stably integrated in the genome (pKB8).

One-step disruption of the *PUT2* gene. To eliminate the possibility of having cloned a suppressor of the *put2-57* mutation, it was neces-

sary to prove that the *PUT2*-complementing activity carried on plasmid pKB1 was due to the presence of the bona fide *PUT2* gene. This was done by using the one-step gene disruption method described by Rothstein (34). The plasmid pKB8 was used for these manipulations since it lacked 2μ DNA and was capable of stable integration into the *S. cerevisiae* genome. The DNA of pKB8 was cut with the restriction enzyme *Bgl*II and purified to remove the 340-bp fragment. A 1.7-kb *Bam*HI fragment carrying the *S. cerevisiae HIS3* gene was ligated to the *Bgl*II-cut plasmid pKB8 to form plasmid pKB10 (Fig. 2A). Given the results from transformations with plasmid pKB5, pKB10 was presumed to be unable to complement *put2*$^-$ mutations due to the deletion of 340 bp between the *Bgl*II sites. DNA from plasmid pKB10 was cut with the restriction enzyme *Sac*I, resulting in two fragments of approximately equal size (7.0 kb), one carrying the disrupted *put2* gene (*put2*::*HIS3*) and the other carrying pBR322 DNA and the *URA3* gene. Without further purification, this DNA (100 to 150 ng) was used to transform a *his3*$^-$ *PUT2*$^+$ *S. cerevisiae* strain (W301-18A) with selection for His$^+$ transformants. Since DNA ends have been shown to be recombinogenic, leading to integration of linear molecules at regions of DNA homology (31), it was expected that the *put2*::*HIS3* region would

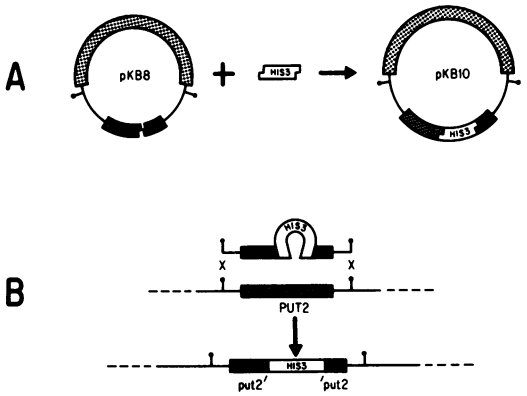

FIG. 2. Formation of the *put2* gene disruption. (A) Plasmid pKB8 was cut with the restriction enzyme *Bgl*II, and the small 340-bp piece was removed. A 1.7-kb *Bam*HI fragment carrying the *HIS3* gene was ligated to the remainder of plasmid pKB8 to form plasmid pKB10. (B) Plasmid pKB10 DNA was cut with restriction enzyme *Sac*I, and the linear DNA fragments were used to transform a *his3*$^-$ *PUT2*$^+$ *S. cerevisiae* strain to *His*$^+$. One transformant had replaced the chromosomal *PUT2* region with the *put2*::*HIS3* disruption, as shown, to become His$^+$ Put$^-$. Symbols: Cross-hatched region, *PUT2* gene; stippled region, YEp24 vector DNA; thin line, *S. cerevisiae* DNA; ●, *Sac*I site; X, sites of cross-overs.

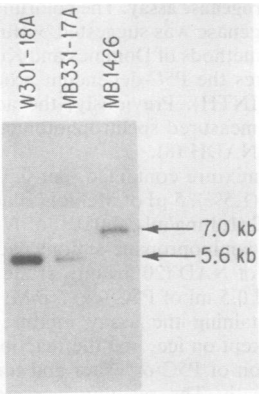

FIG. 3. Southern analysis of wild-type and *put2*-disrupted *S. cerevisiae* strains. Yeast genomic DNA was digested with the restriction enzyme *Sac*I and treated as described in the text. The DNAs were probed with the nick-translated 5.6-kb *Sac*I fragment from plasmid pKB8. Strain MB331-17A is *put2-57*. Strain W301-18A is *PUT2* and the parent of MB1426, which carries the *put2*::*HIS3* gene disruption. The insertion of the *HIS3* gene into the *PUT2* region caused the *Sac*I fragment to increase in size by ca. 1.4 kb.

stably integrate at the locus of the chromosomal *PUT2* gene (Fig. 2B).

