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Roles of *URE2* and *GLN3* in the Proline Utilization Pathway in *Saccharomyces cerevisiae*

SHIWEI XU, DARLENE A. FALVEY, AND MARJORIE C. BRANDRISS*

Department of Microbiology and Molecular Genetics, UMD-New Jersey Medical School and Graduate School of Biomedical Sciences, Newark, New Jersey 07103

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The yeast *Saccharomyces cerevisiae* can use alternative nitrogen sources such as arginine, urea, allantoin, γ-aminobutyrate, or proline when preferred nitrogen sources like glutamine, asparagine, or ammonium ions are unavailable in the environment. Utilization of alternative nitrogen sources requires the relief of nitrogen repression and induction of specific permeases and enzymes. The products of the *GLN3* and *URE2* genes are required for the appropriate transcription of many genes in alternative nitrogen assimilatory pathways. GLN3 appears to activate their transcription when good nitrogen sources are unavailable, and URE2 appears to repress their transcription when alternative nitrogen sources are not needed. The participation of nitrogen repression and the regulators GLN3 and URE2 in the proline utilization pathway was evaluated in this study. Comparison of *PUT* gene expression in cells grown in repressing or derepressing nitrogen sources, in the absence of the inducer proline, indicated that both *PUT1* and *PUT2* are regulated by nitrogen repression, although the effect on *PUT2* is comparatively small. Recessive mutations in *URE2* elevated expression of the *PUT1* and *PUT2* genes 5- to 10-fold when cells were grown on a nitrogen-repressing medium. Although PUT3, the proline utilization pathway transcriptional activator, is absolutely required for growth on proline as the sole nitrogen source, a *put3 ure2* strain had somewhat elevated *PUT* gene expression, suggesting an effect of the *ure2* mutation in the absence of the *PUT3* product. *PUT1* and *PUT2* gene expression did not require the GLN3 activator protein for expression under either repressing or derepressing conditions. Therefore, regulation of the *PUT* genes by URE2 does not require a functional GLN3 protein. The effect of the *ure2* mutation on the *PUT* genes is not due to increased internal proline levels. URE2 repression appears to be limited to nitrogen assimilatory systems and does not affect genes involved in carbon, inositol, or phosphate metabolism or in mating-type control and sporulation.

The proline utilization pathway in *Saccharomyces cerevisiae* enables cells to use proline as the sole source of nitrogen when preferred nitrogen sources are not available in the environment. The proline utilization enzymes proline oxidase and Δ^1-pyrroline-5-carboxylate dehydrogenase, encoded by the nuclear genes *PUT1* and *PUT2*, respectively, convert proline to glutamate in mitochondria (9–11). The expression of the *PUT* genes is regulated by the PUT3 activator protein, which responds to the presence of proline in the medium and increases the transcription of the *PUT1* and *PUT2* genes (7, 8, 67). The PUT3 protein constitutively binds to the upstream activation sequences in the promoters of both *PUT1* and *PUT2* genes in vitro and in vivo but activates transcription only in the presence of proline (2, 44, 45, 58).

Early studies on the proline utilization pathway concluded that the structural genes *PUT1* and *PUT2* were not regulated by nitrogen repression (10). We speculated that the proline transporters were inactive in the presence of good nitrogen sources and proline could not enter the cell, a conclusion subsequently proved by Jauniaux et al. (35). From our measurements of low proline oxidase activity in cells grown on urea (a nonrepressing nitrogen source), we concluded that nitrogen repression did not operate on the *PUT* genes and that inducer exclusion was sufficient to explain the regulation of the *PUT* pathway. Recently, however, Daugherty et al. (25) reported that under their growth conditions and in their strain background (a *dal80* mutant), *PUT1* and *PUT2* were sensitive to nitrogen repression as measured by steady-state RNA levels.

These workers also reported that *PUT1* and *PUT4*, the proline-specific permease, but not *PUT2*, were controlled by GLN3, a regulator of many nitrogen assimilatory pathways. GLN3 activates the expression of *GLN1* (glutamine synthetase), *GDH2* (NAD-dependent glutamate dehydrogenase), genes in the urea, allantoin, and arginine catabolic pathways (*DUR1,2*, *DUR3*, *DAL1* through *DAL5*, *DAL7*, *CAN1*, and *CAR1*), and *GAP1* (general amino acid permease) (23, 25, 48, 50). GLN3 binds to a DNA sequence containing a GATA motif found in the promoters of these genes (43, 48).

The *URE2* gene product is believed to be a negative regulator that prevents the expression of genes in alternative nitrogen assimilatory pathways when preferred nitrogen sources are present. Mutations in *URE2* elevate expression of *GDH2*, *CAR1*, *DUR1,2*, *DAL1*, *GLN1*, and *PUT4* (23, 26–29, 31). Magasanik and coworkers hypothesized that URE2 opposed the action of the GLN3 transcriptional activator in the regulation of *GDH2* and *GLN1* expression, allowing for expression of an alternative nitrogen source only when preferred nitrogen sources were unavailable (23, 48). Coschigano and Magasanik (22) demonstrated that the URE2 protein is required for the inactivation of glutamine synthetase in the presence of glutamine and suggested that the target of URE2 is the GLN3 protein.

With the goal of identifying *trans*-acting factors that play a negative role in the regulation of the proline utilization pathway, a scheme was designed to isolate mutant strains that produced the PUT enzymes in an ammonia-containing medium in the absence of proline (44). Most of the mutants

* Corresponding author. Mailing address: Department of Microbiology and Molecular Genetics, UMD-New Jersey Medical School, 185 S. Orange Ave., Newark, NJ 07103. Phone: (201) 982-6261. Fax: (201) 982-3644. Electronic mail address: brandris@umdnj.edu.

