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Regulation of Expression of *GLT1*, the Gene Encoding Glutamate Synthase in *Saccharomyces cerevisiae*

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Saccharomyces cerevisiae **glutamate synthase (GOGAT) is an oligomeric enzyme composed of three 199-kDa identical subunits encoded by *GLT1*. In this work, we analyzed *GLT1* transcriptional regulation. *GLT1-lacZ* fusions were prepared and *GLT1* expression was determined in a *GDH1* wild-type strain and in a *gdh1* mutant derivative grown in the presence of various nitrogen sources. Null mutants impaired in *GCN4*, *GLN3*, *GAT1*/*NIL1*, or *UGA43*/*DAL80* were transformed with a *GLT1-lacZ* fusion to determine whether the above-mentioned transcriptional factors had a role in *GLT1* expression. A collection of increasingly larger 5′ deletion derivatives of the *GLT1* promoter was constructed to identify DNA sequences that could be involved in *GLT1* transcriptional regulation. The effect of the lack of *GCN4*, *GLN3*, or *GAT1*/*NIL1* was also tested in the pertinent 5′ deletion derivatives. Our results indicate that (i) *GLT1* expression is negatively modulated by glutamate-mediated repression and positively regulated by Gln3p- and Gcn4p-dependent transcriptional activation; (ii) two *cis*-acting elements, a $CGGN_{15}CCG$ palindrome and an imperfect poly(dA-dT), are present and could play a role in *GLT1* transcriptional activation; and (iii) *GLT1* expression is moderately regulated by *GCN4* under amino acid deprivation. Our results suggest that in a wild-type strain grown on ammonium, GOGAT constitutes an ancillary pathway for glutamate biosynthesis.**

The existence of two pathways for glutamate biosynthesis has been demonstrated in a variety of organisms. In one pathway, $NADP^+$-dependent glutamate dehydrogenase ($NADP^+$-GDH; EC 1.4.1.4) catalyzes the reductive amination of 2-oxoglutarate to form glutamate (24). The existence of an alternative pathway for the net biosynthesis of glutamate was demonstrated by Tempest et al. (45). In this pathway, glutamate is aminated to form glutamine by glutamine synthetase (GS; EC 1.4.1.13), the amide group of which is then transferred reductively to 2-oxoglutarate by glutamate synthase (GOGAT; EC 1.4.1.13), resulting in the net conversion of ammonium and 2-oxoglutarate to glutamate. The GS-GOGAT pathway has been found in several microorganisms (8, 25, 30, 32, 40) and in higher plants (32). In *Saccharomyces cerevisiae*, besides the $NADP^+$-GDH1 encoded by *GDH1* and GOGAT encoded by *GLT1* (18, 24, 33), there is a third route for glutamate biosynthesis, constituted by a $NADP^+$-GDH1 isozyme ($NADP^+$-GDH3), encoded by *GDH3* (2). Thus, in this microorganism, mutations inactivating *GDH1*, *GLT1*, and *GDH3* are needed in order to attain full glutamate auxotrophy (2).

The presence of multiple pathways for glutamate biosynthesis in several microorganisms has stimulated discussion on the need for several routes for the biosynthesis of the same end product. Since the demonstration of the existence of GOGAT as an alternative pathway for glutamate biosynthesis (45), it was proposed that the role of the GS-GOGAT pathway would be that of ammonium assimilation and glutamate biosynthesis under ammonium limitation (45). In fact, it has been shown that for *Klebsiella aerogenes* this was the case (40). However, in other microorganisms (18, 28, 40), $NADP^+$-GDH is used to incorporate ammonia during either nitrogen limitation or nitrogen excess. Thus, the initial hypothesis suggesting the differential utilization of $NADP^+$-GDH and GS-GOGAT pathways under excess or limiting ammonia does not hold for most of the microorganisms so far studied. Since in most cases $NADP^+$-GDH seems to be the main pathway for glutamate biosynthesis, the role of GOGAT remains unclear. Physiological studies have been performed with either wild-type or mutant strains impaired in GOGAT or in $NADP^+$-GDH activity. In some cases, this approach has allowed the proposal of different roles for GOGAT in different microorganisms (3, 20, 23, 25, 28, 46). Few studies have been done to investigate the regulation of GOGAT-encoding genes; such studies could also provide information on whether this enzyme is involved in glutamate biosynthesis. In the case of *Escherichia coli*, it has been found that the two structural genes (*gltB* and *gltD*) coding for the two *E. coli* GOGAT subunits form an operon, with a third regulatory gene, *gltF* (9), involved in the glutamate-mediated repression of the *gltBDF* operon (10). In addition, the *E. coli gltBDF* operon appears to be transcriptionally regulated by the leucine-responsive regulatory protein (Lrp) (16). In *Bacillus subtilis*, GOGAT gene expression (*gltA* and *gltB*) is dependent on a positive regulator (*gltC*) that is itself transcribed from a divergent but overlapping promoter site (6). It has been postulated that the product of *gltC* is a positive transcription factor that acts at the *gltA* promoter to stimulate transcription under conditions of limiting glutamate (7).

During the last years, our group has been interested in defining and understanding the role of each of the pathways involved in glutamate biosynthesis in *S. cerevisiae* (2, 17, 18). Since in this yeast there are three pathways for glutamate biosynthesis and the precise function of each has not been established, we decided to initiate our study by examining *GLT1* transcriptional regulation in *S. cerevisiae*.

