JOURNAL OF BACTERIOLOGY, Dec. 1995, p. 6910–6918 Vol. 177, No. 23
0021-9193/95/$04.00+0
Copyright © 1995, American Society for Microbiology

Genetic Evidence for Gln3p-Independent, Nitrogen Catabolite Repression-Sensitive Gene Expression in *Saccharomyces cerevisiae*

JONATHAN A. COFFMAN, RAJENDRA RAI, AND TERRANCE G. COOPER*

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

Received 19 July 1995/Accepted 18 September 1995

The expression of many nitrogen catabolic genes decreases to low levels when readily used nitrogen sources (e.g., asparagine and glutamine) are provided in the growth medium; this physiological response is termed nitrogen catabolite repression (NCR). Transcriptional activation of these genes is mediated by the *cis*-acting element UAS_{NTR} and the *trans*-acting factor Gln3p. A second protein encoded by *URE2* possesses the genetic characteristics of a negative regulator of nitrogen catabolic gene expression. A third locus, *DAL80*, encodes a repressor that binds to sequences required for Gln3p-dependent transcription and may compete with Gln3p for binding to them. These observations are consistent with an NCR regulatory pathway with the structure environmental signal → Ure2p → (Gln3p/Dal80p) → UAS_{NTR} operation → NCR-sensitive gene expression. If NCR-sensitive gene expression occurs exclusively by this pathway, as has been thought to be the case, then the NCR sensitivity of a gene's expression should be abolished by a *ure2*Δ mutation. This expectation was not realized experimentally; the responses of highly NCR-sensitive genes to *ure2*Δ mutations varied widely. This suggested that NCR was not mediated exclusively through Ure2p and Gln3p. We tested this idea by assaying *GAP1*, *CAN1*, *DAL5*, *PUT1*, *UGA1*, and *GLN1* expression in single, double, and triple mutants lacking Gln3p, Dal80p, and/or Ure2p. All of these genes were expressed in the triple mutant, and this expression was NCR sensitive for four of the six genes. These results indicate that the NCR regulatory network consists of multiple branches, with the Ure2p-Gln3p-UAS_{NTR} pathway representing only one of them.

Expression of the nitrogen catabolic genes in *Saccharomyces cerevisiae* responds to the quality of the nitrogen source available. The presence of preferred (i.e., readily transported and metabolized) nitrogen sources (e.g., asparagine, glutamine, and ammonia) in the culture medium leads to highly decreased expression of genes encoding catabolic enzymes and transport systems required for uptake and degradation of alternative, i.e., poorly used, nitrogen sources such as proline, allantoin, and γ-aminobutyrate (GABA). This physiological response has been termed nitrogen catabolite repression (NCR) (8, 9, 39). In the absence of preferred nitrogen sources, the alternative nitrogen source pathway genes are expressed at high levels. In addition, some of these pathway genes (e.g., *DAL7*, *DUR*, *UGA*, and *PUT*) require an inducer to reach their maximum levels of expression, while others are inducer independent (e.g., *DAL5*, *CAN1*, and *GAP1*) (3, 8, 9, 24, 32–35, 37).

Three global regulatory factors, Gln3p, DAL80p, and Ure2p, control nitrogen catabolic gene expression. *GLN3* was first identified by Mitchell and Magasanik as a genetic locus required for high-level production of glutamine synthetase activity (30). This was followed by a demonstration that *DAL5* mRNA synthesis required a functional *GLN3* locus (10). Gln3p was found to operate through the *cis*-acting element UAS_{NTR}, first identified upstream of *DAL5* as mediating inducer-independent transcription of the gene (10). Saturation mutagenesis identified UAS_{NTR} as a dodecanucleotide with the sequence GATAA at its core (4). UAS_{NTR} is the necessary and sufficient *cis*-acting element mediating NCR (11). This element was subsequently shown, by work in several laboratories, to be responsible for both inducer-dependent and inducer-independent transcription of many nitrogen catabolic genes (2, 27, 29, 31). The deduced sequence of Gln3p revealed that it contained a zinc finger motif homologous to the GATA family of DNA-binding proteins (28). This homology and the fact that antibody preparations raised against Gln3p were able to precipitate a synthetic polymer containing seven repeats of a GATAA sequence led to the conclusion that Gln3p was a GATA-binding protein (28). Recent experiments using the electrophoretic mobility shift assay (EMSA) to detect DNA-protein complex formation have demonstrated that the product of *GLN3*, expressed in *Escherichia coli*, binds to native DNA fragments containing single UAS_{NTR} elements (36). Gln3p has also been shown to support transcriptional activation when tethered by LexAp upstream of a reporter gene devoid of UAS elements, indicating that it is likely an activator protein (36).