TABLE 1. Yeast strains used in this study

Strain	Genotype	Reference or source
JM1313	*MAT***a** *ura3-52 gal1-152 TRP1*::*PUT2-lacZ*	44
JM1566[a]	*MAT***a** *ure2-1566 ura3-52 TRP1*::*PUT2-lacZ gal1-152*	44
JD237-3A	*MATα put3-75 ura3-52 TRP1*::*PUT2-lacZ*	44
JD414-1A	*MATα put3-4*Δ::*TRP1 ura3-52 trp1*	J. E. Marczak
JD415-2A	*MATα put3-4*Δ::*TRP1 ura3-52 TRP1*::*PUT2-lacZ*	45
MB342-2B	*MAT***a** *car1-107*	12
MB758-5B	*MAT***a** *ura3-52*	57
MB827-5C	*MATα car1-107 TRP1*::*PUT2-lacZ*	This work
MB828-1A	*MAT***a** *ure2-1566 TRP1*::*PUT2-lacZ*	This work
MB828-1B	*MAT***a** *ura3-52 car1-107 ure2-1566 TRP1*::*PUT2-lacZ*	This work
SX18-1A	*MAT***a** *TRP1*::*PUT2-lacZ*	This work
SX18-1B	*MATα ure2-1566 TRP1*::*PUT2-lacZ*	This work
SX18-4A	*MAT***a** *TRP1*::*PUT2-lacZ*	This work
SX18-4D	*MAT***a** *ure2-1566 TRP1*::*PUT2-lacZ*	This work
SX18-5A	*MATα ura3-52 TRP1*::*PUT2-lacZ*	This work
SX18-5C	*MATα ure2-1566 ura3-52 TRP1*::*PUT2-lacZ*	This work
SX18-6A	*MAT***a** *ure2-1566 ura3-52 TRP1*::*PUT2-lacZ*	This work
SX22-1A	*MAT***a** *ure2-1566 ura3-52*	This work
SX22-5D	*MAT***a** *ura3-52*	This work
SX23-4B	*MATα ure2-1566 ura3-52*	This work
SX23-5B	*MATα ura3-52*	This work
SX23-3C	*MATα ura3-52 ure2-1566 ade2*	This work
SX23-6D	*MAT***a** *ade2 ura3-52*	This work
SX24-4A	*MATα ura3-52 put3-4*Δ::*TRP1 ure2-1566 TRP1*::*PUT2-lacZ*	This work
SX25-4A	*MAT***a** *put3-75 ure2-1566 TRP1*::*PUT2-lacZ ade1-41*	This work
SX26-4A	*MAT***a** *trp1*	This work
SX26-6D	*MATα ure2-1566 trp1*	This work
SX26-7D	*MAT***a** *ure2-1566 ura3-52 trp1*	This work
SX26-10A	*MATα ura3-52 trp1*	This work
SX39-5C	*MATα ure2-1566 ura3-52 his4 trp1*::*TRP1-PUT1-lacZ*	This work
SX39-8C	*MATα ure2-1566 ura3-52 trp1*::*TRP1-PUT1-lacZ*	This work
SX39-8D	*MATα ura3-52 trp1*::*TRP1-PUT1-lacZ*	This work
SX42-1A	*MAT***a** *ure2-*Δ*11*::*LEU2 ade2 leu2 trp1*::*TRP1-PUT1-lacZ*	This work
Y271	*MAT***a** *can1-100 his4-519 leu2-3,112 lys2 trp1-1 ura3-52*	51
Y270	*MAT***a** *can1-100 his4-519 leu2-3,112 lys2 trp1-1 ura3-52 ume6*::*LEU2*	51
02322d	*MATα cargRI ura3-52*	E. Dubois
11552a	*MAT***a** *cargRII ura3-52*	E. Dubois
02451c	*MAT***a** *cargRIII ura3-52*	E. Dubois
30.078c	*MATα ura3 uga43*^{o}	21
PM38	*MATα leu2-3,112 ura3-52*	48
PM38Leu^{+}	*MATα leu2-3,112*::YIpLEU2 *ura3-52*	This work
BMY342[b]	*MAT***a** *ade2-102 ura3-52 leu2-3,112 gln3*Δ*4*::*LEU2*	22
BMY344[c]	*MATα ade2-102 ura3-52 leu2-3,112 ure2*Δ*11*::*LEU2*	22
BMY346[d]	*MATα ade2-102 ura3-52 leu2-3,112 gln3*Δ*4*::*LEU2 ure2*Δ*11*::*LEU2*	22
PH5	*MATα ura3-52 leu2-3,112 ure2*Δ*13*::*LEU2*	49

[a] In addition to JM1566, mutant strains JM1403, JM1404, JM1431, JM1559, and JM1582 were used in this study. They are all derived from JM1313 as described by Marczak and Brandriss (44).
[b] Originally named P40-2C.
[c] Originally named P40-3C.
[d] Originally named P40-5D.

isolated in this scheme carried semidominant mutations in *PUT3* (45). Six mutant strains that had recessive, constitutive expression of the *PUT* genes were isolated. In this report, we present the characterization of one of these complementation groups that carried mutations in the *URE2* gene. We provide evidence that the URE2 protein can act to repress gene expression in the proline and γ-aminobutyrate (GABA) utilization pathways but is not a general repressor. Derepression of the *PUT* genes by the *ure2* mutation does not result from increased internal levels of proline. We demonstrate that full induced expression of the *PUT* genes on a proline-containing medium as well as repression by URE2 on a nitrogen-repressing medium can be obtained in the absence of GLN3.

MATERIALS AND METHODS

Strains, plasmids, and media. The *S. cerevisiae* strains used in this study are listed in Table 1. Strains with the prefixes MB, JM, JD, and SX are congenic. The *car1-107* (arginase-defective) mutation was derived from strain MB342-2B (12) and was introduced by crossing into *ure2-1566* strains. Plasmid pRS305 (59) carrying the *S. cerevisiae LEU2* gene was linearized at its unique *Hpa*I site and transformed into strain PM38 with selection for leucine prototrophy. A stable Leu^{+} transformant was isolated and named PM38Leu^{+}. *Escherichia coli* HB101 was used for plasmid amplification. Plasmids are listed in Table 2.

The growth media have been described previously (6). The nitrogen source was ammonium sulfate (0.2%), asparagine (0.1%), proline (0.1%), ammonium sulfate (0.2%) plus proline (0.1%), arginine (0.1%), or GABA (0.1%). Glucose (2%) was the carbon source. In the experiments in Table 7 comparing the *gln3*Δ, *ure2*Δ, *gln3*Δ *ure2*Δ, and wild-type strains, glutamine (30 μg/ml) was added to all media. High- and low-phosphate media were prepared as described by Han et al.

TABLE 2. Plasmids used in this study

Plasmid[a]	Gene or gene fusion	Reference or source
pWB36	*PUT1-lacZ*	67
pABC4	*PUT2-lacZ*	57
pRS46	*CAR1-lacZ*	39
pRR29	*DAL5-lacZ*	53
pJH330	*INO1-lacZ*	41
pSB231	*FUS1-lacZ*	L. Marsh
YCpUGA1-lacZ	*UGA1-lacZ*	B. Andre
p(SPO13)30	*SPO13-lacZ*	14
pDB37	*PUT3*	44
pRB58	*SUC2*	15
p1C-CS	*URE2*	22
YCp50		D. Botstein
YEp24		D. Botstein

[a] Plasmids pWB36, pABC4, pSB231, YCpUGA1-lacZ, p(SPO13)30, and YCp50 contain *CEN* sequences and are maintained in one to two copies per cell. *ARS1*-containing plasmids pRS46 and pRR29 and 2μm-containing plasmids pJH330, pRB58, pDB37, and YEp24 are high-copy-number plasmids.

(32). Inositol-free medium was identical to synthetic complete medium with the omission of inositol (33). α-Factor (Sigma Chemical Co.) was added to a final concentration of 5 μM (62). Carbon catabolite derepression was accomplished by reduction of the glucose concentration from 2 to 0.05% (63).

