Isolation of Constitutive Mutations Affecting the Proline Utilization Pathway in *Saccharomyces cerevisiae* and Molecular Analysis of the *PUT3* Transcriptional Activator

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The enzymes of the proline utilization pathway (the products of the *PUT1* and *PUT2* genes) in *Saccharomyces cerevisiae* are coordinately regulated by proline and the *PUT3* transcriptional activator. To learn more about the control of this pathway, constitutive mutations in *PUT3* as well as in other regulators were sought. A scheme using a gene fusion between *PUT1* (*S. cerevisiae* proline oxidase) and *galK* (*Escherichia coli* galactokinase) was developed to select directly for constitutive mutations affecting the *PUT1* promoter. These mutations were secondarily screened for their effects in *trans* on the promoter of the *PUT2* (Δ^1-pyrroline-5-carboxylate dehydrogenase) gene by using a *PUT2-lacZ* (*E. coli* β-galactosidase) gene fusion. Three different classes of mutations were isolated. The major class consisted of semidominant constitutive *PUT3* mutations that caused *PUT2-lacZ* expression to vary from 2 to 22 times the uninduced level. A single dominant mutation in a new locus called *PUT5* resulted in low-level constitutive expression of *PUT2-lacZ*; this mutation was epistatic to the recessive, noninducible *put3-75* allele. Recessive constitutive mutations were isolated that had pleiotropic growth defects; it is possible that these mutations are not specific to the proline utilization pathway but may be in genes that control several pathways. Since the *PUT3* gene appears to have a major role in the regulation of this pathway, a molecular analysis was undertaken. This gene was cloned by functional complementation of the *put3-75* mutation. Strains carrying a complete deletion of this gene are viable, proline nonutilizing, and indistinguishable in phenotype from the original *put3-75* allele. The *PUT3* gene encodes a 2.8-kilobase-pair transcript that is not regulated by proline at the level of RNA accumulation. The presence of the gene on a high-copy-number plasmid did not alter the regulation of one of its target genes, *PUT2-lacZ*, suggesting that the *PUT3* gene product is not limiting and that a titratable repressor is not involved in the regulation of this pathway.

The proline utilization (Put) pathway of *Saccharomyces cerevisiae* is a model system for studying transcriptional activation and nuclear localization, amino acid transport, mitochondrial protein import, and compartmentation. Proline oxidase, the first enzyme in the pathway and the product of the *PUT1* gene, converts proline to Δ^1-pyrroline-5-carboxylate (P5C); its activity requires a functional electron transport chain (10, 12). The second reaction, conversion of P5C to glutamate, is carried out by P5C dehydrogenase, the product of the *PUT2* gene (9, 10). Both genes have been cloned and sequenced, and their gene products were localized to mitochondria, where proline catabolism takes place (7, 32, 54, 55). Each enzyme is made as a precursor and is processed to its mature form by cleavage of a presequence during mitochondrial import (31; J. Kaput and M. C. Brandriss, unpublished results).

Proline induction of *PUT1* and *PUT2* is mediated by the product of the *PUT3* gene. The *PUT3* protein was identified as a transcriptional activator of the Put pathway on the basis of analysis of two mutant alleles that affected the expression of proline oxidase and P5C dehydrogenase. The semidominant *PUT3^c-68* mutation caused inducer-independent expression of the enzymes and increased basal RNA levels (7, 11, 54); the recessive *put3-75* mutation caused enzyme expression to be noninducible by proline (8, 49).

We were interested in determining whether genes other

than *PUT3* regulate the expression of *PUT1* and *PUT2*. In several other gene systems of *S. cerevisiae*, both activator and repressor proteins play important roles in the control of gene expression (22, 23, 27, 44). In our previous isolation schemes, which involved tedious screening procedures, we did not uncover mutations whose role would be consistent with that of a negative regulator (i.e., dominant noninducible or recessive constitutive phenotypes). To identify putative repressors, as well as additional activators, by their constitutive phenotypes, we developed a selection scheme that uses a gene fusion between the yeast *PUT1* gene and the *Escherichia coli galK* (galactokinase) gene. We isolated at least nine independent *PUT3* constitutive mutants, one dominant constitutive mutant in a gene called *PUT5*, and a group of recessive constitutive mutants with pleiotropic growth defects. Since the predominant class of mutations fell in the *PUT3* gene, we began a molecular analysis of this gene. We cloned the gene, examined its transcriptional regulation, and determined the phenotype of a null allele to confirm the role of the gene in proline utilization.

MATERIALS AND METHODS

Strains. The *S. cerevisiae* strains used (Table 1) are isogenic or congenic apart from their specified genotypes except for strains YM126, MB1433, JD244-24B, JM1312, and MB733-54A. Strain YM126 contains a *gal1* deletion and was obtained from Mark Johnston (28). This strain was crossed with strains JD226-3D and MB1433 to introduce the markers *TRP1::PUT2-lacZ14* (see below) and *ura3-52*, re-

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TABLE 1. S. cerevisiae strains

Strain	Genotype	Source or reference
C75-6D	MATa put3-75 ura3-52 ade1	S.-S. Wang
JD226-3D	MATa TRP1::PUT2-lacZ14 ura3-52 ade2	This work
JD237-3A	MATα put3-75 TRP1::PUT2-lacZ14 ura3-52	This work
JD238-3B	MATα TRP1::PUT2-lacZ14	This work
JD238-5C	MATa gall-152 TRP1::PUT2-lacZ14 ura3-52	This work
JD244-24B	MATα gall-152 TRP1::PUT2-lacZ14 met14 ura3-52	This work
JD253-9D	MATα PUT3ᶜ-68 TRP1::PUT2-lacZ14 ura3-52/pDB30	This work
JM1312	MATα TRP1::PUT2-lacZ14 met14 ura3-52/pDB30	This work
JM1313	MATa gall-152 TRP1::PUT2-lacZ14 ura3-52/pDB30	This work
JM1314	MATa gall-152 TRP1::PUT2-lacZ14 ura3-52/pDB19	This work
JM5007	MATa put3-1::URA3 ura3-52	This work
MB1000	MATα	10
MB1002	MATα ade2	M. C. Brandriss
MB1035	MATa ade1-41	8
MB1057	MATa his4-42	10
MB1433	MATα ura3-52 trp1	7
MB733-54A	MATα cdc16 ura3 leu1	M. C. Brandriss
MB758-5B	MATa ura3-52	49
YM126	MATa gall-152 ura3-52 trp1-289 lys2	28
JD404	MATa/MATα ura3-52/ura3-52 +/trp1	This work

spectively. The resulting strain, JD238-5C, was backcrossed 10 times with derivatives of strain MB1000 (either MB1002, MB1035, or MB1057) to make it congenic. Strain JD238-5C was transformed with plasmid pDB30 (a CEN plasmid containing the PUT1-galK3 gene fusion) and plasmid pDB19 (a CEN plasmid with a promoterless E. coli galK gene), yielding strains JM1313 and JM1314, respectively.