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TABLE 1. *S. cerevisiae* strains

Strain	Genotype	Reference
CLA1	*MATα GDH1 GDH3 GLT1 ura3 leu2*	2
CLA1-0	*MATα GDH1 GDH3 GLT1 ura3 leu2*/YEp363 (2μm *LEU2*)	This study
CLA1-1	*MATα GDH1 GDH3 GLT1 ura3 leu2*/pLOU1(*GLT1-lacZ* 2μm *LEU2*)	This study
CLA-100	*MATα GDH1 GDH3 GLT1 gcn4Δ::URA3 leu2*/pLOU1(*GLT1-lacZ* 2μm *LEU2*)	This study
CLA-101	*MATα GDH1 GDH3 GLT1 gln3Δ::URA3 leu2*/pLOU1(*GLT1-lacZ* 2μm *LEU2*)	This study
CLA-102	*MATα GDH1 GDH3 GLT1 gat1Δ::URA3 leu2*/pLOU1(*GLT1-lacZ* 2μm *LEU2*)	This study
MAR1	*gdh1Δ::URA3 GDH3 GLT1 leu2*	This study
MAR1-0	*gdh1Δ::URA3 GDH3 GLT1 leu2*/YEp363 (2μm *LEU2*)	This study
MAR1-1	*gdh1Δ::URA3 GDH3 GLT1 leu2*/pLOU1 (*GLT1-lacZ* 2μm *LEU2*)	This study
27034b	*MATα GDH1 GDH3 GLT1 UGA43 ura3 leu 2*/pSIM1 (*GLT1-lacZ* 2μm *URA3*)	13
30078c	*MATα GDH1 GDH3 GLT1 uga43Δ ura3*/pSIM1 (*GLT1-lacZ* 2μm *URA3*)	13

In this work, we prepared *GLT1-lacZ* fusions which allowed the study of *GLT1* expression in *GDH1* and *gdh1* strains in the presence of various nitrogen sources. A collection of 5′ deletion derivatives of the *GLT1* promoter was prepared in order to determine the DNA sequences that could be involved in transcriptional regulation. We also studied the role of three transcriptional activators (Gcn4p, Gln3p, and Gat1p/Nil1p) (5, 11, 22, 34, 41) and of a repressor protein (Uga43p/Dal80p) (13, 15) in *GLT1* expression; all of these proteins have been shown to be involved in regulation of expression of genes coding for enzymes of amino acid biosynthesis or of nitrogen catabolism.

Our results indicate that first, under conditions of glutamate excess, *GLT1* expression is governed by both glutamate-mediated repression and Gln3p- and Gcn4p-mediated activation; second, under derepressive conditions, *GLT1* expression could be positively regulated by a Zn_2-Cys_6 binuclear cluster activator, by Gcn4p and Gln3p, and by an imperfect poly(dA-dT) promoter element; and third, under amino acid deprivation, *GLT1* expression is moderately regulated by Gcn4p.

MATERIALS AND METHODS

Strains. Table 1 describes the characteristics of the strains used in this study. Null mutants impaired in *GCN4*, *GLN3*, or *GAT1* were derived from strain CLA1 by gene replacement using the 3.7-kb *Bst*II-*Mlu*I restriction fragment of pM214 (21), *Aat*II-digested pPM62 (34), or plasmid pRR336 previously digested with *Xba*I-*Eco*RI (11), thus obtaining CLA100, CLA101, and CLA102. MAR1 was obtained by *GDH1* gene disruption with pLV3 linearized with *Bgl*II (2).

Growth conditions. Strains were routinely grown on minimal medium (MM) containing salts, trace elements, and vitamins following the formula of yeast nitrogen base (Difco). Filter-sterilized glucose (2%) was used as the carbon source, and 0.2% $(NH_4)_2SO_4$ or 0.1% glutamate, glutamine, asparagine, or proline was used as the nitrogen source. Amino acids needed to satisfy auxotrophic requirements were added at 0.01% (wt/vol). Cells were incubated at 30°C with shaking (250 rpm). For amino acid deprivation experiments, CLA1/pLOU1 or its *gcn4*Δ/pLOU1 derivative was inoculated into 10 ml of YPD, incubated at 30°C with shaking for 6 h, washed twice, and resuspended in MM. An aliquot was inoculated into 100 ml of MM to give at optical density at 600 nm (OD_{600}) of 0.05. This culture was incubated at 30°C with shaking for 6 h, harvested, resuspended in 10 ml of MM, and inoculated into 100 ml of MM to give an OD_{600} of 0.2 and into 100 ml of MM–10 mM 3-aminotriazole (3-AT) to give an OD_{600} of 0.5. After 6 h of incubation at 30°C with shaking (250 rpm), cultures were centrifuged and used for β-galactosidase (β-Gal) determinations.

Determination of GOGAT and β-Gal activities. Yeast total extracts were prepared from cultures inoculated at an OD_{600} of 0.05 and harvested at an OD_{600} of between 0.8 and 1.0. Cells were washed twice with H_2O and once with the corresponding extraction buffer (12, 37). The pellet was stored at −20°C until used. Soluble extracts were prepared by suspending whole cells in their corresponding extraction buffer and grinding them with glass beads in a Vortex mixer. Yeast GOGAT (EC 1.4.7.1) activity was determined by the method described by Cogoni et al. (12). Specific activity was expressed as nanomoles of NADH oxidized per minute per milligram of protein. β-Gal activities were determined by the method described by Rose and Botstein (37). β-Gal specific activity was expressed as nanomoles of *o*-nitrophenol produced per minute per milligram of protein. Protein was measured by the method of Lowry et al. (29), with bovine serum albumin as a standard.

