MOLECULAR AND CELLULAR BIOLOGY, Dec. 1984, p. 2767–2773
0270-7306/84/122767-07$02.00/0

Vol. 4, No. 12

Three Regulatory Systems Control Production of Glutamine Synthetase in *Saccharomyces cerevisiae*

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Received 30 April 1984/Accepted 24 August 1984

Production of glutamine synthetase in *Saccharomyces cerevisiae* is controlled by three regulatory systems. One system responds to glutamine levels and depends on the positively acting *GLN3* product. This system mediates derepression of glutamine synthetase in response to pyrimidine limitation as well, but genetic evidence argues that this is an indirect effect of depletion of the glutamine pool. The second system is general amino acid control, which couples derepression of a variety of biosynthetic enzymes to starvation for many single amino acids. This system operates through the positive regulatory element *GCN4*. Expression of histidinol dehydrogenase, which is under general control, is not stimulated by glutamine limitation. A third system responds to purine limitation. No specific regulatory element has been identified, but derepression of glutamine synthetase is observed during purine starvation in *gln3 gcn4* double mutants. This demonstrates that a separate purine regulatory element must exist. Pulse-labeling and immunoprecipitation experiments indicate that all three systems control glutamine synthetase at the level of subunit synthesis.

Glutamine synthetase is encoded by the *GLN1* gene in *Saccharomyces cerevisiae* (Mitchell, submitted for publication). This enzyme is the sole route of synthesis of glutamine, because *gln1* point mutations result in an absolute glutamine auxotrophy. Glutamine synthetase levels may vary over a 150-fold range, and these steady-state levels reflect the rate of synthesis of the enzyme subunit (16, 17a). Studies of *gln1* mutants suggest that this regulation responds to glutamine, rather than ammonia availability. Regulation at the level of synthesis is superimposed upon a reversible inactivation reaction (12; Mitchell and Magasanik, J. Biol. Chem. in press).

When *S. cerevisiae* is shifted from a medium containing glutamine to one containing glutamate as the sole nitrogen source, the intracellular levels of a family of proteins and enzymes are greatly increased (17). In addition to glutamine synthetase itself, this family includes four electrophoretically identified proteins and NAD-dependent glutamate dehydrogenase activity. The response to the shift is largely complete within 10 min at 30°C, but these proteins are synthesized continuously in the absence of glutamine and are stable cellular constituents. Although glutamine synthetase and NAD-dependent glutamate dehydrogenase activity function as glutamine biosynthetic enzymes, the cellular role of the other proteins has not been determined.

The coordinate response of this family depends on the *GLN3* product, because *gln3* mutations block the increased expression of all six gene products (17). However, glutamine synthetase expression is still regulated to a limited extent in *gln3* mutants. As a consequence, *gln3* lesions do not cause an absolute glutamine auxotrophy. Here we present an investigation into the nature of this remaining regulatory capacity.

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MATERIALS AND METHODS

Strains. The *S. cerevisiae* strains used in this work are listed in the accompanying paper (17) and in Table 1. All mutations in Σ1278b-derived strains are described elsewhere (17; Mitchell, submitted for publication). *GCN4* and *GCD1* were previously known as *AAS3* and *TRA3*, respectively (13a). Strains carrying either wild-type or mutant alleles at these loci within the S288C genetic background were provided by Alan Hinnebusch, National Institutes of Health, Bethesda, Md. The *his4C* tester 5942-1D was obtained from Cora Styles, Massachusetts Institute of Technology. Strain construction was by standard procedures of crossing, sporulation, tetrad dissection, and meiotic analysis as described previously (16; Mitchell, submitted for publication).

Media and culture conditions. Details of media preparation and composition are described elsewhere (Mitchell, submitted for publication). Ggln, Gglt, GN, and GNglt all contained 2% (wt/vol) glucose, 0.34% yeast nitrogen base (Difco Laboratories, Detroit, Mich.) without amino acids and ammonium sulfate; they contained 0.2% glutamine, 0.128% sodium glutamate, 0.2% ammonium sulfate, and 0.2% ammonium sulfate and 0.128% sodium glutamate, respectively. All supplements were added at 20 mg/liter, except that S288C-derived Leu$^-$ strains received 250 mg of leucine per liter, 65 mg of isoleucine per liter, and 60 mg of valine per liter, and S288C-derived His$^-$ strains were supplemented with 40 mg of histidine hydrochloride per liter. Strains carrying *gcn4-101* exhibit a slight arginine auxotrophy so these strains, as well as *GCN4*$^+$ strains in parallel experiments, received 105 mg of arginine hydrochloride per liter (G. Lucchini, personal communication). 3-Amino-1,2,4-triazole (3AT) was added to 10 mM final concentration (19). C supplements were adenine sulfate, guanine sulfate, uracil, cytosine, histidine hydrochloride, arginine hydrochloride, asparagine, and tryptophan, each added at 20 mg/liter. The composition of YPD is described in the accompanying paper.