DAL80 was first identified as a mutation which resulted in overproduction of inducer-independent allantoin pathway enzymes and transport proteins (6). Dal80p was also required to maintain inducer-dependent allantoin pathway gene expression at a low level in the absence of inducer (8, 9, 15). Grenson and her colleagues subsequently isolated mutants with defects in a locus, *UGA43*, that was required to maintain inducer-dependent *UGA* gene expression at low levels in the absence of inducer (37); when *UGA43* was sequenced, it was found to be identical to *DAL80* (12, 15). The deduced Dal80p sequence contained a GATA zinc finger motif whose sequence was similar to that of the one in Gln3p (12, 15). EMSAs with Dal80p produced in *E. coli* demonstrated that it binds to URS_{GATA} sites consisting of two GATAA-containing sequences separated by 10 to 30 bp, oriented tail to tail or head to tail (16). A broad survey of nitrogen catabolic gene responses to *gln3* and *dal80* null mutations led Daugherty et al. to propose that nitrogen catabolic gene expression was regulated via the op-

* Corresponding author. Phone: (901) 448-6175. Fax: (901) 448-8462.

posing positive and negative regulation of UAS_{NTR}-mediated transcription by Gln3p and Dal80p, respectively (18). A possible mechanism for the opposing Gln3p-Dal80p regulation, namely, competitive protein binding to a common regulatory element, was suggested by the finding that Dal80p bound to the *DAL3* and *UGA4* UAS_{NTR} sequences (17) required for Gln3p-mediated transcription (2, 17). More recently, Gln3p has been shown to bind to the same *DAL3* and *UGA4* sequences previously shown to bind Dal80p (36).

The *URE2* locus was originally identified as one required for ammonia repression (later referred to as NCR); i.e., *ure2* mutants became NCR insensitive (19–21). Coshigano and Magasanik constructed a *ure2 gln3* double mutant and concluded from its phenotype that *gln3* mutations were epistatic to *ure2* mutations (13). This observation and the one that *GLN3* gene expression is not affected in *ure2* mutants led to the suggestion that Ure2p regulated Gln3p activity (14). In a survey of nitrogen catabolic gene responses to deletion of *URE2*, Coffman et al. demonstrated that Ure2p exerts its negative regulation of NCR-sensitive transcription through the UAS_{NTR} element (7). Wickner, in an insightful set of genetic experiments, has recently demonstrated that *URE2* encodes a member of the prion family of proteins (41).

The above information collectively led us to the following working model of NCR-responsive nitrogen regulation (18). Nitrogen catabolic gene expression is activated by the Gln3p that competes with Dal80p for binding to some, but not all, UAS_{NTR} sites (18). The outcome of this competition would then be an important factor influencing transcriptional activation for inducer-independent genes and basal-level transcription of inducer-dependent genes. Ure2p regulates Gln3p function in response to the quality of nitrogen source available to the cell (14). Although speculation about the biochemical mechanism by which Ure2p regulates Gln3p function has been reported (13), no biochemical model is yet supported by experimental evidence.