Genetic analysis. Genetic crosses, sporulation, and tetrad analysis were carried out by using standard procedures (56). Strains Y271, Y270, 02322d, 11552a, 02451c, 30.078c, PM38, and PH5 were used in crosses to various *ure2-1566 PUT2-lacZ* strains to carry out complementation tests (Table 1).

DNA preparation and transformation. Plasmid DNA was prepared from *E. coli* by the alkaline extraction method (4). *E. coli* transformation was performed by the calcium chloride method (17). Yeast transformation was carried out by the lithium acetate method (34).

Enzyme assays. Yeast cell crude extracts were prepared by vortexing exponentially growing cells with glass beads as previously described (9). The β-galactosidase assay was performed by the method of Miller (46). The units of β-galactosidase specific activity are nanomoles of *o*-nitrophenol formed per minute per milligram of protein. Three independent transformants of each strain were assayed. Variation in activity was ≤25%. Acid phosphatase was assayed by the method of Bergman (3). The units of acid phosphatase activity are micromoles of *p*-nitrophenol liberated per minute per unit of optical density at 600 nm of cell culture. Invertase assays were performed by L. G. Vallier (64). The units of invertase activity are micromoles of glucose released per minute per 100 mg (dry weight) of cells. NAD-dependent glutamate dehydrogenase assays were performed as described by Miller and Magasanik (47). Protein concentration was determined by the method of Bradford (5), with crystalline bovine serum albumin as the standard.

RNA preparation and hybridization. Total RNA was prepared by the method of Schmitt et al. (55). RNA was separated in 1.0% agarose gels and transferred to nylon membranes as previously described (7). *PUT1*, *PUT2*, *PUT3*, and yeast actin (*ACT1*) DNAs were labeled by using a Multiprime kit (Amersham). Hybridization was carried out as described by Thomas (60).

To analyze the data in Fig. 2A, the bands were cut out of the membrane and counted in a scintillation counter. For the data presented in Fig. 3, the autoradiographic signal from each band on several different X-ray film exposures was quantitated with a Molecular Dynamics model 300B computing densitometer (volume integration method). The *ACT1* signal was used to normalize the amount of RNA loaded in each lane. Measurements were made on films derived from at least two independent experiments.

Construction of a *trpE-PUT3* gene fusion for overexpression in *E. coli*. The *trpE* gene on the pATH1 vector (38) was used to construct a gene fusion for expression of part of the PUT3 protein in *E. coli*. Codons 680 through 979 of the *PUT3* gene were amplified by PCR with primers designed to put the *PUT3* fragment in frame with the *trpE* gene on plasmid pATH1 cut with *Eco*RI and *Sal*I. The fusion join of the resulting plasmid, pDB208, was checked by DNA sequencing. Induced expression and large-scale preparation of the fusion protein were accomplished as described by Koerner et al. (38). The 69-kDa fusion protein was excised from gels after sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and inoculated into rabbits.

Demonstration of PUT3-specific antibody. The polyclonal antiserum was shown to be specific for PUT3 protein in the following tests. In Western blots (immunoblots), it recognized the gel-purified 69-kDa TrpE-PUT3 protein used for the original inoculation (data not shown) as well as a protein of approximately 111 kDa from yeast extracts. This protein species was absent in extracts from a *put3*Δ strain that lacks almost the entire open reading frame of the *PUT3* gene and was overexpressed in extracts of a yeast strain that carried 10 to 20 copies of the *PUT3* gene (Fig. 1).

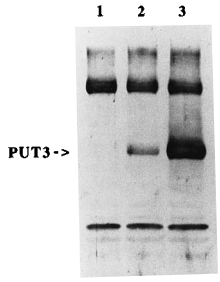

FIG. 1. Specificity of the anti-PUT3 antiserum. Extracts of each strain were made, and immunoblotting was carried out as described in Materials and Methods with antiserum diluted 1:1,000. Lanes: 1, 80 μg of protein from strain JD414-1A (*put3*Δ) carrying plasmid YEp24; 2, 80 μg of protein from strain MB758-5B (*PUT3*) carrying plasmid YEp24; 3, 60 μg of protein from strain MB758-5B carrying plasmid pDB37 (plasmid YEp24 + *PUT3*).

DNA binding assays. Gel mobility shift assays using yeast extracts were carried out as described by Siddiqui and Brandriss (58) except that a 50-fold molar excess of nonspecific 30-nucleotide single-stranded DNA was added to each reaction mixture to eliminate single-stranded binding proteins. The probe was a ^{32}P-5′-labeled 30-bp fragment containing the *PUT3* binding sequence in the *PUT2* promoter (bp −174 to −145). Addition of the anti-PUT3 antibody in the supershift assay was carried out as follows. The yeast extract-DNA mix was prepared and incubated at 30°C for 10 min. Serum (0.5 μl of undiluted preimmune, anti-PUT3, or anti-URE2 antiserum [see below]) was added, and the mixture was incubated for an additional 10 min at 30°C before the samples were loaded on the gel.

The anti-URE2 antiserum was provided by the laboratory of B. Magasanik. The antigen was a TrpE-URE2 fusion protein that contained the entire URE2 protein (43). The polyclonal antiserum, used in immunoblotting experiments, recognized a 40-kDa species in extracts of the wild-type strain that was missing from a *ure2*Δ strain (data not shown).

Immunoblotting. Proteins from extracts of yeast were separated on SDS-polyacrylamide gels as described by Laemmli (40) and transferred to nitrocellulose as described by Towbin et al. (61). The anti-PUT3 antiserum was diluted 1:1,000 for most experiments. The enhanced chemiluminescence protocol (Amersham) was used to detect the proteins as instructed by the manufacturer.

RESULTS

Recessive constitutive mutations affecting proline utilization. Marczak and Brandriss (44) described the isolation of six yeast strains that were able to express the *PUT1* and *PUT2* genes in the presence of ammonia and in the absence of the inducer, proline. Each mutation was recessive to wild type and unlinked to the *PUT3* locus, which encodes a positive activator of *PUT1* and *PUT2* transcription (8). One mutant strain, JM1566, was analyzed in detail.

Strain JM1566 was crossed to an isogenic wild-type strain that carried the same *PUT2-lacZ* gene fusion so that all progeny could be assayed for β-galactosidase activity. The diploid was sporulated, and 15 tetrads were analyzed. In all tetrads, proline-independent expression of β-galactosidase segregated 2:2, indicating that the mutation in JM1566 lay in a single nuclear gene. Two additional outcrosses were made, and the meiotic progeny of the third outcross were used in subsequent studies.

Complementation tests were carried out to determine the number of groups represented among the six original mutant strains. The constitutive mutation in strain JM1403 failed to complement the mutation in strain JM1566. Strains JM1582, JM1431, and JM1559 contained mutations in at least two other groups. The mutant phenotype originally described in strain JM1404 was lost.

