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The *GLN3* Gene Product Is Required for Transcriptional Activation of Allantoin System Gene Expression in *Saccharomyces cerevisiae*

TERRANCE G. COOPER,* DENISE FERGUSON,† RAJENDRA RAI, AND NANDA BYSANI

Department of Microbiology and Immunology, University of Tennessee, Memphis, Memphis, Tennessee 38163

Received 22 August 1989/Accepted 10 November 1989

We show that mutation at the *GLN3* locus results in decreased steady-state levels of *DAL7*, *DUR1,2*, *CAR1*, and *URA3* mRNAs derived from cultures grown in the presence of inducer. Basal levels of these RNA species, however, were not significantly affected by a *gln3* mutation. The *GLN3* product appears to affect gene expression in two ways. The pleiotropic requirement of *GLN3* for induced gene expression probably derives from the need of the *GLN3* product for inducer uptake into the cell and its loss in *gln3* mutants. We also demonstrate that transcriptional activation, mediated by the *DAL5* and *DAL7* upstream activation sequences, requires a functional *GLN3* gene product. This observation identified transcriptional activation as the most likely point of *GLN3* participation in the expression of allantoin system genes.

Saccharomyces cerevisiae can discriminate between various nitrogen sources (3) and selectively utilize those that are most easily assimilated. For example, if glutamate and proline are simultaneously provided to a wild-type culture of *S. cerevisiae*, glutamate will be preferentially used until it is exhausted from the medium. Only then will the less easily assimilated nitrogen source, proline, be transported into the cells and metabolized (18). In a way that is not yet understood, the availability of readily assimilated nitrogen sources (glutamate, in this example) prevents the appearance of enzyme and transport activities needed to assimilate less readily used nitrogen sources. This phenomenon is called nitrogen catabolite repression (NCR) (3). The earliest studies of NCR demonstrated that catabolic enzymes and transport systems such as arginase (*CAR1*), urea amidolyase (*DUR1,2*), and allantoate permease (*DAL5*) were present at high levels when a slowly metabolized compound (proline, allantoin, or ornithine) was provided as the sole nitrogen source (3). In contrast, when a readily used nitrogen source (ammonia, glutamine, or asparagine) was provided, these enzyme activities dropped to undetectable levels (3). The same physiological response was subsequently observed for the steady-state levels of *DAL2*, *DAL3*, *DAL4*, *DAL7*, *DUR1,2*, and *CAR1* mRNAs; they precipitously decreased when a readily used nitrogen source was provided (7, 12, 16, 20). These observations collectively led to the suggestion that NCR was exerted at the level of gene expression (7, 12, 16, 20). This suggestion has been recently confirmed for the *DAL5* and *DAL7* genes, which have been shown to possess upstream activation sequences (UASs) situated 5′ to their transcribed regions. The UASs have been shown to be required for *DAL5* and *DAL7* gene expression (13, 19) and to support transcriptional activation in heterologous expression vectors that is highly sensitive to NCR (4). These results demonstrated that NCR occurs at transcription and identified the *DAL* UAS as the element through which NCR of the *DAL5* and *DAL7* genes is exerted.

The *GLN3* gene product has been proposed to be a component in the NCR regulatory circuit (5). Mutants with lesions at this locus were first isolated by Mitchell and Magasanik (10, 11) as strains possessing low levels of glutamine synthetase. During their genetic characterization of the *gln3* locus, these investigators observed that *gln3* mutants contained low levels of NAD-specific glutamate dehydrogenase activity. They also made the preliminary observation that crude extracts from *gln3* mutants lacked a 204-kilodalton (kDa) protein species under conditions of nitrogen starvation (10, 11). It was suggested that the missing 204-kDa species was urea amidolyase, which had been reported to exhibit a similar apparent molecular mass (15). Subsequently, Courchesne and Magasanik measured enzyme activity levels for NAD-specific glutamate dehydrogenase, glutamine synthetase, and arginase in wild-type and *gln3* mutant cells (5). High-level production of these enzyme activities was observed to require the *GLN3* product (5). However, whether *gln3* functioned at the transcriptional or posttranscriptional level to modulate these enzyme levels was not addressed. This is an important consideration because the *CAR1* gene encoding arginase has been reported to be posttranscriptionally regulated (9).