Procedures for growing, shifting, and harvesting cultures were as described elsewhere (16; Mitchell, submitted for publication).

TABLE 1. Strains

Strain	Genotype
Σ1278b derivatives	
269-5D	α *lys-23 ade2-102*
673-8B	α *ura3-77*
699-1B	a *his4-42*
727-8D	a *ade2-102 gln3-1*
727-17C	a *ade2-102*
734-18B	a
734-19A	a *gln3-2*
735-7D	α *ura3-77 gln3-1*
739-5A	α *gln3-1 gln1-7*
745-1A	α *gln1-37*
S288C derivatives	
AH2	α *leu2-3 leu2-112 his4-519 can1*
5942-1D	α *his4-864*
6288-5A	a *leu2-3 leu2-112 can1*
9043-2C	α-*leu2-3 leu2-112 his4-519 can1 gcd1-101*

Measurement of glutamine synthetase. The preparation of crude extracts and their use in measuring glutamine synthetase transferase specific activity have been described previously (16).

For experiments involving induction kinetics it was more convenient to measure transferase activity in liquid nitrogen-permeabilized cells (1). A culture sample was harvested by filtration onto a 0.45μm membrane filter (Millipore Corp., Bedford, Mass.) and washed with ice water. The filter was submerged in liquid nitrogen for 12 s and then stored overnight in a sealed tube at −70°C. Transferase activity was measured after thawing the tubes for 15 to 30 min at 4°C by adding 0.4 ml of transferase assay mix (16), incubating at 30°C for up to 30 min, and then terminating the reaction with 1.0 ml of stop mix (18). Cells were pelleted for 10 min at top speed in a Sorvall GLC-2B centrifuge, and the absorbance of the supernatant at 540 nm was determined. This assay varied linearly with time and cell number until an absorbance over 0.7 was reached.

Graphs of induction kinetics reflect the differential rate of accumulation of glutamine synthetase, μ, which is the slope of a plot of enzyme activity per ml of culture versus cell density, that is, $E = \mu(K - K_o) + E_o$ where E and K are the enzyme activity per milliliter of culture and the cell density in Klett units, respectively, and E_o and K_o are these quantities at the start of the experiment. Slight differences in K_o can make it difficult to compare two parallel cultures on a single graph. However, dividing both sides of the equation by K_o yields $E/K_o = \mu[K/K_o - 1] + E_o/K_o$. Thus the transferase activity per milliliter axis in our figures is E/K_o, and the culture density axis is K/K_o. It should be noted that all shifts were accomplished during exponential growth between 50 and 80 Klett units.

Measurement of histidinol dehydrogenase. Histidinol dehydrogenase was measured essentially as described by Martin et al. (14). Extracts were prepared by vortexing with glass beads (16) into 20 mM Tris (pH 7.6)–0.1 M NaCl–5 mM dithiothreitol–0.02 mM histidinol hydrochloride containing 1 mM phenylmethylsulfonyl fluoride diluted from a 20 mM stock in dimethyl sulfoxide. Debris was pelleted by centrifugation in an Eppendorf microfuge at 4°C for 15 min, and this supernatant was used for enzyme assays. A 1-ml reaction at 25°C contained 20 to 100 μg of crude extract protein, 0.18 M Tris (pH 8.9), 2.5 mM NAD, 0.5 mM $MnCl_2$, and 2 mM histidinol hydrochloride. The reduction of NAD was monitored continuously at 340 nm, subtracting a blank reaction without histidinol, with a Zeiss PM6 recording spectrophotometer. The activity of a given extract was determined in at least two samples differing by twofold in amount of protein. The two estimates of specific activity agreed to within 20%. Control reactions with extracts of the *his4-42*-bearing strain 699-1B, grown on GN+His, yielded a value of 2 nmol of NADH formed per min per mg of protein, which was less than 1/30 of the specific activity found in MB1000 (*HIS4*) in a parallel culture. The *his4-42* mutation was shown to be *his4C* defective (histidinol dehydrogenase deficient) by its failure to complement the *his4-864* mutation present in strain 5942-1D.

Immunoprecipitation of glutamine synthetase subunits. A description of the immunological isolation of denatured glutamine synthetase subunits with rabbit anti-glutamine synthetase antiserum and protein A–Sepharose CL-4B is presented elsewhere (16; Mitchell, submitted for publication). This procedure was modified to eliminate background from proteins non-specifically adsorbed to Sepharose by treating samples for immunoprecipitation with 20 μl of Sepharose 4B before the addition of antiserum. The Sepharose was pelleted in a Eppendorf microfuge, and the supernatant was used for further analysis.

Miscellaneous. Protein determinations were by the method of Lowry et al. (13). NAD-dependent glutamate dehydrogenase activity was measured as described in the accompanying paper (17). The procedures for pulse-labeling, estimating incorporated radioactivity, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography were described by Mitchell and Magasanik (16).