Construction of *lacZ* fusions. Plasmid Yc14, previously described and sequenced (12, 17), contains 2 kb of the *GLT1* coding sequence, the full *GLT1* promoter and 30 bp of the *UGA3* coding sequence. Yc14 DNA was digested with *Eco*RI and used as template for PCR amplification. Deoxyoligonucleotide F1 contained a *Bam*HI site and 18 bp of the *UGA3* coding region (5′-CGCGCGG GATCCCAATTTCAGCTTCTCCAC-3′). Deoxyoligonucleotide R1 contained a *Sal*I site, 8 bp upstream the *GLT1* coding region, and 3 bp downstream the *GLT1* promoter region (5′-GCGCGCGGTCGACACTGGCATGCT-3′). Deoxyoligonucleotides F1 and R1 were used to amplify the complete *GLT1* promoter. To obtain a 5′ *GLT1* promoter deletion series, the pertinent forward deoxyoligonucleotides were designed based on the *GLT1* promoter sequence. Deoxyoligonucleotide R1 was also used to amplify the full promoter and the 18 individual deletions. The entire family of PCR products was fused in frame to the *E. coli lacZ* gene of YEp363 (2μm *LEU2*) (35), generating 19 fusion plasmids, pLOU1 to pLOU19. The PCR product carrying the full *GLT1* promoter was also fused in frame to the *E. coli lacZ* gene of YEp353 (2μm *URA3*) (35), generating plasmid pSIM1. All fusion plasmids were sequenced with an automated Applied Biosystems 373 DNA sequencer (W. M. Keck Foundation, Yale University).

Yeast transformation. *S. cerevisiae* was transformed by the method described by Ito et al. (26). To generate null derivatives, transformants were selected for uracil prototrophy on MM supplemented with auxotrophic requirements as needed. Pertinent strains were transformed with the *lacZ* fusion plasmids or, when appropriate, with YEp363. Transformants were selected for either leucine or uracil prototrophy on MM supplemented with auxotrophic requirements as needed.

Primer extension RNA analysis. Primer extension reactions were performed by standard procedures (38). To determine chromosomal *GLT1* transcription initiation sites, total RNA was isolated from strain CLA1 grown on MM with 0.2% $(NH_4)_2SO_4$ as the nitrogen source. A deoxyoligonucleotide containing the first 21 nucleotides of the *GLT1* coding region was prepared and used in the primer extension reactions. The transcription initiation sites present in the different *lacZ* fusion constructs were also determined. Primer extension reactions were carried out with total RNA extracted from the pertinent strains grown on MM with 0.2% $(NH_4)_2SO_4$ as the nitrogen source and a deoxyoligonucleotide containing 23 nucleotides of the *lacZ* coding region.

RESULTS AND DISCUSSION

Sequence analysis of *GLT1* promoter region and determination of transcription initiation sites. As stated in the introduction, the role of GOGAT in glutamate biosynthesis and its regulation have not been studied in yeast. To address this matter, we analyzed the nucleotide sequence located upstream of the *GLT1* coding region, which was contained in the previously reported plasmid Yc14 (12). As Fig. 1 shows, *GLT1* was located in opposite orientation, next to the *UGA3* gene, which codes for a transcriptional activator of the genes involved in γ-aminobutyrate catabolism (1). Intragenic sequences may act as sites for *trans*-acting regulatory elements of either of the two divergent genes, *GLT1* and *UGA3*. Such sequences could face or partly overlap sites with the opposite orientation in the complementary DNA strand that regulate the alternative divergent gene. One could expect that simple occupancy of either sequence by its cognate high-affinity regulator may interfere with regulation of the alternative divergent gene. The study of *UGA3* expression may help define this matter.

Most of the genes encoding amino acid biosynthetic enzymes in *S. cerevisiae* are subject to a cross-pathway regulatory system known as the general amino acid control that stimulates their