It is difficult to rigorously assess at this time whether the *GLN3* product plays a direct role in NCR. However, if it does, that role should be assayable at the level of *DAL* and *CAR* gene expression. This hypothesis prompted us to determine the steady-state levels of *DUR1,2*, *DAL7*, *DAL5*, and *CAR1* mRNAs in wild-type and *gln3* mutant strains. These experiments were followed by assays that measured the ability of the allantoin (*DAL*) system UAS to mediate transcriptional activation in wild-type and *gln3* mutant strains. The results we obtained suggest that the *GLN3* product is required for transcriptional activation of the *DAL* system genes mediated by the *DAL* UAS.

(A preliminary report of this work has already appeared [T. Cooper, D. Street, R. Rai, F. Genbauffe, and H. Yoo, 14th Int. Conf. Yeast Gen. Mol. Biol., Helsinki, Finland, 8 to 13 August 1988, abstr. no. L11, Yeast **4**:S323].)

MATERIALS AND METHODS

Genotypes of the strains used in this work appear in Table 1 (10, 11). Wild-type (AM100) and *gln3* mutant (AM201) strains of *S. cerevisiae* were grown in minimal glucose (0.6%)-proline (0.1%) medium with or without inducer (0.25 mM oxalurate). Strain AM201 possessed growth characteristics in minimal glucose allantoin medium that were similar to those observed for *dal81* mutants (17). Growth in glucose-

* Corresponding author.
† Present address: Viral Antigens, Inc., Memphis, TN 38134.

TABLE 1. Strains used in this study

Strain	Genotype
AM100	MATa *his4-42* *lys-23* + / MATα + + *met13-25*
AM201	MATa *his4-42* *lys-23* + *gln3-1* / MATα + + *met13-25* *gln3-1*
M1-2B	MATα *trp1-289* *ura3-52*
M1662-12b	MATa *trp1-289* *ura3-52* *gln3-1*

proline medium was similarly poor. When *CAR1* gene expression was measured, the nitrogen sources were glutamate (0.1%) and arginine (0.1%) for uninduced and induced conditions, respectively. Poly(A)$^+$ RNA was isolated from each of the cultures as previously described (16). These RNA preparations were subjected to agarose gel electrophoresis under denaturing conditions, and the resolved species were transferred to nitrocellulose paper (16). The RNA blots were then hybridized with two radioactive probes, one containing the gene whose product was to be assayed and a second containing a gene whose expression was not subject to regulation by NCR. The latter probe provided a standard for RNA transfer and hybridization efficiency. Plasmids used as probes in this work are shown in Fig. 1. Probes were radioactively labeled by means of the nick translation reaction. An LKB laser densitometer was used to perform the densitometric scans of autoradiographs (see Fig. 2 to 4).

Wild-type M1-2b and *gln3* mutant M1662-12b strains were used for the transformation experiment outlined in Table 2. Strain M1662-12b was constructed by crossing a *gln3* segregant obtained after sporulation of strain AM201 to strain M1-2b. Construction and detailed characteristics of the

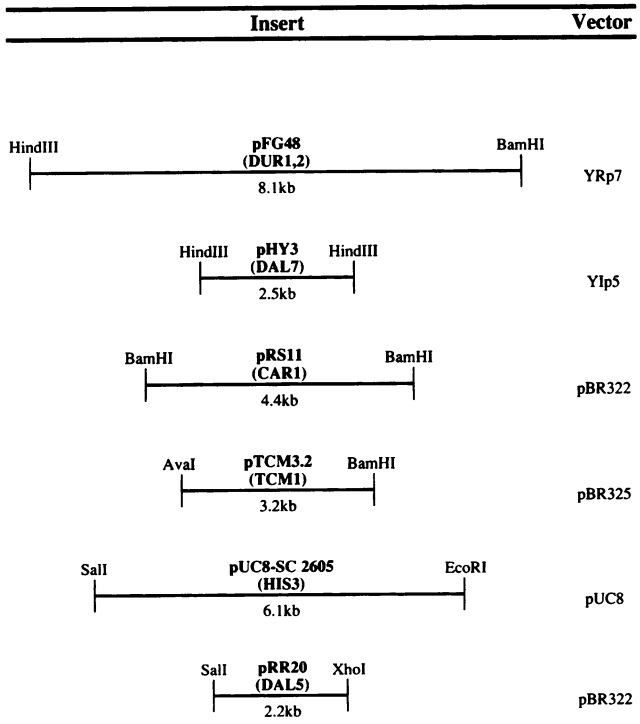

FIG. 1. Plasmids used as hybridization probes in this work. Detailed descriptions of plasmids pFG48, pHY3, pRS11, and pTCM3.2 appear in references 7, 20, 16, and 6, respectively.