The PUT2-lacZ14 gene fusion (9) containing the promoter and first 14 codons of PUT2 fused to the eighth codon of the E. coli lacZ gene was integrated into the genome at the TRP1 locus and used as a reporter gene to monitor expression from the PUT2 promoter. A DraI fragment from plasmid pKB25 (9) containing this fusion was inserted 3' to the TRP1 gene between the sites StuI and NaeI of plasmid pJHW1 to form plasmid pDB11. (Plasmid pJHW1, obtained from John Hill, is a pUC18 vector [57] with the yeast TRP1-ARS1 locus inserted into the EcoRI site of the polylinker.) An EcoRI fragment containing TRP1::PUT2-lacZ14 was isolated from plasmid pDB11 and transformed into MB1433 (MATα ura3-52 trp1) to replace the resident trp1 copy by selection for Trp⁺ (one-step gene replacement technique [46]).

E. coli HB101 was used for plasmid amplification.

Growth media. Minimal and YPD media were described previously (6). When it was necessary to make subtle distinctions in growth of Put mutants on proline-containing media, agarose (SeaKem) was substituted for agar (Difco Laboratories). The agarose apparently lacks trace amounts of nitrogen sources found in agar, making it easier to score the Put⁻ phenotype.

The carbon source was glucose (2%) or galactose (2%), and the nitrogen source was ammonium sulfate (0.2%), glutamate (0.1%), or proline (0.1%). 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates were prepared as described by Guarente (20), with glucose as the carbon source and ammonium sulfate as the nitrogen source.

Genetic analysis. Mating, sporulation, and tetrad analysis were carried out by standard procedures (48).

Plasmids and yeast genomic libraries. The CEN plasmid pDB19 contained a promoterless E. coli galK gene and was used as a control in studies with strains containing a PUT1-galK3 gene fusion (see below). Plasmid pDB19 was constructed in two steps as follows. The E.coli galK gene was

liberated from plasmid pKMH7 (obtained from J. Davison) by digestion with DdeI and the ends were blunted with avian myeloblastosis virus reverse transcriptase (Life Sciences, Inc.), followed by digestion with EcoRI. This fragment was inserted into plasmid YCp50 (28) digested with EcoRI and NruI to form plasmid pDB15. Plasmid pDB15 was digested with SmaI and BamHI, and the fragment containing galK and URA3 was ligated into plasmid YCp19 (52) digested with SmaI and BamHI to form plasmid pDB19. This plasmid contains the following elements: a promoterless galK gene, URA3, TRP1, ARS1, CEN4, ampᴿ, and ori (origin of replication in E. coli).

Plasmid pDB30 was constructed in two steps. Plasmid pDB19 was digested with XhoI (removing CEN4), and a XhoI-SalI fragment of PUT1 containing the promoter and first three codons of PUT1 was inserted to form plasmid pDB29 (constructed by S.-S. Wang). This construction formed an in-frame PUT1-galK3 fusion capable of encoding a hybrid protein carrying the first three amino acids of PUT1 and five amino acids encoded by the polylinker preceding the second residue of galactokinase. Digestion of plasmid pDB29 with XhoI and SmaI liberated the PUT1-galK fusion on a fragment that also contained URA3. This fragment was inserted into plasmid YCp19 digested with SalI and SmaI to form plasmid pDB30, which contains the following elements: PUT1-galK3, URA3, TRP1, ARS1, CEN4, ampᴿ, and ori.

The yeast genomic DNA library constructed in plasmid YEp24 (4) was provided by the laboratory of D. Botstein (13). Plasmids pDB35, pDB36, pDB37, and pDB38 were independent plasmids obtained from this library. Plasmids pDB42, pDB46, pDB48, and pDB51 were constructed from plasmid pDB36 by deleting the area indicated in Fig. 1 and blunting the ends with either mung bean nuclease (New England BioLabs, Inc.) or the large fragment of DNA polymerase I (Klenow fragment; New England BioLabs) when necessary before ligation. Plasmids pDB41 and pDB50 were constructed by digesting plasmid pDB36 with PvuII or SalI, respectively (Fig. 1), and religating adjoining vector sequences to the insert. A PvuII fragment from plasmid pDB36 (containing vector sequences adjacent to the insert) was inserted into plasmid YCp50 digested with SmaI to create plasmid pDB60 or into plasmid YEp24 digested with
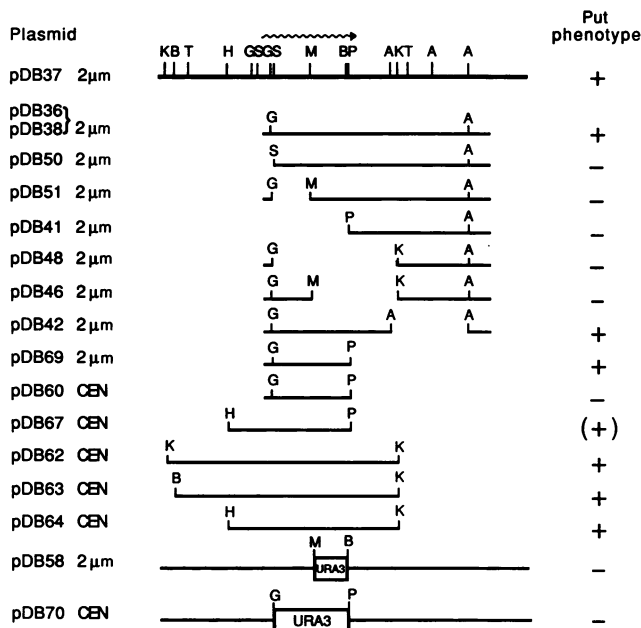
FIG. 1. Restriction map of the *PUT3* region and subcloning of the gene. Plasmids pDB36 and pDB38 contain indistinguishable 7-kb inserts that overlap the 11-kb insert found in plasmid pDB37 as indicated. Subclones were constructed as described in Materials and Methods. All plasmids were introduced into strain C75-6D (*put3-75*). Their ability to complement the Put⁻ phenotype was scored by growth on glucose-proline-adenine sulfate (20 mg/liter) in agarose plates or in liquid medium. Abbreviations and symbols: A, *Sac*I; B, *Bam*HI; G, *Bgl*II; H, *Hpa*I; K, *Kpn*I; M, *Mlu*I; P, *Pvu*II; S, *Sal*I; T, *Bst*XI; +, growth indistinguishable from that of the wild type; −, growth indistinguishable from that of the *put3-75* mutant; (+), a Put⁺ phenotype with a doubling time slightly higher than that of the wild type (4.17 versus 3.25 h); ⌒⌒⌒, approximate position of the *PUT3* transcript.

