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# Gln3p Is Capable of Binding to $UAS_{NTR}$ Elements and Activating Transcription in *Saccharomyces cerevisiae*

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**When readily used nitrogen sources are available, the expression of genes encoding proteins needed to transport and metabolize poorly used nitrogen sources is repressed to low levels; this physiological response has been designated nitrogen catabolite repression (NCR). The *cis*-acting upstream activation sequence (UAS) element $UAS_{NTR}$ mediates Gln3p-dependent, NCR-sensitive transcription and consists of two separated dodecanucleotides, each containing the core sequence GATAA. Gln3p, produced in *Escherichia coli* and hence free of all other yeast proteins, specifically binds to wild-type $UAS_{NTR}$ sequences and DNA fragments derived from a variety of NCR-sensitive promoters (*GDH2*, *CAR1*, *DAL3*, *PUT1*, *UGA4*, and *GLN1*). A LexA-Gln3 fusion protein supported transcriptional activation when bound to one or more LexAp binding sites upstream of a minimal *CYC1*-derived promoter devoid of UAS elements. LexAp-Gln3p activation of transcription was largely independent of the nitrogen source used for growth. These data argue that Gln3p is capable of direct $UAS_{NTR}$ binding and participates in transcriptional activation of NCR-sensitive genes.**

Selective nitrogen source utilization in *Saccharomyces cerevisiae* is accomplished through a physiological process designated nitrogen catabolite repression (NCR) (13, 14, 57). NCR occurs at the level of transcriptional activation of genes encoding the permease and catabolic enzyme systems needed to degrade poor nitrogen sources (e.g., allantoin, proline, and γ-aminobutyrate). When readily used nitrogen sources (e.g., Asn, Gln, or ammonia in some strains) are available, NCR-sensitive genes are expressed only at low levels (14). Upon depletion of these repressive nitrogen sources, NCR is relieved and transcription of NCR-sensitive genes dramatically increases (17). Five observations have influenced current views of the mechanism by which NCR is accomplished. (i) The upstream activation sequence (UAS) $UAS_{NTR}$ element, containing the core sequence GATAA, occurs upstream of all NCR-sensitive genes (16, 46, 47). (ii) *gln3* null mutants grow slowly on poor nitrogen sources and exhibit reduced expression of catabolic pathway genes associated with these compounds (39, 40). (iii) *ure2* null mutants are NCR insensitive; i.e., they express genes associated with catabolism of poor nitrogen sources even when readily used nitrogen sources are available (25, 26). (iv) Ure2p and Gln3p were proposed to be in the same regulatory pathway because *gln3* mutations are epistatic to *ure2* mutations (19). (v) Ure2p exerts its negative control of nitrogen catabolism through the $UAS_{NTR}$ element (10).

One working model relating these observations proposes that the presence of preferred nitrogen sources positively regulates Ure2p at a level other than transcription (4). Ure2p in turn negatively regulates Gln3p in some way other than by affecting transcription of its cognate gene. Since the Gln3p sequence contains a zinc finger motif homologous to those of the GATA family of DNA-binding proteins (37), it has been concluded that Gln3p binds to the $UAS_{NTR}$ elements and activates transcription of NCR-sensitive genes (5, 38, 58).

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Several observations are consistent with this model. First, a footprint is situated over the GATAA sequence of the *DAL5* $UAS_{NTR}$ after incubation with a crude, wild-type cell extract, demonstrating that one or more yeast proteins can bind to it (44). Second, Gln3p has been shown to be required for expression of many NCR-sensitive genes and for transcriptional activation supported by $UAS_{NTR}$ sequences cloned into a heterologous expression vector (15, 18, 24, 36, 37, 48, 55). Third, a polyclonal antibody preparation against partially purified Gln3p produced in *Escherichia coli* immunoprecipitates a DNA-protein complex containing a double-stranded oligonucleotide consisting of seven consecutive GATAA sequences (37). Most recently, a partially purified preparation of Gln3p has been reported to footprint a *GDH2* DNA fragment containing the sequence GATTAG (5).

Although these observations support the proposed role for Gln3p in NCR, five observations argue for a more unambiguous evaluation of Gln3p binding. (i) The yeast genome encodes at least four different proteins containing zinc finger motifs homologous to the one in Gln3p, and at least one of them, Dal80p, has been shown to bind directly to the GATAA sequences of some $UAS_{NTR}$ elements (20, 21, 23). (ii) A footprint formed over the GATAA sequence of the *DAL5* $UAS_{NTR}$ element is observed not only when the source of protein for the assay is wild-type cells but also when it is a *gln3 dal80* double-null mutant (44). (iii) NCR-sensitive transcription is observed for a wide variety of genes (*DAL5*, *CAN1*, *GAP1*, *UGA1*, *UGA4*, *PUT1*, *PUT2*, etc.) in *gln3*Δ single (11, 24, 51, 58), *gln3*Δ *ure2*Δ double (11, 58), and *gln3*Δ *ure2*Δ *dal80*::*hisG* triple (11) mutants. (iv) A newly identified GATA family protein (Gat1p) has been shown to be required along with Gln3p for maximal expression of NCR-sensitive genes (11, 12, 52). Finally, no data other than the Gln3p requirement for NCR-sensitive gene expression have been reported in support of Gln3p being capable of activating transcription. Therefore, we performed experiments to test whether Gln3p, in the absence of other yeast proteins, was able to bind $UAS_{NTR}$ elements situated upstream of NCR-sensitive genes and whether it was capable of transcriptional activation. A preliminary account of this work has appeared elsewhere (54).