TABLE 2. Requirement of *GLN3* gene product for transcriptional activation supported by the *DAL* system UAS

Plasmid	β-Galactosidase production (U) in:	
	Wild-type strain (M1-2b)	*gln3* mutant strain (M1662-12b)
pRR26 (*DAL5-lacZ* fusion)	431	32
pNG48 (*DAL5* UAS in pNG15)	209	39
pHY129 (*DAL7* UAS in pHY100)	187	23
pNG15 (*CYC1-lacZ* expression vector)	12	46

plasmids listed in Table 2 have been described elsewhere (13, 19; N. Bysani and T. G. Cooper, manuscript in preparation). Plasmid pRR26 is a *DAL5-lacZ* fusion consisting of *DAL5* sequences between positions −413 and +28 fused to the *lacZ* reporter gene. Plasmid pNG48 contained the sequence 5′-TCG ATT TGC TGA TAA GGT GCT ACA GCG CGC TCC TGC CGC ACG CTT TGT TCC TTG CTG ATA AGG TGC CCT-3′ cloned into *CYC1-lacZ* expression vector pNG15 (13). The first four bases of this strand were a 5′ extension, while the other strand contained a 5′ extension of 3′-CCGG-5′ for cloning purposes. Plasmid pHY129 contained two copies of the sequence 5′-TCG ACT TTG CTT TTC TTA TCA G-3′ cloned into the *CYC1-lacZ* expression vector pHY100. The first four bases of this sequence were a 5′ extension for cloning purposes. Expression vectors pHY100 and pNG15 are the same with two exceptions: (i) a polylinker was engineered into plasmid pNG15, whereas plasmid pHY100 possesses a single *Xho*I site at this same position, and (ii) plasmid pNG15 contains a 14-base-pair deletion of DNA between positions −242 and −228. Plasmid pHY100 does not contain this deletion. We have never detected any phenotypic difference in expression supported by these two expression vectors. Methods for the transformation and assay of β-galactosidase production are the same as those reported earlier (13, 19). Cultures used for the experiment in Table 2 were grown in minimal glucose-proline medium.

Wild-type (strain AM100) and *gln3* mutant (strain AM201) strains were grown in Wickerham minimal glucose medium containing 0.1% proline as the sole nitrogen source and 30 μg of glutamine per ml (to cover the glutamine auxotrophic phenotype of strain AM201) to a cell density of 45 to 60 Klett units. Half of the cultures were devoid of inducer, while the remainder received oxalurate at a final concentration of 0.25 mM or 0.1% arginine. Poly(A)$^+$ RNA samples were prepared, and 12 μg of sample per lane was resolved into component species on denaturing gels as described earlier (16). After the separated RNA species were transferred to nitrocellulose paper, the blots were probed with radioactive DNA (labeled by nick translation) from plasmid pFG48 plus pTCM3.2 (Fig. 2, lanes A to H), plasmid pRS11 (Fig. 2, lanes I to L), and plasmid pUC8-SC2506 (Fig. 2, lanes M to P). Plasmid pUC8-SC2506 was used in the latter instance instead of plasmid pTCM3.2 because *CAR1* and *TCM1* mRNAs are the same size. The first and second panels of Fig. 2 were generated by short and long exposures of the same blot. The two probes were both present at the same time during hybridization. The third and fourth panels of Fig. 2 were generated from the same blot, but the two probes were hybridized sequentially. The third panel was probed with plasmid pRS11 alone. After a series of autoradiographs was generated from the blot, it was reprobed with plasmid