*Pvu*II to create plasmid pDB69. Plasmid pDB67 was constructed by inserting the *Hpa*I-*Pvu*II fragment from plasmid pDB37 into plasmid YCp50 digested with *Sma*I. Plasmid pDB62 was constructed by digesting plasmid YCp50 with *Kpn*I and inserting the *PUT3*-containing *Kpn*I fragment derived from plasmid pDB37. The resulting plasmid was digested with *Xho*I (the site was in the backbone) and *Bst*XI located in the insert, blunting the ends with mung bean nuclease, and ligating the ends to form plasmid pDB63. Plasmid pDB62 was digested with *Xho*I, the ends were blunted, and the plasmid was then digested with *Mlu*I. A *Hpa*I-*Mlu*I fragment from plasmid pDB37 was inserted to create plasmid pDB64. The deletion plasmid pDB58 was the result of digesting plasmid pDB37 with *Mlu*I, blunting the ends with the large fragment of DNA polymerase I, and subsequently digesting the plasmid with *Bam*HI. The *URA3* gene on a *Bam*HI-*Sma*I fragment from plasmid pJHU1 was inserted and oriented such that the 5' end of its transcript was close to the *Bam*HI site. (Plasmid pJHU1, obtained from John Hill, contains the yeast *URA3* gene inserted into the *Hind*III site of the polylinker of plasmid pUC18.) Plasmid pDB70 was constructed by inserting the same *URA3* fragment into plasmid pDB62 digested with *Pvu*II and partially digested with *Bgl*II such that the 5'-most *Bgl*II site of the insert was still present. The *URA3* gene is oriented such that the 5' end of its transcript is close to the *Bgl*II site.

DNA preparation and transformation. Isolation of plasmid and genomic DNAs from *S. cerevisiae* was performed by the

method of Hoffman and Winston (24). Plasmid DNA from *E. coli* was prepared by the method of Birnboim and Doly (3) or by the cesium chloride gradient method (37).

E. coli transformation was done by the calcium chloride method (16). Yeast transformation was performed by the lithium acetate method of Ito et al. (26).

Electrophoresis of DNA, transfer to nylon, and hybridization. Electrophoresis, transfer to nylon membranes (Nytran; Schleicher & Schuell, Inc.), and hybridization were done by the method of Southern (51). The probes were labeled by using the Multiprime kit (Amersham Corp.).

RNA preparation. Total RNA from *S. cerevisiae* was isolated by the method of Needleman et al. (43). Poly(A)-containing RNA was purified by chromatography on oligo(dT)-cellulose (Collaborative Research, Inc.) as described previously (7).

Electrophoresis of RNA, transfer to nylon, and hybridization. Total or poly(A)-containing RNA was separated in 1.0% agarose gels and transferred to nylon membranes as previously described (7). A *Bgl*II-*Nco*I fragment containing 2.4 kilobase pairs (kb) of *PUT3* DNA adjacent to 0.6 kb of *URA3* DNA was isolated from plasmid pDB60 and labeled by using the Multiprime kit (Amersham). Hybridization was carried out as described by Thomas (53).

S1 mapping. S1 mapping by the method of Berk and Sharp (2) was carried out as described previously (54). 5' end labeling was done by digesting plasmid pDB37 with *Mlu*I, dephosphorylating with bovine intestinal phosphatase, and then phosphorylating with T4 polynucleotide kinase and [γ-³²P]dATP by the method of Maxam and Gilbert (41). The labeled DNA was digested with *Kpn*I, and two labeled fragments of 4.5 and 3.2 kb were isolated. Plasmid pDB36 was digested with *Mlu*I and 3' end labeled by blunting the ends with DNA polymerase I large fragment and adding [α-³²P]dCTP (56). The end-labeled DNA was digested with *Kpn*I and *Nru*I to generate two 3'-labeled fragments, 3.2 kb (*Kpn*I-*Mlu*I) and 1.8 kb (*Nru*I-*Kpn*I). The *Nru*I site was part of the vector sequences adjacent to the inserted yeast DNA.

Mutant isolation. Strain JM1313 (*MAT*a *gall-152 ura3-52 TRP1::PUT2-lacZ14*) carrying plasmid pDB30 (*PUT1-galK3 URA3*) was grown to mid-log phase in glucose-glutamate broth and mutagenized with ethyl methanesulfonate (EMS) by the method of Fink (19). The 1- to 2-h exposure gave 35 to 60% killing. The mutagenized culture was split into five tubes and grown overnight in glucose-ammonia medium. Samples from each tube were spread on galactose-ammonia plates and allowed to grow for 3 to 4 days at 30°C. UV mutagenesis was performed by spreading strain JM1313 on galactose-ammonia plates and immediately exposing the plates to a General Electric germicidal UV lamp (model G15T8; 15 W) with an ouptut measured at 11 ergs/s per mm² for 5 s. This exposure gave approximately 10% killing.

A total of 337 mutants capable of growing on galactose-ammonia medium were picked. Of these, 8 mutants were isolated after UV mutagenesis and the remaining 329 were isolated after EMS mutagenesis. Three independent EMS mutageneses were done, and the 329 mutants were derived from 15 different tubes. Therefore, the EMS-induced mutants derived from a single tube were not necessarily independent. Since we cannot distinguish mutations that arose spontaneously before EMS mutagenesis from induced mutations, strains carrying mutations resulting in similar phenotypes isolated from different tubes are also not necessarily independent of one another.