Allelism to *URE2*. The recessive, constitutive phenotype of the mutations in strains JM1566 and JM1403 was reminiscent of several previously described recessive, constitutive muta-

TABLE 3. Complementation of the JM1566-derived mutation by the *URE2* gene

Strain (genotype)	Gene on plasmid[a]	Reporter[b]	β-Galactosidase sp act[c]
SX39-8D (*URE2*)		*PUT1-lacZ*	1,084
SX39-8C (*ure2-1566*)		*PUT1-lacZ*	9,554
SX39-8C (*ure2-1566*)	*URE2*	*PUT1-lacZ*	783
SX18-5A (*URE2*)		*PUT2-lacZ*	71
SX18-6A (*ure2-1566*)		*PUT2-lacZ*	464
SX18-6A (*ure2-1566*)	*URE2*	*PUT2-lacZ*	56

[a] Plasmids YCp50 and p1C-CS (*URE2* in YCp50) were used.
[b] The *PUT1-lacZ* and *PUT2-lacZ* gene fusions were integrated into the genome at the *TRP1* locus.
[c] See Materials and Methods. Transformants were grown in an ammonia-containing medium.

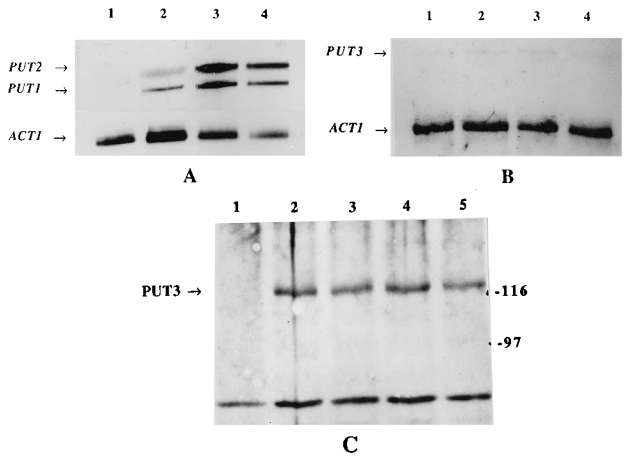

FIG. 2. Analysis of *PUT* gene expression in wild-type and *ure2-1566* mutant strains. (A) Total RNA (7 μg per lane) from cells grown in ammonia or proline medium was extracted and probed with α-^{32}P-labeled *PUT1* and *PUT2* DNAs as described in Materials and Methods. A duplicate membrane was probed with *ACT1* DNA. (B) The RNAs were probed with α-^{32}P-labeled *PUT3* and *ACT1* DNAs. Lanes: 1, SX18-1A (wild-type) cells grown in ammonia medium; 2, SX18-1B (*ure2-1566*) cells grown in ammonia medium; 3, SX18-1A (wild-type) cells grown in proline medium; 4, SX18-1B (*ure2-1566*) cells grown in proline medium. (C) Protein (100 μg per lane) from cells grown in ammonia or proline medium was extracted and immunoblotted with the anti-PUT3 antiserum as described in Materials and Methods. Lanes: 1, MB415-2A (*put3*Δ) cells grown in ammonia medium; 2, SX18-1A (wild-type) cells grown in ammonia medium; 3, SX18-1B (*ure2-1566*) cells grown in ammonia medium; 4, SX18-1A (wild-type) cells grown in proline medium; 5, SX18-1B (*ure2-1566*) cells grown in proline medium. Sizes are indicated in kilodaltons.

tions affecting arginine (*car80* [*cargRI*, *ume6*], *car81* [*cargRII*], and *car82* [*cargRIII*]), allantoin and GABA (*dal80*/*uga43*), and glutamate (*ure2*) assimilatory pathways (16, 27, 51, 65, 68). Wild-type and JM1566-derived mutant haploid strains containing *PUT2-lacZ* were crossed to strains of different backgrounds carrying a *car80*, *car81*, *car82*, *uga43* (*dal80*) or *ure2* mutation (Table 1). Expression of the reporter gene was measured in cells grown on an ammonia-containing medium.

All diploids except the *ure2*/JM1566 diploid gave wild-type values. In the *ure2*/JM1566 diploid, *PUT1* and *PUT2* expression was elevated two- to fourfold above wild-type levels (data not shown). The increase in expression suggested a lack of complementation between the mutations in these two strains. Possibly, the difference in strain backgrounds and the likelihood that the mutation in JM1566 was a missense mutation contributed to the small effect. Within one genetic background, the effect of *ure2* was greater (see below). However, introduction of the *URE2*-bearing plasmid p1C-CS (22) into the haploid mutant cells restored the expression of both *PUT1* and *PUT2* to wild-type levels (Table 3).

Allelism between the mutation in strain JM1566 and *ure2* was confirmed by examination of tetrads from a JM1566/*ure2*Δ *PUT1-lacZ*/*PUT1-lacZ* diploid (SX39-5C × SX42-1A). In five of five tetrads, constitutive expression of *PUT1* segregated 4:0 (data not shown). The mutation in strain JM1566 was named *ure2-1566*.

Effect of *ure2-1566* on expression of *PUT1* and *PUT2*. We examined the steady-state levels of *PUT1* and *PUT2* RNAs in isogenic *ure2-1566* and *URE2* strains under noninducing/repressing (ammonia as the sole nitrogen source) and inducing/derepressing (proline as the sole nitrogen source) growth conditions. Under noninducing/repressing conditions, expression of *PUT1* and *PUT2* in the *ure2-1566* strain was substantially higher than that in the wild-type strain, which was barely detectable (Fig. 2A, lanes 1 and 2). Under inducing/derepressing conditions, *PUT1* and *PUT2* were maximally expressed (Fig. 2A, lanes 3 and 4). Immunoprecipitation of β-galactosidase from a strain carrying *ure2-1566* and *PUT2-lacZ* showed a comparable increase in the level of protein made on ammonia-containing medium compared with that in an isogenic *URE2* strain (30).

To quantitate these findings, we introduced into *ure2-1566* and *URE2* strains a low-copy-number plasmid carrying either the *PUT1-lacZ* (plasmid pWB36) or *PUT2-lacZ* (plasmid pABC4) gene fusion and measured the activity of β-galactosidase under the same two conditions. Under noninducing/repressing conditions, *PUT1* and *PUT2* expression increased 10-fold over that in the wild-type strain. Under inducing/derepressing conditions, levels in the mutant were similar to those in the wild-type strain (Table 4). The recessive constitutive phenotype of the *ure2-1566* mutation suggests that the *URE2* gene product serves as a negative regulator in the proline utilization pathway.

Epistasis of *PUT3* and *URE2*. Recessive mutations in the *PUT3* gene prevent growth of *S. cerevisiae* strains on a medium containing proline as the sole source of nitrogen by preventing transcription of *PUT1* and *PUT2*. Recessive mutations in *URE2* cause high-level expression of the *PUT* genes in the absence of proline. To investigate the epistatic relationship between these two opposing elements, double mutants were constructed. Growth on proline and the expression of *PUT2-lacZ* were examined. Two types of *put3* mutations were used: a complete deletion, *put3*Δ, that removes almost the entire open reading frame of *PUT3* and makes no *PUT3* mRNA and no detectable protein, and a missense mutation, *put3-75*, carrying a glycine→aspartic acid change at position 409 (45) that results in production of stable PUT3 protein that fails to bind DNA containing the proline-responsive element (30).