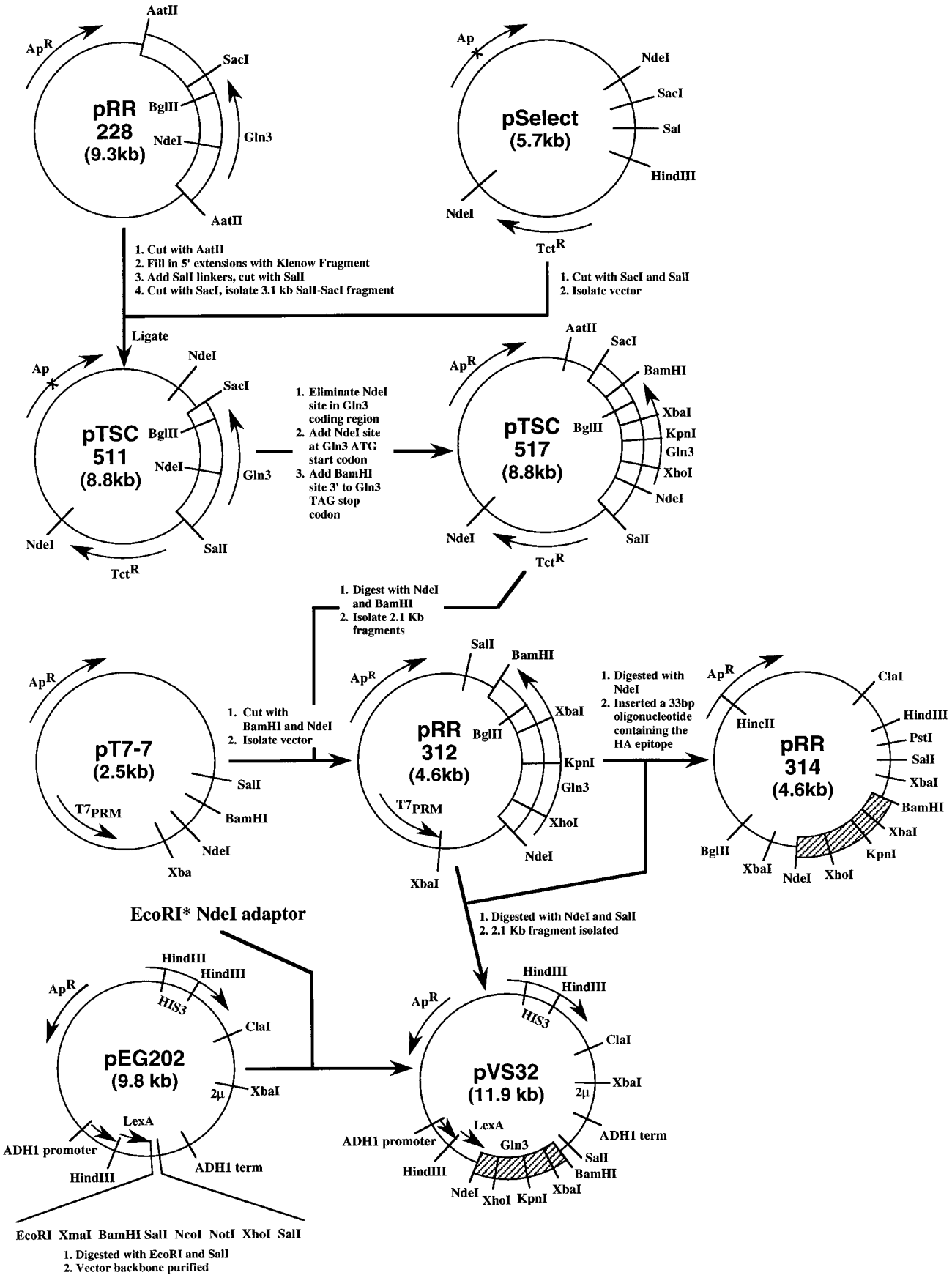


FIG. 1. Strategies through which plasmids used in this work were constructed.

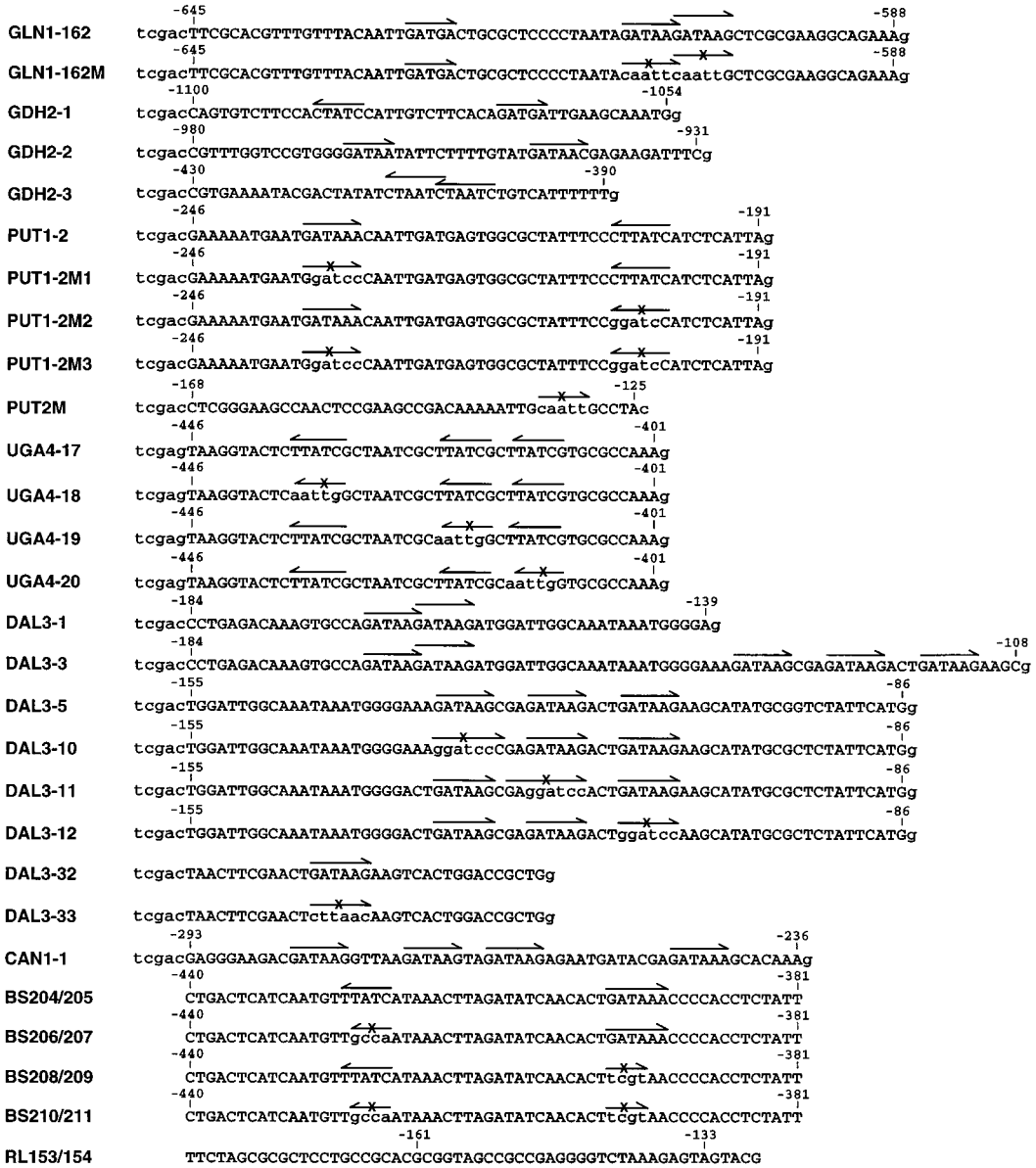


FIG. 2. Nucleotide sequences of DNA fragments used in this work. Only one strand of each double-stranded fragment is shown. Lowercase letters at the termini of the DNA sequences designate nucleotides added to the gene sequences to permit cloning. Lowercase letters in the interior of sequences represent mutated positions.