Fourteen transformants were obtained. One, MB1426, had become simultaneously His$^+$ and *put2*$^-$ and did not contain any pBR322 DNA. In subsequent genetic analysis, it was demonstrated that *HIS3*$^+$ and *put2*$^-$ were tightly linked (9 parental ditype:0 nonparental ditype:0 tetratype) and that *HIS3*$^+$ was not at its normal chromosome 15 location (data not shown). When genomic DNAs from MB1426 and its parent W301-18A were analyzed by the method of Southern (35), the 5.6-kb *Sac*I band of W301-18A was found to be replaced by a larger band of 7.0 kb, corresponding to the size of the *Sac*I fragment of plasmid pKB10 carrying the *put2*::*HIS3* region (Fig. 3). Therefore, in MB1426, the native *PUT2* region was replaced by the plasmid-borne *put2*::*HIS3* region which integrated by homologous recombination. The formation of such a transformant proved that the cloned *PUT2*-complementing activity was due to the presence of the bona fide *PUT2* gene and not to a suppressor of the *put2* mutation.

Of the remaining transformants, 1 resulted from a gene conversion of the *his3*$^-$ gene to *HIS3*$^+$, and 12 were found to contain pBR322 DNA. The latter class was believed to have arisen from the integration of partially digested full-length linear plasmid pKB10 molecules, cut only once with *Sac*I, and yielding a tandem duplication of the *put2* locus with intervening pBR322 DNA.

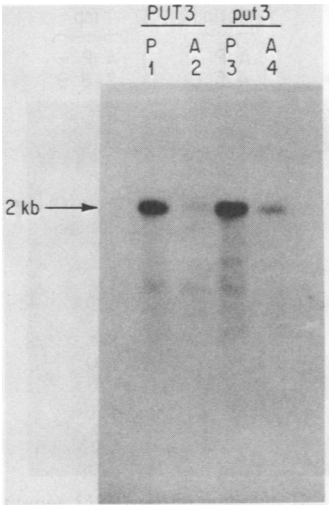

FIG. 4. Northern analysis of the *PUT2* gene. RNA was isolated from wild-type (MB1000 *PUT3*) and constitutive (MB214-18B *put3*) strains, and Northern analysis was carried out as described in the text. Each lane contained 1 μg of poly(A)-containing RNA from either the wild-type strain grown with proline (lane 1) or ammonia (lane 2) or the constitutive strain grown with proline (lane 3) or ammonia (lane 4). The probe was the nick-translated 5.6-kb *Sac*I fragment from plasmid pKB1. The *PUT2*-specific message is ca. 2 kb.

Steady-state mRNA levels. Total RNA and poly(A)-containing RNA were prepared from strain MB1000 grown on either a minimal-ammonia medium or a minimal-proline medium. Proline is an inducer of P5C dehydrogenase activity (8), so these RNA preparations represent, respectively, the uninduced and induced conditions.

The RNAs were subjected to electrophoresis on an agarose gel and transferred to nitrocellulose paper. RNAs homologous to *PUT2* DNA were detected by hybridization to radioactively labeled *PUT2* DNA fragments. A single band corresponding to an RNA of ca. 2 kb in length was seen when either total or poly(A)-containing RNA from proline-grown cells was probed with the 340-bp *Bgl*II fragment (data not shown). The levels of *PUT2*-specific mRNA found in induced and uninduced cells were compared in a similar manner. *PUT2*-specific mRNA was induced approximately 15-fold over the uninduced level as judged by densitometry measurements (Fig. 4). (The faint bands visible in Fig. 4 are due to use of a larger probe, the entire 5.6-kb *Sac*I fragment of plasmid pKB1, which hybridized to additional RNAs outside the *PUT2* region.)

Regulation of the proline degradative enzymes was shown to be altered in strains carrying the *put3* regulatory mutation, which resulted in inducer-independent production of both proline oxidase and P5C dehydrogenase (9). To determine whether the *put3* mutation affected the RNA levels, Northern analysis was carried out. The autoradiogram is shown in Fig. 4. Densitometric measurements showed that there was approximately threefold more *PUT2*-specific message in the *put3* strain than in the wild-type strain when both were grown under noninducing conditions. This result agrees with the threefold difference in P5C dehydrogenase enzyme activity reported earlier (9). The results described here are consistent with the idea that proline induction of P5C dehydrogenase activity occurs at the level of RNA synthesis or stability.