In both double-mutant strains, the cells cannot use proline as the sole source of nitrogen (Table 5), indicating that *PUT3* is epistatic to *URE2* for growth on proline. However, these strains had threefold-elevated expression of *PUT2-lacZ* under noninducing conditions which was insensitive to the addition of proline. The *ure2* mutation can elevate *PUT* gene transcription in the absence of a functional *PUT3* gene, although to a level that is incompatible with growth on proline, indicating that the *ure2-1566* mutation is epistatic to *put3* under repressing conditions. Therefore, there appear to be both PUT3-dependent and PUT3-independent effects of the *ure2* mutation.

To determine whether *URE2* affects *PUT3* at the level of steady-state RNA or protein, *PUT3*-specific RNA and PUT3 protein were measured in *ure2-1566* and *URE2* strains. RNA

TABLE 4. Effects of the *ure2-1566* mutation on nitrogen catabolic genes

Strain (genotype)	Plasmid	Gene fusion	β-Galactosidase sp act[a]	
			Noninduced/ repressed	Induced/ derepressed
SX23-5B (*URE2*)	pWB36	*PUT1-lacZ*	86	3,975
SX23-4B (*ure2-1566*)	pWB36	*PUT1-lacZ*	873	4,158
SX23-5B (*URE2*)	pABC4	*PUT2-lacZ*	14	1,095
SX23-4B (*ure2-1566*)	pABC4	*PUT2-lacZ*	164	926
SX26-4A (*URE2*)	pRS46	*CAR1-lacZ*	36	2,426
SX26-6D (*ure2-1566*)	pRS46	*CAR1-lacZ*	499	4,618
SX26-10A (*URE2*)	pRR29	*DAL5-lacZ*	654	2,496
SX26-7D (*ure2-1566*)	pRR29	*DAL5-lacZ*	5,974	8,481
SX22-5D (*URE2*)	YCpUGA1-lacZ	*UGA1-lacZ*	197	3,117
SX22-1A (*ure2-1566*)	YCpUGA1-lacZ	*UGA1-lacZ*	443	5,083

[a] See Materials and Methods. For noninduced/repressed conditions, cells were grown in a minimal medium with ammonium sulfate as the sole nitrogen source. For induced/derepressed conditions, cells were grown in a minimal medium with proline as the sole nitrogen source for pWB36, pABC4, and pRR29 transformants, arginine as the sole nitrogen source for pRS46 transformants, and GABA as the sole nitrogen source for YCpUGA1-lacZ transformants.

hybridization analysis showed that steady-state levels of *PUT3* RNA were not altered by the *ure2-1566* mutation or, as previously reported (44), by the presence of proline (Fig. 2B). Western blotting indicated that the levels of PUT3 protein in these two strains grown on either an ammonia- or a proline-containing medium were similar (Fig. 2C). From these results, we conclude that it is unlikely that the normal function of the *URE2* gene product is to regulate the amount of *PUT3* message or protein.

To determine if increased dosage of the *PUT3* gene could reverse the effect of the *ure2-1566* mutation on expression of *PUT1* and *PUT2*, the high-copy-number plasmid pDB37 carrying the *PUT3* gene was introduced into *ure2-1566* and *URE2* strains. This plasmid was shown previously to increase production of PUT3 protein (58) (Fig. 1). Specific activities of β-galactosidase from a genomic copy of a *PUT2-lacZ* gene fusion were 160 nmol of *o*-nitrophenol formed per min per mg of protein in the wild-type strain SX18-5A and 482 nmol of *o*-nitrophenol formed per min per mg of protein in the *ure2-1566* strain SX18-5C. In this wild-type background, it appears that *PUT2-lacZ* expression is elevated about twofold when PUT3 levels are elevated (compare with Table 3). However, this level does not increase further in the *ure2* mutant with increased levels of PUT3 (compare with Table 3). The explanation for this result is not clear, although one could speculate that in the wild-type strain, excess PUT3 titrates URE2 (or another negative regulator), leading to escape synthesis of *PUT2* message. Removal of URE2 could lead to a maximum level of expression under repressing conditions, independent of the number of copies of PUT3. We conclude that increased dosage of PUT3 protein can not overcome the effect of the *ure2-1566* mutation.

***URE2* does not affect internal proline or arginine levels.** One hypothesis for increased *PUT* gene expression is that the *ure2* mutation increases the internal concentration of the inducer, proline. Increased levels of the arginine-degradative enzymes or a change in the flux of vacuolar arginine, a major source of stored nitrogen, could result in an increase in cellular arginine, which in turn leads to proline accumulation (12).

To test this possibility, a mutation in the *CAR1* gene, the structural gene for arginase, was introduced into the *ure2* strain. If increased internal arginine levels were responsible for high levels of *PUT* gene expression in the *ure2* strain, the double mutant would be unable to degrade arginine and unable to accumulate high levels of proline. As shown in Table 6, a *ure2 car1* strain had elevated levels of the *PUT* genes comparable to those seen in the *ure2* strain. Wild-type and *car1* strains had low levels of *PUT2* expression on an ammonia-containing medium. Therefore, we conclude that *ure2* does not affect internal proline or arginine levels.

Pleiotropic effects of the *URE2* mutation. The *ure2-1566* strain exhibited other defects in addition to an alteration in the regulation of the proline utilization pathway. As previously reported by Coschigano and Magasanik (22), we also observed that the mutant strain grew more slowly than the isogenic wild-type strain on a glucose-ammonia medium (doubling times of 4.1 h versus 3.0 h) or on a glucose-proline medium (5.3 h versus 4.7 h). The ability of a homozygous *ure2-1566/ure2-1566* diploid to sporulate was slightly, but reproducibly, impaired relative to that of an isogenic wild-type diploid (10 to

TABLE 5. Epistasis of *PUT3* and *URE2*

Strain	Relevant genotype	β-Galactosidase sp act[a] from a *PUT2-lacZ* gene fusion[b]		
		Ammonia	Ammonia + proline	Proline
JM1313	*PUT3 URE2*	58	235	2,448
SX18-5C	*PUT3 ure2-1566*	426	1,078	2,413
JD237-3A	*put3-75 URE2*	35	46	NG[c]
SX25-4A	*put3-75 ure2-1566*	101	107	NG
JD415-2A	*put3Δ URE2*	25	23	NG
SX24-4A	*put3Δ ure2-1566*	98	115	NG

[a] See Materials and Methods.
[b] The *PUT2-lacZ* gene fusion was integrated into the genome at the *TRP1* locus.
[c] NG, no growth.