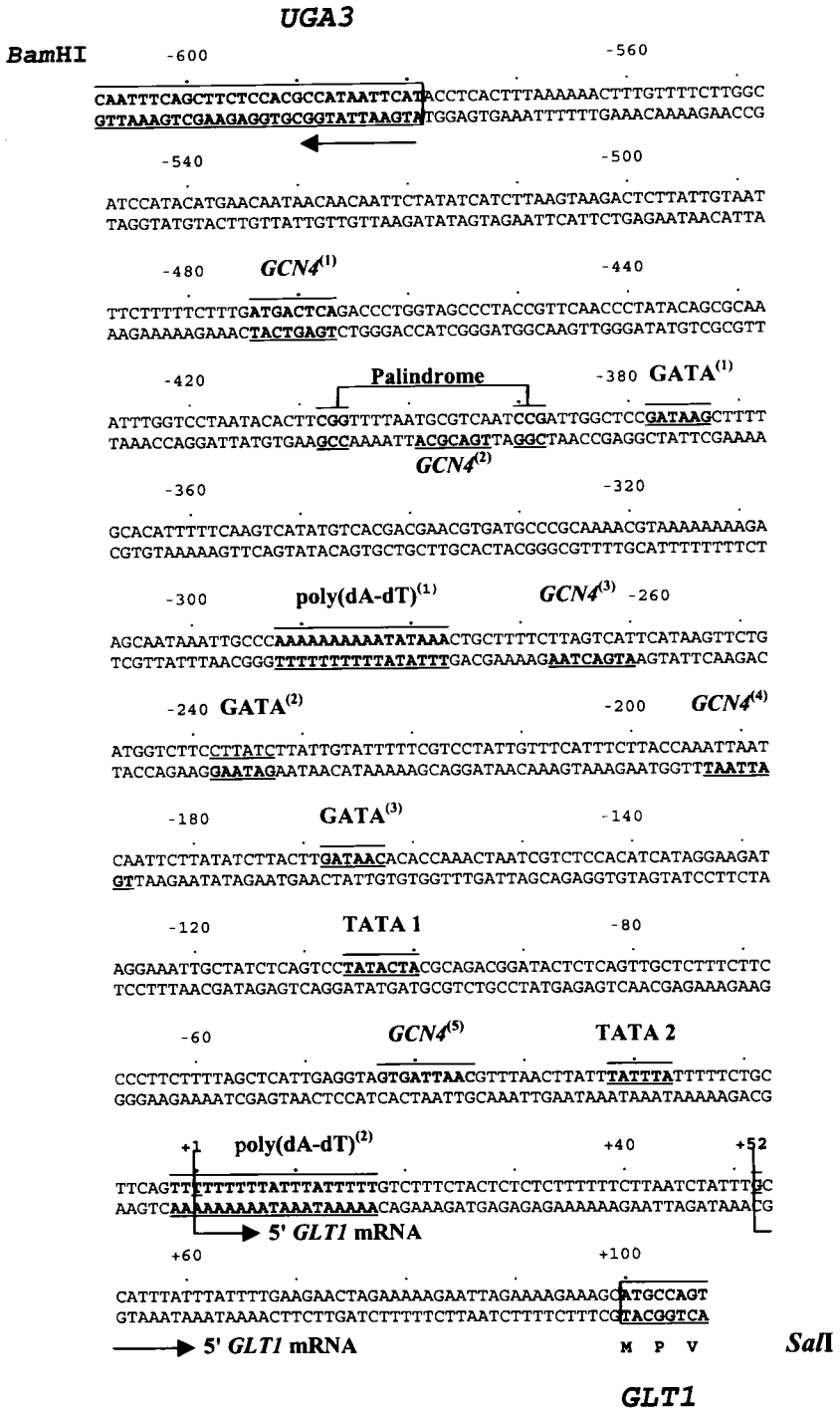


FIG. 1. *GLT1* promoter sequence. Putative Gcn4p (*GCN4*), Gln3p, and Gat1p binding sites (GATA), $CGGN_{15}CCG$ palindrome, and poly(dA-dT) regions are boxed and numbered starting from the most 5′. Two putative TATA boxes (TATA 1 and TATA 2) as well as the two transcription initiation sites, at positions +1 and +52, are indicated. The 714-bp fragment shown includes a 30-bp sequence of the *UGA3* coding region. *Bam*HI and *Sal*I sites were added and used to clone this fragment into the 2μm *LEU2 lacZ* vector YEp363, generating plasmid pLOU1.

expression under conditions of amino acid starvation. Gcn4p is the direct positive regulator of gene expression in this system (22). Examination of the *GLT1* promoter revealed a canonical Gcn4p binding site ATGACTC [$GCN4^{(1)}$] (Fig. 1) located between positions −477 and −466. The *GLT1* promoter also carries four noncanonical binding sites (Fig. 1) with low affinity for Gcn4p (31, 44): TGCGTA from positions −399 to −393 [$GCN4^{(2)}$], TTAGTCAT from −267 to −260 [$GCN4^{(3)}$], ATT AATCA from −193 to −186 [$GCN4^{(4)}$], and GTGATTAAC from −43 to −35 [$GCN4^{(5)}$].

The *GLT1* promoter also contained three GATAA sequences, GATAAG from positions −377 to −372 [$GATA^{(1)}$],

CTTATC (complementary, GATAAG) from −238 to −233 [GATA$^{(2)}$], and GATAAC from −168 to −164 [GATA$^{(3)}$] (Fig. 1), which can constitute the *cis*-acting element, UAS_{NTR} (41). UAS_{NTR} has been proposed as a binding site for two transcriptional activators, Gln3p and Gat1p/Nil1p (4, 11, 34, 42), which regulate the expression of nitrogen-modulated genes.

Down-regulation of nitrogen-controlled gene expression is accomplished by the action of the GATA family member Dal80p/Uga43p. The Dal80p binding site, URS_{GATA}, consists of a pair of GATA-containing sequences oriented tail to tail or head to tail (15). As can be seen in Fig. 1, the *GLT1* promoter harbors two of the above-mentioned GATA sequences oriented tail to tail; these could constitute a Dal80p binding site, although the distance between them (63 bp) is larger than that previously reported (15 to 35 bp) (15).

