Analysis of Constitutive and Noninducible Mutations of the PUT3 Transcriptional Activator

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The *Saccharomyces cerevisiae PUT3* gene encodes a transcriptional activator that binds to DNA sequences in the promoters of the proline utilization genes and is required for the basal and induced expression of the enzymes of this pathway. The sequence of the wild-type *PUT3* gene revealed the presence of one large open reading frame capable of encoding a 979-amino-acid protein. The protein contains amino-terminal basic and cysteine-rich domains homologous to the DNA-binding motifs of other yeast transcriptional activators. Adjacent to these domains is an acidic domain with a net charge of −17. A second acidic domain with a net charge of −29 is located at the carboxy terminus. The midsection of the PUT3 protein has homology to other activators including GAL4, LAC9, PPR1, and PDR1. Mutations in *PUT3* causing aberrant (either constitutive or noninducible) expression of target genes in this system have been analyzed. One activator-defective and seven activator-constitutive *PUT3* alleles have been retrieved from the genome and sequenced to determine the nucleotide changes responsible for the altered function of the protein. The activator-defective mutation is a single nucleotide change within codon 409, replacing glycine with aspartic acid. One activator-constitutive mutation is a nucleotide change at codon 683, substituting phenylalanine for serine. The remaining constitutive mutations resulted in amino acid substitutions or truncations of the protein within the carboxy-terminal 76 codons. Mechanisms for regulating the activation function of the PUT3 protein are discussed.

The expression of individual eukaryotic genes is often sensitive to alterations in the physiological conditions and needs of individual cells. In many cases, these changes in gene expression are mediated by activator proteins that increase the transcription of their target genes in response to environmental signals. Such regulated expression can be accomplished by a variety of mechanisms, including modulating the amount of the activator protein present in the cell (effects on its transcription or translation) or changing its ability to localize to the nucleus, bind DNA, or interact with other proteins including those of the transcription apparatus.

The proline utilization pathway of *Saccharomyces cerevisiae* is an example of a regulated system that responds to environmental changes via a transcriptional activator. The function of this pathway is to convert proline to glutamate for use as a nitrogen source when preferred sources of nitrogen are unavailable. The presence or absence of intracellular proline is sensed by the transcriptional activator, the product of the *PUT3* gene, whose role is to coordinate the expression of *PUT1* and *PUT2*, the structural genes that encode proline oxidase and Δ^1-pyrroline-5-carboxylate dehydrogenase, respectively (12, 14–16).

Recent studies reported the cloning and molecular analysis of the *PUT3* gene. *PUT3* steady-state mRNA (45) and protein (63) are present in comparable amounts under noninducing (ammonia as the sole source of nitrogen) or inducing (proline as the sole source of nitrogen) conditions. The PUT3 protein is required for basal as well as induced levels of *PUT1* and *PUT2* gene products and binds a proline-specific upstream activation sequence in the promoters of *PUT1* and *PUT2* in the presence or absence of proline in vitro (63) as well as in vivo (2). These studies show that the transcriptional activation of *PUT1* and *PUT2* is not due to

changes in *PUT3* transcription, translation, nuclear localization, or DNA binding and must therefore be due to changes in the activity of the DNA-bound PUT3 protein.

Current studies are focused on understanding how the PUT3 protein activates transcription of its target genes only in the presence of proline. In this study we report the sequences of the wild-type *PUT3* gene and of mutant genes that activate transcription aberrantly. One open reading frame capable of encoding a 979-amino-acid protein was identified. The amino terminus of the deduced amino acid sequence shows homology to the well-characterized cysteine-rich DNA-binding motif seen in many other yeast activator proteins. The overall domain structure has striking similarity to that of the galactose utilization pathway *trans*-activator, GAL4. A *put3* mutation leading to noninducible expression of *PUT1* and *PUT2* lies outside the cysteine-rich DNA-binding motif. Six mutations leading to constitutivity of *PUT1* and *PUT2* were localized to the carboxy terminus of PUT3 and are either point mutations or truncations of the protein. A seventh mutation was found more internal to the protein. Mechanisms for modulating the activation function of PUT3 are discussed.

MATERIALS AND METHODS

Strains and genetic analysis. The *S. cerevisiae* strains used are isogenic or congenic, apart from their specified genotypes, and are shown in Table 1. Strains JM1370, JM1521, JM1579, JM1594, JM1641, and JM1657 have been described previously (45). These strains were isolated after ethyl methanesulfonate or UV light mutagenesis of strain JM1313 (strain JD238-5C carrying plasmid pDB30; 45). (For a discussion of the independence of these mutations, see reference 45.) Strain MB1447 was isolated as a ureidosuccinic acid-resistant derivative (3) of strain MB1000 (11); the mutation to uracil auxotrophy was shown by complementation

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Source or reference
C74-6D	*MATa PUT3^c-68 ura3-52 his4-42*	S.-S. Wang
C75-6D	*MATa put3-75 ura3-52 adel*	45
JD237-3A	*MATα put3-75 TRP1::PUT2-lacZ14 ura3-52*	45
JD238-5C	*MATa gal1-152 TRP1::PUT2-lacZ14 ura3-52*	45
JD414-13C	*MATa put3-4::TRP1 ura3-52 trp1*	This work
JD415-2A	*MATα put3-4::TRP1 TRP1::PUT2-lacZ14 ura3-52*	This work
JD416-3A	*MATa put3-3::URA3 ura3-52 trp1*	This work
JM1313	*MATa gal1-152 TRP1::PUT2-lacZ14 ura3-52/pDB30*	45
JM1370	*MATa PUT3^c-1370 gal1-152 TRP1::PUT2-lacZ14 ura3-52*	45
JM1521	*MATa PUT3^c-1521 gal1-152 TRP1::PUT2-lacZ14 ura3-52*	45
JM1579	*MATa PUT3^c-1579 gal1-152 TRP1::PUT2-lacZ14 ura3-52*	45
JM1594	*MATa PUT3^c-1594 gal1-152 TRP1::PUT2-lacZ14 ura3-52*	45
JM1641	*MATa PUT3^c-1641 gal1-152 TRP1::PUT2-lacZ14 ura3-52*	45
JM1657	*MATa PUT3^c-1657 gal1-152 TRP1::PUT2-lacZ14 ura3-52*	45
JM5010	*MATα put3-4::TRP1 ura3-52 trp1*	This work
JD414	*MATα/MATa put3-4::TRP1/put3-3::URA3 ura3-52/ura3-52 trp1/trp1*	This work
MB758-1C	*MATα ura3-52 trp1*	M. C. Brandriss
MB1000	*MATα*	11
MB1447	*MATα ura3*	M. C. Brandriss

test to be in the *URA3* gene. Mating, sporulation, and tetrad analysis were carried out by standard procedures (62).

Strain JD415-2A contains a disruption of the *PUT3* gene which was constructed as follows. Strain MB758-1C was transformed with a *Sna*BI fragment containing the *put3-4::TRP1* null mutation from plasmid pDB101 (see below), with selection for Trp$^+$. DNA hybridization analysis (66) was used to verify that the genomic wild-type *PUT3* gene had been replaced with the disrupted copy. The resulting strain, JM5010, was crossed to strain JD416-3A (*MATa put3-3::URA3 ura3-52 trp1*) that carried another disruption (described in reference 45) that replaced *PUT3* DNA from bp +90 to +2894 with the *URA3* gene. The diploid strain, JD414, was used to determine genetically that the disrupted DNA had integrated at the proper locus. In this cross, the Ura$^+$ and Trp$^+$ phenotypes segregated as alleles, and in 26 tetrads, all spores were Put$^-$, indicating proper localization of the disruption to the *PUT3* locus on chromosome XI.

