

## The *Saccharomyces cerevisiae* *PUT3* Activator Protein Associates with Proline-Specific Upstream Activation Sequences

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The *PUT1* and *PUT2* genes encoding the enzymes of the proline utilization pathway of *Saccharomyces cerevisiae* are induced by proline and activated by the product of the *PUT3* gene. Two upstream activation sequences (UASs) in the *PUT1* promoter were identified by homology to the *PUT2* UAS. Deletion analysis of the two *PUT1* UASs showed that they were functionally independent and additive in producing maximal levels of gene expression. The consensus *PUT* UAS is a 21-base-pair partially palindromic sequence required in vivo for induction of both genes. The results of a gel mobility shift assay demonstrated that the proline-specific UAS is the binding site of a protein factor. In vitro complex formation was observed in crude extracts of yeast strains carrying either a single genomic copy of the *PUT3* gene or the cloned *PUT3* gene on a 2 $\mu$ m plasmid, and the binding was dosage dependent. DNA-binding activity was not observed in extracts of strains carrying either a *put3* mutation that caused a noninducible (*Put*<sup>-</sup>) phenotype or a deletion of the gene. Wild-type levels of complex formation were observed in an extract of a strain carrying an allele of *PUT3* that resulted in a constitutive (*Put*<sup>+</sup>) phenotype. Extracts from a strain carrying a *PUT3-lacZ* gene fusion formed two complexes of slower mobility than the wild-type complex. We conclude that the *PUT3* product is either a DNA-binding protein or part of a DNA-binding complex that recognizes the UASs of both *PUT1* and *PUT2*. Binding was observed in extracts of a strain grown in the presence or absence of proline, demonstrating the constitutive nature of the DNA-protein interaction.

The transcriptional regulation of genes encoding proteins that function in a particular pathway in *Saccharomyces cerevisiae* is generally mediated through homologous upstream activation sequences (UASs) present in their promoters (14, 15, 17, 19, 21, 32, 36). For many of the regulated pathways in yeast cells, these UASs have been shown to be the binding sites of their respective regulatory proteins (13, 15-17, 19, 23, 28, 30).

The unlinked and coordinately regulated *PUT1* and *PUT2* genes encode the enzymes of the proline degradative pathway in yeast cells (5, 8, 9, 39, 40). The presence of proline in the growth medium increases the accumulation of mRNA of both genes and causes a corresponding increase of proline oxidase and  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenase activities, respectively (5, 8, 39). The enzymes are synthesized in the cytoplasm and are then imported into the mitochondria, where the reactions take place (7, 8, 10, 40). A *trans*-acting positive regulator, the product of the *PUT3* gene, mediates proline induction (6, 9). *PUT3* was defined genetically as a transcriptional activator based on the analysis of two mutant alleles that affected the levels of RNA accumulation and enzyme activity of the *PUT* genes (5, 6, 9, 35, 39). Promoter deletion analysis demonstrated the presence of a proline-specific UAS in a 40-base-pair (bp) sequence that was required for transcription of the *PUT2* gene under inducing and noninducing growth conditions. The *PUT3* gene product was shown to act either directly or indirectly through this sequence (35).

The aim of this study was to identify sequences responsible for proline induction in the *PUT1* promoter and to study the interaction of these UASs with the positive activator of the pathway, the *PUT3* gene product. 5' and internal promoter deletions constructed in vitro in the *PUT1* promoter identified a 21-bp partially palindromic sequence homolo-

gous to the previously published *PUT2* UAS. This sequence, which is present once in the 819-bp *PUT2* promoter (35), is found twice in 458 bp of the *PUT1* promoter. Gel mobility shift assays demonstrated that the *PUT* UASs are the recognition sites of a DNA-binding complex that includes the *PUT3* product.

### MATERIALS AND METHODS

**Strains and media.** The *S. cerevisiae* strains used are listed in Table 1. The *Escherichia coli* strains used were HB101 (4) and JM101 (41). The media used for growing cells have been described previously (5). The nitrogen source was ammonium sulfate (0.2%; noninducing medium), ammonium sulfate plus proline (0.2 and 0.1%, respectively; partially inducing medium), or proline (0.1%; inducing medium); ammonia and proline give the lowest and highest levels, respectively, of expression of the genes in this system. Glucose (2%) was the carbon source. When needed, uracil was supplemented (20  $\mu$ g/ml) in the growth medium.

**Plasmids.** Plasmids pWB38 and pWB40 are low-copy-number (*CEN*) yeast shuttle vectors with 458- and 293-bp sequences, respectively, of the *PUT1* promoter and 25 codons of *PUT1* ligated to the eighth codon of *lacZ* (40). Plasmid pDB37 is a high-copy-number (2 $\mu$ m) yeast shuttle vector that carries an 11-kilobase-pair (kb) genomic fragment containing the *PUT3* gene, isolated by functional complementation of a *put3* mutation (26). Plasmid YEp353 (27) is a 2 $\mu$ m plasmid that carries a truncated *lacZ* gene with a polylinker sequence upstream of its eighth codon. Plasmid pDB72 is plasmid YEp353 with a 5.3-kb *Bam*HI fragment containing the *PUT3* promoter and almost the entire open reading frame inserted at the *Bam*HI site of the polylinker to form a *PUT3-lacZ* gene fusion (J. E. Marczak, unpublished results).