At least 79 fungal transcription-activating factors containing a Zn_2-Cys_6 binuclear cluster have been found (39). DNA targets for several members of this family of proteins have two inverted CGG half-sites separated by a spacing characteristic of the particular protein that recognizes it (27). The two inverted CGG half-sites separated by 15 bp present in the *GLT1* promoter (Fig. 1) could also constitute a binding site for members of the Zn_2-Cys_6 binuclear cluster family of proteins.

Many yeast promoters contain homopolymeric (dA-dT) sequences (43). Analysis of the function of these sequences in transcriptional activation has suggested that perfectly homopolymeric sequences function by virtue of their intrinsic structure. For imperfect poly(dA-dT) tracts, it has been proposed that the transcriptional effects might be mediated in part or completely by specific DNA-binding proteins (47). The *GLT1* promoter also bears two poly(dA-dT) sequences: one composed of a 16-poly(dA-dT) tract with two imperfections located from positions −292 to −276 [poly(dA-dT)^1 in Fig. 1], and another consisting of a 19-poly(dA-dT) tract with two imperfections located from −2 to +17 [poly(dA-dT)^2 in Fig. 1].

Primer extension analysis (Fig. 2) defined two transcription initiation sites in *GLT1*, which are shown in Fig. 1 at positions +1 and +52. The results presented in Fig. 2 indicate that the +1 initiation site is stronger than the +52 site. Two putative TATA boxes differing from the TATAAA canonical sequence were also found (Fig. 1). Either the TATACTA or TATTTA sequence can substitute for TATAAA in transcription initiation (19). Constructions from pLOU14 to −17 were able to initiate transcription only from +52. It is possible that each of these initiation sites together with TATA$^{(1)}$ or TATA$^{(2)}$ can signal transcription under different physiological conditions. If this were the case, the first element would direct transcription regulated by glutamate-mediated repression and by Gcn4p-, Gln3p-, and putative Zn_2-Cys_6 binuclear cluster-mediated activation. The second element would direct transcription mediated by the poly(dA-dT) element by itself or together with a glutamate-sensitive activator.

Regulation of *GLT1* expression. It has been previously observed that mutants impaired in *GDH1* display increased GOGAT activity (2), suggesting that *GLT1* can be negatively modulated by glutamate and that in a *gdh1* mutant, glutamate limitation can result in *GLT1* derepression. To determine whether *GLT1* expression was regulated by the nature of the nitrogen source, we determined GOGAT and β-Gal activities in a wild-type strain and in a *gdh1* mutant. Both strains harbored either plasmid pLOU1, containing the *GLT1* promoter fused to the complete β-Gal coding region, or the vector YEp363 (see Materials and Methods). As expected, in the presence of YEp363, no β-Gal activity was detected, and

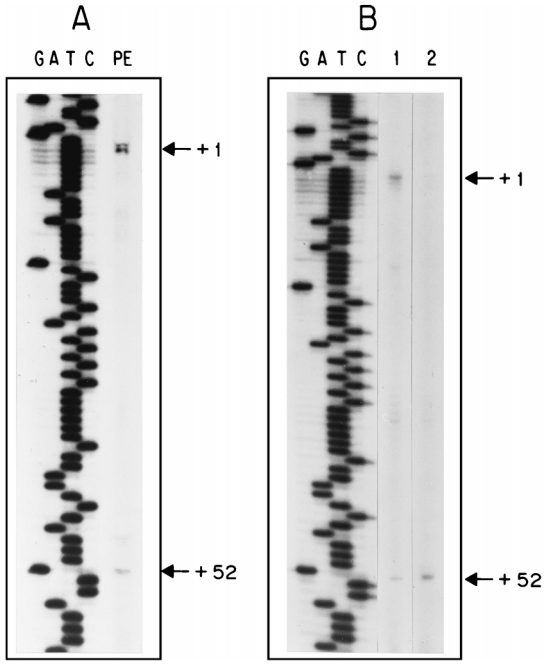


FIG. 2. Primer extension analysis. (A) Assay of transcription initiation sites (lane PE) of the *GLT1* gene, carried out with total RNA obtained from the wild-type strain CLA1. (B) Representative results of primer extension analysis carried out with total RNA obtained from strain CLA1 transformed with plasmid pLOU1, -3, -4, -6, -8, -9, -10, or -11 (lane 1) or with pLOU14, -15, -16, or -17 (lane 2). The sequence ladder was produced with the same deoxyoligonucleotide used for the primer extension reaction (described in Materials and Methods).

GOGAT activity values were similar to those found in the presence of pLOU1 (Table 2). As Table 2 and Fig. 3 (row 1) show, GOGAT and β-Gal activities were higher in the *gdh1* mutant strain grown on ammonium or proline as the sole nitrogen source than in the wild-type strain grown under similar conditions. In the presence of glutamate, glutamine, or asparagine, both GOGAT and β-Gal activities decreased and achieved similar values in extracts obtained from either the wild-type or *gdh1* strain (Table 2). These results indicate that *GLT1* expression was repressed in the presence of glutamate-rich nitrogen sources.