To construct a strain that contained both the *put3-4::TRP1* disruption and the integrated *PUT2-lacZ* reporter gene fusion (described in reference 45), strain JD414-13C, a segregant from the cross described above, was crossed to strain JD237-3A, and a meiotic segregant JD415-2A (Table 1) was isolated. DNA hybridization analysis demonstrated that this strain contained the *put3-4::TRP1* disruption (data not shown). All markers in this cross segregated as expected.

Plasmid constructions. To construct a complete deletion of *PUT3*, plasmid pDB64 (45), carrying a 6.0-kb *Hpa*I-*Kpn*I *PUT3* fragment, was digested with *Sac*II and *Pvu*II. After the *Sac*II end was filled in by using the large fragment of DNA polymerase I (Klenow fragment; New England Bio-Labs), the fragment was ligated to a *Sma*I-*Stu*I fragment

carrying the *TRP1* gene from plasmid pJHW1, modified so that it no longer contained any *Eco*RI sites. (Plasmid pJHW1, obtained from John Hill, is a pUC18 vector [73] with the yeast *TRP1-ARS1* locus inserted into the *Eco*RI site of the polylinker.) The recombinant plasmid, pDB101, was deleted for sequences between −190 and +2896 with respect to the translational initiation site. The *TRP1* gene was inserted so that the direction of transcription of *TRP1* was opposite that of *PUT3*.

Plasmids for sequencing were constructed using the exonuclease III-mung bean nuclease deletion kit (Stratagene). Plasmid pDB80 contained a 4-kb *Hpa*I-*Pvu*II fragment of *PUT3* (45) inserted into the filled-in *Eco*RI site of plasmid pBS-KS$^+$ (supplied by Stratagene). This plasmid was cut with *Bst*XI and *Bam*HI, and exonuclease III-mung bean nuclease digestion was carried out as described by the manufacturer to create nested deletions.

Plasmid pDB107 contains the cloned library *PUT3* gene (from strain DBY939 [17]) and was constructed by inserting the 3.7-kb *Sna*BI fragment from plasmid pDB37 (described in reference 45) into plasmid pDB104 (a YCp50 [33] derivative in which one *Bgl*II and one *Sal*I site were destroyed) such that the *PUT3* and *URA3* genes were tail to tail. The *Sna*BI fragment from plasmid pDB112 (see below) carrying the *put3-75* allele was inserted into the *Sma*I site of the high-copy-number plasmid YEp24 in the same orientation with respect to *URA3* as described above, resulting in plasmid pDB193. Plasmids pDB67 (45) and pDB194 contain a *Hpa*I-*Pvu*II fragment of the *PUT3* gene inserted into the *Sma*I site of plasmid YCp50 (low copy number) or plasmid YEp24 (high copy number), respectively, in the same orientation as described above.

Plasmid pDB99 contains a *Sal*I fragment of 5′-truncated *PUT3*-containing DNA (45) inserted at the *Sal*I site of plasmid YIp5 (73) such that the *PUT3* and *URA3* genes are tail to head.

To construct plasmid pDB72 carrying a *PUT3-lacZ* gene fusion, a 5.3-kb *Bam*HI fragment of *PUT3* DNA carrying the *PUT3* promoter and codons 1 to 962 was inserted into the *Bam*HI site of the high-copy-number plasmid YEp353 (50) to form an in-frame fusion to the *Escherichia coli lacZ* gene.

DNA sequencing. Approximately 4 kb of *PUT3*-containing DNA was sequenced by the double-strand dideoxynucle-otide chain termination method (61) with the Sequenase kit used according to the manufacturer's instructions (United States Biochemical Corp.). Plasmids were sequenced (coding strand) with the universal M13 primer supplied in the Sequenase kit. DNA not represented in the exonuclease III-mung bean nuclease deletion series, including the DNA on the noncoding strand, was sequenced with oligonucleotide primers synthesized on an Applied Biosystems DNA synthesizer (Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey [UMDNJ], Newark) or purchased from United States Biochemical Corp. (M13 and reverse primers).

All the DNA on both strands was sequenced, excluding 280 bases at the 5′ end in which only the bottom strand was sequenced and 57 bases at the 3′ end in which only the top strand was sequenced. These regions were outside the predicted open reading frame. The *PUT3* wild-type, consti-tutive, and noninducible alleles isolated by plasmid rescue were sequenced with the same primers.

Isolation of wild-type, constitutive, or noninducible *PUT3* alleles from *S. cerevisiae* genome. Plasmid pDB99 was line-arized 3′ to *PUT3* by digestion with *Kpn*I and integrated into strains JD238-5C, MB1447, C75-6D, C74-6D, JM1370,

JM1521, JM1579, JM1594, JM1641, and JM1657 by homologous integration. DNA hybridization analysis was done to confirm that the plasmid integrated properly. Genomic DNA was isolated from each strain and digested with ClaI. ClaI digestion liberates a DNA fragment that contains the genomic PUT3 (wild-type, constitutive, or noninducible) allele, which, upon ligation and transformation into E. coli, can form a yeast-integrating shuttle vector useful for testing the function of the isolated gene and determining its sequence. These plasmids were pDB174, pDB166, pDB112, pDB130, pDB131, pDB132, pDB106, pDB120, pDB123, and pDB191.

Plasmids isolated by this method were digested with SnaBI, and a 3.7-kb fragment was inserted into the SmaI site of the low-copy-number plasmid pDB104 such that the PUT3 and URA3 genes were tail to tail. This resulted in the construction of plasmids pDB109, pDB113, pDB126, pDB128, pDB133, pDB134, pDB135, pDB186, pDB187, and pDB192.

To locate the position of each mutation, a series of plasmids was constructed that contained hybrid PUT3 genes in which restriction fragments from the wild-type and constitutive PUT3 genes were interchanged. The constructions took advantage of unique restriction enzyme sites or used recipient plasmids in which certain sites had been destroyed. PUT2-lacZ-carrying strains were transformed with these plasmids for subsequent analysis of β-galactosidase activity levels.

Growth media. Minimal and YPD media were described previously (11). The carbon source was glucose (2% [wt/vol]), and the nitrogen source was ammonium sulfate (0.2%) or proline (0.1%).

DNA preparation, transformation, and hybridization. Isolation of genomic DNA from S. cerevisiae was carried out by the method of Hoffman and Winston (29). E. coli plasmid DNA was prepared by the method of Birnboim and Doly (8) or by the cesium chloride gradient method (44). DNA hybridizations were done by the method of Southern (66) with nylon membranes (Nytran; Schleicher & Schuell, Inc.). The probes were labeled by using the Multiprime kit (Amersham Corp.).

Extract preparation and β-galactosidase assays. β-Galactosidase assays were done on either crude extracts or whole cells as described previously (13). The assays were performed by the method of Miller (49). The units of specific activity are nanomoles of o-nitrophenol formed per minute per milligram of protein. Protein concentrations of crude extracts were determined by the Bradford method (10; Bio-Rad Protein Dye Mix), using crystalline bovine serum albumin as the standard.

Protein homology database searches and codon usage. Sequence analysis software of the University of Wisconsin Genetics Computer Group (UWGCG; 23) and Bionet (supported by Public Health Service grant P41RR01685 from the National Institutes of Health) were used to run the FastA program (57) to determine amino acid sequence homologies of PUT3 to other proteins. Databases searched include NBRF (release number 20.0), SwissProt (release number 10.0), and EMBL (release number 19.0). The entire PUT3 amino acid sequence as well as four contiguous overlapping regions (amino acids 1 to 300, 200 to 500, 400 to 700, and 600 to 979) of this sequence were used to search the databases using a ktup of 2. The RDF program (42), which used 200 shuffles (randomly permuted versions) of the homologous sequence compared with PUT3, was used to determine

statistical significance, with a ktup of 1 and a uniform window.