TABLE 6. Lack of effect of the *ure2* mutation on internal proline levels

Strain	Relevant genotype	β-Galactosidase sp act[a]
SX18-5A	*URE2 CAR1*	69
MB827-5C	*URE2 car1*	69
MB828-1A	*ure2 CAR1*	327
MB828-1B	*ure2 car1*	356

[a] See Materials and Methods. Cells were grown in a medium containing ammonia (0.2%), glucose (2%), and uracil (30 mg/liter). Each strain contains a *PUT2-lacZ* gene fusion integrated at the *TRP1* locus.

15% versus 25 to 35%). Exposure of the *ure2-1566* strain to heat shock (45°C, 3 h) resulted in reduced ability to recover at 30°C compared with an isogenic wild-type strain.

Genes in two additional nitrogen assimilatory pathways, allantoin and GABA, were examined for regulation by *URE2*. The utilization of allantoin as a nitrogen source requires the expression of eight genes (reviewed in references 18 and 42). The product of each is repressed by growing the cells in a medium containing preferred nitrogen sources such as ammonia or glutamine. In the presence of the inducer of the allantoin system, allophanate or its analog oxalurate, some genes are induced, while others are constitutively expressed. *DAL5* encoding allantoate permease is an example of a gene that is repressed by ammonia but does not respond to the inducer (53).

The degradation of GABA provides *S. cerevisiae* cells with glutamate. The production of γ-aminobutyrate-α-ketoglutarate transaminase, encoded by *UGA1*, succinic semialdehyde dehydrogenase, encoded by *UGA2*, and the GABA permease, encoded by *UGA4*, is increased in the presence of the inducer, GABA (66). The *UGA3* gene product is required for the GABA-dependent induction of the *UGA1*, *UGA2*, and *UGA4* genes (1). The GABA system and the allantoin system share common elements, the negative regulator *DAL80* (*UGA43*) and the positive regulator *DAL81* (*UGA35*) (13, 20, 21, 24).

β-Galactosidase activities from plasmid-borne *DAL5-lacZ* and *UGA1-lacZ* gene fusions were measured in isogenic *ure2-1566* and *URE2* strains under different growth conditions. Levels of *DAL5* and *UGA1* expression under noninducing/repressing conditions were elevated approximately 9- and 2-fold, respectively, in the *ure2-1566* strain (Table 4). Regulation of *CAR1*, previously shown to be regulated by *URE2* (23), was included as a positive control. Its expression was elevated 14-fold in the mutant (Table 4). Under inducing/derepressing conditions, the *ure2-1566* mutation caused slight hyperinduction of *CAR1* and *UGA1* and over threefold derepression of *DAL5*. We conclude that *URE2* affects the regulation of representative genes in the proline, allantoin, and GABA utilization pathways, as well as those in the arginine and glutamate pathways.

Involvement of URE2 and GLN3. Two laboratories have reported that GLN3 regulates *CAR1*, *DAL5*, and *PUT1* but does not control *PUT2* and *UGA1* (19, 23, 25). Since the data in Table 4 show that the *PUT* genes are regulated by URE2, we reexamined the role of GLN3 in proline utilization and its relationship to URE2.

We used a set of related strains (wild type, *gln3*Δ, *ure2*Δ, and *gln3*Δ *ure2*Δ) constructed in the laboratory of B. Magasanik in which we could also assess the individual roles of URE2 and GLN3. We measured β-galactosidase activities from single-copy plasmids carrying *PUT1-lacZ* or *PUT2-lacZ* reporter genes under three conditions: asparagine (noninducing and repressing), GABA (derepressing and noninducing for the *PUT* genes), and proline (derepressing and inducing for the *PUT* genes). (Asparagine rather than ammonia was used in this experiment to obtain balanced growth of the double mutant.) *PUT1* expression under repressing conditions in the *gln3*Δ strain was comparable to that seen in the wild-type strain (Table 7). Removal of URE2 caused *PUT1* expression to increase 16-fold. A strain lacking both proteins had levels of expression 10-fold higher than those in the wild-type strain, less than 2-fold different from results for the *ure2*Δ strain. This result suggests that GLN3 plays little or no role in *PUT1* expression under repressing conditions. Under derepressing conditions, with or without inducer (proline or GABA), the removal of GLN3 and URE2 proteins had no effect on *PUT1* expression, in agreement with the results presented in Tables 4 and 5. *gln3*Δ strains maintained balanced growth in proline-containing medium and produced equivalent levels of β-galactosidase (Table 7) and RNA (Fig. 3, lanes 1 and 2). The *ure2*Δ mutation had no effect on *PUT1* expression under derepressing conditions (Table 7; Fig. 3, lanes 1 and 3). There is some variation in enzyme activity in the *ure2*Δ and *ure2*Δ *gln3*Δ strains, which we think is not significant.

TABLE 7. Effects of *GLN3* and *URE2* deletions on *PUT1* and *PUT2* expression

Strain	Relevant genotype	Gene fusion on plasmid[a]	β-Galactosidase sp act[b]		
			Asparagine	Proline	GABA
PM38Leu$^+$	+	*PUT1-lacZ*	5	2,168	200
BMY342	*gln3*Δ	*PUT1-lacZ*	4	2,419	203
BMY344	*ure2*Δ	*PUT1-lacZ*	80	1,174	181
BMY346	*gln3*Δ *ure2*Δ	*PUT1-lacZ*	46	3,956	430
PM38Leu$^+$	+	*PUT2-lacZ*	4	582	16
BMY342	*gln3*Δ	*PUT2-lacZ*	6	713	39
BMY344	*ure2*Δ	*PUT2-lacZ*	15	490	64
BMY346	*gln3*Δ *ure2*Δ	*PUT2-lacZ*	15	720	79

[a] The plasmids were pWB36 (*PUT1-lacZ*) and pABC4 (*PUT2-lacZ*) (see Table 2).

[b] See Materials and Methods. The growth medium contained 2% glucose, adenine sulfate (20 mg/liter), and glutamine (30 μg/ml). The nitrogen source was asparagine (0.1%), proline (0.1%), or GABA (0.1%).

PUT2 expression paralleled that of *PUT1*. On asparagine-containing medium, removal of GLN3 did not affect expression, while removal of URE2 caused a fourfold increase (Table 7). The double mutant resembled the *ure2*Δ mutant. On a proline-containing medium, within experimental variation, the three mutant strains resembled the wild type (Table 7; Fig. 3). On GABA-containing medium, the *gln3* strain had twofold-elevated expression compared with the wild type, an effect that we do not understand. As in the case of *PUT1*, the *ure2*Δ *gln3*Δ mutant resembled the *ure2*Δ mutant on this medium as well.