This picture of nitrogen catabolic gene regulation explains much, but not all, of the available experimental data derived from studies of nitrogen metabolism in *S. cerevisiae*. Several observations suggest that the above working model may need revision. If one argues that NCR is mediated exclusively through Gln3p, as has been thought until now, then the response of a given gene's expression to deletion of *GLN3* should be as strong as its NCR sensitivity. This expectation failed when *UGA1* expression, which is highly NCR sensitive, was found to be Gln3p independent (7). A similar correlation was expected for deletion of *URE2*; i.e., if Ure2p was the exclusive route for transmitting NCR control (via Gln3p), then deletion of *URE2* should result in uniform abolishment of NCR sensitivity for genes responding to this control. In contrast to expectation, what was observed experimentally was a wide variation of responses to deletion of *URE2*; they ranged from nearly complete NCR insensitivity to NCR sensitivity (7). These observations led us to conclude that the environmental nitrogen signal → Ure2p → Gln3p → UAS_{NTR} → NCR-sensitive gene expression control pathway was not exclusive (7). A similar conclusion was more recently reached by Xu et al. for *PUT1* and *PUT2* expression (42).

The above considerations prompted us to ask two questions. What is the phenotype of nitrogen catabolic gene expression in strains that lack all three known global transcription factors responding to nitrogen source supply? If expression occurs in these mutant strains, is it NCR sensitive? The present work answers these questions and leads to the conclusion that NCR-sensitive expression of nitrogen catabolic genes is mediated by at least two sets of transcription factors.

TABLE 1. Strains used in this study

Strain	Description
S. cerevisiae	
TCY1	*MAT*α *lys2 ura3*
JCY125	*MAT*α *lys2 ura3 ure2*Δ::*URA3*
JCY23	*MAT*α *lys2 ura3 ure2*Δ::*URA3 dal80*::*hisG*
JCY37	*MAT***a** *lys2 ura3 ure2*Δ::*URA3 gln3*Δ::*hisG*
RR91	*MAT*α *lys2 ura3 gln3*Δ::*hisG*
RR92	*MAT*α *lys2 ura3 gln3*Δ::*hisG dal80*::*hisG*
JCY48	*MAT*α *lys2 ura3 ure2*Δ::*URA3 gln3*Δ::*hisG dal80*::*hisG*
E. coli	
HB101	*supE44 hsdS20* (r_B m_B) *recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 mtl-1*
DH5α	F′ *endA1 hsdS20* (r_K^- m_K^+) *supE44 thi-1 recA1 gyrA* (Nal^+) *relA1* Δ(*lacZYA-argF*)*U169* [φ80d*lac*Δ(*lacZ*)M15]

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this work are listed in Table 1. Yeast cultures from which polyadenylated [poly(A)$^+$] RNA was isolated were grown in Wickerham's minimal medium (40) supplemented with 0.6% glucose and a nitrogen source at a final concentration of 0.1%. The auxotrophic requirements of the strains were provided as needed: uracil, L-lysine, and L-glutamine at 20, 40, and 30 μg/ml, respectively. The media used for growth of all *gln3*Δ strains, with proline or GABA as the nitrogen source, contained L-glutamine at a final concentration of 30 mg/liter. The media used for yeast transformation included yeast extract-peptone-glucose for cell growth and yeast nitrogen base (YNB; Difco Laboratories) supplemented with ammonium sulfate and Casamino Acids (final concentrations, 0.5%) for selective growth following transformation. Standard bacterial growth conditions were employed throughout.