Histidinol hydrochloride and 3AT were purchased from Aldrich Chemical Co., Inc., Milwaukee, Wis., and glutamine was A grade from Calbiochem-Behring, La Jolla, Calif. Other chemicals were from standard commerical sources.

RESULTS

In *S. cerevisiae*, the level of glutamine synthetase is at least 100-fold higher during growth on glutamate medium than it is in the presence of exogenous glutamine. Mutations at the *GLN3* regulatory locus, unlinked to the enzyme's structural gene, reduce this derepressed glutamine synthetase level by a factor of 10. This level of expression may be still further reduced by providing the mutants with exogenous glutamine. The remaining regulatory capability is probably not due to partial functioning of *gln3* products because it is exhibited by strains carrying the amber-suppressible *gln3-1* allele (17).

We considered the possibility that the derepression of the enzyme observed when the *gln3* mutants were grown on glutamate was due to systems responding to the levels of glutamine-derived metabolites, such as purines, pyrimidines, and the amino acids arginine, asparagine, histidine, and tryptophan, rather than to glutamine itself. Indeed, when a *gln3-1* mutant was shifted from Ggln to Gglt, with or without various supplements, the differential rate of glutamine synthetase accumulation was reduced twofold by providing either a mixture of purines and pyrimidines or the four glutamine-derived amino acids (Fig. 1). When all of these compounds were provided together, glutamine synthetase was almost completely repressed, although a wild-type growth rate was not restored. Thus it appears that glutamine-derived metabolites can repress glutamine synthetase in a *gln3* background.

If such *GLN3*-independent regulatory systems existed, then starvation for one of the relevant glutamine-derived metabolites should derepress glutamine synthetase even if

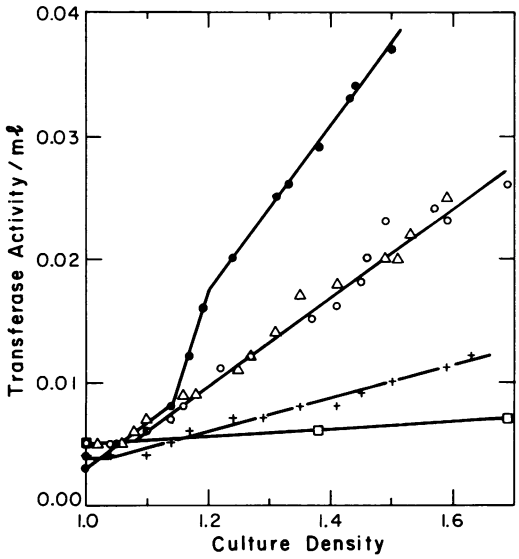

FIG. 1. Effects of glutamine-derived metabolites on *gln3-1* glutamine synthetase expression. 570-3C (*gln3-1*) was grown to the midexponential phase on Ggln, and culture samples were shifted to fresh Ggln (□), Gglt (●), Gglt-Ade-Gua-Ura-Cyt (△), Gglt-Arg-Asn-His-Trp (○), or Gglt with all eight supplements (+). Transferase activity was measured in permeabilized cells and plotted against the cell density increase.

the *GLN3* system is inactivated by mutation or by providing exogenous glutamine. This prediction was used to identify specific regulatory circuits comprising the alternative glutamine synthetase regulatory systems.

Regulation by purines and pyrimidines. The participation of purine control in glutamine synthetase regulation was investigated as follows. Adenine-requiring strains of genotype *GLN3*$^+$ *ade2* or *gln3-1 ade2* were shifted from Ggln-Ade-Gua to Ggln, with or without adenine and guanine, and glutamine synthetase levels were monitored (Fig. 2). Purine starvation of either strain resulted in a 10-fold increase in the rate of glutamine synthetase accumulation. The activity that accumulated was not due to the expression of a glutamine synthetase isozyme because it was not found in adenine-starved *gln1* mutants, whose lesions lie in the structural gene for the one identified glutamine synthetase (data not shown). Therefore purine starvation causes *GLN3*-independent derepression of glutamine synthetase. Providing *ade2* mutants with guanine alone did not block the derepression (data not shown). Thus AMP or a derivative is likely to be the metabolite to which this system responds.