These results agree in part with those of Daugherty et al. (25), who reported that GLN3 had little or no effect on the expression of *PUT2*. However, these workers reported that steady-state RNA levels of *PUT1* were dramatically lower in a *gln3* strain grown on either GABA or proline than in the wild-type strain. We find no significant decrease in *PUT1* expression under these conditions (Fig. 1). We demonstrated that the strain was a bona fide *gln3* mutant in two ways: we monitored the growth rate in the presence or absence of glutamine to show that it was a glutamine bradytroph, and we measured NAD-dependent glutamate dehydrogenase activity. The *gln3* strain had NAD-dependent glutamate dehydrogenase activity about one-fifth of that found in the wild-type strain (data not shown), in good agreement with the levels reported by Coschigano and Magasanik (22) for this strain. Therefore,

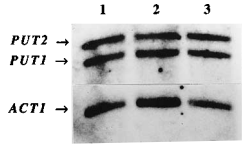

FIG. 3. Analysis of *PUT1* and *PUT2* RNAs in wild-type, *ure2*, and *gln3* mutant strains. Total RNA (7 μg per lane) was isolated from cells that were grown on proline. The RNA was hybridized with α-^{32}P-labeled *PUT1* and *PUT2* DNAs, and the membranes were stripped and reprobed with *ACT1* DNA as described in Materials and Methods. Lanes: 1, strain PM38Leu$^+$ (wild type); 2, strain BMY 342 (*gln3*Δ); 3, strain BMY344 (*ure2*Δ).

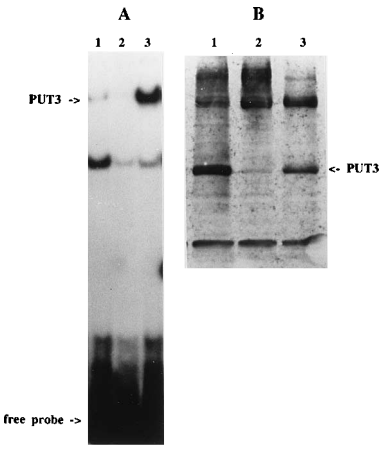

FIG. 4. DNA binding and protein stability of PUT3 in *ure2* and wild-type strains. Extract preparation, gel shift assays, and immunoblotting were carried out as described in Materials and Methods. (A) DNA binding. The unknown faster-migrating species varies in intensity from experiment to experiment and, since it is found in the *put3*Δ strain, is unrelated to PUT3. (B) Immunoblot. Lanes: 1, strain SX18-5C (*ure2-1566*); 2, JD414-1A (*put3*Δ); 3, JM1313 (wild type).

FIG. 5. Effect of addition of the anti-URE2 antiserum to PUT3-DNA complexes. Gel shift and supershift assays were carried out as described in Materials and Methods. Lanes: 1, probe alone; 2, extract of strain JD414-1A (*put3*Δ); 3, extract of strain MB758-5B (*PUT3*); 4, as in lane 3, but with anti-PUT3 antiserum added; 5, as in lane 3, but with anti-URE2 antiserum added; 6, as in lane 3, but with preimmune serum added. The faster-migrating species observed in lanes 2 to 5 is unrelated to PUT3. The unlabeled arrow indicates the position of the supershifted PUT3 complex.

we conclude that maximum expression of *PUT1* and *PUT2* can be achieved in the absence of the GLN3 product and that repression by URE2 also does not require a functional GLN3 product.

Nitrogen repression. Nitrogen repression was assessed by comparing the levels of reporter gene activity in cells grown in a nitrogen-derepressing medium with those of cells grown in a nitrogen-repressing medium. The pathway-specific inducer was omitted in all media tested. Nitrogen repression of the *PUT* genes was measured by comparing β-galactosidase activities in cells grown on GABA (derepressing) medium and in cells grown on asparagine (repressing) medium. The data in Table 7 show 40- and 4-fold repression of *PUT1* and *PUT2* expression, respectively. Steady-state RNA levels of the *PUT* genes in the wild-type strain grown on repressing and derepressing media supported these results (data not shown).

The *ure2* mutation reduced the nitrogen repression ratio (GABA/asparagine) for *PUT1* from 40- to 2-fold, almost completely abolishing nitrogen repression. For *PUT2*, the *ure2* mutation increased expression beyond that seen on GABA so that the nitrogen repression ratio remained fourfold.

Does URE2 interact with PUT3? The effect of the URE2 protein on the ability of PUT3 to bind DNA and the stability of the PUT3 protein were compared in isogenic wild-type and *ure2* mutant strains. Gel shift assays of *ure2* cells grown in a repressing medium demonstrated that the protein-DNA complex containing PUT3 appeared to be slightly less abundant or less stable than in the wild-type strain (Fig. 4A, lanes 1 and 3). PUT3 protein in a *ure2* mutant is as stable as in the wild-type, as demonstrated by immunoblotting (Fig. 4B, lanes 1 and 3).

A supershift gel mobility shift assay was carried out to determine if the URE2 protein was part of the PUT3-DNA complex. The PUT3-DNA complex is the most slowly migrating species in the gel shift assay shown in Fig. 5, lane 3. This species is absent in extracts from a *put3*Δ strain (Fig. 5, lane 2). Lane 4 demonstrates that the addition of the anti-PUT3 antiserum causes the formation of a complex too large to enter the gel. Addition of preimmune serum has no effect on the PUT3-DNA complex (compare lanes 3 and 5). Addition of anti-URE2 antiserum provided by the laboratory of B. Magasanik also did not affect the mobility of the PUT3-DNA complex. This negative result suggests, but does not prove, that PUT3 and URE2 do not form a stable complex. It is still possible that a loose complex forms but does not survive the gel shift assay conditions or that the epitope(s) recognized by the anti-URE2 antibody is hidden in a putative PUT3-URE2 complex. The latter explanation is less likely since the antiserum is polyclonal.

URE2 does not affect genes in pathways not involved in nitrogen assimilation. To determine whether *URE2* regulates gene expression in pathways unrelated to nitrogen assimilation, we examined the expression of representative genes in other pathways, including carbon source utilization, phosphate utilization, inositol biosynthesis, mating-type control, and sporulation. The *SUC2* (invertase) gene is subject to glucose repression, and its expression is derepressed in low-glucose conditions (52). The *PHO5* (acid phosphatase) gene is regulated by the level of phosphate in the growth medium and is actively transcribed only in response to phosphate starvation (54). *INO1* (inositol-1-phosphate synthase) transcription is reduced in the presence of inositol (33). The *FUS1* gene product is required for efficient cell fusion during the mating process. *FUS1* is haploid cell specific, and its expression is stimulated by mating pheromone of the opposite mating type (62). *SPO13* is required for proper chromosome segregation at meiosis I and is expressed early in meiosis (14). The results shown in Table 8 indicate that expression of these genes is unaffected by the *ure2-1566* mutation, either in repressing/noninducing or in derepressing/inducing conditions. We conclude that the *URE2* gene product is not a negative regulator of pathways that do not involve nitrogen assimilation.