To analyze whether Gln3p, Gat1p/Nil1p, Gcn4p, or Dal80p/Uga43p had a role in *GLT1* expression, plasmid pLOU1 was transformed into *gcn4*Δ, *gln3*Δ, *gat1*Δ, and *uga43*Δ mutant strains, and β-Gal activity was determined (Table 1; Fig. 4, row 1). It was found that with ammonium or proline as the nitrogen source, the lack of Gln3p severely diminished β-Gal activity; impairment of Gcn4p had a slight effect on this activity, while the lack of Gat1p had no effect. Extracts obtained from cultures of the *gln3*Δ or *gcn4*Δ mutant showed decreased β-Gal activity compared to extracts obtained from the wild type when either strain was grown on glutamate, glutamine, or asparagine. These results suggested that *GLT1* transcription of yeast cells grown in glutamate-rich nitrogen sources was down-regulated by glutamate repression and up-regulated by transcriptional activators Gln3p and Gcn4p. The capacity of Gln3p and Gat1p to activate transcription appears to be nitrogen regulated in such a way that *GLN3* stimulates transcription on glutamate and proline and *GAT1* does so on ammonium and urea (42). However, neither Gln3p or Gat1p promotes the expression of nitrogen-regulated genes on glutamine (42). Our results indicate (i) that *GLT1* is not regulated by *GAT1* and (ii)

that *GLN3* activates *GLT1* expression with all nitrogen sources tested, including glutamine. Possibly the *GLT1* promoter has a higher affinity for the *GLN3* inactive form that has been postulated to be present in glutamine (5). Also, as has been observed in other cases (36), *GLN3* may act in combination with the positive activator that should bind the CGG palindrome. Maybe in the case of *GLT1* expression, a less active form of *GLN3* has an important effect on glutamine when assisted by another activator.

Isogenic strains carrying the wild-type *UGA43*/*DAL80* gene or the null allele were also transformed with pLOU1. As Table 2 shows, lack of Uga43p/Dal80p did not result in derepressed *GLT1* expression in the presence of glutamate, indicating that glutamate-mediated repression was not *UGA43*/*DAL80* dependent. Further experiments will be required to determine the nature of the *cis*- and *trans*-acting elements which mediate glutamate repression.

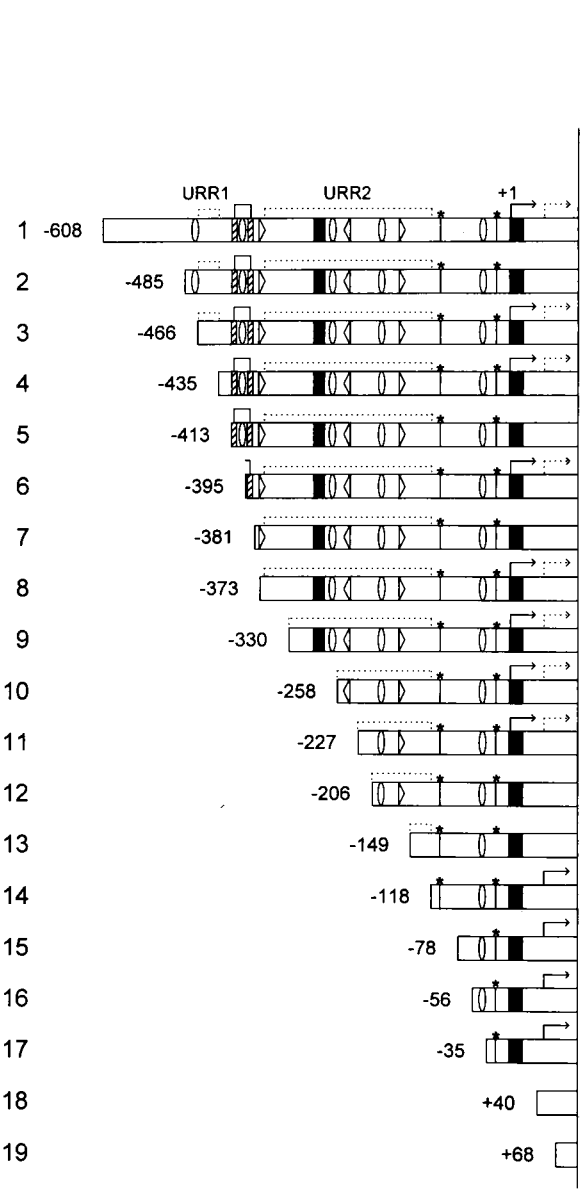
To address if *GLT1* expression was regulated during amino acid deprivation by the general amino acid control mediated by Gcn4p (22), β-Gal was determined in extracts from cultures of the wild-type strain and of the *gcn4*Δ mutant grown in the presence and absence of 3-AT, a competitive inhibitor of His3p. In the presence of this analog, cells become deprived for histidine. β-Gal activity was twofold higher in extracts obtained from the wild-type strain grown in the absence of 3-AT compared to that found in its presence (1,280 versus 2,170 nmol min^{-1} mg^{-1}). This increment was not observed in the *gcn4*Δ mutant strain (600 versus 550 nmol min^{-1} mg^{-1}). These results indicate that *GLT1* expression was increased during amino acid deprivation and that this increase was Gcn4p dependent (22). Since glutamate is a precursor in the biosynthesis of most amino acids, the genes coding for the enzymes involved in its biosynthesis would likely have to be responsive to starvation of a number of amino acids. However, our results indicate that GOGAT (*GLT1*) is not strongly regulated by Gcn4p. The analysis of whether *GDH1* or *GDH3* transcription responds to amino acid limitation will be very useful to fully understand the pathway(s) through which glutamate biosynthesis could be increased during amino acid starvation. The exact binding site(s) for Gcn4p on *GLT1* promoter remains to be determined. However, our 5′ deletion analysis suggests that the canonical *GCN4* binding site [$GCN4^{(1)}$] plays no role in *GLT1 GCN4*-dependent transcriptional activation, since when it is deleted (pLOU3), *GLT1* transcription is not decreased. It is clear that pLOU4-dependent β-Gal activity is decreased in a null *gcn4*Δ derivative, indicating that the *GCN4* binding site [$GCN4^{(2)}$] could play a more important role than [$GCN4^{(1)}$] in *GCN4*-mediated transcriptional activation. It is also possible that the $GCN4^{(3)}$ putative binding site plays a role in *GLT1* gene activation together with the poly(dA-dT)$^{(1)}$, since it has been suggested that during gene activation of promoters harboring both a poly(dA-dT) tract and a *GCN4* binding site, transcription can be either hindered or promoted through chromatin reorganization (47).