Extract preparation and enzyme assays. Crude extracts were prepared by vortexing the cells with glass beads as

described previously (9). Galactokinase was assayed by the method of Rymond et al. (47), using D-[1-^{14}C]galactose (Amersham). Units of specific activity are expressed as nanomoles of galactose-1-phosphate formed per minute per milligram of protein. Since the measurements were made in GAL1 GAL10 strains, the values underestimate slightly the actual levels. β-Galactosidase assays were done by the method of Miller (42) on either crude extracts or whole cells. The specific activity of β-galactosidase in crude extracts is expressed as nanomoles of o-nitrophenol formed per minute per milligram of protein; variation was approximately 20%. The activities of glucose-6-phosphate dehydrogenase (15) and cytochrome c oxidase (38) were determined in both the cytosolic and crude mitochondrial fractions. Protein concentrations of crude extracts were determined by the method of Bradford (5), with crystalline bovine serum albumin used as the standard.

Cellular fractionation. Yeast cells were fractionated into cytosolic and crude mitochondrial fractions by the method of Daum et al. (17) as described previously (55). The growth medium contained glucose and proline.

RESULTS

Isolation of constitutive regulatory mutations affecting proline utilization. To isolate additional constitutive alleles of the PUT3 gene as well as mutations in other as yet unidentified regulatory genes, we modified a selection scheme for constitutive mutations described by Heusterspreute et al. (25) and also used by Rymond et al. (47). We fused the promoter and first three codons of the PUT1 gene to the E. coli galK gene. The galK gene can complement a gall mutation in S. cerevisiae. In a gall deletion strain, the gene fusion provides the only source of galactokinase for the cell, and its expression is now under proline regulation. Therefore, in this strain galactose can be used as a carbon source only when proline is present in the medium. Mutations leading to constitutive expression of the PUT1 promoter were selected by demanding growth on a medium containing galactose as the carbon source and ammonia as the nitrogen source.

The parent strain JM1313 (MATa gall-152 ura3-52 TRP1:: PUT2-lacZ14) carried a CEN plasmid, pDB30, that contained the PUT1-galK3 gene fusion and the wild-type URA3 gene. cis- or trans-acting mutations obtained from the selection scheme could be distinguished by measuring the β-galactosidase activity made from the genomic PUT2-lacZ reporter gene or by measuring galactokinase activity after segregation of the original plasmid and retransformation with a naive plasmid.

Before strain JM1313 was used to isolate constitutive mutants, it was necessary to demonstrate that galactokinase activity was now under proline regulation. Furthermore, because the PUT1 gene product is a mitochondrially localized protein, it was important that the PUT1-galK-encoded hybrid protein remain in the cytoplasm. Import of the PUT1-galK fusion protein into the mitochondria would separate galactokinase from its substrate, galactose, in the cytoplasm, interfering with the selection scheme. It was therefore necessary to determine the cellular location of the hybrid protein.

The regulation of galactokinase was determined by assaying JM1313 and control strains for galactokinase activity under various growth conditions (Table 2). Strain JD238-3B carried a wild-type GAL1 gene and produced galactose-inducible and glucose-repressible galactokinase, as expected

TABLE 2. Galactokinase levels in wild-type and gall deletion strains containing the PUT1-galK3 gene fusion

Strain	Relevant genotype	Plasmid[b]	Galactokinase sp act[a]			
			Glu + Amm	Glu + Pro	Gal + Amm	Gal + Pro
JD238-3B	GAL1		0	0	340	457
JM1314	gall-152	pDB19	0	0	NG	NG
JM1313	gall-152	pDB30	8	149	NG	ND

[a] Expressed as nanomoles of galactose-1-phosphate formed per minute per milligram of protein. The growth medium contained either 2% glucose (Glu) or 2% galactose (Gal) as a carbon source and either 0.2% ammonium sulfate (Amm) or 0.1% proline (Pro) as a nitrogen source. NG, No growth; ND, not determined.

[b] Plasmid pDB19 is a CEN vector carrying a promoterless galK gene; plasmid pDB30 is a CEN vector carrying the PUT1-galK3 gene fusion.

(40; for a review, see reference 27). Strain JM1314 contained the gall-152 deletion and carried a CEN plasmid (pDB19) with a promoterless galK gene. Without galactokinase, this strain was unable to grow on media containing galactose as a carbon source. Plasmid pDB30, carrying the PUT1 promoter installed in plasmid pDB19 so that the gene fusion encoded a proline-inducible galactokinase, enabled strain JM1313 to grow on a medium containing galactose only if proline was present. Its doubling time on galactose-proline medium was 5.7 h, compared with 3.25 h for the wild-type strain. This difference in doubling times probably reflects the relative strengths of the native GAL1 and PUT1 promoters. As expected, this strain could not grow on a galactose-ammonia medium.

A fractionation experiment was carried out as described in Materials and Methods to determine where the majority of galactokinase activity was found in the cell. More than 95% of the galactokinase activity fractionated with the cytoplasmic marker glucose-6-phosphate dehydrogenase (Table 3). The small portion fractionating with the mitochondrial marker, cytochrome c oxidase, was probably due to cytoplasmic contamination of the mitochondrial pellet. Therefore, the fusion protein was not being targeted into the mitochondria.

Strain JM1313 was mutagenized and spread on galactose-ammonia plates, and large Gal$^+$ colonies were isolated as described in Materials and Methods. To screen for mutants that were trans acting and affected both PUT1 and PUT2 expression, the mutants were replica plated to X-Gal indicator plates to monitor expression of the PUT2-lacZ reporter gene. Colonies with a more intense blue color than the wild type exhibited on glucose–ammonia–X-Gal medium were chosen for further analysis.

β-Galactosidase activity was assayed from liquid cultures grown with ammonia as the nitrogen source (noninducing conditions for PUT2). Of 76 strains tested, 51 from nine different tubes treated with EMS and one from a UV mutagenesis showed increased levels of expression under

TABLE 3. Subcellular distribution of enzymes in the PUT1-galK3 strain JM1313

Enzyme	% Activity in:	
	Cytosol	Pellet
Glucose-6-phosphate dehydrogenase	90	10
Cytochrome c oxidase	4	96
Galactokinase	98	2

TABLE 4. Analysis of semidominant and dominant
constitutive mutants

Strain	Tube[a]	β-Galactosidase sp act[b]		Linkage to metl4[c] (P:N:T)	Linkage to put3-75[d] (P:N:T)
		Haploid	Diploid		
JM1313		58	60		
JM1323	B	461	169	6:0:1	ND
JM1370	D	543	208	7:0:0	8:0:0
JM1372	D	1,277	534	13:0:0	ND
JM1503	E	130	111	2:2:2	1:3:4
JM1521	C	463	157	7:0:0	8:0:0
JM1579	A	727	143	7:0:0	4:0:0
JM1594	O	355	259	6:0:1	8:0:0
JM1599	F	488	258	7:0:0	ND
JM1607	N	683	324	6:0:1	9:0:0
JM1641	B	512	108	6:0:1	7:0:0
JM1657	UV	977	330	6:0:1	ND
JM1857	G	776	326	5:0:2	ND

[a] After EMS mutagenesis, the culture was split into nine tubes and grown 12 to 16 h (see Materials and Methods). One mutant resulted from UV mutagenesis.