RNA mapping. The location of the *PUT2* gene within the 6.5-kb insert DNA of plasmid pKB1 was determined by the S1 nuclease mapping method of Berk and Sharp (4). Since deletion of the 340-bp region between the *Bgl*II sites resulted in loss of *PUT2*-complementing activity in *S. cerevisiae*, this region was chosen as one to which *PUT2* RNA would be expected to hybridize. The initial strategy involved end labeling the DNA of the *PUT2* region at the *Bgl*II sites only, followed by restriction digestion with the enzyme *Sac*I (see Fig. 5). The DNA was denatured

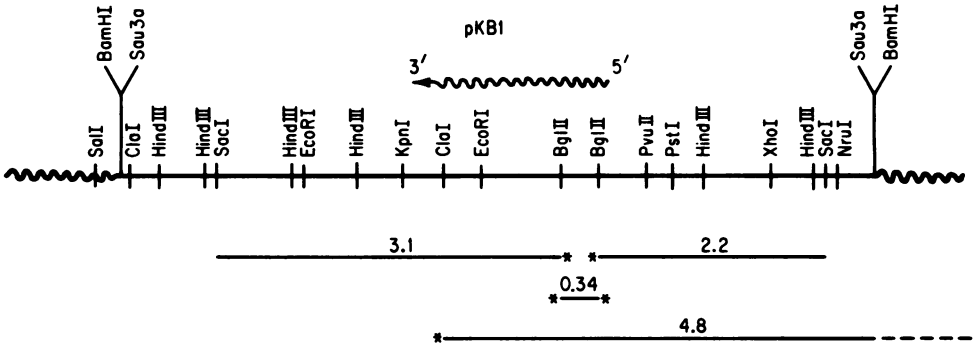

FIG. 5. Strategy for mapping *PUT2* RNA. The fragments of DNA shown were end labeled (either 5' or 3') as indicated by the asterisk and described in the text. The arrow indicates the direction of transcription and location of the *PUT2* gene.

and allowed to hybridize with poly(A)-containing RNA isolated from proline-grown or ammonia-grown yeast cultures (see above).

RNA from proline-grown yeast cultures protected 1.7 kb of the DNA from the 3.1-kb *Sac*I-*Bgl*II fragment only when it was labeled at the 3′ end of the *Bgl*II restriction site (Fig. 6). The complementary single-stranded 340-bp *Bgl*II fragment was fully protected by RNA when it was labeled at the 3′ (not shown) or 5′ (Fig. 7) end. No protected DNA could be detected from the 5′-end-labeled 2.2-kb *Bgl*II-*Sac*I fragment under similar conditions, presumably due to the small size of the hybrid formed (Fig. 6). These results suggested that the *PUT2* gene extends from the *Bgl*II restriction site to the *Kpn*I site, as indicated in Fig. 5.

A second experiment was performed to test this result. Plasmid pKB1 was cut with *Cla*I, and the 4.8-kb fragment shown in Fig. 5 was isolated and labeled at its 5′ ends. RNA protected a fragment of DNA ca. 1.3 kb in length, putting the 5′ end of the *PUT2* gene between the *Pvu*II and *Bgl*II sites, very close to *Bgl*II (Fig. 8).

RNA from ammonia-grown cells protected the DNA fragments to variable extents (Fig. 6, 7, and 8), although always less than the RNA from proline-grown cells. The appearance of such protection is believed to be due, in part, to the vast excess of RNA present in the S1 reaction and overexposure of the films.

The *PUT2* gene is, therefore, contained within the 3-kb *Hin*dIII sites of plasmid pKB1 (Fig. 5). To determine whether all the regulatory signals were present within this 3-kb region, enzyme levels were measured under various conditions as described below.