Starvation of a *ura3* mutant for pyrimidines also derepressed glutamine synthetase, but this response was entirely *GLN3* dependent (Fig. 3). In the reciprocal experiment, glutamine synthetase was produced at the same rate in a *gln3-1* mutant shifted from Ggln to either Gglt or Gglt-Ura-Cyt (data not shown). One possible explanation for these observations is that the *GLN3* system senses both pyrimidine and glutamine levels and responds to a drop in either one by stimulating glutamine synthetase production. An alternative hypothesis takes into account the extreme feedback sensitivity of carbamyl phosphate synthetase (CPSase), the first enzyme of pyrimidine biosynthesis, and its use of glutamine as a substrate (11). Specifically, pyrimidine starvation would relieve the feedback inhibition, causing accelerated glutamine consumption. The resulting depletion of the glutamine pool would then lead to *GLN3*-mediated derepression of glutamine synthetase. The latter explanation predicts that pyrimidine starvation of Ura$^-$ mutants that lack the pyrimidine-specific CPSase would not derepress glutamine synthetase because such mutations eliminate the means to accelerate depletion of glutamine reserves. According to the first hypothesis, pyrimidine starvation of any Ura$^-$ mutant should derepress glutamine synthetase.

Glutamine synthetase levels were therefore examined in several independently isolated Ura$^-$ mutants subjected to pyrimidine starvation (Fig. 4). CPSase is encoded by the bifunctional *URA2* locus, and a previous study indicated that only two-thirds of all Ura$^-$ mutations at *URA2* eliminate this activity (4). In fact, 5 of 12 *ura2* mutants failed completely to raise glutamine synthetase levels during pyrimidine starvation, and an additional 5 mutants were partially defective in this response. In each of the three most extremely impaired mutants (leftmost bars in Fig. 4A), glutamine synthetase levels were normal during growth on

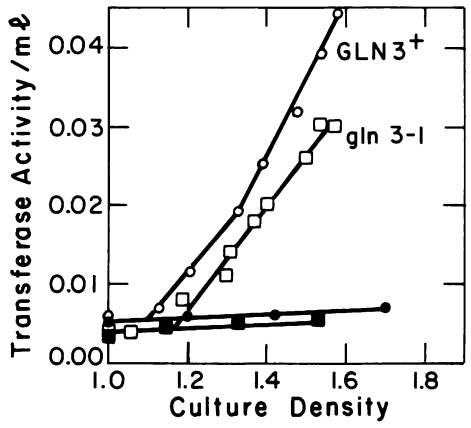

FIG. 2. Derepression of glutamine synthetase during purine starvation. 727-8D (*ade2-102, gln3-1*; □ and ■) and 727-17C (*ade2-102 GLN3*$^+$; ○ and ●) were grown to the midexponential phase in Ggln-Ade-Gua and shifted to Ggln-Ade-Gua (■ and ●) or to Ggln (□ and ○). Transferase activity was measured in permeabilized cells and plotted against the cell density increase.

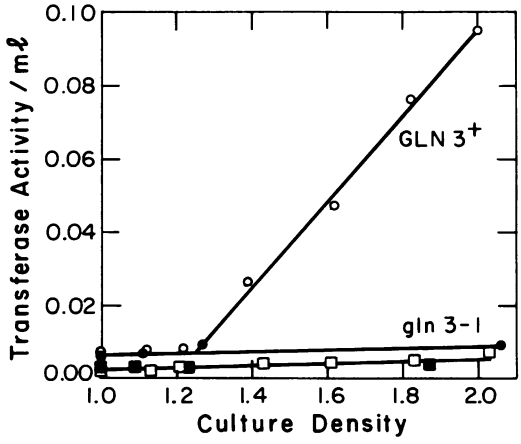

FIG. 3. Derepression of glutamine synthetase during pyrimidine starvation. 735-7D (*ura3-77 gln3-1*; □ and ■) and 673-8B (*ura3-77 GLN3*$^+$; ○ and ●) were grown to the midexponential phase in Ggln-Ura and shifted to Ggln-Ura (■ and ●) or to Ggln (□ and ○). Transferase activity was measured in permeabilized cells and plotted against the cell density increase.

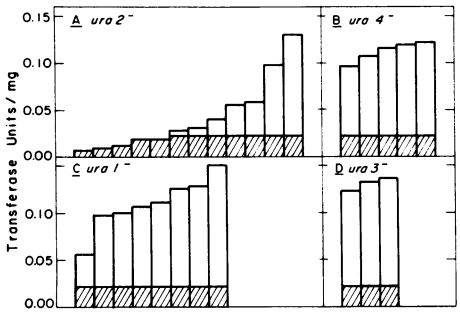

FIG. 4. Glutamine synthetase derepression upon pyrimidine starvation of different Ura⁻ mutants. Twenty-eight independent ethylmethane sulfate-induced, uracil-requiring mutants derived from 269-5D were grown to the midexponential phase in Ggln-Ura-Lys-Ade and shifted to Ggln-Lys-Ade for 4 to 5 h, and then transferase specific activity was determined in crude extracts. Each bar represents the results obtained with a single mutant. The shaded area represents the specific transferase activity of 269-5D after the same treatment. The mutants carried lesions at the loci (A) *URA2*, (B) *URA4*, (C) *URA1*, and (D) *URA3*.