The hydropathy analysis was done using the SPAC program, a software package for molecular biological analysis modified by G. Cleaves (UMDNJ-R. W. Johnson Medical School) for use on a Hewlett Packard HP1000 computer. The algorithm of Hopp and Woods (31) was used for hydropathic analysis. A window of 20 residues was used in calculating a residue-specific hydrophobicity index. The α-helical secondary-structure determinations were done by using the UWGCG package (23). α-Helical secondary structure was determined by the method of Garnier et al. (27) or Chou and Fasman (20), using the default parameters. The codon usage index was calculated by the method of Bennetzen and Hall (6).

Nucleotide sequence accession number. The PUT3 sequence has been entered in the EMBL database under acquisition number X55384.

RESULTS

As the first step to understanding how PUT3 works, the gene was sequenced to determine the structure of its product and, by comparing the sequence with those of other characterized activator proteins, to deduce information about its functional domains. The sequences of eight mutations in PUT3 that lead to either constitutive or noninducible expression of its target genes were also determined to begin a functional analysis of the protein.

Nucleotide sequence analysis of PUT3 gene. A DNA fragment approximately 4 kb in length containing the PUT3 gene that complemented the recessive, noninducible put3-75 allele (45) was sequenced as described in Materials and Methods. This wild-type gene is referred to as the library PUT3 gene, since it was cloned (45) from the DNA library constructed from strain DBY939 (17). The complete nucleotide sequence and deduced amino acid sequence of the library PUT3 gene are shown in Fig. 1.

The sequenced DNA fragment contained one large open reading frame capable of encoding a 979-amino-acid protein with a predicted molecular weight of 111, 357. The size of this putative protein correlates well with the previously identified 2.8-kb mRNA, and the open reading frame lies within the region determined to encode this mRNA (45). The codon bias of the PUT3 gene is low (0.08), which is typical for yeast transcriptional activators.

Upstream from the first in-frame ATG (the A is +1), a weak match to the TATA consensus sequence lies at position -132. Two closer matches to the TATA consensus sequence are located at -210 and -455. Since the PUT3 gene is expressed relatively poorly, at approximately one mRNA molecule per cell (45), it is possible that the weak match functions as the PUT3 TATA box. Also in this region 5' to the open reading frame are two sets of closely situated direct repeats, two 10-bp direct repeats (AATGACTGAA) located at -761 and -729, and three 7-bp direct repeats with the sequence TCACGTG located at -294, -283, and -265. Tests to determine the function of these sequences have not yet been carried out. No sequences were found to be homologous to the conserved residues identified in the three PUT upstream activation sequences of PUT1 and PUT2 (63).

Downstream of the open reading frame there is no obvious tripartite terminator TAG. . .TAGT. . .TTT (74) or a sequence comparable to the consensus polyadenylation signal AATAAA (25).

The only other open reading frame of significant length in

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-900                                                                                    TTTAACTCCT TGGAAACTAA
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-880    GATTCTGTCT CCTTGGCGTT GGTCATTGCG TGCTTTTCAA AAGACTCAAT CACTTGTAAT TGTTTGGAGG CATCGATTTT GACCGGCTTA TATTGCTTAA CGTATGATTC

-770    TATCTTGTCA ATGACTGAAG TGTTTTTCAA CACAGATCTA TAATGACTGA AATCTACTTC GGTGGGTTGA CTTTGCAGCT CTAGTAATTG TCTACGTGCC TCATCGTTTC

-660    TCTTTTTAAA ACTCGATAAT TGAGTGGCAG TCGACCCTGT TATACGTAGA GAAGAGATGA CTTTTGCCCA GTCAAGTTTG TTAGCAGCGG ATTTGGCCAA AGACATAGTC

-550    GAACAAAAAT ATAATTAGGT GATTCTGCAA TTAAGTGCAC GAAAAATTTC TTTTAAATCC TAAGATTGTT CTTTTGCGTT ACGTGTCAAT ATGAATATAT AAACTTATAT

-440    AGAAAAGTAT TGTACTAGTA GAAATATGTT TTACACCTAC GCTAAGCAGC AAGTGGTAAT TGGTGTATCT TTTACTTTTT TGGGGGCATC AAGACAAATA TCCAATCAAA

-330    TCGAAGAGAA AATAATTCTT GTAACCCGCT CATTAGTCAC GTGGTCATCA CGTGAGCATA ATATATCACG TGATATACAC CCATACCTCG TTTATGGGAT TGAGTCATCG

-220    GATATGTCAT TATAAACGAT ATTTCTCCGC GGCAATGAGG TGGCGAATAT TGGAGTAAAA ATGAAAACAT CCACCAGGTG CTTGATGATA TACTTGACTT CGGCGGCAAT

-110    TTAAAGCTTT CTAAGCAGCA ATTAGTAAGT AACCATTACA ATAACGATAA ATACGACACT GGTCCAACTA ACTCATTGAT AGGATAATTG CAGTGATCTT AACGAGTTTA
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   1    ATG GTG ACC GAC CAA GGC AGT AGG CAT TCG ATA CAA TCT AAG CAA CCA GCC TAC GTT AAT AAA CAA CCG CAA AAA AGG CAG CAG AGA TCT
        met val thr asp gln gly ser arg his ser ile gln ser lys gln pro ala tyr val asn lys gln pro gln lys arg gln gln arg ser
                          5              10             15             20             25             30

  91    TCT GTC GCA TGC CTT TCT TGT AGG AAA CGT CAT ATA AAA TGT CCT GGT GGT AAC CCT TGC CAA AAA TGT GTT ACG AGC AAT GCC ATA TGT
        ser val ala cys leu ser cys arg lys arg his ile lys cys pro gly gly asn pro cys gln lys cys val thr ser asn ala ile cys
                         35             40             45             50             55             60

 181    GAG TAC TTG GAG CCG TCA AAA AAA ATT GTT GTG TCG ACA AAG TAT CTG CAA CAA CTG CAA AAA GAC TTG AAT GAT AAA ACT GAA GAG AAT
        glu tyr leu glu pro ser lys lys ile val val ser thr lys tyr leu gln gln leu gln lys asp leu asn asp lys thr glu glu asn
                         65             70             75             80             85             90

 271    AAC CGC CTG AAA GCT TTG CTC TTG GAG AGA CCA GTG AGT GTA CGT GGT AAG GAT AAC AGC GAT GAT GAC GAG AGG CAT ATA AAC AAT GCA
        asn arg leu lys ala leu leu leu glu arg pro val ser val arg gly lys asp asn ser asp asp asp glu arg his ile asn asn ala
                         95             100            105            110            115            120

 361    CCC TCA AGT GAC ACA TTG GAA GTA TCC AGC GCT CCG GCG GCT CCT ATA TTT GAC CTC ATG TCC AAT AGT AAC ACT GCG TCC GAT AAC GAT
        pro ser ser asp thr leu glu val ser ser ala pro ala ala pro ile phe asp leu met ser asn ser asn thr ala ser asp asn asp
                         125            130            135            140            145            150

 451    AAC GAC GAT GAC AAC AGC AAC AGA ATC ACA AAT AAT AGG AGC TAT GAT CAT AGT TTG GAA AAA TAC TAC AAA AAG GCC ATC AGC ATC TTT
        asn asp asp asp asn ser asn arg ile thr asn asn arg ser tyr asp his ser leu glu lys tyr tyr lys lys ala ile ser ile phe
                         155            160            165            170            175            180

 541    AAA CAA CCA GCT AAT GCT AAT GGC GAA AAT GGC AAC GGT GCC AAT GGT CAT GAG GAT GAT GAT GAA GAT GAT GAA GAA ATA TCA ACA AAT
        lys gln pro ala asn ala asn gly glu asn gly asn gly ala asn gly his glu asp asp asp glu asp asp glu glu ile ser thr asn
                         185            190            195            200            205            210