## MATERIALS AND METHODS

**Plasmid construction and DNA fragments.** Plasmids used in this work are shown in Fig. 1. All DNA manipulations were performed by standard methods (49).

**EMSA.** Electrophoretic mobility shift assays (EMSAs) were performed as described earlier (20, 21, 23); total crude protein extract when present in reaction mixtures was at 1 μg per reaction. DNA fragments used as probes or competitors are shown in Fig. 2. Highly sheared calf thymus DNA was used in all reaction mixtures as a nonspecific competitor DNA (final concentration, 200 μg/ml).

**Western blotting (immunoblotting).** Procedures for electrophoresis, transfer, and immunoassay of *E. coli*-produced yeast proteins were essentially as described earlier (49). The primary antibody (directed against influenza virus hemagglutinin [HA] epitope 12CA5 [27, 33]) was used at a 1:100 dilution of the supernatant fluid derived following growth of cell line 12CA5. In all cases, 2 μg of protein extract was analyzed.

**Construction of the *gln3* mutants.** N-terminal deletion of Gln3p (plasmid pVS32-4) was generated by digesting plasmid pVS32 with *Xho*I and *Nde*I. The resulting 11.4-kb DNA fragment was isolated, and the *lexA* and truncated *gln3* DNA fragments were religated by using an *Nde*I-*Xho*I adapter produced by annealing the oligonucleotides TCGAGAGCGTAGTCTGGGACGTCGTAT GGGTACA and TATGTACCCATACGACGTCCCAGACTACGCTC. The *gln3* deletion that removed amino acid residues 471 to 669 (plasmid pVS32-2) was constructed by digesting plasmid pVS32 with *Eco*RI. The resulting 11.2-kb DNA fragment was isolated and religated. The C-terminal deletion (plasmid pVS32-3) was generated by digesting plasmid pVS32-2 with *Eco*RI and *Sal*I. The resulting 11-kb fragment was isolated and religated by using an *Eco*RI-terminator-*Sal*I adapter containing four in-frame termination codons. This adapter was produced by annealing the oligonucleotides AATTGATAGTAGTGACGC TATG and TCGACATAGCGTCACTACTATC.

To construct *gln3* mutants for complementation assays, plasmid pVS316 was constructed by inserting a 3.1-kb *Sal*I-*Sac*I fragment from plasmid pTSC517 (20) (Fig. 1) into plasmid pRS316 (50) previously digested with *Sac*I and *Sal*I. Plasmid pVS316-2, which contained sequences for the N-terminal portion of Gln3p, was generated by subcloning the 3.1-kb *Sac*I-*Sal*I fragment from plasmid pTSC517 into plasmid pBluescript II KS+ to yield plasmid pVS3-7. Plasmid pRR312 (Fig. 1) was digested with *Nde*I and *Xho*I, and the resulting 4.1-kb fragment was isolated and religated by using the above-mentioned *Nde*I-*Xho*I adapter to yield plasmid pVS3-8. The 1.6-kb fragment derived from plasmid pVS3-8 following digestion with *Nde*I and *Bam*HI was inserted into the 4-kb *Nde*I-*Bam*HI derivative of plasmid pVS3-7 to yield plasmid pVS3-9. Plasmid pVS3-9 was digested

with *Sac*I and *Sal*I, and the resulting 2.6-kb fragment was inserted into plasmid pRS316 to yield plasmid pVS316-2. The *gln3* Cys-306→Ser mutant was generated by using a pSelect site-directed mutagenesis protocol. A 3.1-kb *Sac*I-*Sal*I fragment from plasmid pTSC517 was cloned into the pSelect vector. The mutagenic primer GTCTTGAAAGTTTTACAATTGAAACTTTGTATCAGAGG TTT was used according to the manufacturer's recommendations. After the desired mutation was confirmed by sequencing, the 3.1-kb *Sac*I-*Sal*I mutant fragment was cloned into plasmid pRS316 to yield plasmid pVS316-3. Plasmids pVS316, pVS316-2, and pVS316-3 were transformed into *gln3* deletion strain RR91, and their abilities to complement the *gln3* growth phenotype were assayed by comparing the doubling times of the transformants growing in liquid medium (Difco yeast nitrogen base minimal glucose medium) supplemented as necessary for auxotrophic requirements and containing either 0.1% glutamine or 0.1% asparagine as the sole nitrogen source.

## RESULTS

**Expression of *GLN3* in *E. coli*.** Problems with other proteins contaminating or influencing Gln3p binding assays prompted us to seek a method of Gln3p preparation that would yield only a single yeast protein. Therefore, we cloned an HA epitope-tagged version of *GLN3* (pT7-GLN3E) into *E. coli* expression vector pT7-7 (Materials and Methods). Extracts were prepared from *E. coli* BL21(DE3) transformed with either plasmid pT7-GLN3E or control plasmid pT7-7, and the proteins that they contained were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Extract from a pT7-7 transformant did not contain protein that reacted with a monoclonal antibody prepared against the HA epitope tag fused to Gln3p (Fig. 3A, lanes A and E). Extract derived from a pT7-GLN3E transformant contained two major and many minor protein species that reacted with the HA epitope tag-specific antibody (lanes B and D). Although not visible in this blot exposure, a small amount of full-length (calculated molecular weight, 79,342) Gln3p was detected. *E. coli*-produced, epitope-tagged Dal80p was used as a control and yielded a single major band (lane C). The presence of multiple species of epitope-tagged Gln3p indicated that the protein or its message was present in fragmented form in the *E. coli* cells; this has also been observed for other large yeast proteins expressed in *E. coli* (9).

**Specific Gln3p-dependent binding to *DAL3* DNA fragments containing GATAA sequences.** Although dismayed by the extent of Gln3p degradation, we persisted with this approach because of past successful experiences reported by other investigators working with GATA-binding proteins. Fu and Marzluf observed specific binding of *Neurospora crassa* Nit2p to GATAA-containing DNA fragments in assays using small (ca. 229-amino-acid) zinc finger-containing peptide fragments (29). Omichinski et al. (41, 42) used a zinc finger DNA-binding domain of only 66 residues to determine the three-dimensional structure of the chicken Gata1p-DNA complex. These data demonstrated that little more than the zinc finger of GATA family proteins is needed to specifically bind GATAA-containing DNA sequences. These were critically important precedents for us because so little full-length Gln3p was present in our *E. coli*-produced protein preparations.