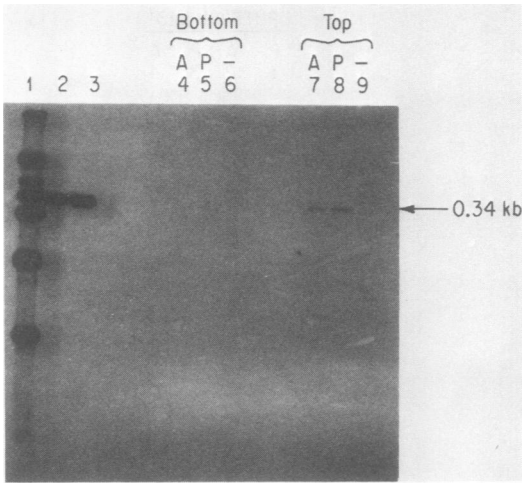

FIG. 7. S1 mapping of the *PUT2* gene: the 5′ end. The 340-bp fragment shown in Fig. 5 was isolated, labeled at the 5′ ends, and strand separated. S1 mapping experiments were performed with the separated strands. Lane 1, Radioactively labeled marker fragments of pBR322 digested with *Hin*fI. Lane 2, Top strand as marker. Lane 3, Bottom strand as marker. Lanes 4, 5, and 6, S1 mapping experiments with the bottom strand as probe and either RNA from ammonia (A) or proline (P) cultures or no additional yeast RNA (–). No protected DNA is visible. Lanes 7, 8, and 9, S1 mapping experiments with the top strand as probe and either RNA from ammonia (A) or proline (P) cultures or no additional yeast RNA (–). The entire 340-bp piece was protected in lanes 7 and 8.

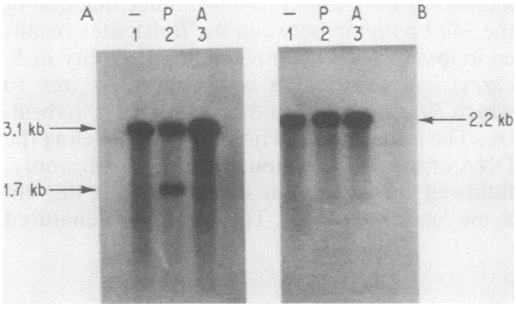

FIG. 6. S1 mapping of the *PUT2* gene: the 3′ end. S1 mapping experiments were carried out with the probes shown in Fig. 5 and poly(A)-containing RNA from proline-grown or ammonia-grown *S. cerevisiae* cells. (A) With the 3′-labeled 3.1-kb probe, a 1.7-kb piece of DNA was protected by RNA from proline-grown cells (lane 2). The 3.1-kb band represents some of the input DNA which reannealed to itself and is therefore S1 nuclease resistant. No DNA was protected in the absence of added yeast RNA (lane 1). A faint band of protected DNA is visible with RNA from ammonia-grown cells (lane 3). (B) With the 3′-labeled 2.2-kb probe, no protected DNA is seen with RNA from proline-grown (lane 2) or ammonia-grown (lane 3) cultures or without additional yeast RNA (lane 1).

Regulation of the plasmid-borne *PUT2* gene. P5C dehydrogenase levels were measured under noninducing and inducing conditions in a set of isogenic strains carrying various forms of the cloned *PUT2*$^+$ gene (Table 2). The haploid strain MB331-17A (*ura3-52 put2-57*) was the recipient for several *PUT2*$^+$ plasmids which are described below. To compare enzyme levels, it was necessary to construct a *PUT2*$^+$ haploid strain, otherwise isogenic to MB331-17A. This was accomplished by replacing the *put2-57* mutation in strain MB331-17A with its wild-type allele by first integrating a plasmid carrying *PUT2*$^+$ at that site ("targeting"; 31) and later selecting for homologous recombination events which removed the vector sequences and substituted the *PUT2*$^+$ for the *put2-57* gene. Such recombinants are expected to be Ura$^-$ due to the loss of the *URA3*$^+$ gene; these Ura$^-$ cells can be isolated on a medium containing ureidosuccinic acid, as described by Bach and Lacroute (1). Ura$^-$ colo-