 631    TTT GCT CAA AGA AGT GGT AGG CTC ATA GAA TCT CAC AAT GGA TTC CAT TAT TTT GTT GGA TCT TCT TCA ATG ACA TTA TTT GGG TTA GAA
        phe ala gln arg ser gly arg leu ile glu ser his asn gly phe his tyr phe val gly ser ser ser met thr leu phe gly leu glu
                         215            220            225            230            235            240

 721    ATT CAA TCT TTG GTT ACA AAG TAT ATT TCG GTC AAG AAT TTT CGA CCT TTG CCA ATA AAC ACC AAA AAC AAG ATT TTA AAT TCA AAC CTG
        ile gln ser leu val thr lys tyr ile ser val lys asn phe arg pro leu pro ile asn thr lys asn lys ile leu asn ser asn leu
                         245            250            255            260            265            270

 811    AAC CCG GCT ATA AGT TCT TTT ATC AAT TCA AAC AAC TAT CTT TTC TCG TCC TAT AAT TTT TTG AAC CCT ATA TCG ACT ATT GTC AAC TTA
        asn pro ala ile ser ser phe ile asn ser asn asn tyr leu phe ser ser tyr asn phe leu asn pro ile ser thr ile val asn leu
                         275            280            285            290            295            300

 901    AAC TCA ATC AAC GAT AAT TTA TCT CCA TTG ATG TTT AAG ATA ATT TTG AAA AGC GAT ACG GAT GGT AGT AGT GGC CAG GAA GAG GTA ATA
        asn ser ile asn asp asn leu ser pro leu met phe lys ile ile leu lys ser asp thr asp gly ser ser gly gln glu glu val ile
                         305            310            315            320            325            330

 991    CAG TTT CAG TTG CCT TCG TAT AAT TAC ACA AAA CTA TTA ATT GAC TGC TTT ATA AAT TAT AAC GAT GGT TGT TTT TAC TTT TTC AAC GAA
        gln phe gln leu pro ser tyr asn tyr thr lys leu leu ile asp cys phe ile asn tyr asn asp gly cys phe tyr phe phe asn glu
                         335            340            345            350            355            360

1081    GGC CTG GTT AAA TGT GGC ATT AAC AAA TTG TAC TTG GAA AAT AAA TGG CTA TAC TAT GAT AAT ACC AAA AAA GCT TTG GAC AAT GAG AAT
        gly leu val lys cys gly ile asn lys leu tyr leu glu asn lys trp leu tyr tyr asp asn thr lys lys ala leu asp asn glu asn
                         365            370            375            380            385            390

1171    GAT CCA ATT TTA CAA GCT GTT TGG TTT TGC AAA ATT TTG TTA ATT TTA GCA GTA GGT GAA ATG TAT CTG GGT TCC ATC AAT AAT GAA ATG
        asp pro ile leu gln ala val trp phe cys lys ile leu leu ile leu ala val gly glu met tyr leu gly ser ile asn asn glu met
                         395            400            405            410            415            420

1261    TTG AAG AAT TAT TCT AAT CAG CCA AAA TTG CCC GGT TCA AAA TTT TTT CAA ATG GGT TCT AAA ATT TTT AAT TGT TTG TTC TCA AGT GAA
        leu lys asn tyr ser asn gln pro lys leu pro gly ser lys phe phe gln met gly ser lys ile phe asn cys leu phe ser ser glu
                         425            430            435            440            445            450

1351    AGA TTA GAA AAT GTG ACT AAG AAA GGC GGT ATT GAA GTC CTG TTG TTA TAC GCG TTC TTC TTA CAG GTG GCT GAT TAC ACT TTA GCC TCT
        arg leu glu asn val thr lys lys gly gly ile glu val leu leu leu tyr ala phe phe leu gln val ala asp tyr thr leu ala ser
                         455            460            465            470            475            480

1441    TAT TTT TAC TTC GGT CAA GCC CTA AGG ACG TGC TTA ATT CTA GGC TTA CAT GTT GAT TCT CAA AGC GAC ACT CTA TCC AGG TAT GAA ATC
        tyr phe tyr phe gly gln ala leu arg thr cys leu ile leu gly leu his val asp ser gln ser asp thr leu ser arg tyr glu ile
                         485            490            495            500            505            510

1531    GAG CAT CAT AGG AGA CTT TGG TGG ACA GTT TAC ATG TTT GAA CGA ATG CTT AGC TCA AAA GCT GGG TTA CCA TTA AGT TTC ACT GAT TAT
        glu his his arg arg leu trp trp thr val tyr met phe glu arg met leu ser ser lys ala gly leu pro leu ser phe thr asp tyr
                         515            520            525            530            535            540
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FIG. 1. Nucleotide and deduced amino acid sequences of three wild-type *PUT3* genes. The entire sequence found in strain DBY939 (library gene) and the deduced amino acid sequence are shown. +1 refers to the start of translation. Potential TATA boxes are overlined, direct repeats are underlined, the small arrow represents the start of the small open reading frame, and the asterisk indicates the stop codon of the small open reading frame. At codons 746, 751, 769, 811, 816, 818, 820, 859, 880, and 936, nucleotide changes in the *PUT3* genes found in wild-type strains MB1447 and JD238-5C are indicated with the amino acid change where appropriate.

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1621  ACA ATC TCT ACA GCA CTG CCA GCG GAT ATT GAT GAT GAA ACT ATC GAA GAG AAA AAT AGT CAC TAT GTT TTC AGA AAG GCA GAA TTG ATT
      thr ile ser thr ala leu pro ala asp ile asp asp glu thr ile glu glu lys asn ser his tyr val phe arg lys ala glu leu ile
                      545                 550                 555                 560                 565                 570

1711  TCT AAC TGC GTT ACT ATT GTG AAA ATC AAT GCA CAA ATT TTG AGC AAA TTA TAT CAA AGG CAA CCT GAG ACA AAC ATC ATA ATT ACT TTG
      ser asn cys val thr ile val lys ile asn ala gln ile leu ser lys leu tyr gln arg gln pro glu thr asn ile ile ile thr leu
                      575                 580                 585                 590                 595                 600

1801  AAA GTT GTC ATC AAG CAG TTG TTG GAA TGG AGG AAC AAT TTG TCC GAT TCC TTA CAG GTG GAT TTT ACG CAA AAG GAT GAA GAT TTC AAA
      lys val val ile lys gln leu leu glu trp arg asn asn leu ser asp ser leu gln val asp phe thr gln lys asp glu asp phe lys
                      605                 610                 615                 620                 625                 630

1891  ATA TCG AGA TTG TCA ACC AAT ATG TTT ACG GAA TAT TTT CAA GGA ATA AAC TTG GCC GTG AGA CCT TTA TTA TTT CAT TTT GCA TCC ATT
      ile ser arg leu ser thr asn met phe thr glu tyr phe gln gly ile asn leu ala val arg pro leu leu phe his phe ala ser ile
                      635                 640                 645                 650                 655                 660

1981  CAA TTG AAA AGG TTC AAA ACG AGC AAT ACT TTC GTC AAC TTA CAA AAC TAT TCT GCC ACA ATA TCT TCC TTA TTA ACA TGT TCT TTG CAT
      gln leu lys arg phe lys thr ser asn thr phe val asn leu gln asn tyr ser ala thr ile ser ser leu leu thr cys ser leu his
                      665                 670                 675                 680                 685                 690

2071  GCT TCT GTG AAT ACT ATT AGG TCT CTG TGG AGT TTA TTA CAG AAT AGT ATG CTT GCT ATG TTT AGT TAT ATG GAC AGA GAG TAT CTT TTT
      ala ser val asn thr ile arg ser leu trp ser leu leu gln asn ser met leu ala met phe ser tyr met asp arg glu tyr leu phe
                      695                 700                 705                 710                 715                 720