[b] β-Galactosidase assays were done on haploid strains grown under noninducing conditions (glucose plus ammonium sulfate); activity is expressed as nanomoles of o-nitrophenol formed per minute per milligram of protein. Each haploid was grown in YPD medium to segregate the plasmid. The resulting strain was crossed to strain JD244-24B (MATα metl4 gall-152 TRP1::PUT2-lacZ14 ura3-52) to form the diploid that was assayed.

[c] Each diploid was sporulated, and tetrad analysis was performed to determine linkage of metl4 to the constitutive allele.

[d] Linkage to put3-75 was determined after tetrad analysis of diploids formed from crosses of the constitutive strains to strain JD237-3A (MATα put3-75 TRP1::PUT2-lacZ14 ura3-52). ND, Not determined.

noninducing conditions. β-Galactosidase values of these mutants, expressed as described in Materials and Methods, ranged in specific activity from 100 to 1,277, whereas the specific activity in the parental strain JM1313, under the same conditions, was 58 (Tables 4 and 5). The highest value measured was similar to that observed in strain JM1313 grown under fully inducing conditions (specific activity of 1,126). By comparison, the specific activity of β-galactosidase in strain JD253-9D carrying the previously isolated PUT3[c]-68 constitutive mutation (11) was 425 under noninducing conditions.

To verify that the mutants had increased expression of the PUT1-galK gene fusion, the original plasmid in several strains was segregated, and the strains were retransformed with plasmid pDB30. Galactokinase activity in these strains grown on galactose-ammonia medium increased about 15-fold over the level found in strain JM1313 (data not shown).

To determine whether a mutation was dominant or recessive, β-galactosidase activity was measured under noninducing conditions in heterozygous diploids resulting from

TABLE 5. Analysis of recessive constitutive mutants[a]

Strain	Tube	β-Galactosidase sp act		Linkage to metl4 (P:N:T)
		Haploid	Diploid	
JM1313		58	60	
JM1403	A	385	68	ND
JM1404	A	319	68	ND
JM1431	E	100	48	ND
JM1559	E	130	54	0:0:6
JM1566	B	337	58	1:0:2
JM1582	B	128	77	ND

[a] See footnotes to Table 4.

crosses to a Put⁺ wild-type strain, JM1312. Of the 52 mutants studied, 6 were recessive to wild type, 1 was fully dominant, and 45 were semidominant (see below). Those that were recessive showed β-galactosidase specific activities in heterozygous diploids at wild-type levels (Table 5). These mutants came from three different tubes, and their haploid β-galactosidase specific activities ranged from 100 to 385. All of the recessive mutants had increased doubling times on YPD and minimal ammonia medium as well as on minimal proline medium. They may be affected in more general regulators involved in the expression of a number of different genes.

Mutations defined as semidominant were those that, when heterozygous in diploids, showed β-galactosidase levels that were significantly higher than the levels in a homozygous wild-type diploid. The specific activities of β-galactosidase in 45 diploid strains varied from 20 to 73% of the haploid values, from 119 to 534. Data from 11 such strains are shown in Table 4. The previously described constitutive allele, PUT3[c]-68, also showed semidominance in a heterozygous diploid (data not shown). One mutant, JM1503, whose haploid-specific activity was 130, had a value of 111 when heterozygous in a diploid; we consider this mutation to be fully dominant.

Fourteen diploid strains were sporulated, asci were dissected, and spore clones were assayed for constitutive expression of β-galactosidase activity from the PUT2-lacZ14 gene present in each strain. In all crosses, the constitutive mutation segregated as a single nuclear gene. Linkage of the constitutive phenotype to the easily scored metl4 marker was used to determine whether the new mutation was linked to PUT3 (Table 4). All of the semidominant mutations were tightly linked to MET14. Several of these constitutive strains were also crossed to a strain (JD237-3A) carrying a noninducible put3-75 allele. The constitutive and noninducible alleles demonstrated tight linkage (Table 4). These mutations are therefore presumed to be in the PUT3 gene, although it cannot be absolutely ruled out that they lie in another tightly linked regulatory gene that also affects proline utilization.

The dominant mutation in strain JM1503 (Table 4) and two of the recessive mutations (in strains JM1566 and JM1559; Table 5) were not tightly linked to MET14 and are mutations in other genes. When strain JM1503 (carrying PUT5[c]-21) was crossed to a strain containing put3-75, the constitutive and noninducible phenotypes did not segregate as alleles. In addition, the Put⁺ phenotype did not segregate 2⁺:2⁻ in this cross but rather gave three tetrad types: 4⁺:0⁻, 3⁺:1⁻, and 2⁺:2⁻. This result suggests that the spores containing both the constitutive and noninducible mutations had a Put⁺ phenotype. The spore clones from one nonparental ditype tetrad (4⁺:0⁻) were each crossed to a wild-type strain (MB1002 or MB1035), the diploids were sporulated, and asci were dissected. Put⁻ spores could be retrieved from half of the diploids analyzed, indicating that they contained the put3-75 mutation apparently masked by the constitutive mutation. Additional analysis of this mutant must be carried out to understand the function of this gene in proline utilization.

Isolation of the PUT3 gene. The predominant class of constitutive mutations derived from the screen described above were in the PUT3 gene, indicating the major role of this gene in the regulation of proline utilization. A molecular analysis was undertaken to understand its role in this process. The wild-type PUT3 gene was cloned by functional complementation of the recessive, noninducible put3-75 mutation. Strain C75-6D (MATa ura3-52 put3-75 adel) was

transformed with a 2μm plasmid yeast genomic DNA library, with selection for uracil prototrophy. Approximately 15,000 independent Ura+ transformants were screened for the ability to grow on a medium containing proline as the sole nitrogen source. Four independent Ura+ Put+ transformants were obtained and subsequently grown on nonselective media to show simultaneous loss of the two plasmid-borne markers. Loss of the plasmid occurred at a frequency expected for 2μm vectors. Plasmid DNA was isolated from these original transformants and used to retransform strain C75-6D. All of the Ura+ transformants were also Put+.