**DNA preparation and transformation of *E. coli* and *S. cerevisiae*.** Plasmid DNA was prepared from *E. coli* by

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TABLE 1. *S. cerevisiae* strains used

Strain	Genotype	Source or reference
MB758-5B	<i>MATa ura3-52</i>	35
C74-4D	<i>MATa ura3-52 PUT3<sup>c</sup>-68</i>	35
C75-2B	<i>MATa ura3-52 put3-75</i>	35
JM5007	<i>MATa ura3-52 put3-1::URA3</i>	J. E. Marczak
JM5008	<i>MATa ura3-52 put3-2::URA3</i>	J. E. Marczak

alkaline extraction (2) or on CsCl gradients (25). *E. coli* transformation was performed by the CaCl<sub>2</sub> method (12). *S. cerevisiae* transformation was carried out by the lithium acetate procedure (20).

**Formation of 5' and internal deletions in the *PUT1* promoter.** The promoter deletions in plasmids pABC24, pABC25, and pABC26 required a two-step construction. The source of the *PUT1* DNA for plasmids pABC24 and pABC25 was a 425-bp *EcoRI-SphI* fragment of plasmid pWB39 (40) carrying the 293-bp promoter. The 425-bp fragment was digested with *Sfa*NI (at -272 bp) and the ends were filled in with Klenow fragment of DNA polymerase I, followed by digestion with *Pst*I. This resulted in a 375-bp fragment containing the 248-bp promoter. The 375-bp fragment was cloned into a 2- $\mu$ m *lacZ* expression vector (plasmid YEp356 [27]) that had been digested with *Sma*I and *Pst*I to generate plasmid pABE2. The promoter and *PUT1-lacZ* sequences were moved into plasmid pABC1 (35), a low-copy-number expression vector containing a promoterless *lacZ* gene, by cutting with *Eco*RI and *Cla*I (in *lacZ*) to generate plasmid pABC25.

The 425-bp linear fragment from plasmid pWB39 was digested with *Rsa*I (at -188 bp), followed by isolation of the 310-bp fragment carrying 186 bp of *PUT1* promoter sequences. The 310-bp *Rsa*I-*Sph*I fragment was first moved into plasmid YEp356 to generate plasmid pABE1, and then an *Eco*RI-*Cla*I fragment was moved into plasmid pABC1 to yield plasmid pABC24.

The source of *PUT1* DNA for constructing plasmid pABC26 was the 310-bp *Rsa*I-*Sph*I fragment from plasmid pABC24. After digestion with *Dde*I (at -149 bp) and end filling, the DNA was digested with *Pst*I, resulting in a 266-bp fragment carrying 141 bp of the promoter sequences. The 266-bp fragment was first cloned into plasmid YEp356 to make plasmid pABE3, and then an *Eco*RI-*Cla*I fragment was moved into plasmid pABC1 to generate plasmid pABC26.

The internal deletion in plasmid pABC27 was constructed as follows. The 1.3-kb *Eco*RI-*Cla*I fragment containing 248 bp of the promoter sequences and part of the *PUT1-lacZ25* gene fusion was isolated from plasmid pABE2 and cloned into a 7.5-kb *Bst*EII-*Cla*I fragment of plasmid pWB38. Both the *Eco*RI and *Bst*EII ends were filled in with Klenow fragment of DNA polymerase I. Sequences between -288 and -248 bp in the *PUT1* promoter were removed and replaced by the polylinker sequence CCCATGGCTCG AGCTTA. The endpoints of the promoter deletions in plasmids pABC24, pABC25, pABC26, and pABC27 were verified by the dideoxy-nucleotide chain termination method (33), using the Sequenase kit (United States Biochemical Corp.).

**Formation of an internal deletion in the *PUT2* UAS.** A 14-bp internal deletion (-142 to -129 bp) in the 40-bp *PUT2* UAS (-168 to -128 bp [35]) was generated by using an oligonucleotide-directed in vitro mutagenesis (38) system (Amersham Corp.) according to the instructions of the manufacturer. The template was bacteriophage M13mp18 with a

200-bp *PUT2* insert containing 168 bp of promoter sequences. A 30-nucleotide-long oligonucleotide (CCCTTATA TAGTAGGGTCCGGCTTCGGAGTT) containing 15 flanking nucleotides on either side of the desired deletion was synthesized on an Applied Biosynthesis DNA synthesizer (Department of Microbiology and Molecular Genetics, University of Medicine and Dentistry of New Jersey, Newark). The presence of the desired deletion was detected by differential plaque filter hybridization to the oligonucleotide. The endpoints of the deletion were determined by sequencing as described above, and the 200-bp *Eco*RI-*Hind*III fragment carrying the deletion was cloned into plasmid pABC1 to generate plasmid pABC28.