                                                                                                              G
2161  ACT TCT TCT TGT ACT TTA TTA CTA TTC AAC ACT GCT TTT GGT ATT CAT GAA CAA ACA CTA TAT CAT TTG GAT CAT TCT CTG GAA ATT TTC
      thr ser ser cys thr leu leu leu phe asn thr ala phe gly ile his glu gln thr leu tyr his leu asp his ser leu glu ile phe
                      725                 730                 735                 740                 745                 750

      G                                                                          C
2251  ACA CAA ATG AGA AAC TTA GGC AAC ATT CCA GCA GGC TTA AGA AGA GCA CAA TTA TTA ACA TTA ATG GCA AAT TTG GAT TTC CAC GGC ATA
      thr gln met arg asn leu gly asn ile pro ala gly leu arg arg ala gln leu leu thr leu met ala asn leu asp phe his gly ile
                      755                 760                 765                 770                 775                 780

2341  ATG AAT GAC TTG ATT ACT AAA TAT AAC GAT ATT TTG AAA TTT GAT TCT ATG AAT TGT GAA AAC GAT AAC ATA GTA GAA GAT AGC AAT GAA
      met asn asp leu ile thr lys tyr asn asp ile leu lys phe asp ser met asn cys glu asn asp asn ile val glu asp ser asn glu
                      785                 790                 795                 800                 805                 810

      ala                       arg thr
      G               G     C   A
2431  CCC AAA AGA GAA ACC GAA AAG TGT AAA CCT CAC AAA GAT GGC GAT CGC ATT GAC CCT TCA ATT ATA GAC TGT GAT AAA TCA AAC ACC AAT
      pro lys arg glu thr glu lys cys lys pro his lys asp gly asp arg ile asp pro ser ile ile asp cys asp lys ser asn thr asn
                      815                 820                 825                 830                 835                 840

                                                                  ser
                                                                  A
2521  ACA AAT ATG ATC AAG AAC GAA TCT ATA TCG AAC ATT GTT AGC ATA CTT CCG GAA GGC GCG AAA CCA ACG CTG ACT GAT TAT AGT AAT GGT
      thr asn met ile lys asn glu ser ile ser asn ile val ser ile leu pro glu gly ala lys pro thr leu thr asp tyr ser asn gly
                      845                 850                 855                 860                 865                 870

                                  C
2611  AAT AAT GAT GTT AAT GAT ATT AAC GTC AAT AAC TCG GAA CCT TCT ACT TTT TTT GAT ATC ATA ACT GCG AGC TTG GAA AAT TCA TAC CAA
      asn asn asp val asn asp ile asn val asn asn ser glu pro ser thr phe phe asp ile ile thr ala ser leu glu asn ser tyr gln
                      875                 880                 885                 890                 895                 900

2701  ACC ACG CTA ACT GAA AAG GGC TCT CAG GTG ATG GAA AAA AAC ATG GAT CAG TTG GAT TCC GTT CAT AAT CTA AAT GAT GAC GAT TTA CAA
      thr thr leu thr glu lys gly ser gln val met glu lys asn met asp gln leu asp ser val his asn leu asn asp asp asp leu gln
                      905                 910                 915                 920                 925                 930

                          G
2791  CAA TTG TTG GAG GAT TTA GGC AAT ATT GAT CAT TCC GAT GAA AAA CTT TGG AAG GAA ATC ACT GAT CAA GCA ATG TGG CTG GGA AAT ACT
      gln leu leu glu asp leu gly asn ile asp his ser asp glu lys leu trp lys glu ile thr asp gln ala met trp leu gly asn thr
                      935                 940                 945                 950                 955                 960

2881  ATG GAT CCA ACT GCA GCT GCT GGT AGT GAA ATT GAC TTT ACT GAT TAT TTA GGA CCA
      met asp pro thr ala ala ala gly ser glu ile asp phe thr asp tyr leu gly pro
                      965                 970                 975

2938      TAA CACCATTACA AAGACAAAGA AGAAAATACT GATTTCTTTG TTTTCTTATG CATATATTAT ACATTTCGCA CATTATATAG AAAAGTGGAC ATTTAATTCT

3041  TCAAATCTTA TTTAAAATAT CTATCACAAG ACAGCTCATT TACGTAGCCT CTTGACAACA TTGTCGCTAT CACCTTCTGT CCATAATATT AGCCTTTTT
```

the 4-kb fragment was located in the 5' region (reverse complement) beginning at −554 and ending at −884, with the potential to encode a 109-amino-acid protein. It is not known if this region is transcribed. When the predicted amino acid sequence encoded by this open reading frame was compared to proteins in the EMBL, SwissProt, and NBRF databases, no significant homologies were found.

The PUT3 protein sequence is shown in Fig. 1. Hydropathic analysis of this protein as determined by Hopp and Woods (31) predicted that the amino and carboxy termini would be hydrophilic and the internal region of the protein would be hydrophobic (data not shown). The amino-terminal 7% (residues 1 to 82) of the protein is basic, with a net charge of +10. This region contains six cysteine residues that may constitute a single metal-binding structure that is more similar to a binuclear-metal-ion cluster, as determined for GAL4 (56), than a zinc finger, as described for transcription factor IIIA from *Xenopus* oocytes (48).

Adjacent to the basic region in PUT3 is a region (residues 110 to 240) rich in acidic residues with a net charge of −17. This region contains a stretch of nine consecutive aspartic acid or glutamic acid residues (Fig. 1, amino acids 198 to 206). A second acidic region lies at the carboxy terminus. This area (residues 783 to 979) has a net charge of −29 but lacks a long consecutive stretch of acidic residues. The algorithms of Chou and Fasman (20) and Garnier et al. (27)