Three plasmids (pDB36, pDB37, and pDB38; Fig. 1) contained overlapping DNA inserts, as determined by restriction mapping, and were capable of restoring a wild-type growth rate to strain C75-6D on a medium containing proline as the sole source of nitrogen. The fourth plasmid (pDB35) contained DNA with a different restriction map and did not fully restore the wild-type growth rate on proline-containing media (see below).

Plasmids pDB36 and pDB38 contained indistinguishable 7-kb inserts, and pDB37 contained an 11-kb insert (Fig. 1). The high-copy-number 2μm plasmid pDB36 was used for subcloning to localize the PUT3 gene. Deletion of approximately 300 base pairs from the left side of the insert (with respect to the orientation in Fig. 1; plasmid pDB50) resulted in a Put− phenotype when this plasmid was transformed into strain C75-6D. Deletion of DNA immediately upstream or downstream of MluI also resulted in the inability to complement (plasmids pDB51, pDB41, pDB48, and pDB46). However, deletion of the DNA between the SacI sites in plasmid pDB42 did not affect the ability of this fragment to complement the put3 mutation. Similarly, removal of DNA 3′ to the PvuII site (plasmid pDB69) did not alter the Put+ phenotype. However, if this fragment was placed on a low-copy-number vector (plasmid pDB60), it was no longer able to complement the defect. Plasmid pDB67 carrying the longer HpaI-PvuII fragment (derived from plasmid pDB37) restored the Put+ phenotype, although the doubling time of the transformant was slightly higher (4.17 h) than that of the wild-type strain MB758-5B (3.25 h). The addition of DNA downstream of the PvuII site on a CEN plasmid (pDB64) restored the wild-type rate of growth on proline. The large fragments contained on plasmids pDB62 and pDB63 were also capable of fully complementing the put3-75 mutation, as expected. These results suggest that the original clone carried on plasmid pDB36 did not have the entire PUT3 gene but at high copy number was capable of fully complementing the Put− phenotype.

The PUT3-complementing DNA was disrupted on plasmids by insertion of the URA3 gene, replacing either 1.4 kb of PUT3 DNA at the 3′ end of the gene from MluI to BamHI (plasmid pDB58), or by deletion of 2.5 kb of PUT3 DNA from the 3′-most BglII site to PvuII (plasmid pDB70). Plasmids pDB58 and pDB70 were transformed individually into strain C75-6D (put3-75) and were unable to complement the Put− phenotype. The disrupted DNA from pDB58 was introduced by transformation into the yeast genome (one-step gene replacement [46]) to replace the resident wild-type copy in the haploid strain MB758-5B. Viable transformants were obtained, and a Southern blot analysis was done to confirm that the disrupted put3 DNA had integrated and replaced the resident copy (data not shown). The put3-disrupted strain, JM5007, was Put−, failed to complement, and was indistinguishable in phenotype from the put3-75 mutation.

Strain JM5007 was used in mapping studies to verify that the cloned fragment represented the bona fide PUT3 gene. Allelism of the put3-1::URA3 and put3-75 mutations was examined in a cross between strains JM5007 (MATa ura3-52 put3-1::URA3) and JD237-3A (MATα ura3-52 put3-75 TRP1 ::PUT2-lacZ14). In 66 tetrads, the Put− phenotype segregated 0+:4−, indicating allelism of the two genes. Linkage of the gene disruption to MET14 was also tested, since both genes map to chromosome XI. Tetrads from a cross between JM5007 and JD244-24B (MATα ura3-52 met14 TRP1::PUT2-lacZ14) demonstrated tight linkage between URA3 and put3 (27 parental ditypes [P]:0 nonparental ditypes [N]:0 tetratypes [T]) and between URA3 and MET14 in this cross (26 P:0 N:1 T). All of the Ura+ spores were Put−.

To construct a strain carrying a complete put3 deletion, DNA from plasmid pDB70 was integrated into the genome in a diploid strain JD404 to replace one wild-type copy of PUT3. Analysis of seven tetrads from a stable transformant showed wild-type spore viability. Both the Ura+ and Put+ phenotypes segregated 2+:2−. All spores that were Ura+ were Put−, as expected, and the phenotype was indistinguishable from that of the put3-75 mutation. Southern blot analysis of the transformed diploid showed one wild-type PUT3 gene and one disrupted gene (data not shown). As expected, Southern blot analysis of four spores from a single tetrad revealed two spores with a wild-type gene (both spores were Ura− and Put+) and two spores with a disrupted gene (both spores were Ura+ and Put−; data not shown). A Put− spore was crossed to strain C75-6D (put3-75), and 24 tetrads were analyzed. All spores were Put−, whereas URA3 segregated 2+:2−. These results demonstrated that PUT3 is not an essential gene, since the wild-type copy can be disrupted in the haploid genome without loss of viability.

Determination of direction of transcription and message length. The direction of PUT3 transcription and the size of the transcript were determined by 5′ and 3′ S1 mapping, using poly(A)-containing RNA from a PUT3 wild-type strain (MB1000) grown under inducing (proline as the sole nitrogen source) or noninducing (ammonia as the sole nitrogen source) conditions. RNA protected a 1.5-kb fragment of the 4.5-kb 5′-labeled probe under both growth conditions (Fig. 2A). This same protected fragment was seen with poly(A)-containing RNA isolated from the put3 partial deletion strain JM5007. This deletion removes the 3′ half of the gene and has an intact 5′ half that can make a truncated message (see below). RNA protected a 1.3-kb fragment of the 3.2-kb 3′ labeled probe under both growth conditions (Fig. 2B). Taken together, these results indicate that the PUT3 transcript is 2.8 kb in length and that the direction of transcription is from left to right with respect to the map shown in Fig. 1. From the restriction map of the insert in plasmid pDB37, the start site of transcription was predicted to lie between the two BglII sites at a region that may not be present in the two original plasmids, pDB36 and pDB38. These results and the subcloning results (see above) suggest that these plasmids do not contain the entire PUT3 gene. Transcription may be promoted from a fortuitous promoter on the plasmid sequences adjacent to the insert in these clones. The transcript is predicted to end near the PvuII site.