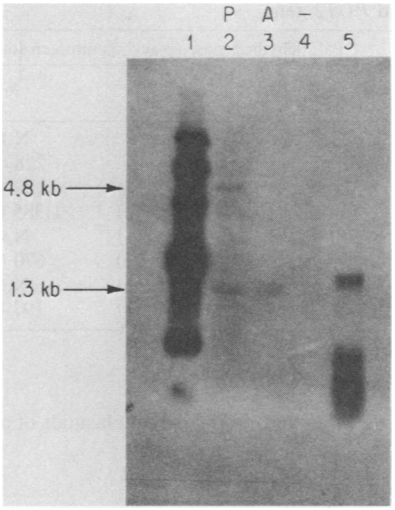

FIG. 8. S1 mapping of the *PUT2* gene: the 5′ end. S1 mapping was carried out as described in the legend to Fig. 7 except with the 5′-end-labeled 4.8-kb fragment shown in Fig. 5 as probe. Lane 1, Radioactively labeled lambda DNA digested with *Hin*dIII. Lanes 2 and 3, RNA from proline-grown (P) and ammonia-grown (A) cells protected a 1.3-kb fragment of DNA. The 4.8-kb band is the reannealed input probe. Lane 4, No additional yeast RNA in the S1 mapping experiments(–). No protected band was seen. Lane 5, Labeled pBR322 DNA digested with *Hin*fI.

nies were identified as *ura3*$^-$ and *PUT2*$^+$ by complementation tests with appropriate tester strains and were shown to lack pBR322 sequences by colony filter hybridization (26). MB1433 was one of these *PUT2*$^+$ MB331-17A derivatives.

The recipient strain MB331-17A had low non-inducible enzyme levels characteristic of the *put2-57* mutation (Table 2, line 1). Although MB331-17A was not isogenic to strain MB1000 in which the original enzyme regulation studies were done (8, 9), it carried the original *put2-57* allele which behaved in a similar manner in both strain backgrounds. Strain MB1433 containing a single *PUT2*$^+$ gene showed enzyme levels and inducibility identical to those of a nontransformed *PUT2* haploid strain: a modest induction (2- to 3-fold) when the cells were grown on a medium containing ammonia and proline and the full 8- to 10-fold induction when proline was the sole nitrogen source (line 2).

With the *PUT2* gene on the multicopy plasmid pKB1, the 10-fold induction ratio was also observed (Table 2, line 4). In addition, there was a fivefold increase above wild-type levels on all media tested.

To test whether the 3-kb *Hin*dIII fragment carrying the *PUT2* gene contained all the regulatory signals necessary for *PUT2* function, the fragment was inserted into the unique *Hin*dIII site in each of two plasmids, pCGS42 and pFW14, described above. The *PUT2*-containing plasmids, pKB11 and pKB13, carry the 3-kb *Hin*dIII fragment inserted in the same orientation in pCGS42 and pFW14, respectively. The presence of each of the two plasmids in MB331-17A allowed growth on proline as the sole nitrogen source. Induction ratios similar to those with plasmid pKB1 were seen (Table 2, lines 6 and 8). The multicopy plasmid pKB11 showed a smaller increase than pKB1, and the *PUT2* gene on the centromere plasmid behaved like a single *PUT2* gene maintained stably on a chromosome.

The presence of the parental plasmids lacking the *PUT2* insert (YEp24, pCGS42, or pFW14) in strain MB331-17A had no effect on the P5C dehydrogenase levels of that strain (Table 2, lines 3, 5, and 7).

Therefore, the 3-kb *Hin*dIII fragment carries the entire *PUT2* gene with sufficient information to allow growth on proline as the sole nitrogen source, and the gene appears to be regulated like the chromosomal gene.

Chromosomal mapping of *PUT2*. Since preliminary mapping attempts indicated that *PUT2* is not linked to any centromere, the 2μ mapping method of Falco and Botstein (Genetics, in press) was employed to indicate which chromosome carried *PUT2*.