**Growth of *S. cerevisiae* and preparation of cell extracts.** Cells were grown to exponential phase (approximately 100 Klett units; Klett-Summerson colorimeter, blue filter) in the appropriate media as described above. The cell extracts for mobility shift assays were made as described previously (1), with the following modifications. Cells were harvested and washed in a modified buffer A (with 1 mM dithiothreitol substituted for  $\beta$ -mercaptoethanol and lacking MgCl<sub>2</sub>). They were then vortexed with glass beads in the same buffer, and the extract was centrifuged for 1 h at 12,000  $\times$  g. The conditions for growing plasmid-bearing strains, preparation of cell extracts for  $\beta$ -galactosidase assays, and protein determinations were as described previously (35).

**Gel mobility shift assays.** The DNA fragments used in the gel mobility shift assays were as follows. The 98-bp DNA fragment containing the *PUT2* UAS was derived from plasmid pSB5, containing a 2-kb *PUT2* DNA fragment carrying promoter sequences and a truncated open reading frame (35). Plasmid pSB5 was digested with *Afl*II (at -228 bp) and *Stu*I (at -130 bp). The ends were filled by using [ $\alpha$ -<sup>32</sup>P]dATP and [ $\alpha$ -<sup>32</sup>P]TTP (25), and the 98-bp labeled fragment was isolated. The source of the 64-bp fragment lacking the UAS was plasmid pABC18 (35), in which *PUT2* UAS sequences between -164 and -129 bp were replaced by a *Kpn*I site. Plasmid pABC18 was digested with *Kpn*I and *Afl*II (at -228 bp), and the 64-bp fragment was isolated. The source of the proximal *PUT1* UAS was the previously described 425-bp *Eco*RI-*Sph*I fragment of plasmid pWB39. The 425-bp fragment was digested with *Rsa*I (at -188 bp), followed by isolation of the 107-bp fragment containing the *PUT1* UAS. The conditions for gel shift assays were as described previously (34), with the following modification. The protein extracts and the end-labeled 98-bp DNA fragment carrying the *PUT2* UAS were mixed for 15 min at 30°C in DNA-binding buffer (containing 1 mM phenylmethylsulfonyl fluoride) with poly(dI-dC) (Boehringer-Mannheim Biochemicals) as the nonspecific DNA and immediately loaded on a 6% polyacrylamide gel. The 15-cm gels were subjected to electrophoresis at room temperature for 2 to 3 h at 150 V in Tris borate buffer (25) and dried. Autoradiograms shown in Fig. 6A are exposures of 40-cm gels run for 6 h at 300 V.

## RESULTS

**Locations of proline-responsive sequences in the *PUT1* promoter.** Our previous studies (35) on the promoter of the *PUT2* gene identified a 40-bp region responsible for proline induction of its gene product,  $\Delta^1$ -pyrroline-5-carboxylate dehydrogenase. To identify similar sequences upstream of the *PUT1* gene encoding proline oxidase, a computer search of the *PUT1* promoter was carried out. Examination of a truncated promoter that contains 293 bp upstream of the start of the open reading frame and responds normally to proline (40) revealed a sequence that was homologous to the

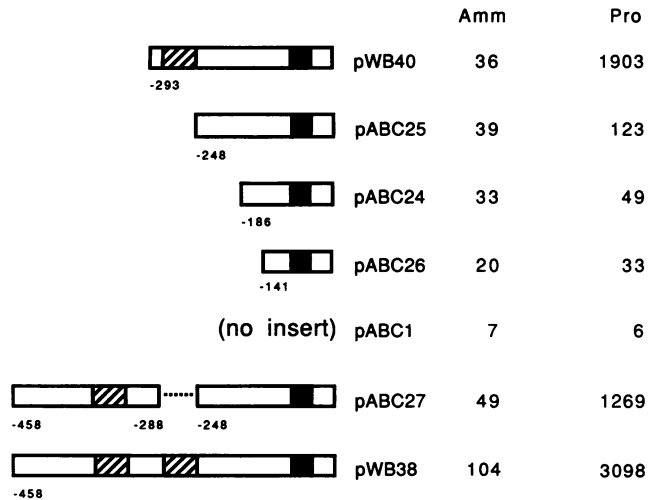


FIG. 1. Effects of 5' and internal promoter deletions on *PUT1* expression. Promoter deletions were generated as described in Materials and Methods, and the fragments were ligated into the low-copy-number expression vector pABC1. Strain MB758-5B carrying the plasmids shown was assayed for  $\beta$ -galactosidase activity under noninducing (ammonia [Amm]) or inducing (proline [Pro]) growth conditions. Values reported are averages of two or more determinations; variation was  $\leq 20\%$ . Shown are the TATA (■) and UAS (▨) elements.

*PUT2* UAS. This sequence, located between  $-284$  and  $-264$  bp, was a 14-of-18 match and was present as the reverse complement. When the  $-293$ -bp promoter was inserted into a *lacZ* reporter plasmid, proline-inducible  $\beta$ -galactosidase was produced (Fig. 1, plasmid pWB40), and the 50-fold induction agreed with previously observed steady-state *PUT1* mRNA levels (39). Removal of 45 bp ( $-293$  to  $-248$ ) resulted in almost complete loss of proline inducibility (Fig. 1, plasmid pABC25). The residual proline induction could have been due to some sequences resembling a *PUT* UAS, although a computer search failed to show any obvious relationship. Removal of additional sequences to  $-186$  bp eliminated residual proline induction (Fig. 1, plasmid pABC24); deletion to  $-141$  bp did not reduce the  $\beta$ -galactosidase levels any further (Fig. 1, plasmid pABC26). These data suggest that there is a *PUT* UAS contained in the region between  $-293$  and  $-248$  bp.