Analysis of PUT3 expression. RNA analysis was performed to confirm the size of the PUT3 transcript and to determine whether PUT3 was regulated by proline at the level of RNA accumulation. Poly(A)-containing RNA from a PUT3 wild-type strain (MB1000) grown under inducing or noninducing conditions was isolated and used in Northern (RNA) blot analysis as described in Materials and Methods. Quantitatively similar amounts of a 2.8-kb PUT3 transcript were seen

in both ammonia- and proline-grown cells (Fig. 3). To confirm that this transcript was that of *PUT3*, RNA was analyzed from ammonia-grown cells of the *put3* deletion strain, JM5007. This strain is missing the 3′ half of the gene, which is predicted to reduce the message to approximately 1.4 kb. Northern blot analysis confirmed this prediction (data not shown). We conclude that *PUT3* transcript accumulation is not regulated by proline. Taking into consideration the relative lengths of the *URA3* and *PUT3* probes used in the experiment shown in Fig. 3, *PUT3* appears to be expressed at about one-fourth the level of *URA3*.

To determine the effect of altering the dosage of the *PUT3* gene on its target genes, we compared *PUT2-lacZ14* expression in strains carrying single or multiple copies of *PUT3*. If, as was found for the *PPR1* (34) and *GAL4* (21, 29) proteins, the *PUT3* protein was limiting or regulated by a titratable repressor, we would expect to see an increase in β-galactosidase levels with increasing levels of *PUT3*. Specific activity of β-galactosidase in strain JD238-5C carrying the *PUT3* gene on a low- or high-copy-number plasmid (pDB62
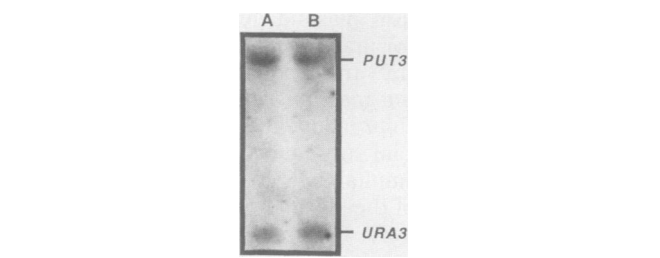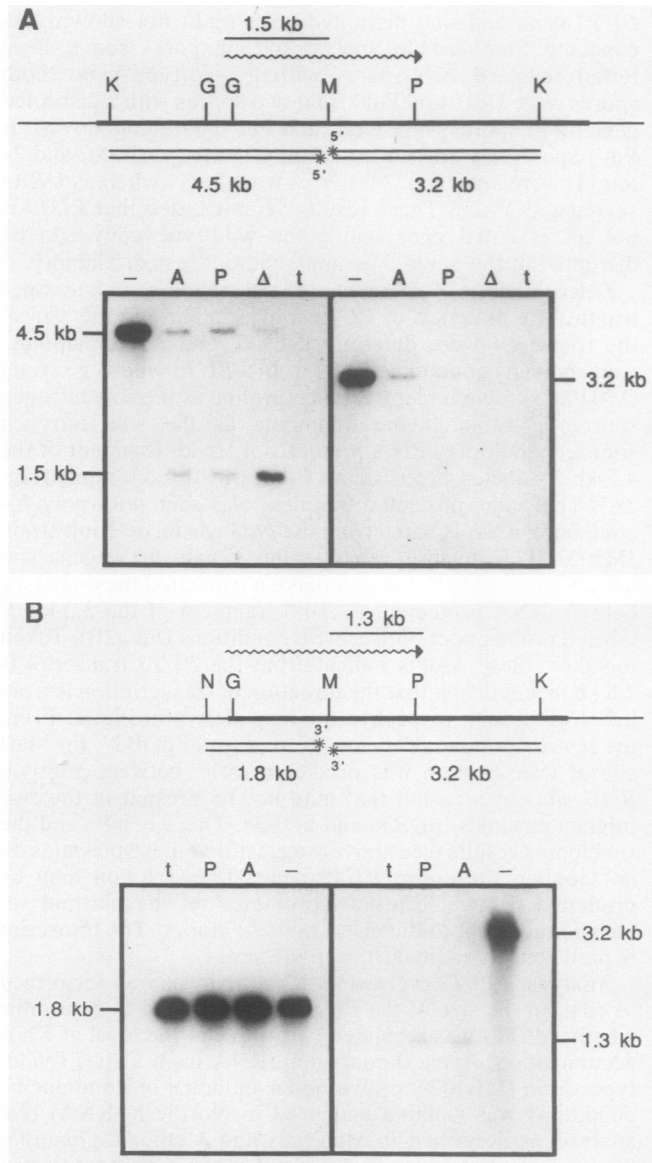
FIG. 3. Northern blot analysis of the *PUT3* gene. A 3.0-kb *BglII-NcoI* fragment of DNA containing 2.4 kb of *PUT3* DNA and 0.6 kb of *URA3* DNA was used as a probe. Each lane contained 3 μg of poly(A)-containing RNA isolated from strain MB1000 grown on ammonia (lane A)- or proline (lane B)-containing medium. The probe detected the 2.8-kb *PUT3* transcript and the 0.9-kb *URA3* transcript under both growth conditions.

or pDB37, respectively; Fig. 1) was 53 or 59, respectively, under noninducing conditions, and 1,342 or 1,198, respectively, under inducing conditions. Strain JD238-5C carrying either of the parent plasmids lacking the *PUT3* gene, YCp50 or YEp24, had similar enzyme levels. Southern blot analysis confirmed that multiple copies of plasmid pDB37 were present in strain JD238-5C (data not shown). That the increased gene dosage resulted in increased protein was demonstrated in recent experiments showing that *PUT3* encodes a protein that either binds DNA directly or is part of a DNA-binding complex (50). A large increase in binding activity in gel shift assays was seen when extracts from a strain carrying the high-copy-number plasmid pDB37 were compared with those from a strain with one copy of *PUT3*. The gene dosage results suggest that the *PUT3* product is not limiting in its activation of the *PUT* enzymes and that a titratable repressor is probably not involved in the regulation.