Deletion analysis of *GLT1* promoter. A collection of 5′ deletions of increasing size affecting the *GLT1* promoter was prepared as described in Material and Methods. When a *GDH1* strain harboring pLOU4, which lacks the most 5′ 173 bp of the *GLT1* promoter, was grown on ammonium, β-Gal activity was slightly higher than that obtained with the *GDH1* strain carrying pLOU1 (Fig. 3, row 4). This increment was more evident when β-Gal was determined in a *gdh1* strain carrying pLOU4, which showed β-Gal activity nearly threefold higher than that found in the *gdh1* strain carrying pLOU1. These results suggested that pLOU4 had lost a target for negative regulation (upstream repressing region 1 [URR1])

TABLE 2. β-Gal and GOGAT specific activities

Nitrogen source	Sp act (nmol min^{-1} mg^{-1})[a]										β-Gal in:	
	CLA1-0 (*GDH1*/YEp363)		CLA1-1 (*GDH1*/pLOU1)		MAR1-0 (*gdh1*Δ/YEp363)		MAR1-1 (*gdh1*Δ/pLOU1)		CLA100 (*GDH1 gcn4*Δ/pLOU1)	CLA101 (*GDH1 gln3*Δ/pLOU1)	CLA102 (*GDH1 gat1*Δ/pLOU1)	27034b (*GDH1 UGA43*/pSIM1)	30078c (*GDH1 uga43*Δ/pSIM1)
	β-Gal	GOGAT	β-Gal	GOGAT	β-Gal	GOGAT	β-Gal	GOGAT					
Ammonium	ND	40	1,280	41	ND	71	3,650	69	600	230	1,150	850	630
Proline	ND	40	1,580	41	ND	53	2,360	55	600	290	1,250	3,850	4,250
Glutamate	ND	22	550	24	ND	24	600	25	200	270	392	910	750
Glutamine	ND	22	520	21	ND	27	710	29	200	60	362	410	340
Asparagine	ND	23	450	25	ND	23	450	24	250	100	308	1,340	1,000

[a] Mean of three independent experiments. Variations were ≤15%. ND, not detected.



	ß-galactosidase (nmol.min^{-1}.mg^{-1})			
	CLA1 *GDH1*		MAR1 *gdh1*Δ	
	ammonium	glutamate	ammonium	glutamate
1	1280	550	3650	600
2	1000	440	4780	890
3	1250	600	3530	540
4	1570	780	9500	1200
5	1410	800	7410	930
6	710	310	4020	710
7	740	400	3860	660
8	660	330	2250	570
9	570	410	4130	540
10	750	620	5020	840
11	560	700	5060	1000
12	1230	1050	5400	1220
13	920	670	7110	1110
14	1440	770	8250	1470
15	2110	1800	8100	1610
16	2320	1990	8530	1780
17	4080	3110	9400	1860
18	80	40	40	10
19	ND	ND	ND	ND

FIG. 3. β-Gal activities of 5′ deletions of the *GLT1* promoter. The *GLT1* full promoter and 5′ deletions were cloned into the 2μm *LEU2 lacZ* vector YEp363, generating plasmids pLOU1 to -19. These plasmids were transformed into either the *GDH1* wild-type strain CLA1 or the *gdh1* mutant strain MAR1. The 5′ region carried in each plasmid is indicated in rows 1 to 19. β-Gal activity was determined in extracts obtained from cells grown on either 0.2% ammonium sulfate or 0.1% glutamate. ND, not detected. Diagrams depict Gcn4p putative binding sites (○), palindrome (⎍), Gln3p putative binding sites (▷, ◁), poly(dA-dT) (▮), putative TATA boxes (T), transcription initiation sites (┌→, ⋮⋯›), and putative URRs.