A search for sequences similar to this UAS further upstream revealed the presence of an additional putative UAS. Located between  $-312$  and  $-292$  bp, it was a 13-of-18 match to the *PUT2* UAS and was also present as the reverse complement. Although no attempt was made to delete specifically this small region, sequences between  $-288$  and  $-458$  bp restored proline inducibility to a promoter missing the first UAS identified above (Fig. 1, plasmid pABC27). These results suggest that there are at least two UASs in the longer ( $-458$  bp) *PUT1* promoter (plasmid pWB38) and that they function independently of each other. The data in Fig. 1 suggest that the two UASs contribute additively to the values for uninduced as well as induced expression observed with the intact  $-458$ -bp promoter.

**Effect of the *PUT3* genotype on *PUT1* UAS activity.** To determine whether expression from these UASs depended on the positive activator of the pathway, the *PUT3* gene product, the promoter deletion plasmids were introduced into strains carrying wild-type, constitutive (*PUT3<sup>c-68</sup>*), or noninducible (*put3-75*) alleles of the *PUT3* gene.

In the constitutive mutant, plasmids with a *PUT1* pro-

TABLE 2. Dependence of UAS activity on the *PUT3* genotype

Plasmid <sup>a</sup>	$\beta$ -Galactosidase sp act <sup>b</sup>		
	Ammonia	Ammonia + proline	Proline
Wild type <sup>c</sup>			
pWB38 ( $-458$ bp)	104	448	3,098
pWB40 ( $-293$ bp)	36	211	1,903
pABC27 ( $\Delta -288/-248$ bp)	49	137	1,269
pABC25 ( $-248$ bp)	39	39	123
pABC24 ( $-186$ bp)	33	29	49
pABC1 (no insert)	7	7	6
Constitutive			
pWB38 ( $-458$ bp)	812	ND	6,369
pWB40 ( $-293$ bp)	493	ND	3,691
pABC27 ( $\Delta -288/-248$ bp)	359	ND	1,825
pABC25 ( $-248$ bp)	82	ND	157
pABC24 ( $-186$ bp)	15	ND	15
pABC1 (no insert)	4	ND	6
Noninducible			
pWB38 ( $-458$ bp)	10	17	NG
pWB40 ( $-293$ bp)	7	13	NG
pABC27 ( $\Delta -288/-248$ bp)	18	21	NG
pABC25 ( $-248$ bp)	16	8	NG
pABC1 (no insert)	5	4	NG

<sup>a</sup> See Materials and Methods for descriptions. The strains were MB758-5B (wild type, *PUT3*), C74-4D (constitutive, *PUT3<sup>c-68</sup>*), and C75-2B (noninducible, *put3-75*).

<sup>b</sup> Expressed as nanomoles of *o*-nitrophenol formed per minute per milligram of protein. Each value represents the average of two or more determinations. Growth conditions are described in Materials and Methods. ND, Not determined; NG, no growth.

<sup>c</sup> Some of the data are also shown in Fig. 1.

motor containing either one (plasmids pABC27 and pWB40) or both (plasmid pWB38) of the UASs produced inducer-independent expression of  $\beta$ -galactosidase (Table 2). As previously published (6, 9, 35), hyperinduction was observed when the plasmid-bearing strains were grown in the presence of proline. A promoter lacking either UAS (plasmid pABC24) did not show constitutive expression. In the noninducible strain, expression from these *PUT1* promoters was noninducible, and the enzyme levels were lower than those in the wild-type strain with the same plasmid. The regulated expression observed with the  $-248$ -bp promoter (plasmid pABC25) was due to the *PUT3* gene product, since uninduced enzyme levels were twofold higher in the constitutive strain (specific activity of 82) and twofold lower in the noninducible strain (specific activity of 16) than in the wild-type strain (specific activity of 39). As previously demonstrated with the *PUT2* gene, expression of *PUT1* required the *PUT3* gene product for both basal and induced expression.

**Delineation of the *PUT* UASs.** A comparison of the three UASs found in the *PUT* promoters suggested that only 20 bp of the 40-bp sequence previously identified as containing the *PUT2* UAS was important for activation. This region of *PUT2*, from  $-168$  to  $-143$  bp, was tested as a UAS. A promoter deleted for the sequence from  $-142$  to  $-129$  (Fig. 2A) was constructed by oligonucleotide-directed mutagenesis as described in Materials and Methods. This deletion did not alter the regulated expression of  $\beta$ -galactosidase (ratio of proline level to ammonia level), although the overall level of expression was reduced (Fig. 2B, plasmid pABC28). We did not determine whether the reduction in expression was due to moving the UAS too close to the TATA box or to loss of some enhancing function contained within the sequences that were removed. The UAS is partially palindromic, as indicated by the arrows under the sequences (Fig. 3).