Northern (RNA) blot analysis. Yeast cultures used for Northern blot analysis were grown to mid-log phase (55 to 70 Klett units on a Klett-Summerson photoelectric colorimeter equipped with a green filter). Total RNA was isolated by the method of Carlson and Botstein (5), and poly(A)$^+$ RNA was isolated by oligo(dT)-cellulose chromatography (Pharmacia). The poly(A)$^+$ RNAs were resolved in 1.4% agarose (SeaKem; FMC Bioproducts)–6% formaldehyde–morpholinepropanesulfonic acid (MOPS) gels and transferred to Gene Screen Plus Nylon 66 membranes (Dupont, New England Nuclear Research Products) by capillary transfer with 1.5 M NaCl–0.15 M sodium citrate (10× SSC). The membranes were hybridized with DNA probes radiolabeled by the polynucleotide kinase reaction (New England Biolabs, Inc., or Boehringer Mannheim) or by random priming (Boehringer Mannheim or Promega). Hybridization was performed at 42°C for 12 to 20 h in a solution containing 50% deionized formamide, 1 M NaCl, and 1% sodium dodecyl sulfate (SDS). Membranes were washed as described in the Gene Screen Plus manual.

It is important to emphasize that comparisons of the quantities of various RNA species are only possible if the species compared were present on the same blot. In particular, it is difficult to directly compare the data obtained in this work with that published earlier (7, 18) because the experiments described here focused, for the most part, on the small amounts of gene expression present in *gln3*Δ strains. As a result, the present autoradiograms were exposed longer than in earlier works. In most cases, hybridization to the standard histone H4 probe was carried out separately from that in which test gene expression was assayed. This permitted the use of optimal exposures for each signal assayed.

Construction of deletion stains. The *ure2*Δ strain JCY125 (7), *gln3*Δ strain RR91 (18), and *dal80*::*hisG* strain TCY17 (15) have been described earlier. The *gln3*Δ *dal80*::*hisG* double mutant strain RR92 was constructed by transformation with a linear 6-kb *Eco*RI DNA fragment derived from plasmid pTSC326 (15) into *gln3*Δ strain RR91 (18). The *gln3*Δ *ure2*Δ double mutant strain JCY37 was constructed by transformation of a linear *Sal*I-*Xba*I DNA fragment isolated from plasmid pJC12 (7) into strain RR91. The same DNA fragment was used to construct *ure2*Δ *dal80*::*hisG* double mutant strain JCY23 and *ure2*Δ *dal80*::*hisG gln3*Δ triple mutant strain JCY48 by transformation into strains TCY17 and RR92, respectively. Recombinants were selected for growth on YNB medium, and genomic Southern blot analysis was used to confirm the deletions and disruptions. Yeast transformations were performed as described by Ito et al. (23).

RESULTS

NCR-sensitive gene expression in *gln3* deletion strains. Two observations led us to determine if all NCR-sensitive gene expression is mediated through the *URE2-GLN3* transcription control pathway: (i) several genes whose expression is NCR