```
PUT3     33  A C L S C R K R H I K C P . . . G G N P . . C Q K C V T S N A I . C E Y L
PDR1     45  S C D N C R K R K I K C N . . . G K F P . . C A S C E I Y S C E . C T F S
LEU3     36  A C V E C R Q Q K S K C D A H . E R A P E P C T K C A K K N V P . C I L K
PPR1     33  A C K R C R L K K I K C D . . . Q E F P . S C K R C A K L E V P . C V S L
ARGRII   20  G C V T C R G R K V K C D . . . L R H P . H C Q R C E K S N L P . C G G Y
GAL4     10  A C D I C R L K K L K C S . . . K E K P . K C A K C L K N N W E . C R Y S
LAC9     94  A C D A C R K K K W K C S . . . K T V P . T C T N C L K Y N L D . C V Y S
QUTA     48  A C D S C R S K K D K C D . . . G A Q P . I C S T C A S L S R P . C T Y R
MAL63     7  S C D C C R V R R V K C D . . . R N K P . . C N R C I Q R N L N . C T Y L
MAL6R     7  S C D C C R V R R V K C D . . . R N K P . . C N R C I Q R N L N . C T Y L
qa-1F    75  ? C D Q C R A A R G K C D . . . G I Q P . A C F P C V S Q G R S . C ? ? ?
amdR     19  A C V H C H R R K K R C D A R L V G L P . . C S N C R S A G K T D C Q I H
```

FIG. 2. Sequence alignment of the cysteine-rich region of PUT3 and other transcriptional activator proteins. The conserved sequences are boxed. The numbers following the names of the proteins refer to the position of the first amino acid listed in the protein. Dots represent gaps in the sequence for better alignment. References are as follows: PDR1 (4), LEU3 (26, 75), PPR1 (37), ARGRII (47), GAL4 (41), LAC9 (59), QutA (7), MAL63 (39), MAL6R (64), qa-1F (5), and amdR (1).

predict that small α-helical regions form within these acidic domains. Acidic domains that may form α helices have been identified in numerous activator proteins, and for GAL4 and GCN4 proteins, these regions constitute the transcriptional activation domains (30, 43).

Proteins homologous to PUT3. Two regions of the PUT3 protein exhibited significant homologies with other proteins, identified by the FastA program (57). The amino terminus of the PUT3 protein, including the putative metal-binding domain, has homology to the *S. cerevisiae* proteins LEU3 (26, 75), ARGRII (47), PPR1 (37), and GAL4 (41); the *Kluyveromyces lactis* GAL4 analog, LAC9 (59, 72); the *Aspergillus nidulans* protein QutA (7); and a protein encoded by a gene linked to β-glucosidase (*GCA1*) in *Candida pelliculosa* (40). Figure 2 shows the putative metal-binding-domain homology between PUT3 and other fungal regulatory proteins, indicating the conservation of many amino acids in addition to the cysteines. Some of the proteins show extended homology to PUT3, although the cysteine-rich domain itself has the highest degree of homology. The regions surrounding zinc finger or binuclear-metal-ion cluster motifs are normally basic; it is not surprising that extended homology exists. The function of this region has been best characterized in GAL4, where it is known to bind zinc and participate in DNA binding (34, 38, 55, 56). By analogy to GAL4, we predict that the amino terminus of PUT3 contains its DNA-binding domain.

The second region of PUT3 that showed homology to other proteins extends from residue 431 to 720 of the PUT3 protein. These homologies range in statistical significance from 5.4 to 12.0 standard deviations above the mean after randomized testing using the RDF program (42). Chasman and Kornberg (18) have recently identified this region of homology between GAL4, LAC9, PPR1, PDR1, QutA, LEU3, and PET111. Figure 3 shows the alignment of these sequences and the consensus sequence derived by Chasman and Kornberg (18). The PUT3 homologous region is also aligned, and the addition of this sequence allows the consensus sequence to be revised, as shown in Fig. 3.

Sequence of two additional wild-type *PUT3* genes. The *PUT3* genes from two other *S. cerevisiae* strains, MB1447 and JD238-5C (see Table 1), congenic to each other and unrelated to strain DBY939, were also cloned and sequenced as described in Materials and Methods. These are the parents of the *PUT3ᶜ* and *put3* mutations described in this report.

The sequences of these wild-type genes were identical to each other but differed in 10 positions from that of the library gene. These changes and the four amino acid substitutions that resulted are indicated in Fig. 1. All the nucleotide changes were localized to the 3' third of the gene. Four of the sequence changes fell within a small region of the gene from codons 811 to 820.

Although some of the predicted amino acid substitutions appear to be nonconservative changes (Fig. 1), all three of the wild-type genes were able to regulate the expression of an integrated *PUT2-lacZ* gene fusion. A 3.7-kb *SnaBI* fragment from each gene was inserted into plasmid YCp50 at the *SmaI* site such that the *PUT3* and *URA3* genes were tail to tail. These plasmids were transformed into the *put3Δ* strain JD415-2A (*MATα ura3-52 put3-4::TRP1 TRP1::PUT2-lacZ*), and β-galactosidase activity was measured under inducing and noninducing conditions. All three strains had basal activ-

```
consensus     ESGS..L... .ALLLL..Y. .........T .W...G.A.R .A.SLGLNR.
Put3          ENVTKKGGI. EVLLLYAFFL QVADYTL A  SYFYFGQALR TCLILGLHVD
Gal4          ESGSIIL..V TALHLLSRYT QWRQKTN..T SYNFHSFSIR MAISLGLNRD
Lac9          ETGSTDL..T IALILLTHYV QKMHKPN..T AWSLIGLCSH MAISLGLHRD
Pprl          FSSSDRLEAL AGTLIMVIYS IMRPNQP..G VWYTMGSVLR LTVDLGLHSE
Pdrl          VDFTCDITHL EQLLYFLDLL FWLSEIY..G FEKVLNVAVH FVSRVGLSRW
QutA          ESGTYQLGHI QALLILSLIK LGQQDCA..A AWMLVGQAVR SAQSLGLNDP
Leu3          LNVA.SVYSV QAFLLYTFWP PLTSSLSADT SWNTIGTAMF QALRVGLNCA
Pet111        .......... .......... .......... YIKYCKVKP  QMISLGLN..
new consensus ESGS..L... .ALLLL..Y. Q........T SY...G.A.R .AISLGLHRD
                                                    W              N