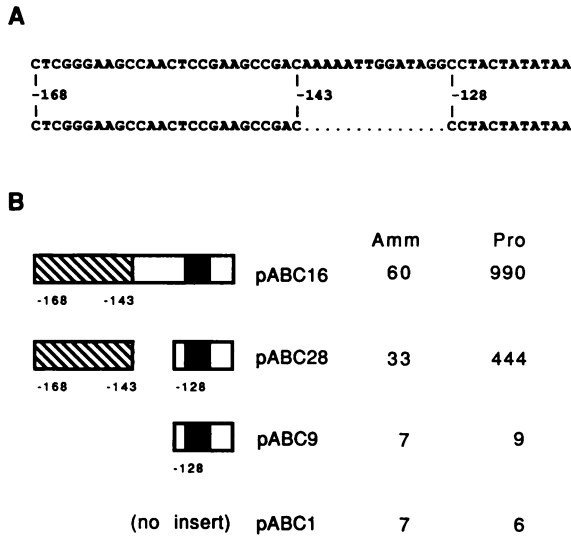


FIG. 2. (A) Comparison of nucleotide sequences of the wild-type (top line) and deleted (bottom line) UAS regions of *PUT2*; (B) effect of the 14-bp deletion on *PUT2* expression. Measurements and symbols are as described in the legend to Fig. 1.

The *PUT* UAS is a protein-binding site. To determine whether the *PUT* UASs were the binding sites for proteins, a gel mobility shift assay (1, 34) was used. As described in Materials and Methods, a crude extract of a wild-type yeast strain carrying the cloned *PUT3* gene on the high-copy-number plasmid pDB37 was used as the source of binding activity. The probe was a 98-bp fragment of the *PUT2* promoter (-228 to -130 bp) that contained the unique *PUT2* UAS. A major DNA-protein complex and several minor complexes were observed (Fig. 4A, lane 2). A competition assay was carried out to determine the specificity of the complex. Addition of a 100-fold molar excess of unlabeled 98-bp UAS-containing fragment completely inhibited the formation of the major complex without affecting the minor complexes (Fig. 4A, lane 3). At a 200-fold molar excess, a 64-bp fragment (-228 to -164 bp) lacking the *PUT2* UAS failed to inhibit formation of the major complex (Fig. 4A, lane 4). These results demonstrate that a protein binds specifically to the *PUT2* UAS.

To determine whether the protein factor also recognized the *PUT1* UAS sequence, a competition assay using the homologous *PUT1* and *PUT2* UASs was carried out. A 107-bp fragment containing the proximal *PUT1* UAS (-293 to -186 bp; see Materials and Methods) was used to compete for binding of the *PUT2* UAS-protein complex. A 5- to 100-fold molar excess of unlabeled fragment containing

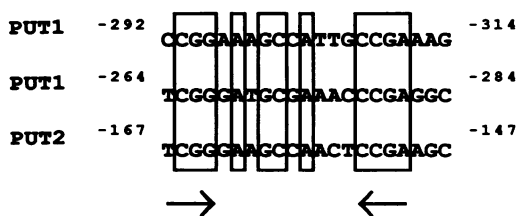


FIG. 3. Sequence homologies of the *PUT* UASs. Coordinates are given relative to the translation initiation of each gene. The *PUT1* gene has two such sequences, which are present as the reverse complement of the previously published *PUT2* UAS (35). Exact matches are boxed. Arrows indicate the partially palindromic nature of the UAS.

either the *PUT1* UAS (Fig. 4B, lanes 9 to 13) or the *PUT2* UAS (Fig. 4B, lanes 4 to 8) successfully competed for formation of the specific complex. An excess of the fragment that lacked a UAS failed to compete for binding (Fig. 4B, lane 3). We conclude that the proline-specific UASs are the binding sites for a protein factor.

DNA binding is proportional to the amount of *PUT3* product. Previous work identifying the *PUT3* gene product as the transcriptional activator of the *PUT1* and *PUT2* genes (6) led us to suspect that *PUT3* might be directly or indirectly involved in the observed DNA binding. We therefore compared in vitro complex formation, using extracts from a wild-type strain carrying a single genomic copy of the *PUT3* gene, with that from a strain carrying *PUT3* on a multicopy plasmid. A clear effect of *PUT3* copy number can be seen in Fig. 5 (compare lane 2 with lanes 3 to 5). The effect was due specifically to the presence of the *PUT3* gene, since extracts from a strain carrying the parent plasmid YE24 (3) did not bind this DNA fragment (Fig. 5, compare lanes 9 and 10).

We examined the ability of strains carrying noninducible and constitutive alleles of the *PUT3* gene to bind the *PUT2* UAS. Constitutive strains express the *PUT1* and *PUT2* gene products in the absence of the inducer proline (Table 2; 6, 9, 35). Noninducible mutants are proline nonutilizing and fail to make both gene products under any growth conditions (6, 35). Gel mobility shift assays were performed with extracts made from strains carrying these mutations. Wild-type levels of the specific complex were observed in extracts of the constitutive *PUT3<sup>c-68</sup>* strain (Fig. 5, lane 6), whereas extracts of a strain carrying the noninducible *put3-75* allele failed to show the DNA-binding activity (Fig. 5, lane 7). At present, the nature of these two alleles has not been determined.