We first ascertained whether our fragmented Gln3p preparations could specifically bind to a DNA fragment containing known $UAS_{NTR}$ elements. A small DNA fragment from the *DAL3* gene (DAL3-5) was initially used because it contains three well-studied $UAS_{NTR}$ elements (20, 21–23). When the DAL3-5 fragment was used as radioactive probe in an EMSA, a prominent Gln3p-dependent band (consisting of several closely spaced bands) was observed (Fig. 3B, lanes A to C). To determine the specificity of this DNA-protein complex, we used several DNA fragments as competitors of DAL3-5 for Gln3p-dependent complex formation. DNA fragment CAN1-1, previously shown to support Gln3p-dependent, NCR-sensitive reporter gene expression (32), effectively competed with fragment DAL3-5 for Gln3p binding (Fig. 3D, lanes A to G). A similar result was obtained with competitor fragment DAL3-3, the one from which DAL3-5 was derived (21) (Fig. 3E, lanes A to G). *CAR1* DNA fragment RL153/154 (35), containing multiple protein-binding sites but no $UAS_{NTR}$ elements (Fig. 2), was unable to serve as a competitor (Fig. 3D, lanes G to M). A similar result was obtained with a *PUT2* fragment containing a mutant GATAA sequence (PUT2M) (Fig. 2 and Fig. 3E). We have also demonstrated that HA epitope-tagged Gln3p will supershift the DAL3-3 fragment relative to native untagged Gln3p (22). Together, these data argue that complex formation between *E. coli*-produced Gln3p fragments and DNA fragments containing $UAS_{NTR}$ elements was specific.

We previously determined individual contributions of the three GATAA-containing sequences of DNA fragment DAL3-5 toward DAL80p binding, reporter gene transcription, and DAL80-mediated transcriptional regulation (21). Mutation of the 5′- and 3′-most DAL3-5 GATAA sequences reduced DNA-Dal80p complex formation below detectable levels, indicating that Dal80p binding absolutely required both sequences (21). To compare requirements for Dal80p and Gln3p binding, we performed analogous assays using Gln3p. Mutation of the central GATAA sequence of DNA fragment DAL3-5 (fragment DAL3-11) had no demonstrable effect on DNA-Gln3p complex formation (Fig. 3B, lanes G to I). In contrast, mutation of the 3′-most GATAA sequence (fragment DAL3-12) greatly reduced Gln3p binding but did not eliminate it (Fig. 3B, lanes J to L). Mutation of the 5′-most GATAA sequence (fragment DAL3-10) only slightly decreased Gln3p binding to the mutant DNA fragment (Fig. 3B, lanes D to F). To ascertain whether Gln3p bound to a single GATAA sequence, we used a DNA fragment (DAL3-32) in which the 3′-most GATAA sequence from DNA fragment DAL3-5 was embedded in plasmid pBR322 DNA (Fig. 2). As shown in Fig. 3C, lanes A to C, a weak Gln3p-dependent signal was observed. This complex disappeared when the GATAA sequence was mutated from GATAAG to cttaac (fragment DAL3-33).

**Gln3p-dependent binding to wild-type and mutant DNA fragments derived from *GLN1*.** We evaluated Gln3p binding to pertinent promoter fragments from the most frequently studied nitrogen catabolic genes. Mutation of the *GLN3* locus decreases glutamine synthetase enzyme activity and *GLN1* mRNA (4, 10, 15, 36, 38–40, 45). Therefore, we assayed Gln3p binding to the wild-type and mutated *GLN1* DNA fragments used for our earlier heterologous expression experiments (45). We observed a strong, Gln3Ep-dependent signal when DNA fragment GLN1-162 was used as a probe (Fig. 4A, lanes A to C), but no binding occurred when the GATAA sequence of this fragment was mutated (fragment GLN1-162M) (Fig. 2 and 4A, lanes D to F). From this result, we concluded that Gln3p binding required the GLN1-162 GATAA sequence previously shown to be required for Gln3p-dependent reporter gene expression (45).

**Gln3p-dependent binding to wild-type and mutant *PUT1* DNA fragments.** Expression of the *PUT* genes encoding proline degradative enzymes is NCR sensitive (10, 24, 58). Of the two *PUT* loci, *PUT1* expression is most NCR sensitive and Gln3p dependent (11, 24, 45). A *PUT1* DNA fragment (PUT1-2; positions −246 to −191) containing a Put3p-binding site (3, 6, 56, 58) and $UAS_{NTR}$-homologous sequences (45) was shown to support high-level, Gln3p-dependent, heterologous reporter gene expression only when both elements were present (45). These observations argued that Gln3p should be capable of binding to one or more of the three $UAS_{NTR}$-homologous sequences, GATAAA, GATAG, and CTTATC (GATAAG), 3′ of the Put3p-binding site. We tested this in-