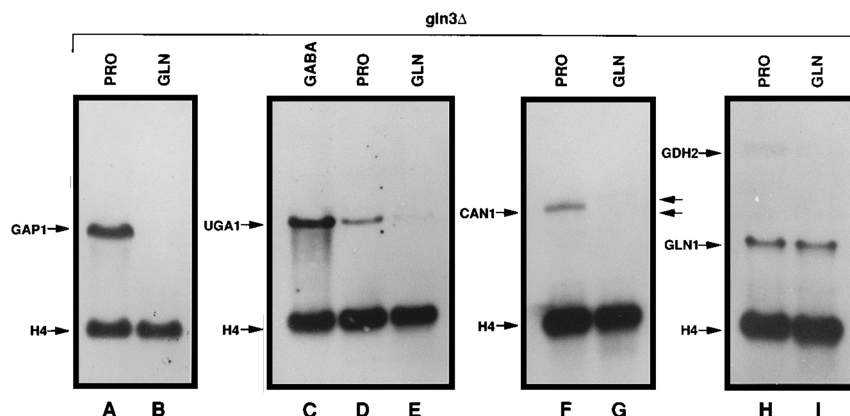

FIG. 1. Effects of *gln3*Δ on steady-state *GAP1*, *UGA1*, *CAN1*, *GDH2*, and *GLN1* mRNA levels. Strain RR91 (*gln3*Δ) was grown as described in Materials and Methods with proline (PRO), glutamine (GLN), or GABA as the nitrogen source. Following resolution by electrophoresis, poly(A)$^+$ RNA immobilized on a nylon membrane (10 μg per lane) was hybridized with DNA probes rendered radioactive by random priming. The plasmids used were pPL257 (25), pRD102 (17), and pTSC289 (22), containing *GAP1* (lanes A and B), *UGA1* (lanes C, D, and E), and *CAN1* (lanes F and G) DNA, respectively. In lanes H and I, 5′-labeled synthetic oligonucleotides complementary to mRNA for the *GDH2* (positions 81 to 130) (27) and *GLN1* (positions 825 to 874) (29) genes were used as hybridization probes. In lanes A through I, a 5′-labeled synthetic oligonucleotide complementary to histone H4 sequences (positions 1301 to 1350) (32) was used as the probe to determine loading and transfer efficiency.

sensitive were transcribed in *gln3* deletion strains (18), and (ii) highly varied responses of nitrogen catabolic gene expression were observed in a *ure2* deletion strain growing in medium containing the repressive nitrogen source asparagine (7). From these results, we predicted that it should be possible to demonstrate Gln3p-independent, NCR-sensitive nitrogen catabolic gene expression. This prediction was tested by comparing the steady-state levels of nitrogen catabolic gene mRNA produced in *gln3* deletion strain RR91 growing in either minimal glucose-proline or glucose-glutamine medium. As shown in Fig. 1, *GAP1* mRNA was present in strain RR91 growing in glucose-proline medium (lane A) but was undetectable when glutamine replaced proline as the nitrogen source (lane B); i.e., this *GAP1* expression was NCR sensitive but Gln3p independent. The same result was obtained for *UGA1* (Fig. 1, lanes D and E), *CAN1* (lanes F and G), and *GDH2* (lanes H and I) mRNAs. Although it is not clearly visible in this photograph, two species of mRNA with different electrophoretic mobilities were observed when a poly(A)$^+$ RNA preparation derived from glucose-glutamine-grown cells was hybridized with the *CAN1* DNA fragment. Note also that very little *GDH2* mRNA was produced even in proline-grown cells, although the *GDH2* expression detected appeared to follow the same pattern of control in this experiment as that of *GAP1* and *CAN1* (Fig. 1, lanes H and I). Finally, Gln3p-independent expression of *GLN1* did not respond to the substitution of glutamine for proline in the growth medium, as observed for the other genes whose expression was assayed (Fig. 1, lanes H and I). This presumably results from the fact that transcription factors unrelated to the NCR control pathway participate in *GLN1* expression along with Gln3p.

Expression of *GAP1* in nitrogen-regulatory mutants. Three regulatory proteins have been reported to participate in controlled expression of nitrogen catabolic genes: Gln3p, Dal80p, and Ure2p. To determine if the loss of either Dal80p or Ure2p affected NCR-sensitive, Gln3p-independent mRNA production, we first constructed a *gln3*Δ *dal80*::*hisG* double mutant and compared steady-state *GAP1* mRNA levels in this mutant (RR92) with those found in wild-type (TCY1) and *gln3*Δ (RR91) strains. As shown in Fig. 2, lanes B and C, *gln3*Δ and *gln3*Δ *dal80*::*hisG* mutants have the same amount of *GAP1* mRNA, which is about two- to fourfold less than that observed in the wild type (lane A). *GAP1* expression in the double mutant was undetectable, however, when asparagine was used in place of the nonrepressive nitrogen source proline (Fig. 2, lanes D and E). Finally, a *gln3*Δ *dal80*::*hisG ure2*Δ triple mutant strain (JCY48) was constructed. *GAP1* mRNA levels were determined in this strain growing in minimal medium with proline, asparagine, or glutamine (Fig. 2, lanes F to H, and Fig. 3, lanes E and F). *GAP1* mRNA was produced in cells provided with proline as the nitrogen source but not with asparagine or glutamine. The latter observation suggested that the loss of *GAP1* expression in cells provided with a repressive nitrogen source was derived from NCR rather than specific negative regulation due to the presence of a particular amino acid, e.g., the recently reported glutamine-specific effects associated with transcriptional activation mediated by the *cis*-acting sequence 5′-TTTGTTTAC-3′ (31). These data also show that Ure2p is not the negative regulator in this NCR-sensitive expression; otherwise, *GAP1* expression should have been observed in this *ure2*Δ strain (JCY48) provided with glutamine or asparagine as the nitrogen source.