consensus     ......D... .E....RRRL WW..Y..... LAS..GRP.. ..
Put3          SQSDTLSRYE IEH...HRRL WWTVYMFERM LSSKAGLPLS F
Gal4          LPSSFSDSSI LEQ...RRRI WWSVYSWIEQ LSLLYGRSIQ L
Lac9          LPNSTIHD.. QQL...RRVL WWTIYCTGCD LSLETGRPSL L
Pprl          KINKNYDAFT REI...RRRL FWCVYSLDRQ ICSIFGRPFG I
Pdrl          EFYVGLDENF AFR...RRNL WWKAFYFEKT LASKLGYPSN I
QutA          SDATGVEK.T AGR...SKHV FLGCFVLETL VAAKLGLLPS V
Leu3          GFSKEYASAN SELVNEQIRT WICCNVVSQT VASSFGEPAY V
Pet111        .......... .......... .......... .......... .
new consensus ..S...D... .E....RRRL WW.VY..E.. LSSK.GRP.. .
                                                    A
```

FIG. 3. Homology between PUT3 and other activators. Chasman and Kornberg (18) identified the consensus sequence on the top line among the seven activators listed below. The addition of PUT3 to this list permits the consensus to be extended, as shown on the bottom line.

TABLE 2. Analysis of *PUT3* constitutive and noninducible mutants

Plasmid[a]	Allele	Mutation[b]	β-Galactosidase sp act[c]	
			Amm	Pro
YCp50			39	NG
pDB107	*PUT3* (DBY939)	Wild type	100	1,622
pDB187	*PUT3* (JD238-5C)	Wild type	135	1,595
pDB109	*PUT3^c-1579*	W (TGG)-956 → stop (TAG)	488	1,544
pDB126	*PUT3^c-1594*	L (CTA)-903 → R (CGA)	372	2,022
pDB128	*PUT3^c-1641*	W (TGG)-956 → stop (TGA)	526	1,499
pDB134	*PUT3^c-1370*	W (TGG)-956 → stop (TGA)	515	1,650
pDB135	*PUT3^c-1521*	W (TGG)-956 → stop (TGA)	492	1,501
pDB192	*PUT3^c-1657*	N (AAC)-914 → I (ATT)	765	2,737
pDB186	*PUT3* (MB1447)	Wild type	111	1,110
pDB133	*PUT3^c-68*	S (TCC)-683 → F (TTC)	504	2,149
pDB113	*put3-75*	G (GGT)-409 → D (GAT)	34	NG
pDB193	*put3-75* (2μm)	G (GGT)-409 → D (GAT)	21	NG

[a] Low-copy-number plasmids derived from plasmid YCp50 unless specified as high-copy-number plasmid (2 μm).

[b] See text for further explanation of mutant sequence changes. Amino acids are represented by the single-letter code (D, aspartic acid; F, phenylalanine; G, glycine; I, isoleucine; L, leucine; N, asparagine; R, arginine; S, serine; W, tryptophan; stop, nonsense codon).

[c] β-Galactosidase assays were done under noninducing (ammonia [Amm]) or inducing (proline [Pro]) conditions. The specific activity is expressed as nanomoles of *o*-nitrophenol formed per minute per milligram of protein. Each value is an average of two or more determinations, with deviations of less than 8%. Plasmids were transformed into the *put3Δ* strain JD415-2A (*MATα ura3-52 put3-4::TRP1 TRP1::PUT2-lacZ*). NG, no growth.

ity levels ranging from 100 to 135; induced activity levels ranged from 1,110 to 1,622 (Table 2, plasmids pDB107, pDB186, and pDB187). Wild-type alleles from strains MB1447 and JD238-5C (Table 2, plasmids pDB186 and pDB187, respectively) caused a 10- to 12-fold induction, while the library gene (Table 2, plasmid pDB107) caused a 16-fold induction in *PUT2* expression. The 5' regions upstream of the open reading frame of the genes from strains MB1447 and JD238-5C have not been sequenced; we do not know if any nucleotide changes occur in this region.

Isolation of constitutive *PUT3* alleles. Identification in mutants of sequence changes that alter the function of transcriptional activators has proven useful in determining their critical functional domains (9, 19, 22, 34, 36, 54, 58, 60, 70). *PUT3^c* alleles in strains JM1370, JM1521, JM1579, JM1594, JM1641, and JM1657 (45) and their wild-type parental *PUT3* gene in strain JD238-5C were retrieved from the genome by plasmid rescue as described in Materials and Methods. The *PUT3^c-68* allele isolated in a previous mutagenesis (15) and its parent gene in strain MB1447 were treated in a similar manner.

To determine the location of the mutation in each cloned gene, restriction fragments from the wild-type and mutant genes were exchanged, forming hybrid genes. The ability of each hybrid gene to activate transcription was measured as a function of *PUT2-lacZ* expression. In each case, the expression of *PUT2-lacZ* as measured by β-galactosidase specific activity was equivalent to that of wild type or the constitutive allele (see below). Therefore, the smallest region identified as being responsible for the constitutive phenotype contains the mutation(s); if additional sequence changes in other regions of the gene exist, they do not contribute to the phenotype.

For each of the constitutive alleles, the smallest region identified that was able to confer constitutivity (see Materials and Methods) was sequenced and compared with the wild-type parental gene (either strain JD238-5C or strain MB1447). In all but one case, a single nucleotide change was identified for each mutant allele. A summary of the results is shown in Table 2. The seven mutations fell into two separate regions, six at the carboxy terminus within 76 codons of the end of the protein and one located more internal to the

protein. Three of the mutations caused amino acid substitutions. The mutation in allele *PUT3^c-1594* was a nucleotide change in codon 903 that replaced a leucine residue with an arginine residue (CTA → CGA). The mutation in allele *PUT3^c-1657* altered two consecutive nucleotides, replacing asparagine (AAC) at residue 914 with isoleucine (ATT). The mutation in allele *PUT3^c-68* was located more interiorly in the protein and replaced serine (TCC) at position 683 with phenylalanine (TTC).

Four of the mutations leading to a constitutive phenotype cause a truncation of the protein at residue 956, resulting in a protein 23 residues shorter than the wild type. The tryptophan codon (TGG) at this position was changed to a TAG termination codon in allele *PUT3^c-1579* or to a TGA termination codon in alleles *PUT3^c-1370*, *PUT3^c-1521*, and *PUT3^c-1641*.

Each gene on a low-copy-number plasmid was used to transform a *put3Δ* strain (JD415-2A). The ability of the cloned *PUT3* gene to activate transcription was measured as a function of β-galactosidase expression from a *PUT2-lacZ* gene fusion. The specific activities are shown in Table 2. The leucine-to-arginine change at residue 903 resulted in almost threefold-greater *PUT2-lacZ* expression under noninducing conditions than the wild-type gene (Table 2, plasmid pDB126). Replacement of asparagine with isoleucine at residue 914 resulted in a strain that had the highest specific activity under noninducing conditions, close to sixfold greater than that of wild type (Table 2, plasmid pDB192). The mutant carrying the serine-to-phenylalanine change at position 683 had fivefold-higher enzyme activity than its parent under noninducing conditions (Table 2, plasmid pDB133).

All the nonsense mutants produced the identical truncated PUT3 protein and had comparable β-galactosidase values under noninducing conditions, approximately fourfold greater than that of wild type under the same conditions (Table 2, plasmids pDB109, pDB128, pDB134, and pDB135).

To determine if the mutations also affected the induced level of expression, β-galactosidase activity was assayed in strains grown on proline-containing medium. The missense alleles *PUT3^c-1594*, *PUT3^c-1657*, and *PUT3^c-68* led to hy-

TABLE 3. Analysis of in vitro-constructed *PUT3*
deletion mutants

Plasmid[a]	Allele	Mutation	β-Galactosidase sp act[b]	
			Amm	Pro
YCp50			39	NG
pDB107	*PUT3* (DBY939)	Wild type	100	1,622
pDB67	*Hpa*I-*Pvu*II[c]	Δ966-979 + 11[d]	29	674
pDB194	*Hpa*I-*Pvu*II[c] (2μm)	Δ966-979 + 11[d]	32	994
YEp353			51	NG
pDB72	*PUT3-lacZ* (2μm)[e]	Δ963-979 + *lacZ*	169	1,514

[a] Plasmids YCp50, pDB107, and pDB67 are low-copy-number plasmids. Plasmids pDB194, YEp353, and pDB72 are high-copy-number plasmids.

[b] Assays were carried out as described in Table 2, footnote c. Plasmids were transformed into the *put3*Δ strain JD415-2A. NG, no growth; Amm, ammonia (noninducing conditions); Pro, proline (inducing conditions).

[c] The doubling time of this strain on a minimal proline medium is 50% greater than that of the wild-type strain.

[d] The 11 additional amino acids are GVITDIIKLKL.

[e] The amount of β-galactosidase produced from the *PUT3-lacZ* fusion (50 U) has been subtracted from the total β-galactosidase activity.

perinducible expression of *PUT2-lacZ* when proline served as the sole source of nitrogen (Table 2, plasmid pDB126, pDB192, and pDB133). The four truncated proteins caused induced levels comparable to those of the wild-type allele (Table 2, compare plasmids pDB109, pDB128, pDB134, and pDB135 with the wild-type plasmid pDB187).

Since amino acid substitutions or truncation of the PUT3 protein at the carboxy terminus causes constitutive expression of its target genes, two in vitro-made carboxy-terminal deletions of the *PUT3* gene were tested to see if they resulted in a comparable phenotype. Plasmid pDB67 contains a *Hpa*I-*Pvu*II fragment from the library *PUT3* gene (45) inserted into plasmid YCp50 at the same site and in the same orientation as the constitutive and wild-type alleles described above. In this construction, the entire promoter of the gene is present, but this fragment lacks the carboxy-terminal 14 codons. Since the *PUT3* fragment lacks a terminator, the open reading frame extends into the plasmid sequences and codes for an additional 11 residues (GVITDI-IKLKL). A similar plasmid was constructed in which the same *PUT3* fragment was inserted into the same site and orientation in a high-copy-number plasmid (pDB194).