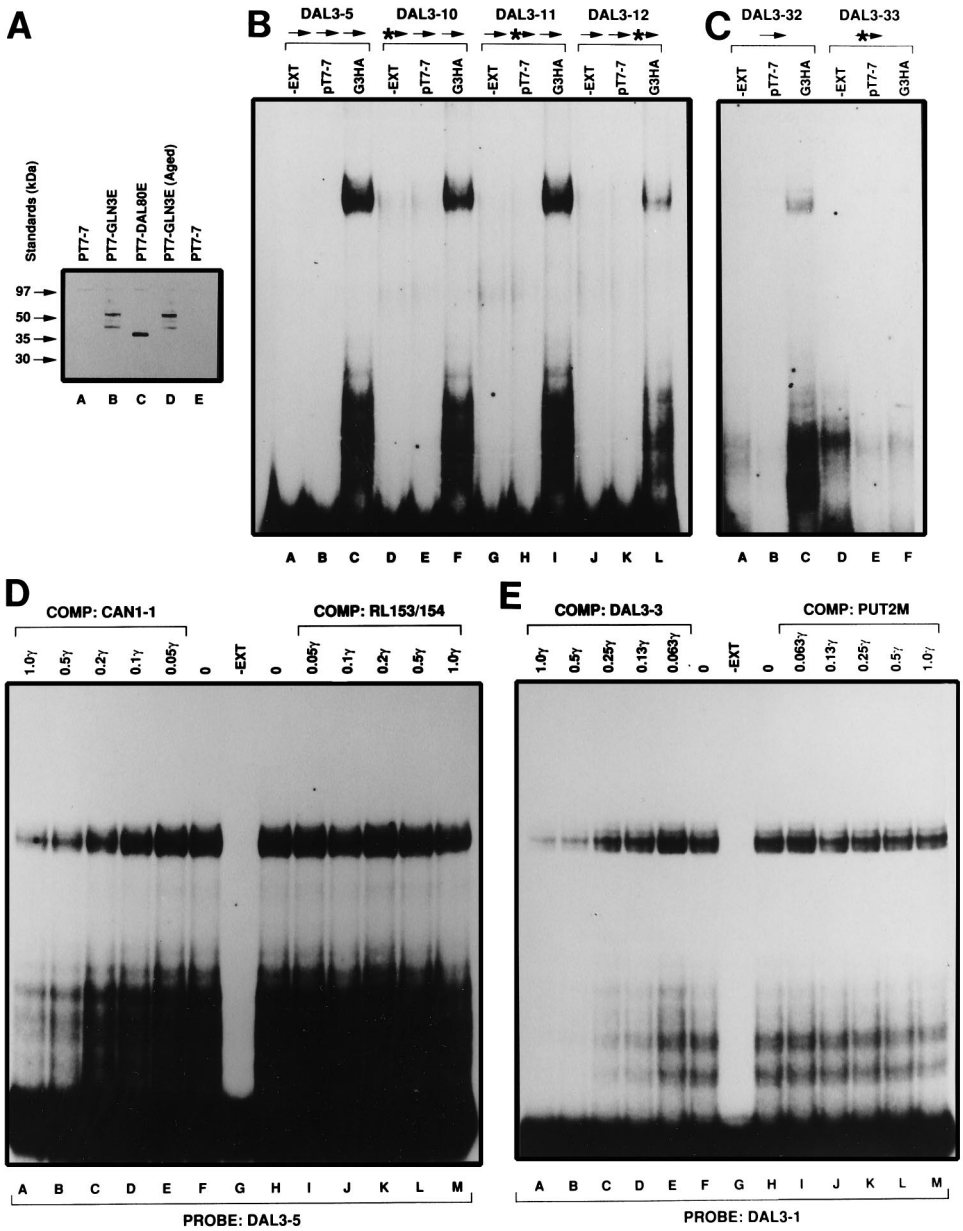


FIG. 3. (A) Western blot analysis of *E. coli*-produced proteins. Protein extract preparation and immunoblotting were performed as described in Materials and Methods. Each lane contained 2 μg of extract. Lanes A and E, B, and C contained extracts from strain BL21(DE3) transformed with plasmids pT7-7, pRR314(Gln3E), and pTSC419(*DAL80*), respectively; lane D contained extract identical to that in lane B except that it was stored at −80°C for approximately 1 month. (B and C) EMSAs performed with GATAA-containing probes from the *DAL3* gene. Lanes A, D, G, and J contained no added protein extract (EXT); lanes B, E, H, and K contained reaction mixtures with 1 μg of extract produced by *E. coli* DL21(DE3) transformed with plasmid pT7-7; lanes C, F, I, and L (G3HA) contained reaction mixtures with 1 μg of extract produced by *E. coli* BL21(DE3) transformed with plasmid pRR314 (Gln3E). (D and E) Competition EMSAs performed with GATAA-containing *DAL3* DNA fragments (DAL3-5 [D] or DAL3-1 [E]) as $^{32}$P-labeled hybridization probes. (D) Lanes F to A and H to M contained increasing amounts ($\gamma$ = μg) of unlabeled DNA fragment CAN1-1 and RL153/154, respectively; lane G contained no added protein. (E) Lanes F to A and H to M contained increasing amounts of unlabeled DNA fragment DAL3-3 or PUT2M, respectively; lane G contained no added protein. COMP, competitor.

ference by incubating *E. coli*-produced Gln3p with wild-type DNA fragment PUT1-2 and found a strong Gln3p-dependent DNA-protein complex (Fig. 4B, lanes A to C). While mutation of the 5′ GATAAA sequence (fragment PUT1-2M1) resulted in only a modest decrease in DNA-protein complex formation (Fig. 4B, lane F), mutation of the 3′ GATAAG sequence (fragment PUT1-2M2) diminished it to a barely detectable level (Fig. 4B, lane I). A doubly mutated DNA fragment (PUT1-2M3) lacking both the GATAAA and GATAAG sequences but still containing the GATAG sequence did not exhibit demonstrable protein binding (Fig. 4B, lane L). These data correlate with earlier Dal80p binding studies concluding that the sequence GATAAG binds to Dal80p more avidly than GATAAA (20, 21).

**Gln3p-dependent binding to wild-type and mutant *UGA4* DNA fragments.** A *UGA4* DNA fragment containing three tandem $UAS_{NTR}$ sequences (i) binds Dal80p, (ii) supports NCR-sensitive, Gln3p-dependent reporter gene transcription, and (iii) responds to *DAL80* disruption (1, 14, 23, 24). To test Gln3p binding, we used the same wild-type and mutant *UGA4*