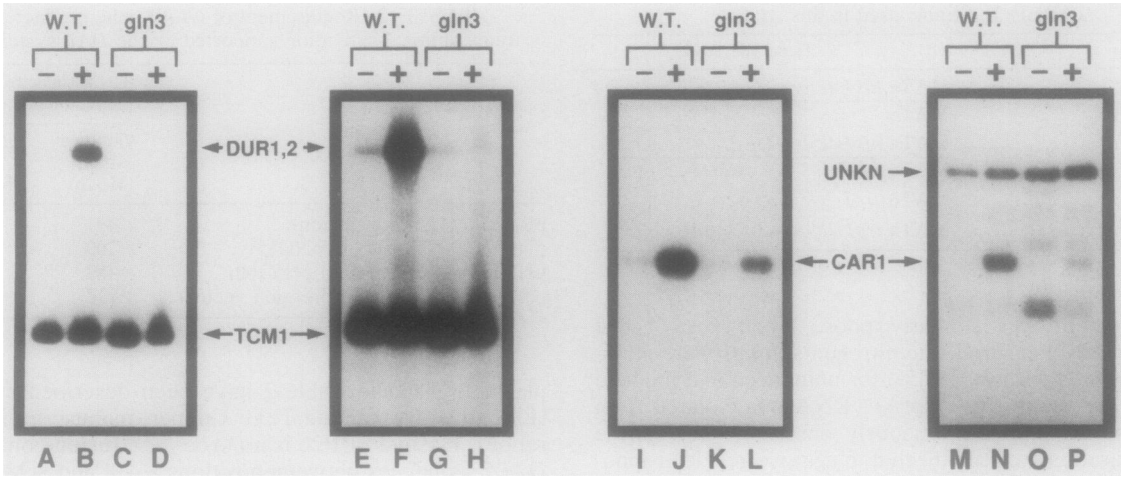

FIG. 2. Effects of mutation at the *GLN3* locus on expression of the *DUR1,2* and *CAR1* catabolic genes. Wild-type (W.T.) (strain AM100) (lanes A, B, E, F, I, J, M, and N) and *gln3* mutant (strain AM201) (lanes C, D, G, H, K, L, O, and P) strains were grown as described in Materials and Methods. Lanes M to P require a further note of explanation. Two pUC8-SC2506-specific species were observed: the uppermost band (UNKN) is an unidentified species that reproducibly hybridizes to pUC8 DNA and has not been observed to be influenced by any of the physiological conditions we have used; the smallest species is also unknown. *HIS3* mRNA levels were too low to be detected on this blot.

pUC8-SC2506, and the autoradiograph in the last panel was generated.

RESULTS

Steady-state mRNA levels in wild-type and *gln3* mutant strains. To assess whether the *GLN3* product was required for *DAL* and *CAR* gene expression, we measured the levels of allantoin and arginine pathway mRNAs in wild-type and *gln3* mutant cells grown in the presence or absence of inducer. *DUR1,2*-specific RNA was found to be inducible in the wild-type strain (Fig. 2, lanes A, B, E, and F), but this high inducibility was not detected in *gln3* mutant preparations (Fig. 2, compare lanes B and D). Overexposure of the autoradiograph (Fig. 2, lanes E to H) revealed that basal levels of *DUR1,2*-specific RNA were not severely affected by the *gln3* mutation (Fig. 2, compare lanes E and G). This qualitative observation was confirmed by densitometric analysis of the autoradiographs (Table 3). The induced level of *DUR1,2* mRNA was 15-fold lower in the *gln3* mutant than in the wild type, while the uninduced levels were the same or slightly higher. A similar result was observed for *CAR1*-specific RNA (Fig. 2, lanes I to L). Here, the basal level was decreased twofold in the *gln3* mutant, while the induced mRNA level dropped sixfold (Fig. 2) (Table 3). For *DAL7*, another inducible gene of the allantoin pathway, we again observed a loss of induced *DAL7* mRNA (Fig. 3, compare lanes A and B with lanes C and D). However, an easily measurable signal was observed with RNA preparations derived from both the wild-type and mutant strains grown in the absence of inducer (Fig. 3, compare lanes A, C, E, and G and Table 3). Again, the effect of the *gln3* mutation was observed more strongly at the level of induction; the basal levels of *DAL7*-specific RNA were affected less than twofold in the *gln3* mutant. In all cases, the amounts of *DAL7* mRNA observed in preparations derived from the *gln3* mutant strain were significantly greater, under all conditions, than those previously observed in preparations derived from cells grown under conditions of NCR (20). This was true of the

TABLE 3. Estimates of relative steady-state levels of allantoin and arginine system mRNAs in wild-type and *gln3* mutant strains

Gene	Relative mRNA levels[a] of:			
	Wild-type strain (AM100)		*gln3* mutant strain (AM201)	
	− Inducer	+ Inducer	− Inducer	+ Inducer
DUR1,2	1.2	13.9	1.6	0.9
DAL7	1.9	6.3	1.2	1.6
DAL5	3.6	4.8	0.7	0.6
CAR1	0.9	8.2	0.4	1.4
URA3	1.8	7.2	2.1	2.7

[a] Values were obtained from densitometric analysis of autoradiographs depicted in Fig. 2 to 4. Values for each allantoin system gene were normalized to the *TCM1* signal. The *CAR1* values were derived by normalization to the unknown transcript at the top of the gel (Fig. 2, lanes M through P).