Identity of the DNA-binding protein. To determine whether the *PUT3* product itself is part of the protein-DNA complex, we sought to alter the mobility of the complex by altering the molecular weight of the *PUT3* product. This was accomplished by using a *PUT3-lacZ* gene fusion (see Materials and Methods) that made a bifunctional hybrid protein. In the mobility shift assays with extracts from the *PUT3-lacZ* strain, at least two new complexes were observed with slower mobility than that of the wild-type DNA-protein complex (Fig. 6A, lane 4). These complexes were absent in extracts made from strains carrying multiple copies of either *PUT3* (Fig. 6A, lane 2) or a truncated *lacZ* gene (Fig. 6A, lane 3). The formation of slower-moving complexes was inhibited by a 100-fold molar excess of unlabeled fragment containing either the *PUT1* UAS (Fig. 6A, lane 5) or the *PUT2* UAS (Fig. 6A, lane 6) but not by a 200-fold molar excess of the fragment lacking a *PUT* UAS (Fig. 6A, lane 7). Strains carrying either a partial or a complete deletion of the *PUT3* gene failed to show the specific DNA-protein complex present in the wild-type strain (Fig. 6B, compare lane 2 with lanes 3 and 4). We conclude that the *PUT3* protein is directly involved either alone or as part of a complex in binding to the *PUT* UAS.

In vitro *PUT3* binding is not proline inducible. Since the *PUT1* and *PUT2* mRNAs and proteins are proline inducible, it was of interest to determine whether formation of the DNA-protein complex was regulated by proline. To detect any differences in complex formation under inducing and noninducing conditions, gel mobility shift assays were performed by using extracts made from the wild-type strain grown with ammonia, ammonia and proline, or proline alone as the sole source(s) of nitrogen. Quantitatively similar amounts of DNA-protein complex were observed in extracts from all growth conditions when either single (Fig. 7, lanes 5

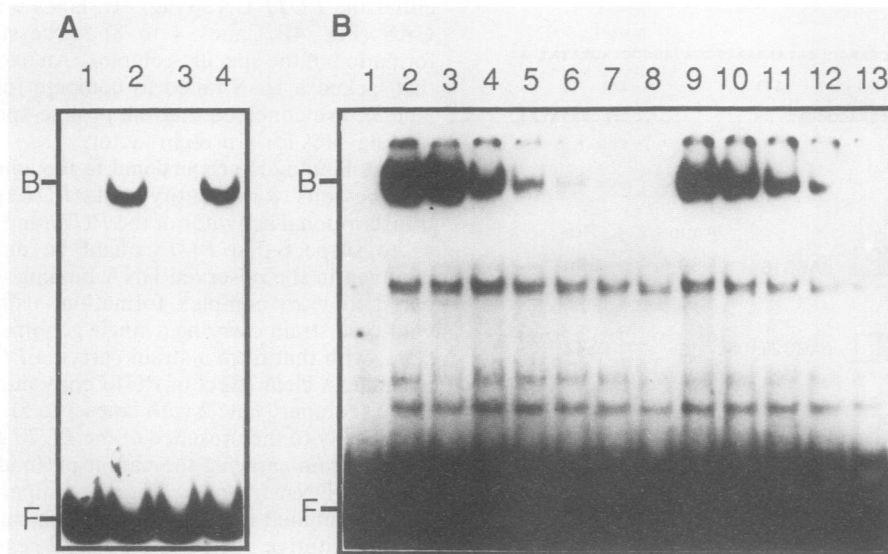


FIG. 4. (A) Demonstration that the *PUT* UASs are the binding sites of a protein factor. Crude cell extracts were made from a wild-type yeast strain carrying the cloned *PUT3* gene on a multicopy plasmid (pDB37). The cells were grown on a medium containing proline as the nitrogen source (see Materials and Methods). The binding reactions contained 7.5  $\mu$ g of protein extracts and 1 ng of a  $^{32}$ P-end-filled 98-bp fragment containing the *PUT2* UAS. Positions of free probe (F) and bound (B) specific complex are indicated to the left of each panel. Binding reactions were performed with no extract (lane 1), no competitor (lane 2), a 100-fold molar excess of the unlabeled 98-bp fragment containing the *PUT2* UAS (lane 3), and a 200-fold molar excess of 64-bp fragment lacking the *PUT2* UAS (lane 4). (B) Demonstration that both *PUT1* and *PUT2* UASs are protein-binding sites. Cells were grown on a medium containing ammonia and proline as nitrogen sources. Competitive DNA-binding reactions were performed with various amounts of unlabeled fragments carrying either the *PUT1* or *PUT2* UAS. Lanes: 1, no extract; 2, no competitor; 3, 200-fold molar excess of the 64-bp fragment lacking a UAS; 4 to 8, 5, 10, 25, 50, and 100-fold molar excess of the 98-bp fragment carrying the *PUT2* UAS; 9 to 13, 5, 10, 25, 50, and 100-fold molar excess of the 107-bp fragment carrying the *PUT1* UAS.

to 7) or multiple (Fig. 7, lanes 2 to 4) copies of the *PUT3* gene were present. Thus, the association of the *PUT3* protein with DNA is not proline inducible.