Both plasmids were transformed into *put3*Δ strain JD415-2A, and β-galactosidase activity was measured under noninducing and inducing conditions to determine the ability of this mutant PUT3 protein to regulate transcription. When ammonia was used as the nitrogen source (noninducing conditions), the β-galactosidase activity measured from strains containing either plasmid was significantly below wild-type levels (Table 3, 29 versus 100). Since the PUT3 protein is required for basal as well as induced expression of its target genes (63), this finding indicates that the protein is not functioning as well as the wild-type protein. When proline was substituted for ammonia in the medium, there was an increase in doubling time of strains carrying either plasmid (7 h) compared to that of the wild-type strain (4 h). Induction still occurred, but the maximum specific activity was 2.4-fold lower than that of wild type in strains carrying the low-copy-number plasmid (674 versus 1,622) and 1.6-fold lower than that of wild type for the strain carrying the high-copy-number plasmid (Table 3, plasmids pDB67 and pDB194). Clearly, these constructions did not result in a

constitutive phenotype. We do not know whether the poor activating function of this protein resulted from removal of the carboxy terminus, less stable mRNA or protein, or the presence of 11 additional residues.

We also studied the activation function of a *PUT3-lacZ* gene fusion that was capable of binding the upstream activation sequences of *PUT1* and *PUT2* (63). This gene fusion contained the entire *PUT3* promoter and open reading frame, excluding the carboxy-terminal 17 codons, fused in-frame to *lacZ* at its eighth codon. *PUT3-lacZ* on a high-copy-number plasmid (pDB72) transformed into the *put3*Δ strain JD415-2A enabled it to utilize proline as the sole source of nitrogen. Since this strain contained two *lacZ* gene fusions (*PUT3-lacZ* and *PUT2-lacZ*), the total β-galactosidase activity is the sum of activity from each fusion. The *PUT3-lacZ* gene fusion produced 50 U of activity under each condition in a strain lacking a *PUT2-lacZ* gene fusion. The net values shown in Table 3 (plasmid pDB72) indicate that the level of *PUT2-lacZ* activity under noninducing conditions is slightly higher than that of the wild type and under inducing conditions is comparable to the levels of a wild-type *PUT3* strain. We conclude that replacement of the carboxy-terminal 17 residues of PUT3 with *lacZ* sequences from the plasmid appears to have little effect on the protein's ability to regulate transcription.

Analysis of a noninducible allele. Strains carrying the *put3-75* mutation fail to grow on proline as the sole source of nitrogen, cannot induce the enzymes of the proline utilization pathway, and have reduced basal and induced levels of *PUT1-lacZ* and *PUT2-lacZ* expression (12). Furthermore, extracts made from a *put3-75* strain lack the ability to bind the upstream activation sequence of *PUT2* (63). The *put3-75* allele from strain C75-6D and its wild-type parent gene in strain MB1447 were isolated by plasmid rescue as described in Materials and Methods. The mutation was localized to the 5' half of the *PUT3* gene by construction of hybrid genes as described above. Sequence analysis of the 5' half of the mutant gene revealed a single nucleotide change within codon 409 resulting in replacement of glycine (GGT) with aspartic acid (GAT). This mutation does not lie within the putative DNA-binding or acidic domains. The mutant allele on plasmid pDB113 (Table 2) does not activate transcription and does not permit growth on proline as the sole source of nitrogen.

Without anti-PUT3 antisera, it is impossible to determine if this mutant protein is made in wild-type amounts and fails to activate gene expression because it cannot bind the proline-specific upstream activation sequence or if the mutation causes the message or protein to be highly unstable in vivo. If stability were the problem, we reasoned that overproduction of the mutant message and protein might restore wild-type basal-level activity (*PUT2-lacZ* specific activity, 111) or allow some growth on minimal proline medium. Previous studies showed that the wild-type *PUT3* gene on a 2μm vector resulted in a dramatic increase in the formation of slower moving complexes in gel mobility shift assays (63), indicating that the PUT3 protein can be overproduced. (The overproduction of wild-type PUT3 protein does not alter the regulation of *PUT1* and *PUT2* [45].) When the *put3-75* gene was placed on a high-copy-number vector and transformed into the *put3*Δ strain, no compensation for the Put⁻ defect was observed. Basal-level expression was still reduced, and the strain could not grow on proline as the sole nitrogen source (Table 2, plasmid pDB193). This suggests, but does not prove, that the *put3-75* mutation leads to interference

with DNA binding rather than instability of the PUT3 protein.

DISCUSSION

The regulator of the proline utilization pathway is a 979-amino-acid protein with domains that are homologous in sequence to those of other well-characterized fungal transcriptional activators. Its amino-terminal basic and cysteine-rich region has the consensus sequence of a binuclear-metal-ion cluster, recently described by Pan and Coleman (55, 56) for the GAL4 protein. In GAL4, this region is known to bind zinc and is involved in binding DNA; most missense noninducible mutations mapped to this region of the protein and prevented DNA binding (32, 34). PUT3 binds DNA, either directly or as part of a complex in vitro (63) and in vivo (2), and on the basis of the sequence homology, we predict that the amino terminus of PUT3 forms the same type of structure as is seen in GAL4. However, the single noninducible put3 allele that was sequenced did not fall into the putative DNA-binding region (as might have been expected on the basis of the GAL4 prototype) but rather fell in a region of the protein that lacks similarity to domains that have been characterized in other regulatory proteins.

Two acidic stretches of the PUT3 protein are located in positions analogous to those in GAL4 (43), suggesting that they can activate transcription. Deletion studies of PUT3 to verify this role have not yet been performed. PUT3 mutations that lead to an activator-constitutive phenotype resemble those of GAL4: carboxy-terminal deletions and amino acid substitutions.

Since PUT3 binds its target promoters in the absence of proline (2, 63), it sits poised on the DNA ready to respond to environmental signals when culture conditions change. Therefore, transcriptional activation must occur by modulation of the activity of the DNA-bound protein. Our study of mutational changes in PUT3 leads us to suggest several possible mechanisms for regulation of PUT3 activity. (i) PUT3 could bind proline, resulting in a conformational change that alters its contacts with proteins of the transcriptional apparatus. (ii) A posttranslational modification of the protein in response to the presence of proline could alter its activation function. (iii) PUT3 could interact with one or more proteins that repress its activity in the absence of proline or induce its activity in the presence of proline.

To our knowledge, a proline-binding domain has not yet been identified in any protein that reacts with proline. Our comparisons of proline-binding proteins (S. cerevisiae proline oxidase [PUT1; 71] and proline permease [PUT4; 69], E. coli proline permease [putP; 53], and A. nidulans proline permease [PrnB; 65]) did not reveal a linear sequence common to these proteins, suggesting that a "proline pocket" may form from the teritiary conformation of each protein. In this model, the binding of proline to PUT3 is predicted to expose an activation domain or prevent the tail of PUT3 from serving as its own repressor.

The isolation of an activator-constitutive mutation that converted Ser-683 to Phe in PUT3 has led us to consider the possibility of protein modification, specifically phosphorylation. Phosphorylation can provide a reversible means of modulating both conformation and catalytic activity of a protein (67). PUT3 lacks known consensus recognition sites for phosphorylation (21, 24, 46). However, recognition sequences can be quite variable, and it is possible that phosphorylation sites are present in PUT3. Phosphorylation of ADR1, the regulator of the yeast glucose-repressible alcohol dehydrogenase, prevents transcriptional activation of ADH2 when glucose is present. Constitutive mutations in ADR1 map to the phosphorylation recognition site and prevent this modification from occurring (19, 22, 68). In contrast to the phosphorylation of ADR1, the phosphorylation of GAL4 correlates well with its ability to activate transcription. Mutations resulting in an activator-defective phenotype alter the profile of phosphorylated forms of GAL4 (51, 52).

The spectrum of mutations leading to an activator-constitutive phenotype in PUT3 bears a striking resemblance to that seen in GAL4 (36, 43, 60), raising the possibility that the tail of PUT3 interacts with a protein that has a repressor (Gal80-like) function. One prediction of such a model (fulfilled by GAL4 [28, 35]) is that an increase in the dosage of the activator should titrate out the repressor, leading to constitutive expression of the target genes. This is not seen with PUT3; overexpression of the PUT3 gene does not alter the level or regulation of target gene expression (45). Furthermore, the expected class of mutations with a phenotype consistent with the role of a proline-specific repressor (constitutive and recessive or dominant and noninducible, with effects limited to proline utilization) has not yet been uncovered in our systematic searches. However, we recently identified a class of recessive mutants with pleiotropic growth defects that expressed constitutive levels of the PUT genes and did not map in PUT3 (45). Given these results, we believe there is a high probability that PUT3 does interact with other proteins that may be relatively abundant and play regulatory roles in other pathways as well. Experiments to distinguish these possibilities are in progress.

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