DISCUSSION

Recent work by Magasanik and coworkers (22, 42, 43) and Daugherty et al. (25) points toward the importance of the GLN3 protein as a positive regulator of many nitrogen assimilation genes when conditions require the utilization of nonpreferred sources of nitrogen. GLN3 was first identified as a protein required for full expression of glutamine synthetase

TABLE 8. Genes unaffected by the *ure2-1566* mutation

Strain (genotype)	Gene	Assay[a]	Sp act in the indicated growth conditions[a]	
			High P_i	Low P_i
SX18-4A (+)	*PHO5*	Acid phosphatase	8.6	201
SX18-4D (*ure2-1566*)	*PHO5*	Acid phosphatase	6.9	159
			2% Glucose	0.05% Glucose
SX18-5A (+)	*SUC2*	Invertase	76	1,389
SX18-5C (*ure2-1566*)	*SUC2*	Invertase	64	1,042
			+Inositol	−Inositol
SX22-5D (+)	*INO1*	*INO1-lacZ*	376	1,242
SX22-1A (*ure2-1566*)	*INO1*	*INO1-lacZ*	345	839
			−α-Factor	+α-Factor
SX22-5D (+)	*FUS1*	*FUS1-lacZ*	0.28	19
SX22-1A (*ure2-1566*)	*FUS1*	*FUS1-lacZ*	0.38	21
			Vegetative	Sporulation
SX26-10A/SX23-6D (+/+)	*SPO13*	*SPO13-lacZ*	<0.5	2.0
SX22-1A/SX23-3C (*ure2-1566*/*ure2-1566*)	*SPO13*	*SPO13-lacZ*	<0.5	2.5

[a] See Materials and Methods. β-Galactosidase activity was measured from the *INO1-lacZ* gene fusion on plasmid pJH330, the *FUS1-lacZ* gene fusion on plasmid pSB231, and the *SPO13-lacZ* gene fusion on plasmid p(SPO13)30.

and NAD-dependent glutamate dehydrogenase in cells growing on glutamate, a nonpreferred nitrogen source (50). Subsequently, GLN3 was reported to be required for expression of genes and transporters in arginine, allantoin, urea, and proline utilization pathways and the general amino acid permease under nitrogen-derepressing conditions (23–25). Many of these genes are also negatively regulated by URE2, which represses their transcription when preferred nitrogen sources are available (23, 26–29, 31). Magasanik and coworkers have postulated that together GLN3 and URE2 are responsible for the phenomenon of nitrogen repression and that the URE2 protein antagonizes the action of GLN3. They have hypothesized that URE2 works through GLN3, possibly by a mechanism involving posttranslational modification (22, 42, 43).

Daugherty et al. (25) recently examined the relationship between genes that were regulated by nitrogen repression and those that were regulated by GLN3. They found that among nitrogen-regulated genes, not all required GLN3 for maximum expression on nonpreferred nitrogen sources. *PUT2* and *UGA1*, in particular, did not require GLN3. Therefore, it seems clear that the situation is more complex than previously envisioned.

Our finding that URE2 negatively regulates the *PUT* genes led us to examine whether this effect was mediated by GLN3. Contrary to the report by Daugherty et al. (25), we find that fully induced or derepressed *PUT1* expression can be obtained in the absence of GLN3, as steady-state *PUT1* RNA levels and expression of β-galactosidase from a *PUT1-lacZ* reporter gene are comparable to wild-type levels on all media tested. This result is consistent with the ability of a *gln3*Δ strain to grow on proline, since high levels of *PUT1* and *PUT2* expression are required for production of proline oxidase and Δ^1-pyrroline-5-carboxylate dehydrogenase. Our results concerning the GLN3 independence of *PUT2* expression on derepressing media agree with those of Daugherty et al. (25). Our studies of the double *gln3*Δ *ure2*Δ mutant grown on a repressing medium allowed us to assess whether URE2 repression could occur in the absence of GLN3. Since expression of both *PUT* genes was elevated in the double mutant and similar to the levels measured in the *ure2*Δ single mutant, we conclude that URE2 can repress even in the absence of GLN3.

The most likely explanation for the difference in the GLN3 requirement for *PUT1* expression between our results and those of Daugherty et al. (25) is a difference in strain backgrounds. Magasanik (42) referred to the existence of a novel regulator, the product of the *NIL1* gene, that is responsible for activation of *GAP1* (general amino acid permease) and other genes in a *gln3*Δ strain. In the absence of GLN3 protein, NIL1 could activate transporters for proline, allowing the inducer into the cell. Perhaps URE2 interacts with another protein (e.g., NIL1) in the absence of GLN3 that permits nitrogen repression of the *PUT* genes. Whether NIL1 is capable of also activating the *PUT* genes remains to be experimentally determined. It is possible that the Magasanik laboratory strains contain two regulators with overlapping functions that control expression of specific nitrogen assimilatory genes. In the Cooper laboratory strain, GLN3 may be the sole nitrogen activator responsible for *PUT1*.

Our observation that the PUT3-DNA complex appears less stable in a *ure2* strain than in the wild type may be a reflection of the in vitro conditions rather than its in vivo state. We find a similar effect in gel shift assays using extracts of strains containing constitutive mutations that map in the *PUT3* gene itself (30). These findings suggest that PUT3 associates with another protein that may protect it from proteolysis. There is precedence for this observation in the galactose pathway, where it is known that GAL80 binds the tail of GAL4 (reviewed in reference 36): GAL4-DNA complexes in *gal80* mutant extracts exhibit less stability than those from wild-type extracts (37).

We report several new findings concerning URE2. The number of nitrogen pathways regulated by URE2 now includes genes in the proline and GABA utilization pathways. We demonstrated that URE2 is not a general repressor since it does not affect representative genes in pathways unrelated to nitrogen assimilation. We have also shown that URE2 does not affect internal proline levels, either from higher levels of arginine-degradative enzymes or from increased flux of vacuolar arginine. Removal of URE2 permits a small but significant increase in *PUT* gene expression even in the absence of the PUT3 activator, but growth on proline as the sole nitrogen source absolutely requires PUT3.

It is tempting to speculate that URE2 interacts with the transcriptional activators of nitrogen assimilatory pathway genes to repress transcription when preferred nitrogen sources are present. At present, however, we have no evidence that PUT3 and URE2 form a stable complex. The URE2 protein sequence lacks similarity to known DNA-binding proteins, and no ability to bind DNA has been reported to date (22, 43). Its ability to repress a large number of genes suggests analogy to the CYC8 (SSN6)-TUP1 repressor complex, which functions to repress genes in a variety of pathways by association with specific DNA-binding proteins. For example, to achieve glucose repression, the MIG1 repressor associates with the CYC8-TUP1 complex (reviewed in reference 36). However, URE2's role appears to be much less widespread since it does not affect genes unrelated to nitrogen assimilation; to date, no protein partners have been identified. In a recent report, Wickner (69) suggests that the URE2 protein in a mutant form (URE3) may act like a prion analog in *S. cerevisiae*, adding further complexity to our understanding of the role of this protein.

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