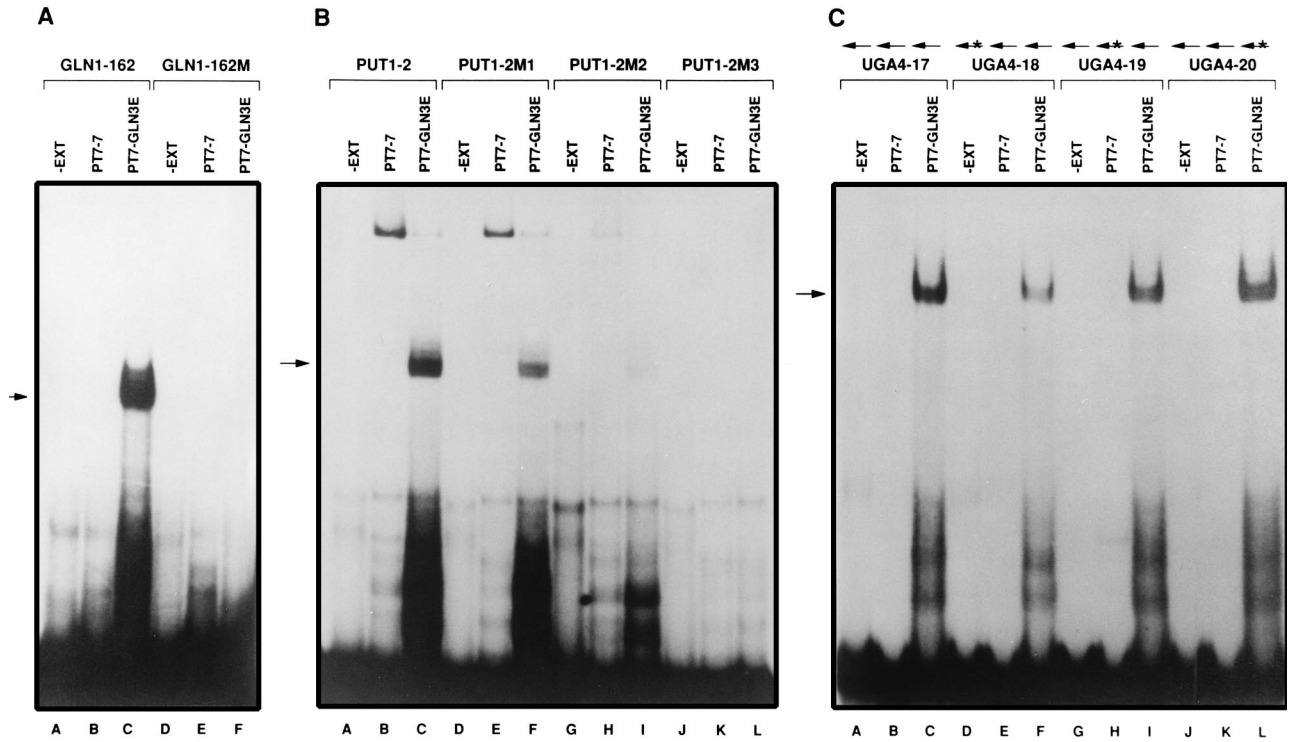


FIG. 4. (A) EMSA performed with wild-type (GLN1-162) and mutant (GLN1-162M) *GLN1* promoter fragments as radioactive hybridization probes; (B) EMSA performed with wild-type (PUT1-2) and mutant (PUT1-2M1, PUT1-2M2, PUT1-2M3) *PUT1* promoter fragments as radioactive hybridization probes; (C) EMSA performed with wild-type (UGA4-17) and mutant (UGA4-18, UGA4-19, and UGA4-20) *UGA4* promoter fragments as radioactive hybridization probes. All reaction conditions were as described for Fig. 3. Arrows denote specific Gln3p-DNA complexes. EXT, extract.

DNA fragments previously assayed for Dal80p binding (23). Wild-type DNA fragment UGA4-17, which contained three CTTATC (GATAAG) sequences, formed a strong Gln3p-dependent protein-DNA complex (Fig. 4C, lanes A to C). Mutation of the 5′-most GATAAG sequence (fragment UGA4-18) dramatically decreased DNA-protein complex formation, loss of the center GATAAG sequence (fragment UGA4-19) modestly decreased complex formation, and loss of the 3′ GATAAG sequence (fragment UGA4-20) was without demonstrable effect (lanes D to L).

**Gln3p-dependent binding to wild-type and mutant *GDH2* DNA fragments.** Expression of the *GDH2* gene, whose product is primarily responsible for degrading glutamate to ammonia and α-ketoglutarate, exhibits strong Gln3p dependence (24, 36), and its 5′ region contains multiple $UAS_{NTR}$-homologous sequences (36). One of these sequences in particular, GAT TAG, has been singled out as important (36) because of its homology to a sequence 5′ of *GLN1* (38); it is the one reported to bind to partially purified Gln3p (5). We therefore determined whether Gln3Ep could bind to DNA fragments in the 5′ region of *GDH2*. The first fragment tested (fragment GDH2-1) contained the sequences CTATC (GATAG) and GATGA but was not found to form a Gln3p-dependent DNA-protein complex (Fig. 5A, lanes A to C); DAL3-5 DNA was used as a positive control (lanes J to L). DNA fragment GDH2-2 contained the sequences GATAAT and GATAAC and formed a Gln3p-dependent DNA-protein complex, though the amount of complex formed was significantly less than that obtained with the DAL3-5 control (lanes D to F). The third *GDH2* fragment tested (GDH2-3) contained the sequence CTAAT CTAATC (GATTAGATTAG) and formed only a barely detectable DNA-protein complex compared with fragments GDH2-2 and DAL3-5 (lanes G to I).

**Gln3p-dependent binding to wild-type and mutant *CAR1* DNA fragments.** *CAR1* expression possesses limited NCR sensitivity which has been assumed to be mediated by two $UAS_{NTR}$-homologous sequences at positions −426 to −421 and −399 to −395 (34, 53). To test whether these sequences actually bind to Gln3p, wild-type fragment BS204/205 (*CAR1* sequences −440 to −381 [Fig. 2]) was tested for its ability to form a Gln3p-dependent protein-DNA complex. A strong complex was observed (Fig. 5B, lanes A to C). When the 5′ *CAR1* element TTTATC (GATAAA) was mutated (fragment BS206/207), complex formation modestly decreased (lanes D to F). In contrast, when the 3′ GATAAA element was mutated (fragment BS208/209), the signal drastically decreased (lanes G to I). In the double mutant (fragment BS210/211), no DNA-protein complex was detected (lanes J to L). These data suggest that Gln3p is able to specifically bind to both of the GATAA-containing sequences upstream of *CAR1*. Binding to the two elements with their common GATAAA cores was not the same, however, indicating that the Gln3p-binding site consists of more than GATAA.

**Reporter gene expression mediated by LexAp-Gln3p.** Gln3p is required for production of glutamine synthetase enzyme activity (4, 39, 40), NCR-sensitive gene expression (24), and $UAS_{NTR}$-mediated reporter gene expression (15), observations consistent with Gln3p functioning as a positive regulator of transcription. Indeed, on the basis of these data, Gln3p has been claimed to be a transcriptional activator (5, 36, 38, 58). Unfortunately, no extant data distinguish between the situations of Gln3p acting remotely as a positive regulator and