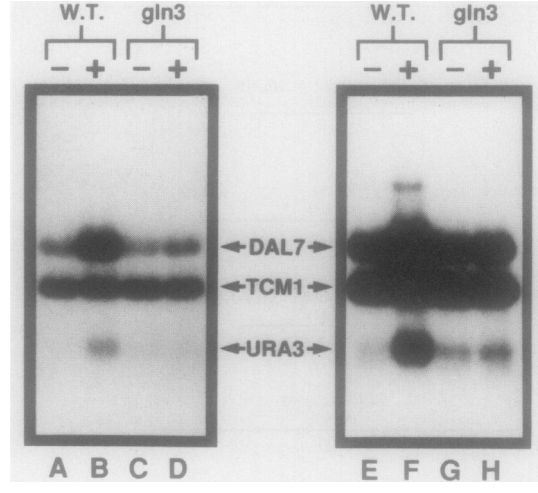

FIG. 3. Effect of mutation at the *GLN3* locus on expression of the *DAL7* and *URA3* genes. The experimental conditions for this experiment were as described in the text for Fig. 2, lanes A to H, except that the blot was probed with plasmids pHY3 and pTCM3.2. The two panels were generated by short and long exposures of the same blot. W.T., Wild type.

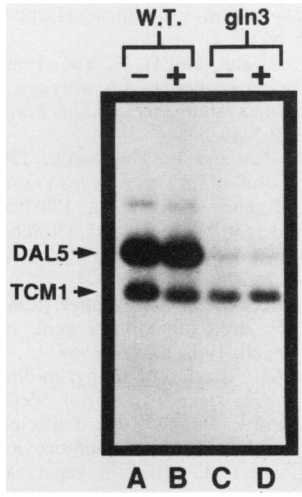

FIG. 4. Effect of mutation at the *GLN3* locus on expression of the *DAL5* gene. The experimental conditions for this experiment were as described in the text for Fig. 2, lanes A to H, and Fig. 3, except that the blot was probed with plasmids pRR20 and pTCM3.2. W.T., Wild type.

DUR1,2, *DAL5*, and *CAR1* mRNA species as well (7, 12, 20).

We also observed induction of *URA3*-specific mRNA in wild-type cells (Fig. 3, lanes A, B, E, and F). This mRNA species was detected because the plasmid used to probe the blot for *DAL7* mRNA also contained the *URA3* gene. Oxalurate-mediated induction of *URA3* gene expression has been previously reported (2). Oxalurate is an analog of dihydroorotate, the native inducer of *URA3* gene expression (2), as well as allophanate, the native inducer of the *DAL* system (14). Induction of *URA3* expression was lost in the *gln3* mutant (Fig. 4, lanes G and H), as was observed for the other genes we assayed. This was an unexpected result because the *URA3* gene encodes the last enzyme of uracil biosynthesis (orotidine 5'-phosphate decarboxylase), and biosynthetic enzymes have never been reported to be subject to NCR (8). This observation raised the possibility that the loss of induction observed in the *gln3* mutant derived from a loss of inducer uptake in this strain. To assess this possibility, we measured oxalurate uptake directly and found that it was not accumulated above background levels in the *gln3* mutant (data not shown).

At face value, these observations argue that the loss of induced gene expression described above and in the work of others (5) probably derived trivially from the failure of the inducer (oxalurate) to be transported into the cell. However, this explanation would not account for the loss of the 204-kDa protein species observed in *gln3* mutants by Mitchell and Magasanik (10, 11). If the 204-kDa species they observed was, in fact, urea amidolyase, then its loss in the *gln3* mutant could not have derived from a failure to transport inducer into the cell because in that instance, induction would have derived from the starvation-induced release of vacuolar pools of arginine and allantoin and subsequent internal induction of urea amidolyase production resulting from their degradation, as shown earlier (21). The data of Mitchell and Magasanik, although tenuous (the identity of the 204-kDa species was not determined), argued that *GLN3* probably played a role in *DAL* system gene expression.

To distinguish a rigorously demonstrable role of the *GLN3* product in *DAL* system gene expression from effects on inducer uptake, we ascertained whether *GLN3* was required for *DAL5* gene expression. This gene, which encodes a component of the allantoate permease, is expressed at high levels and does not respond to inducer (12). Therefore, a loss of inducer uptake in *gln3* mutants would not affect the expression of this gene. High-level *DAL5* gene expression required a wild-type *GLN3* allele (Fig. 4). However, as described above, low levels of the mRNA remained in preparations derived from the *gln3* mutant (Fig. 4) (Table 3).