Plasmid pKB1 carrying *PUT2* and 2μ DNA was cut with either *Kpn*I or *Xho*I, and the linearized plasmid DNA was used to transform MB331-17A (*ura3-52 put2-57*) to Ura$^+$. Stable Ura$^+$ transformants (less than 1 Ura$^-$ colony in 1,000 colonies) were screened and subsequently mated to the three *ura3*$^-$ strains (23) listed in Table 1, each marking a different subset of the 17 *S. cerevisiae* centromeres with a known mutation. After growth on yeast extract-peptone-glucose medium, the diploids showed instability for the Ura$^-$ character, segregating Ura$^-$ cells at a frequency of 4 to 8%. This phenomenon is believed to be due to interactions between the 2μ plasmid and the 2μ fragment on the chromosome (19). In diploids from only one of the three matings (with strain K381-15C), the requirement for uracil was accompanied (40 to 80% of the colonies) by a requirement for arginine, due to uncovering of the *arg4* marker on the other chromosome. These results indicated that *PUT2* was located on the same chromosome as *arg4*, namely, chromosome VIII.

Classical meiotic analysis was performed in diploids carrying *arg4*, *thr1*, *put2*, *cup1*, and *pet3* mutations. The markers have the following origins: *arg4* and *thr1* from X3127-2A, *pet3* from SP2-3C, *cup1* from JW913-4D, and *put2-57* from MB211-3B (see Table 1). *PUT2* is located between *THR1* and *CUP1* on chromosome VIII,

TABLE 2. Regulation of cloned *PUT2* gene

Expt	Strain	Location of *PUT2* gene	P5C dehydrogenase sp act[a] on nitrogen source[b]:		
			Amm	Amm + Pro	Pro
1	MB331-17A	Chromosome (defective)	33	32	N.G.[c]
2	MB1433	Chromosome	29	62	228
3	MB331-17A(YEp24)[d]	Chromosome (defective)	29 (96%)[e]	35 (95%)	N.G.
4	MB331-17A(pKB1)	Multicopy plasmid	130 (89%)	398 (89%)	1,385 (85%)
5	MB331-17A(pCGS42)[d]	Chromosome (defective)	30 (92%)	36 (92%)	N.G.
6	MB331-17A(pKB11)	Multicopy plasmid	56 (81%)	165 (79%)	670 (86%)
7	MB331-17A(pFW14)[d]	Chromosome (defective)	32 (99%)	40 (98%)	N.G.
8	MB331-17A(pKB13)	Single-copy plasmid	20 (96%)	52 (95%)	197 (95%)

[a] Nanomoles of product formed per minute per milligram of protein.
[b] Ammonium sulfate (Amm) was supplied at 0.2%; proline (Pro) was supplied at 0.1%.
[c] N.G., No growth.
[d] Plasmids YEp24, pCGS42, and pFW14 do not contain any *PUT2* DNA and are the parent plasmids of pKB1, pKB11, and pKB13, respectively.
[e] Percent Ura$^+$ (plasmid-carrying) cells in culture.

approximately 11 centimorgans from *THR1* (Table 3). The order and distances in centimorgans between these genes are: *arg4*–16–*thr1*–11–*put2*–15–*cup1*–>30–*pet3*.

DISCUSSION

The *PUT2* gene was isolated on a recombinant DNA plasmid and identified by its ability to complement a *put2* mutation in *S. cerevisiae*. In the absence of a defined map position for the gene or an in vitro method for identification of plasmid-made gene products, a gene disruption and chromosome replacement technique (34) was employed to demonstrate that the bona fide *PUT2* gene had been cloned. Since plasmid integration occurs by homologous recombination in *S. cerevisiae* (31), the existence of a yeast transformant in which the chromosomal *PUT2* gene had been replaced by the *put2*::*HIS3* fragment proved that the *PUT2* gene, and not a suppressor of the *put2-57* mutation, had been cloned.

The *PUT2* gene is unique in the *S. cerevisiae* genome as indicated by Southern analysis and is transcribed to form an mRNA of ca. 2 kb in length. Steady-state RNA levels are induced 15-fold when proline is the sole nitrogen source, and therefore at least one aspect of the regulation of proline utilization occurs at the level of RNA synthesis or stability. The increase in RNA correlates well with the observed 10-fold increase in enzyme levels (8). An *S. cerevisiae* strain carrying the regulatory *put3* mutation which resulted in inducer-independent enzyme activities (9) had threefold-elevated *PUT2*-specific mRNA. Although Northern analysis cannot be very quantitative, this increase is nevertheless in excellent agreement with the threefold increase in enzyme activities reported for strains carrying this mutation, under these growth conditions.