Glt, demonstrating the specificity of the defect, and six of six Ura⁻ meiotic progeny from crosses to a Ura⁺ strain were defective in pyrimidine starvation-dependent glutamine synthetase derepression, demonstrating the genetic identity of the two phenotypes (data not shown). Of 16 mutants with blocks later in pyrimidine biosynthesis, only one was impaired in derepression (Fig. 4B, C, and D). This phenotype did not cosegregate with the Ura⁻ phenotype through meiosis and therefore was due to a coincidental secondary mutation. These results support the second hypothesis: that glutamine synthetase derepression during pyrimidine starvation is a consequence of glutamine degradation by CPSase.

Regulation by amino acids. Initially, we observed increased glutamine synthetase levels in response to either histidine or leucine starvation. Because leucine has no biosynthetic relation to glutamine, a signal more global than simply the levels of glutamine-derived amino acids was implicated. Yeast cells possesses a general amino acid control system which couples derepression of a diverse set of amino acid biosynthetic enzymes to starvation for any one of several amino acids, including histidine and leucine (reviewed in reference 9). Therefore we examined the effects of the *gcn4-101* mutation, which blocks derepression through general amino acid control, and the *gcd1-101* mutation, which renders the derepression constitutive (19).

Treatment of wild-type strains with 3AT causes histidine starvation and results in a 10-fold elevation of glutamine synthetase levels within one generation, in the presence of exogenous glutamine (Table 2). The *gcn4-101* mutation blocked this response, indicating that it depends on the general amino acid control system. This was corroborated by the finding that *gcd1-101* prevented complete repression of glutamine synthetase. The *gcn4-101* mutation did not block the elevated expression of the enzyme during growth on Ggln; the slightly reduced glutamine synthetase activity in the mutant did not cosegregate with *gcn4-101* through meiosis (data not shown). Thus accumulation of glutamine synthetase specifically during histidine starvation depends on general amino acid control.

Glutamine synthetase was also derepressed in *gln3* mutants after treatment with 3AT, but the extent of derepression was more limited than in *GLN3*⁺ strains (Table 2). All transferase activity was due to the one identified glutamine synthetase, because it was abolished by the *gln1-37* mutation. *GLN3* does not play a global role in general control, because histidinol dehydrogenase, an enzyme regulated solely by general control (5, 9), is derepressed normally in a *gln3* background (Table 3). In fact, glutamine starvation of a strain carrying the leaky *gln1-7* mutation had little effect on histidinol dehydrogenase (Table 3, GNglt containing C supplements and without glutamine). This suggests that *GLN3* activity does not influence general amino acid control directly.

How does a general control response stimulate the *GLN3* system? One simple explanation is that there is a reduction in the size of the cytoplasmic glutamine pool, potentially through effects on metabolism, compartmentation, or uptake. This effect would be more pronounced in a *gln1* mutant because it is incapable of endogenous glutamine production. To assess *GLN3* activity in a *gln1* mutant, we made use of the *gln1-7* mutation. This blocks almost all glutamine biosynthetic activity but does not eliminate the enzyme's transfer-

TABLE 2. Response of glutamine synthetase to general amino acid control stimuli[a]

Strain	Relevant genotype	General control phenotype	GS transferase (U/mg)		
			Ggln	Ggln plus 3AT[b]	Glt
S288C derivatives					
6288-5A	*GCN4*⁺	Wild type	0.03	0.29	1.39
L869	*gcn4-101*	Nonderepressible	0.02	0.02	0.86
AH2	*GCD1*⁺	Wild type	0.06		2.25
9043-2C	*gcd1-101*	Constitutively derepressed	0.38		1.46
Σ1278b derivatives					
739-6C	*GLN3*⁺		0.02	0.21	
739-13C	*gln3-1*		0.01	0.04	
734-19A	*gln3-2*		0.01	0.04	
745-1A	*gln1-37*		0.01	0.01	

[a] Midexponential cultures growing on the media indicated were harvested, and glutamine synthetase (GS) specific activity was determined in crude extracts.
[b] Duration of drug treatment was 0.6 generations.

TABLE 3. Role of *GLN3* and glutamine in general amino acid control[a]

Strain	Relevant genotype	Histidinol dehydrogenase (nmol of NADH per min per mg)			
		Ggln		GNglt plus C supplements	
		−3AT	+3AT	+Gln	−Gln
734-18B	*GLN3*⁺	38	118		
734-19A	*gln3-2*	49	94		
739-6C	*GLN1*⁺	36	103	32[b]	23[b]
739-1D	*gln1-7*	34	92	32[b]	37[c]

[a] Midexponential cultures growing on the media indicated were harvested, and histidinol dehydrogenase specific activity was determined in crude extracts. The C supplements included arginine, asparagine, histidine, tryptophan, adenine, guanine, uracil, and cytosine.
[b] Doubling time was 120 min.
[c] Doubling time was 270 min.