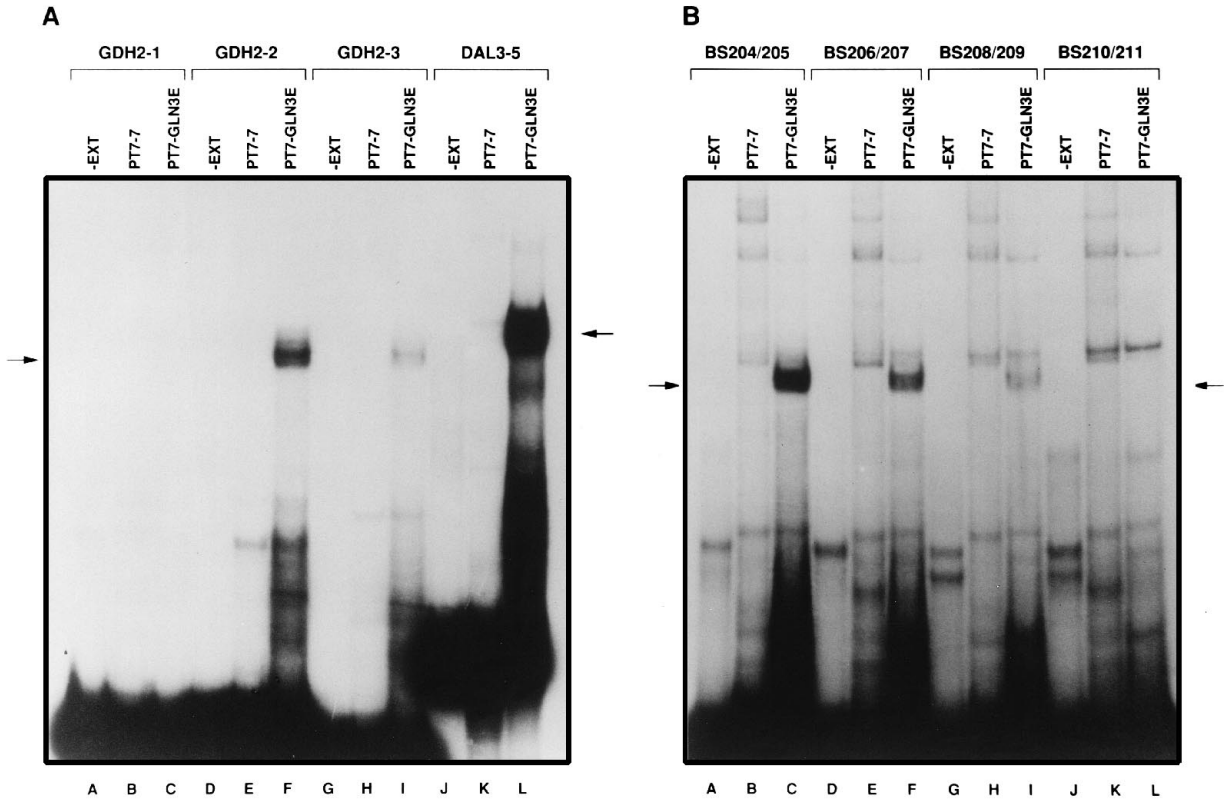


FIG. 5. (A) EMSA performed with wild-type *GDH2* promoter fragments as radioactive hybridization probes (see Fig. 2 for sequences of the DNA fragments); (B) EMSA performed with wild-type (BS204/205) and mutant (BS206/207, BS208/209, and BS210/211) *CAR1* promoter fragments as radioactive hybridization probes. All reaction conditions were as described for Fig. 3. Arrows denote specific Gln3p-DNA complexes. EXT, extract.

Gln3p being a transcriptional activator. This lack of data prompted us to determine whether Gln3p was capable of supporting reporter gene transcription when tethered to a site upstream of a minimal promoter devoid of UAS elements. We used a *lexA* fusion and reporter plasmid system (2, 28, 30) in which we fused a DNA fragment encoding the 202-amino-acid LexAp 5′ of full-length *GLN3* (plasmid pVS32; Materials and Methods and Fig. 1). Plasmid pVS32 or control plasmid pEG202, containing only the *lexA* fragment, was used along with reporter plasmid p1840, containing a single *lexA*-binding site 5′ of a *lacZ* reporter gene, to transform recipient strain EGY48 (Fig. 6). In contrast to a transcriptionally inert *lexA* insert (plasmid pEG202), a *lexA-GLN3* fusion (plasmid pVS32) supported high-level reporter gene expression (Fig. 6). Plasmid pRFHM1, containing a DNA fragment encoding a polypeptide (bicoid 2-160) reported to be incapable of supporting transcriptional activation, was used as an additional negative control (7, 31). These data demonstrated that Gln3p, tethered upstream of a minimal promoter, supported high-level reporter gene activation (Fig. 6). When this experiment was repeated with a second reporter plasmid (pSH18-34) containing four *lexA* operators, Gln3p supported approximately four- to fivefold-greater reporter gene activation (Fig. 6); background expression, however, remained minimal.

To estimate the relative strength of reporter gene expression supported by tethered Gln3p, we transformed yeast strain InvSc1 with plasmid pVS32 or pSH17-4, containing full-length *GLN3* or residues 74 to 881 of the strong activator Gal4p (7), respectively. The *GLN3* construct supported about 50% higher levels of reporter gene expression than did the *GAL4* construct in transformants provided with either Asn or Gln as a nitrogen source (Fig. 6); i.e., activation by the LexAp-Gln3p fusion was not significantly affected (less than threefold) by nitrogen source quality.

**Assay of *gln3* mutant alleles for the ability to support reporter gene expression.** When the Gln3p sequence was reported, a highly acidic N-terminal region (residues 1 to 150) was noted (37, 38). Therefore, we deleted the N-terminal region to test whether it was important for Gln3p to support transcriptional activation. Deletion of amino acid residues 470 to 670 or 470 to the C-terminal end of the protein did not affect its ability to support reporter gene expression (Fig. 6). In contrast, deletion of the first 150 Gln3p residues decreased reporter gene expression 15-fold.

To assess whether the zinc finger and N-terminal acidic regions of Gln3p were important to its function in vivo, we constructed two mutant alleles and assayed their abilities to complement a *gln3*Δ allele. In the zinc finger mutant (pRS316-derived centromeric plasmid pVS316-3), DNA encoding Gln3p residue Cys-306 was mutated to encode Ser, while in the second mutant (centromeric plasmid pVS316-2), DNA encoding the Gln3p acidic region (residues 2 to 150) was deleted (50). The growth rate in glucose-Asn medium supported by a plasmid-borne wild-type *GLN3* allele (3.1-kb *Sal*I-*Sac*I from plasmid pTSC517 carried in plasmid pRS316 [plasmid pVS316]) was two to three times lower than that of *gln3*Δ strain RR91 or strain RR91 transformed with plasmid pRS316, pVS316-3, or pVS316-2. Strain TCY1, containing a chromosomal wild-type *GLN3* allele, grew two to three times faster in glucose-Asn medium (110-min doubling time) than one in