The results of S1 mapping experiments placed the *PUT2* gene entirely within a 3-kb *Hin*dIII fragment on pKB1. Although the exact location of the transcriptional start has not yet been determined, there was no indication of the presence of large introns in the *PUT2* gene. Furthermore, the 2-kb message size estimate correlates well with a preliminary observation from sodium dodecyl sulfate-polyacrylamide gels that the protein monomer is approximately 60,000 molecular weight (unpublished data).

The induction ratios of the *PUT2* gene as measured by P5C dehydrogenase activity are maintained whether the gene is present in single copy on chromosome VIII, on a centromere plasmid, or on a multicopy plasmid. When compared with strains carrying a single copy of the *PUT2* gene, all strains carrying multicopy *PUT2* plasmids had elevated P5C dehydrogenase levels on all media tested. The presence of plasmid pKB1 in MB331-17A resulted in a fivefold increase, whereas the presence of pKB11 resulted in a two- to threefold increase in P5C dehydrogenase activity (Table 2). This finding may be explained by position effects due to changes in the local DNA environment (plasmid DNA-*PUT2* gene junction) (36) or to gene dosage effects. According to Toh-e and Wickner (38), the native 2μ yeast plasmid and recombinant 2μ-containing plasmids can coexist in the yeast cell, and the total 2μ copy number is ca. 50. It is possible that in MB331-17A(pKB1) the apparent number of copies of pKB1 maintained per cell may be five, whereas in MB331-17A(pKB11) the number of copies of pKB11 may be two to three. Whatever the cause, it is significant that the induction ratios remain constant. Although there is insufficient evidence to determine whether the

TABLE 3. Chromosomal mapping of *PUT2*

Markers[a]	PD	NPD	T[b]	Total[c]	Map distance[d]
arg4-thr1	40	1	11	52	16
arg4-put2	30	1	21	52	26
arg4-cup1	22	4	27	53	48
arg4-pet3	5	10	37	52	Unlinked
thr1-put2	40	0	11	51	11
thr1-cup1	31	2	19	52	30
thr1-pet3	4	10	37	51	Unlinked
put2-cup1	41	1	10	52	15
put2-pet3	7	10	34	51	Unlinked
cup1-pet3	8	9	36	53	Unlinked

[a] Data from MB638:

MATa	*arg4*	*thr1*	+	*cup1*	+
MATα	+	+	*put2-57*	+	*pet3*

[b] PD, Parental ditype; NPD, nonparental ditype; T, tetratype ascal types. Close linkage of two genes is indicated by an excess of parental ditype to nonparental ditype tetrads.

[c] Includes 28 four-spored and 27 three-spored tetrads. Tetrads with gene conversions were omitted for individual pairs of markers.

[d] Distance in centimorgans equals [100(T + 6NPD)]/[2(PD + NPD + T)] (see footnote *b* for T, PD, and NPD) by the equation derived by Perkins (32).

genes involved in proline utilization are positively or negatively regulated, this result also suggests that no regulatory elements are limiting when the gene is present in at least five times its normal number.

In the initial studies on proline degradation (8), we attributed the threefold increase in enzyme activity (seen as induction by proline in the presence of ammonia) to limited proline transport (i.e., inducer exclusion). This was based on studies of proline transport (8) as well as enzyme levels in the *put3* regulatory mutant (9). The same threefold induction is seen in Table 3, superimposed on the increase in basal levels of expression. Although this result can still be explained by limited proline transport and low internal proline levels, the possibility that P5C dehydrogenase is sensitive to ammonia catabolite repression cannot be excluded.

The mapping method of Falco and Botstein (in press) for cloned genes on 2μ vectors worked well for the initial determination of the chromosomal location of *PUT2*. The distances between previously mapped genes on chromosome VIII, determined from the tetrad analysis data (Table 3) (e.g., *arg4*, *thr1*, and *cup1*), agreed well with published data (30). No linkage was detected between *pet3* and *cup1* in this cross, although slight linkage was seen in other crosses (data not shown). On the basis of the map presented by Mortimer and Schild (30), these two genes are approximately 30 centimorgans apart, and such linkage should have been detected.

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