TABLE 4. Physiological contribution to the general control response of glutamine synthetase[a]

Strain	Relevant genotype	GS transferase (U/mg)		NAD-dependant glutamate dehydrogenase (nmol/min per mg)	
		Ggln	Ggln plus 3AT	Ggln	Ggln plus 3AT
739-13C	*gln3-1 GLN1*$^+$	0.02	0.09	2	6
739-5A	*gln3-1 gln1-7*	0.02	0.07	2	4
739-6C	*GLN3*$^+$ *GLN1*$^+$	0.03	0.41	2	5
739-1D	*GLN3*$^+$ *gln1-7*	0.03	1.21	3	15

[a] Midexponential cultures growing on the media indicated were harvested, and glutamine synthetase GS and NAD-dependent glutamate dehydrogenase specific activities were determined in crude extracts.

ase activity. Transferase activity has no known physiological function but served as a measure of *GLN1* expression.

In a *gln3-1* background, the *GLN1*$^+$ and *gln1-7* enzymes responded to the same extent to 3AT treatment (Table 4), indicating that the *GLN3*-independent component of general control was unaffected by *gln1-7*. However, in the *GLN3*$^+$ background, expression of the *gln1-7* enzyme was stimulated to a threefold greater extent than that of the *GLN1*$^+$ enzyme by treatment with 3AT. That the increased transferase activity actually represented increased glutamine synthetase antigen levels was confirmed by immunological blotting (data not shown).

To be certain that the hyper-derepression of the *gln1-7* enzyme was not due to some unusual *cis*-acting feature of this allele, NAD-dependent glutamate dehydrogenase levels were also measured (Table 4). This enzyme is under both general control (2, 3, 9) and *GLN3* control (17). Indeed, two- or threefold stimulation of the activity by 3AT was observed in the *gln3-1* strains and in the *GLN3*$^+$ *GLN1*$^+$ strain, but treatment of the *GLN3*$^+$ *gln1-7* strain with 3AT caused a fivefold elevation of the activity. No such phenomenon was observed with histidinol dehydrogenase, which is under general control but not *GLN3* control (Table 3). These observations support the view that *GLN3* is stimulated by reduced glutamine pools during a general control response.

To examine independence of purine control from both general control and the *GLN3* system, we crossed strains carrying *gcn4-101* and *gln3-1* and measured the extent of glutamine synthetase derepression after adenine starvation in their meiotic progeny. Several segregants of each genotype were examined to avoid focusing on the contribution of non-isogenicity of the parent strains. All progeny accumulated increased glutamine synthetase levels during adenine starvation, including the *gln3-1 gcn4-101* double mutants (Fig. 5). Therefore the response to purine starvation operates independently of the other two systems.

Regulation at the level of synthesis. To see whether glutamine synthetase derepression through these alternative systems involved de novo synthesis of glutamine synthetase subunits, *GLN3*$^+$ and *gln3-1* strains were pulse-labeled with [^{35}S]methionine during adenine starvation or 3AT treatment. Extracts were examined by immunoprecipitation with anti-glutamine synthetase antiserum, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography (Fig. 6). Adenine starvation resulted in a 10- to 20-fold increase in the rate of glutamine synthetase synthesis in both strains, and 3AT brought about 20- and 3-fold increases in the *GLN3*$^+$ and *gln3-1* strains, respectively. The agreement between these values and the increases in enzyme activity indicate that both general amino acid control and purine control

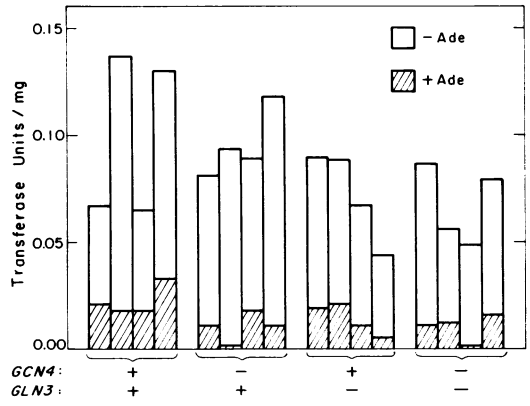

FIG. 5. Derepression of glutamine synthetase after purine starvation of *gln3* and *gcn4* mutants. *ade2*-bearing meiotic segregants from a cross of L869 (*gcn4-101 ADE2*$^+$ *GLN3*$^+$) and 736-17A (*GCN4*$^+$ *ade2 gln3-1*) were grown to the midexponential phase in Ggln-Ade-Leu-Ile-Val (leucine, isoleucine, and valine were included to supplement other auxotrophies). One sample of each culture was removed to determine transferase specific activity (□), and the remainder was shifted to Ggln-Leu-Ile-Val for 5 h before a second transferase measurement was made (□). Each bar represents an experiment with a different segregant. The alleles at the *GLN3* and *GCN4* loci of each segregant are indicated under the figure. It should be noted that the parent strains were not isogenic.