## DISCUSSION

We have demonstrated that similar 21-bp partially palindromic sequences, required for proline induction, are present in the promoters of the *PUT1* and *PUT2* genes. The *PUT* UAS is present once in the 819-bp *PUT2* promoter and twice in the 458 bp *PUT1* promoter. The two UASs present in the *PUT1* promoter appear to be functionally independent of one another and behave additively in producing maximal expression. Like the *PUT2* gene, both the UAS and a functional *PUT3* product are required for regulated *PUT1* expression.

The *PUT* UAS was shown to be the recognition site for binding of either the *PUT3* gene product itself or a complex with which the *PUT3* protein is associated. The DNA-binding activity was absent in extracts of strains carrying either a partial or a complete deletion of *PUT3* or carrying a *PUT3* mutation that caused a noninducible phenotype. New slower-moving complexes specific for the *PUT* UAS were observed in extracts of a strain carrying a *PUT3-lacZ* gene fusion. Because these experiments were carried out with crude extracts of yeast cells, we do not know whether the *PUT3* protein binds DNA directly or participates in formation or stabilization of a complex that includes other proteins.

A growing number of transcriptional activators in yeast cells have been shown to be DNA-binding proteins that recognize their respective UASs (15–17, 19, 23, 28, 30). The well-characterized transcriptional activators seem to fall into one of two major groups. The first class includes activators

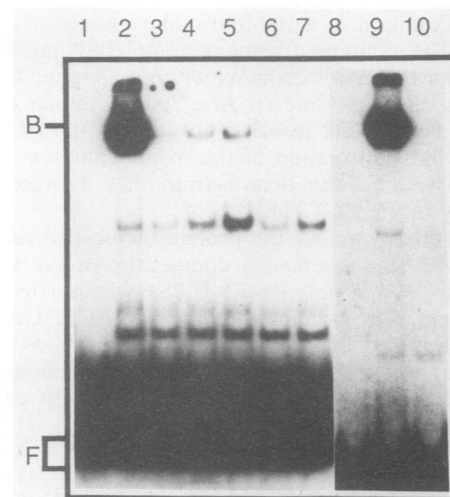


FIG. 5. Effect of *PUT3* gene dosage on DNA binding. The strains were grown on a medium containing ammonia and proline as the nitrogen sources. Lanes: 1, no extract; 2, 7.5  $\mu$ g of extract of strain MB758-5B with plasmid pDB37 (multiple copies of the *PUT3* gene); 3, 4, and 5, 10, 20, and 30  $\mu$ g of extract of strain MB758-5B (single genomic copy of *PUT3*); 6, 21  $\mu$ g of extract of strain C74-4D (*PUT3<sup>c</sup>-68*; genomic constitutive mutation); 7, 24  $\mu$ g of extract of strain C75-2B (*put3-75*; genomic noninducible mutation); 8, no extract; 9, 7.5  $\mu$ g of extract of strain MB758-5B carrying plasmid pDB37 (multicopy plasmid with the *PUT3* gene); 10, 7.5  $\mu$ g of extract of strain MB758-5B carrying plasmid YEp24 (multicopy plasmid with no insert). The probe used is explained in the legend to Fig. 4.