***DAL* system UAS functions in wild-type and *gln3* mutant strains.** The requirement of a functional *gln3* allele for *DAL5* gene expression suggested that it is required for transcriptional activation mediated by the *DAL* system UAS (13). To test this hypothesis, we transformed wild-type and *gln3* mutant strains with three different plasmids: one containing a *DAL5-lacZ* promoter fusion and two consisting of oligonucleotides, previously shown to be the *DAL5* and *DAL7* UAS elements, cloned into a *CYC1-lacZ* expression vector (13, 19). These transformants were grown in glucose-proline medium and assayed for β-galactosidase production. Expression of the reporter gene in the *DAL5-lacZ* fusion plasmid (pRR26) was lost in the *gln3* mutant strains, and β-galactosidase production was down 10-fold (Table 2). Similar results were observed with expression vectors containing only the *DAL5* (plasmid pNG48) or *DAL7* (plasmid pHY129) UASs.

DISCUSSION

The data presented in this report demonstrate that the *GLN3* gene product is required for induced expression of the *DUR1,2*, *DAL7*, and *URA3* genes. Induced gene expression can be divided into two processes for purposes of analysis: entry of inducer molecules into the cell and participation of intracellular inducer molecules in bringing about induction of gene expression. Our inability to detect oxalurate uptake into *gln3* mutant cells makes it impossible to conclude that the latter process (induced gene expression) requires a functional *GLN3* product. The more likely interpretation of our observations is that induction was not observed because there was insufficient uptake of the oxalurate provided in the culture medium. This interpretation would also more readily explain the loss of induced *URA3* gene expression in *gln3* mutants because this biosynthetic gene has never been reported to share any of the regulatory characteristics associated with the genes encoding nitrogen catabolic enzymes or transport systems. Unanswered is the issue of why oxalurate uptake was not observed in the *gln3* mutant. It is possible that *GLN3* is required for expression of the genes whose products catalyze oxalurate uptake. This question cannot be addressed at present because these genes have not been identified.

In contrast to the above conclusions, which are tentative, we could demonstrate a clear requirement of the *GLN3* product for transcriptional activation mediated by the *DAL* system UAS. However, an observation made during this study suggests that the *GLN3* product is not the only protein required for *DAL* system UAS-mediated transcriptional activation. Mutation of a gene that plays an exclusive and positively acting role in transcriptional activation of the *DAL* genes would be expected to result in a total loss of *DAL* gene expression. This was not observed experimentally; basal level expression of the inducible *DAL* genes was largely immune to mutation at *gln3*.

Few definitive conclusions relative to participation of the *GLN3* product in the NCR regulatory circuit can be drawn.

The most productive correlation is that both *GLN3* function and NCR appear to affect the same process, transcriptional activation mediated by the *DAL* system UAS. However, the potential participation of more than one protein factor in transcriptional activation as described above does not permit extending the correlation to conclude that NCR operates through or requires the *GLN3* product. In fact, two observations derived from this study can be interpreted to suggest that this is not the case. Mutation of a gene that plays an exclusive and positively acting role in NCR would be expected to generate the same physiological response as NCR itself. NCR has been repeatedly shown to bring about a loss of detectable mRNA derived from NCR-sensitive genes (7, 12, 16, 20). If the *GLN3* gene product acted as the sole means of exerting NCR, then the *gln3* mutant we used would be expected to be unable to produce detectable levels of allantoin and arginine system mRNAs. This was not the case (Fig. 3 and 4). Second, NCR has been repeatedly shown to be exerted on both the basal and induced levels of gene expression (3, 7, 12, 16, 20). This predicted effect was not observed in the case of the *gln3* mutant. The basal levels of the inducible genes tested were only minimally affected (twofold or less) by mutations at *gln3*. Together these observations suggest that if the *GLN3* product participates in NCR, its role is not exclusive.

The response of induced *CAR1* expression seems to parallel that observed for the *DAL7* and *URA3* genes. The nature of the *GLN3* requirement for induced *CAR1* expression cannot be ascertained at present because the detailed structure of the *CAR1* promoter has yet to be reported. The same situation exists for *GLN1* expression, which has been recently reported to require a functional *GLN3* product (1).

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