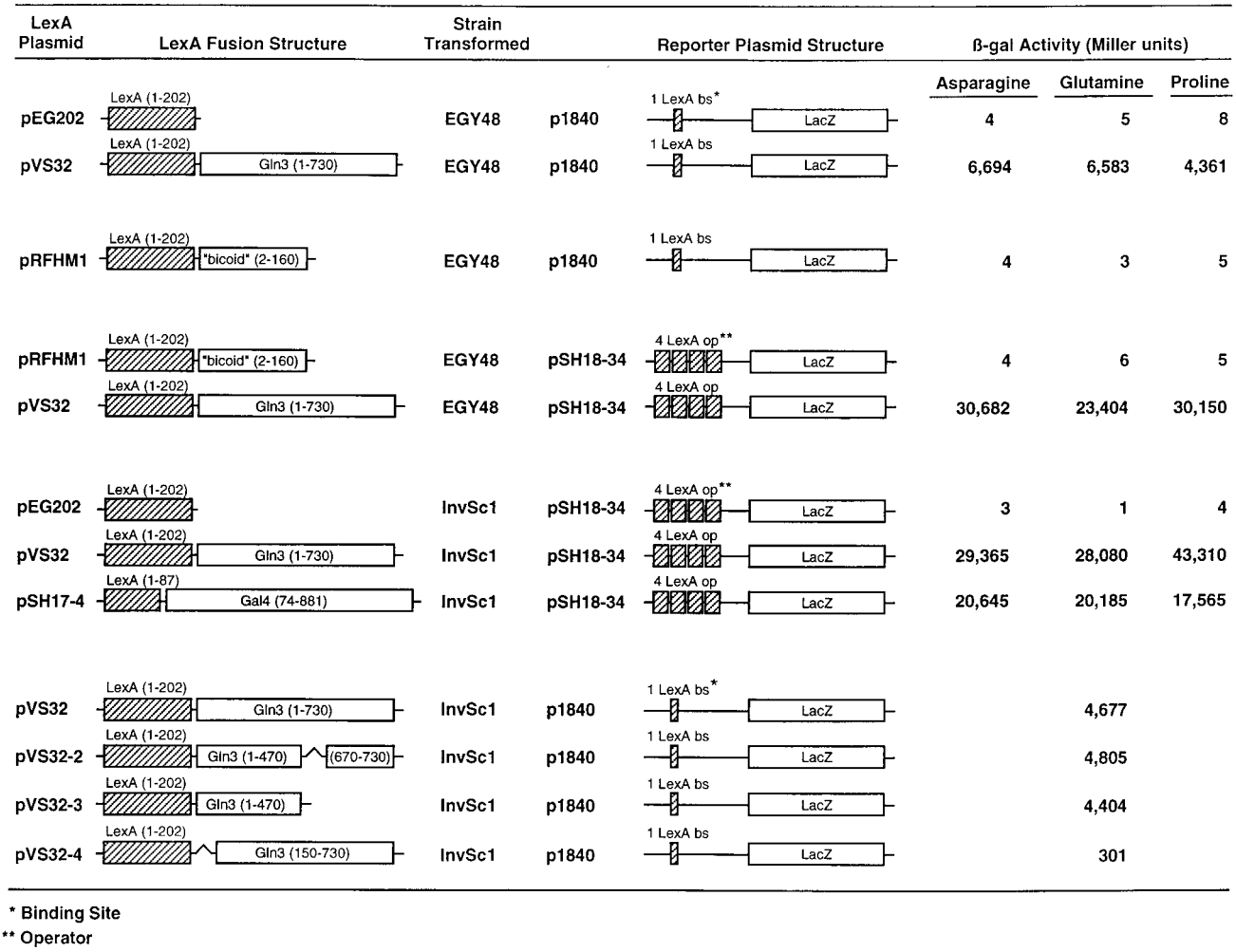


FIG. 6. Assay of abilities of wild-type and mutant LexA-Gln3 fusion proteins to support transcriptional activation from upstream of a *CYC1* minimal promoter devoid of UAS elements but containing a *lexA* operator site. Numbers near the plasmid diagrams indicate the residues of protein encoded by the various plasmids. Plasmids and assay conditions are as described in the text. The strain genotypes are EGY48 (*MAT***a** 3LexAop::*leu2 ura3 trp1 his3*) and Invgc1 (*MAT*α *his3-1 leu2 trp1-289 ura3-52*). β-gal, β-galactosidase.

which the wild-type *GLN3* allele was carried on plasmid pRS316 (plasmid pVS316). These data argued that the integrity of both the Gln3p zinc finger and N-terminal acidic regions was important to its in vivo function. It has been previously reported that increased or decreased amounts of Gln3p in a strain provided with Asn as a nitrogen source profoundly decrease the growth rate (39, 42).

## DISCUSSION

This work demonstrates that Gln3p possesses two characteristics of a nitrogen catabolic gene transcriptional activator: (i) when produced in *E. coli* and hence devoid of other yeast proteins, it specifically binds to DNA fragments containing $UAS_{NTR}$ elements, and (ii) it supports strong transcriptional activation when bound via a heterologous DNA-binding domain to a minimal promoter devoid of UAS elements. We would have preferred to use full-length Gln3p for our experiments. However, earlier investigations (41, 42) indicate that little more than the zinc finger is needed to specifically bind GATA-containing DNA sequences. These observations and the demonstrated binding specificity suggest that Gln3p binds to $UAS_{NTR}$-containing DNA fragments from the NCR-sensitive genes *GLN1*, *CAN1*, *DAL3*, *UGA4*, *GDH2*, *CAR1*, and *PUT1*. Overall Gln3p-binding requirements are somewhat different, however, from those of Dal80, a global nitrogen catabolic gene repressor that binds to some but not all $UAS_{NTR}$ elements (21, 23). The sequence geometry of Gln3p binding is less demanding than that of Dal80p, as evidenced by the fact that Gln3p exhibited strong binding to all DNA fragments previously shown to bind Dal80p and to some fragments that did not bind well to Dal80p. A significant difference in the binding of Gln3p and Dal80p is that optimal DNA binding by the latter requires two GATAA-containing sequences oriented tail-to-tail or head-to-tail 15 to 35 bp apart (21, 23). Our data suggest that Gln3p can bind to single GATAA sequences, though DNA fragments with multiple GATAA sequences yield the highest levels of DNA-Gln3p complex. Previous physiological data also support our conclusion that Gln3p binds to native DNA fragments containing only a single GATAA sequence (45).

Even though this work was not designed to delineate the precise Gln3p-binding site sequence or comparisons between it and those of Dal80p or Gat1p, Gln3p binding to the native

sequences that we used provided some insight into this structure. DNA fragments with multiple, consecutive $UAS_{NTR}$-homologous sequences bind Gln3p best. It also appears that Gln3p binds GATAAG better than GATAAA and does not bind the sequence GATGA or GATAG. Finally, fragment GDH2-3, containing sequences GATTAGATTAG and GATAG, forms only a barely detectable complex with Gln3p compared with fragment GDH2-2, containing the sequences GATAAT and GATAAC. This observation is at variance with a recent report that the sequence GATTAG binds to a partially purified preparation of Gln3p (5).

Our data also demonstrate that Gln3p bound upstream of a minimal promoter is able to support high-level reporter gene activation. Moreover, the N-terminal 150 Gln3p amino acid residues are required for complementation of a *gln3*Δ mutation and high-level transcriptional activation. These characteristics are more consistent with Gln3p acting as transcriptional activator following binding to $UAS_{NTR}$ sites upstream of NCR-sensitive genes than the possibility of Gln3p operating earlier in transmission of the NCR regulatory signal.

While this work contributes to resolving two issues of uncertainty regarding Gln3p function, it does not provide clear insight into the regulation of Gln3 function. If the *lexA* experiments are taken at face value, one would conclude that once Gln3p is bound to DNA, its activity is largely indifferent to the quality of nitrogen source available. This would lead to the conclusion that the regulated step of NCR-sensitive gene expression is Gln3p binding to $UAS_{NTR}$ elements, rather than its operation once bound. However, the alternate argument, i.e., that it is Gln3p function after binding to DNA that is nitrogen source dependent, cannot be ruled out so far because it is known from the work of several other laboratories that when a transcriptional activator is tethered by *lexA* upstream of a core promoter, its in vivo regulatory characteristics may be lost or markedly diminished (43). Having observed a lack of responsiveness to physiological conditions displayed by LexA fusion proteins (reference 43 and this work), one may also have to consider the possibility that a hypothetical regulatory molecule controlling Gln3p function is needed in stoichiometric rather than catalytic amounts and hence may be titrated away by LexA-Gln3 fusion protein expressed from the strong *ADH1* promoter.

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