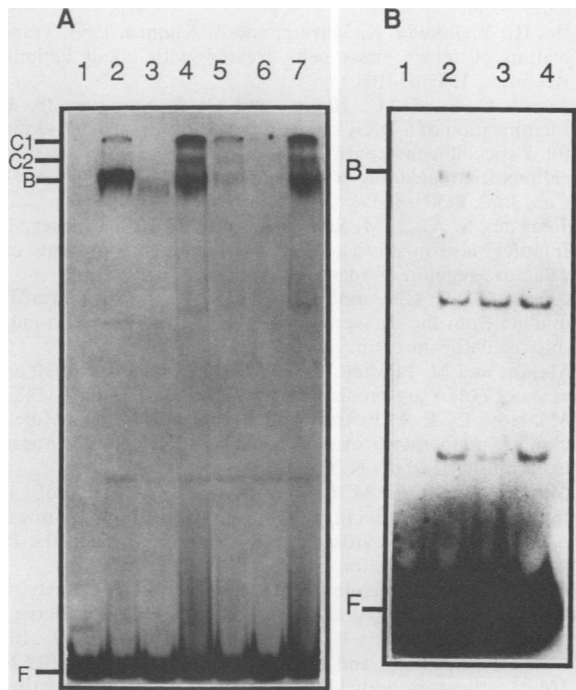


FIG. 6. (A) Demonstration that the *PUT3* product is required for DNA binding. The plasmid-bearing strains were grown on a medium containing ammonia and proline as the nitrogen sources. Lanes: 1, no extract; 2, 7.5  $\mu$ g of extract of strain MB758-5B carrying plasmid pDB37 (*PUT3*); 3, 30  $\mu$ g of extract of strain MB758-5B carrying plasmid YEp353 (*lacZ*); 4 to 7, 30  $\mu$ g of extract of strain MB758-5B carrying plasmid pDB72 (*PUT3-lacZ* gene fusion); 4, no competitor; 5, 100-fold molar excess of the 107-bp fragment carrying the *PUT1* UAS; 6, 100-fold molar excess of the unlabeled 98-bp fragment carrying the *PUT2* UAS; 7, 200-fold molar excess of the 64-bp fragment lacking the *PUT* UAS. Positions of the free probe (F), bound *PUT3-PUT2* UAS complex (B), and bound *PUT3-lacZ-PUT2* UAS complexes (C1 and C2) are indicated on the left. Complex C1 is located just below the material trapped in the wells in lanes 4, 5 (trace), and 7. (B) Absence of binding activity in extracts of strains carrying deletions of the genomic *PUT3* gene. Cells were grown on a medium containing ammonia and proline as nitrogen sources. Lanes: 1, no extract; 2, 20  $\mu$ g of extract of strain MB758-5B (single genomic copy of *PUT3* gene); 3, 20  $\mu$ g of extract of strain JM5007 (3' deletion of genomic *PUT3* gene); 4, 20  $\mu$ g of extract of strain JM5008 (complete deletion of genomic *PUT3* gene).

that bind to their respective UASs only in the presence of an inducer or environmental signal (16, 30). This class includes proteins whose synthesis is regulated (e.g., the *GCN4* protein [18]) as well as proteins that are made constitutively but bind only under specific conditions (e.g., the *GAL4* [17], *HAPI* [29], and *ACE1* [16, 37] proteins). Thus, regulation is at the level of differential binding of the activator to the regulatory sequences. The second class includes activators that bind to their UASs under noninducing as well as inducing growth conditions, such as the *GAL4* (17) and *LEU3* (G. B. Kohlhaw, personal communication) proteins. In this class, transcriptional activation occurs through another mechanism.

Since the *GAL* genes are galactose inducible as well as glucose repressible, *GAL4* activation reflects both types of regulation. Binding of the *GAL4* protein to its UAS is not observed under carbon catabolite-repressing conditions. Under derepressing conditions, the binding occurs with or without galactose. The presence or absence of the repressor *GAL80* protein determines the state of activation of the *GAL*

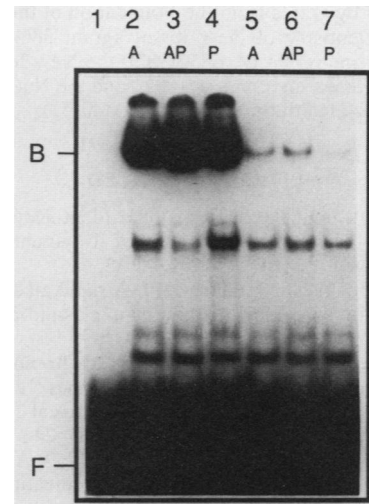


FIG. 7. Demonstration that *PUT3* binding to DNA is independent of the presence of proline. Strains were grown on media containing either ammonia (A; noninducing), ammonia and proline (AP; partially inducing), or proline (P; fully inducing) as the nitrogen source(s) (see Materials and Methods). Lanes: 1, no extract; 2 to 4, 7.5  $\mu$ g of extract of the strain MB758-5B carrying plasmid pDB37 (multiple copies of *PUT3*); 5 to 7, 10 to 12  $\mu$ g of extract of strain MB758-5B (single genomic copy of *PUT3*). The apparent lower intensity of the upper band in lane 7 was due to an inaccuracy in loading the sample and was not observed in repetitions of this experiment.

genes by its interaction with the C-terminal domain of the *GAL4* protein (22, 24). Posttranslational modification (i.e., changes in phosphorylation) has been suggested as the mechanism by which the *ADR1* product activates transcription of the glucose-repressible alcohol dehydrogenase (11).

If the *in vitro* results are true *in vivo*, the *PUT3* product seems to fall into the second category, since binding was shown to be independent of the presence of proline. Given this finding, we can speculate on how proline affects the *PUT3* protein to cause differential transcriptional activation of the *PUT1* and *PUT2* genes. One possibility is that the presence of proline alters the cellular location of the *PUT3* protein from cytoplasmic to nuclear, as in the case of the glucocorticoid receptor (31). A second possibility is that the presence of proline alters the *PUT3* protein conformation such that it contacts different nucleotides or proteins of the transcription apparatus. Alternatively, some form of proline-dependent posttranslational protein modification may change the activation state of the *PUT3* product. A fourth possibility is that the *PUT3* product interacts with an as yet undefined repressor protein in the absence of proline, similar to that observed for the *GAL4-GAL80* proteins (22, 24). To date, we have no strong genetic evidence to suggest the involvement of a proline-specific repressor protein, leading us to discount the fourth model. Once the sequence of the *PUT3* gene is completed, we hope to obtain information on possible protein domains by using homologies to other sequenced regulatory proteins.

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