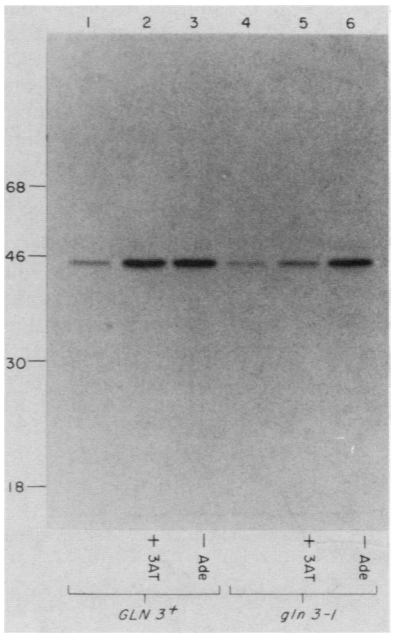

FIG. 6. Regulation of glutamine synthetase subunit synthesis by alternative regulatory pathways. 727-17C (*ade2-102 GLN3*$^+$, lanes 1 through 3) and 727-8D (*ade2-102 gln3-1*, lanes 4 through 6) were grown to the midexponential phase in Ggln-Ade and shifted to Ggln-Ade (lanes 1 and 4), Ggln-Ade-3AT (lanes 2 and 5), or Ggln (lanes 3 and 6). After 0.5 generations, each culture was pulse-labeled with [^{35}S]methionine for 5 min. Samples of 4 × 10^6 cpm of each extract were analyzed by immunoprecipitation, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and fluorography. The exposure time was 4 days. The numbers on the left indicate approximate molecular weight (× 10^3).

TABLE 5. Physiological contribution to end product repression of glutamine synthetase[a]

Strain	Relevant genotype	GS transferase (U/mg) in Gglt plus:				
		0.1% Gln	0.002% Gln	C supplements	Ammonia and C supplements	
					+Gln	−Gln
739-6C	*GLN1*$^+$	0.03	2.59	0.80	0.02	0.41
739-1D	*gln1-7*	0.03	3.48	3.40	0.03	3.45

[a] Cells were grown overnight on Ggln and then pelleted, washed twice with water, and inoculated into the media indicated. After at least 2.5 generations, the cultures were harvested, and glutamine synthetase (GS) specific activity was determined in crude extracts. C supplements included arginine, asparagine, histidine, tryptophan, adenine, guanine, uracil, and cytosine.

regulate glutamine synthetase at the level of de novo synthesis.

Physiological contribution to repression by glutamine-derived metabolites. Glutamine synthetase is derepressed 100-fold by growth on glutamate (Table 5). This reflects the functioning of the *GLN3* regulatory system and, perhaps, some contribution of the systems responding to glutamine-derived metabolites. Indeed, when the medium is supplemented with purines, pyrimidines, and glutamine-derived amino acids (C supplements), the glutamine synthetase level is only 30% of its maximal value. These supplements may affect glutamine synthetase levels through two mechanisms. In addition to direct effects on the enzyme levels through their respective regulatory systems, the supplements act to decrease the rate of glutamine utilization through inhibition and repression of their respective biosynthetic enzymes. A consequent increase in the glutamine pool would reduce the activity of the *GLN3* system and further reduce glutamine synthetase levels.

To assess the magnitude of these two effects, transferase activity was measured in a *gln1-7* mutant supplemented with the glutamine-derived metabolites, but without glutamine. As mentioned above, the *gln1-7* enzyme is defective only in biosynthetic activity. Because the mutant grows slowly unless glutamine itself is provided, any repression of transferase activity by the glutamine-derived metabolites could reflect only a direct regulatory contribution and not a physiological glutamine buildup. The data in Table 5 show that the *gln1-7* enzyme is expressed at its maximal level when the glutamine-derived metabolites are provided. The observation that only exogenous glutamine represses the mutant enzyme suggests that glutamine levels alone are responsible

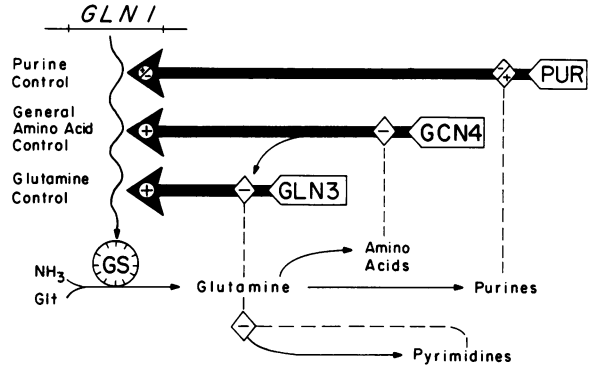

FIG. 7. Regulatory circuits controlling glutamine synthetase synthesis. See the text for details.

for most of the regulation of glutamine synthetase production. This result has been corroborated by the very limited repression of several structurally altered, defective glutamine synthetases during growth on the glutamine-free rich medium YPD (data not shown).