(Fig. 3). Deletions from bp −608 to −413, −608 to −395, −608 to −381, and −608 to −373 (pLOU5 to -8) resulted in decreased β-Gal activity in both *GDH1* and *gdh1* strains, indicating that this region (−413 to −373) could contain DNA binding sites for transcriptional activators. As Fig. 1 shows, this region contained putative binding sites for Gcn4p, Gln3p, and a Zn_2-Cys_6 binuclear cluster activator. To determine whether the observed increase in β-Gal activity, conferred by pLOU4, was *GCN4*, *GLN3*, or *GAT1/NIL1* dependent, pertinent strains were transformed with this plasmid. Increased β-Gal activity was mainly *GLN3* and *GCN4* dependent (Fig. 4, row 2). Fig. 3 also shows that increased β-Gal activity was still glutamate sensitive, indicating that pLOU4 had retained a *cis*-acting region able to respond to glutamate. Further deletions (pLOU9 to -14) resulted in a constant increase of β-Gal activity in the *gdh1* derivatives but practically no changes in β-Gal activity of the corresponding *GDH1* strains. Deletions present in pLOU15 to -17 resulted in a clear increase of β-Gal activity in both *GDH1* and *gdh1* strains, the highest activity being observed after removal of the first 573 bp (pLOU17). β-Gal activity of constructions pLOU1 to -13 was clearly diminished by the presence of glutamate in the medium; however, when β-Gal was determined in strains harboring constructions pLOU14 to -17, although addition of glutamate to the medium reduced β-Gal activity, the values were severalfold higher than those found with the full promoter in cells grown in the presence of glutamate. These results suggested (i) that a glutamate-responsive negative-acting region (URR2) was lo-

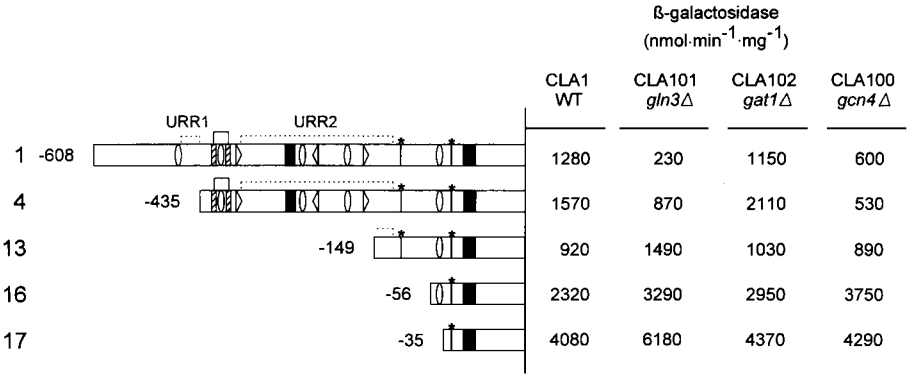


FIG. 4. Effects of *gln3*, *gat1*, and *gcn4* null mutations on β-Gal activity in 5′ deletions of the *GLT1* promoter. Mutant strains CLA101 (*gln3*Δ), CLA102 (*gat1*Δ), and CLA100 (*gcn4*Δ) harboring plasmids pLOU1, pLOU4, pLOU13, pLOU16, and pLOU17 (lines 1, 4, 13, 16, and 17) were grown on 0.2% ammonium sulfate as the nitrogen source, and β-Gal activity was determined. The reported β-Gal activities are averages of values obtained in three independent experiments. *GLT1* promoter regions are represented as in Fig. 3. Variations were <15%.

calized from positions −373 to −119 and (ii) that the region between −35 and +40 contained a target for a *trans*-acting positive regulatory element. As Fig. 3 shows, this DNA segment contained a poly(dA-dT) tract, which has been considered a promoter element able to stimulate transcription (47). In the presence of glutamate, transcription conferred by pLOU17 is diminished, although the β-Gal levels determined in this condition are threefold higher than those found under repressive conditions (MAR1/pLOU1 on glutamate). Thus, it is possible that the as yet undetermined activator, which we propose acts in combination with the poly(dA-dT) tract, could be glutamate inactivated. β-Gal activity fostered by pLOU16 and -17 was also found in *gln3*Δ, *gat1*Δ, and *gcn4*Δ null derivatives (Fig. 4), indicating that the poly(dA-dT) tract acted either independently of activators or was assisted by an as yet unrecognized positive regulatory element. This analysis suggested that *GLT1* transcriptional regulation depended on the action of both negative regulatory regions (URR1 and URR2) and positive-acting elements. Both URR regions could be targets for glutamate-mediated repression, since when they were removed, *GLT1* expression was no longer fully repressed by glutamate. In addition, our results indicate that of the putative *cis*-acting sites depicted in Fig. 1, the following could have a positive role in *GLT1* transcription: (i) the *GCN4*$^{(2)}$ binding site from positions −396 to −390, (ii) the *GLN3* binding site from −377 to −372 [GATA$^{(1)}$], (iii) the CGG palindromic region located from −412 to −388, and (iv) the poly(dA-dT) tract located from −2 to +17. No β-Gal activity was determined in strains carrying constructions present in pLOU18 and pLOU19, indicating that the promoter fragment contained from +40 to +100 was unable to initiate *GLT1* transcription.

In regard to the role of GOGAT in glutamate biosynthesis, our results indicate that (i) under low-glutamate conditions *GLT1* transcription is considerably low, which suggests that GOGAT may have an important role in glutamate biosynthesis under conditions where this amino acid becomes limiting; and (ii) GOGAT could constitute an ancillary pathway furnishing low but sustained glutamate production, even in the presence of $NADP^+$-GDH, i.e., in the presence of a relatively high glutamate pool, suggesting that a high intracellular glutamate pool may be needed for optimal growth. Since it has been reported that null GOGAT mutants grow as well as the wild-type strain on ammonium (2), the high glutamate need could be restricted to certain physiological conditions, such as high external osmolality (14), or during sporulation, since in this condition, both carbon and nitrogen are limiting and this could result in glutamate deprivation.

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