Analysis of a partial complementing clone. Plasmid pDB35 contained DNA with a restriction map different from that of *PUT3* and did not fully restore the wild-type growth rate on proline-containing medium to a strain carrying the *put3-75*

FIG. 2. 5′ and 3′ S1 mapping of the *PUT3* gene. Double-stranded probes (labeled at only one end, 5′ or 3′) used for S1 mapping are shown at the tops of both panels and are described in Materials and Methods. The fragments were isolated from two plasmids that differed in the amount of upstream *PUT3* DNA they contained. The restriction maps reflect the different sites present in the two plasmids. A 5-μg sample of poly(A)-containing RNA isolated from ammonia- or proline-grown cultures was used per reaction. (A) 5′ mapping. RNA protected a 1.5-kb DNA fragment of the 5′-labeled 4.5-kb probe. This DNA was protected with RNA isolated from ammonia (A)- or proline (P)-grown cultures of strain MB1000. The same 1.5-kb protected fragment was also present with RNA isolated from the partial *put3* deletion strain JM5007 (Δ) grown on ammonia as the sole nitrogen source. This deletion removes the 3′ half of the gene but leaves the 5′ half intact. No protected fragment was observed with the 3.2-kb probe, in the absence of S1 nuclease (−), or when tRNA (t) was substituted for poly(A)-containing RNA. (B) 3′ mapping. RNA protected a 1.3-kb DNA fragment of the 3′-labeled 3.2-kb probe in the presence of RNA isolated from ammonia (A)- or proline (P)-containing cultures of strain MB1000. This protected fragment was not present in the absence of S1 nuclease (−), when tRNA (t) was substituted for poly(A)-containing RNA, or with the 1.8-kb probe. Abbreviations and symbols: G, *BglII*; K, *KpnI*; M, *MluI*; N, *NruI*; P, *PvuII*; ▬, insert sequences; ——, vector sequences; ∿, *PUT3* transcript; line above the transcript, protected fragment; ∗, labeled ends.

mutation (5.5 h versus 3.25 h for the wild-type strain MB758-5B).

Plasmid pDB35 was integrated into the genome of strain C75-6D at a site homologous to the insert DNA to determine its chromosomal location. Preliminary mapping results in separate crosses with met14 (strain JD244-24B) and cdc16 (strain MB733-54A) suggested that the integration was targeted to chromosome XI, the same chromosome that carries PUT3. However, the integrated plasmid mapped 33 centimorgans from the centromere-linked MET14 gene and on the same arm as MET14 (linkage of Ura$^+$ to MET14, 6 P:0 N:12 T; linkage of Ura$^+$ to CDC16, 0 P:1 N:23 T), whereas PUT3 is tightly linked to MET14 but on the other arm (8). The identity and function of this gene are not yet known.

DISCUSSION

Mutations in regulatory proteins that result in constitutive enzyme expression have been useful in defining specific domains of two well-characterized transcriptional regulators, GAL4 and ADR1. One set of constitutive GAL4 mutants maps to the carboxy terminus of the protein and affects interaction with the GAL80 repressor (30, 36, 39; for a review, see reference 27). ADR1 constitutive mutations were shown to map to one region of the gene that corresponds to a phosphorylation site in the protein (14). We hoped that a large collection of PUT3 constitutive mutants would help us define critical domains for activation of the PUT genes. Until now, the only existing PUT3 constitutive allele was isolated by screening a large number of colonies for inducer-independent proline oxidase activity (11). It was therefore advantageous to develop a scheme in which constitutive mutations could be directly selected. The PUT1-galK gene fusion method was useful for selecting mutations that increased expression from the PUT1 promoter of the gene fusion. Since putative mutants were then screened for constitutive expression of a PUT2-lacZ gene fusion to eliminate cis-acting mutations, only those mutations that affected both PUT1 and PUT2 genes were studied. Mutations that increase the endogenous proline concentration in the cell would be among those studied further. Other genes that might regulate PUT1 independently of PUT2 would not be included in this study.

The predominant class of mutations isolated by this scheme are believed to be in the PUT3 gene. The ability of these mutations to activate PUT2-lacZ expression under noninducing conditions varied over a large range of β-galactosidase activities, suggesting that many different mutations had been isolated. This characteristic appears to contrast with those of the collection of constitutive mutations isolated in the ADR1 gene; most of these mutations had quantitatively similar activities of alcohol dehydrogenase II, and all map to a single region of the protein (14).

We now have evidence that other genes contribute to the expression of the Put pathway. One dominant, low-level constitutive mutation (PUT5^c-21) that was isolated was determined by genetic analysis to be in a gene other than PUT3. The double put3-75 PUT5^c-21 mutant was able to utilize proline, suggesting that PUT5 is epistatic to PUT3. Based on our current understanding of the role of PUT3, it is unlikely that PUT5 increases endogenous proline levels, since doing so would not be expected to bypass the absence of PUT3 product.

Our selection scheme also yielded strains carrying recessive constitutive mutations. These mutants have pleiotrophic growth defects (slower growth on a rich medium or on minimal media containing ammonia or proline as the sole nitrogen source) and are possibly affected in general regulatory proteins or in the transcription apparatus. At present, very little is known about this group of mutants, including the number of complementation groups that are represented in Table 5. One or more of them may correspond to the locus on chromosome XI identified by the suppressor clone (plasmid pDB35) that could complement the put3-75 mutation only when present in multiple copies.

To learn more about the nature of the PUT3 activator, a molecular analysis was undertaken. The wild-type PUT3 gene was cloned by functional complementation of the recessive, noninducible put3-75 mutation. The complementing DNA was capable of directing integration to the PUT3 locus on chromosome XI. The PUT3 gene is not essential, since a complete deletion of the gene did not affect cell viability on growth media containing nitrogen sources other than proline. The null mutant was indistinguishable in phenotype from the put3-75 allele.

The 2.8-kb PUT3 transcript is not regulated by proline at the level of RNA accumulation. This finding has been supported recently by preliminary results indicating that β-galactosidase activity from a bifunctional PUT3-lacZ gene fusion was not inducible by proline (unpublished results). Therefore, the ability of PUT3 to activate PUT1 and PUT2 is not a function of the appearance of the PUT3 message. The level of the PUT3 transcript is about one-quarter of that seen for URA3 (1). Similar-size transcripts of regulatory genes that do not respond to the same signals that regulate their target genes have been observed for several other positive regulators, including GAL4 (33, 45), PPR1 (34, 35), and ARG81 (ARGRII) (18).

Increased copy number of the PUT3 gene did not alter the regulation of a genomic copy of PUT2-lacZ. This finding suggests that the Put system lacks a repressor that can be titrated out by increased PUT3 protein and that this activator is not limiting. Therefore, proline utilization appears to be regulated differently from galactose utilization (21, 29) or pyrimidine biosynthesis (34). The PUT3 gene product was recently shown to bind directly, or as part of a complex, to a specific DNA sequence in the promoters of the PUT1 and PUT2 genes (50). We are optimistic that our collection of constitutive and noninducible mutations in the PUT3 gene will provide us with sequence information to relate structure to function in the role of this protein in DNA binding and transcription activation.

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