DISCUSSION

The regulation of production of glutamine synthetase by three distinct systems is summarized in Fig. 7. These regulatory circuits reflect the major metabolic fates of glutamine and underscore the large number of biosynthetic pathways in which glutamine is an obligate participant.

Glutamine synthetase levels may vary around 100-fold in response to glutamine control alone. This system is responsible for virtually all of the glutamine synthetase production observed during extreme glutamine limitation. The assignment of *GLN3* to this circuit is based on two observations. One is that *gln3* mutations eliminate most of the range of expression of glutamine synthetase (17), which correlates with the relative contribution of glutamine control. Also, glutamine-derived metabolites are able to repress glutamine synthetase almost completely in a *gln3* background, even though such mutants remain glutamine starved, indicating that it is the limitation for these metabolites that is responsible for regulation of glutamine synthetase when *GLN3* function is lost.

Derepression of glutamine synthetase upon pyrimidine starvation depends on both *URA2* and *GLN3* functions. *URA2* encodes the first and second enzymes of pyrimidine biosynthesis, CPSase and aspartate transcarbamylase (10). Uracil-requiring *URA2* mutants must lack the latter activity, because loss of the *URA2* CPSase alone is compensated by the arginine-specific CPSase encoded by *CPA1* and *CPA2* (4, 9, 11). Thus the finding that two of the *ura2* mutants examined derepressed glutamine synthetase fully upon pyrimidine starvation indicates that aspartate transcarbamylase activity does not influence this derepression. If the response of glutamine synthetase depends on *URA2* but not on aspartate transcarbamylase activity, then CPSase must be the required function. The simplest assembly of these observations is that *URA2* CPSase depletes glutamine pools when its feedback inhibition is relieved, and the reduced glutamine levels stimulate the *GLN3* system.

Derepression of glutamine synthetase through general amino acid control depends entirely on the positively acting *GCN4* element. However, *GLN3* function amplifies the response of glutamine synthetase specifically, suggesting that there is some interaction between the two systems. The hyper-derepression of *GLN3*-controlled functions during the general control response of a *gln1-7* mutant indicates that the amount of intracellular glutamine is decreased when the general control system is activated. Indeed, Messenguy et al. found that starvation of a leaky histidine auxotroph resulted in reduced glutamine+asparagine pools, although this observation was made in a glutamine-free medium (15). We have also observed reduced glutamine uptake rates in response to 3AT (Mitchell, unpublished data). Thus it seems likely that the operation of the *GCN4* and *GLN3* regulatory elements is independent, but the physiological consequences of the general control response activate the *GLN3* system.

A previous study concluded that glutamine synthetase was not regulated by general control (19). Because these observations were made with cells grown on a glutamine-free medium, it is likely that the background of glutamine synthetase derepression through *GLN3* activity obscured any further elevation of enzyme levels in response to 3AT

addition or the *gcd1-101* mutation. Detection of the response in our experiments was facilitated both by using a completely repressing medium, Ggln, and by the amplification of the response through the *GLN3* system.

Purine starvation derepresses glutamine synthetase through a third system. Though no regulatory element has yet been identified, the independence of this response from glutamine and general control is indicated by its expression in *gln3-1 gcn4-101* double mutants. Thus the possibility that adenine limitation only stimulates general control through a resultant histidine starvation is ruled out. Whether these hypothetical *PUR* regulatory proteins act positively or negatively cannot be determined from our results.

One simple model to explain how *GLN1* regulation is achieved is that the *GLN1* region has multiple upstream activation sites (5, 7, 8). These elements promote transcription initiation at nearby TATA boxes with relatively flexible distance requirements. Thus the *GLN1* upstream region should contain three different types of elements, each being a signal for one of the regulatory systems. Recent work on the regulatory region of the yeast *CYC1* gene indicates the presence of two independent upstream elements (6). Current studies in this laboratory on the molecular biology of *GLN1* will test this model directly.

ACKNOWLEDGMENTS

We thank Teresa Keng and Peter Drain for their interest and comments, and Al Hinnebusch, Giovanna Lucchini, and Gerry Fink for their enthusiasm and for making strains and information easily accessible. We are grateful to Elizabeth Jones for her insight into the coupling of glutamine synthetase regulation and pyrimidine biosynthesis. We greatly appreciate the assistance of Hilda Harris-Ransom in the preparation of this manuscript.

This investigation was supported by Public Health Service grants GM07446 and AM13894 from the National Institute of General Medical Sciences and the National Institute of Arthritis, Metabolism and Digestive Diseases, respectively, by grant PCM-7808576 from the National Science Foundation, and by a predoctoral fellowship from the National Distillers and Chemical Foundation.

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