We also investigated the relationship between Ure2p and Dal80p in the presence and absence of Gln3p. The level of *GAP1* expression observed in a *ure2*Δ mutant was the same whether proline or glutamine was provided as the nitrogen source (Fig. 3, lanes A and B). Furthermore, this level of *GAP1* expression was not significantly increased (twofold or less) upon disruption of the *DAL80* locus (Fig. 3, lanes A to D; note that lane C was slightly overloaded, generating the false impression that it contained more *GAP1* mRNA). This pattern of regulation will be important to the discussion below.

There was a clear difference between the mRNA levels observed when *ure2*Δ *dal80*::*hisG* and *ure2*Δ *dal80*::*hisG gln3*Δ mutant cells were provided with glutamine as the nitrogen source. When the *ure2*Δ *dal80*::*hisG* double mutant (Gln3$^+$) growing in glucose-glutamine medium was assayed, it was found that *GAP1* mRNA was produced (Fig. 3, lane D). In contrast, in the *ure2*Δ *dal80*::*hisG gln3*Δ triple mutant (Gln3$^-$) provided with glutamine as the nitrogen source, no *GAP1* mRNA was produced (Fig. 3, lane F). Therefore, the *GAP1* mRNA observed when glutamine was provided to the double mutant was Gln3p dependent (Fig. 3, lanes C and D versus E and F). This mRNA level also required the absence of Ure2p,

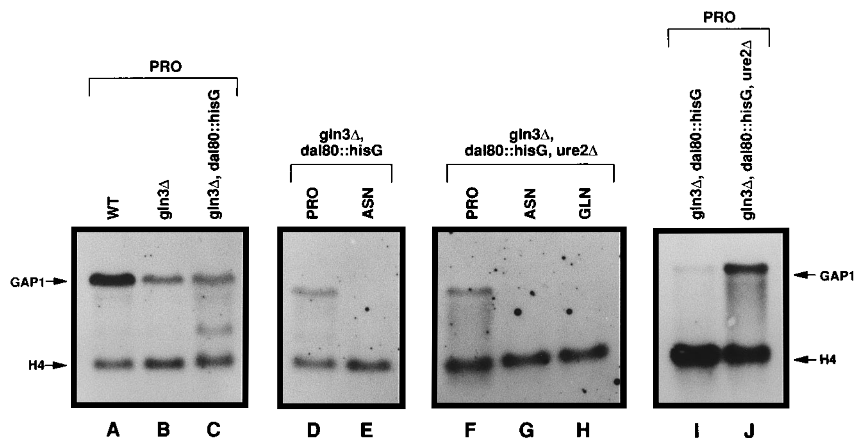

FIG. 2. Steady-state *GAP1* mRNA levels in various mutant strains provided with nonrepressive (proline [PRO], lanes A, B, C, D, and F) or repressive (asparagine [ASN] or glutamine [GLN]; lanes E, G, and H) nitrogen sources. Wild-type (WT) strain TCY1 (lane A), *gln3*Δ strain RR91 (lane B), *gln3*Δ *dal80*::*hisG* strain RR92 (lanes C, D, and E), and *gln3*Δ *dal80*::*hisG ure2*Δ strain JCY48 (lanes F, G, and H) were grown as described in Materials and Methods with the nitrogen source indicated. Poly(A)$^+$ RNA immobilized to a nylon membrane (10 μg per lane) was hybridized with radioactive *GAP1* DNA (see Fig. 1 legend). Histone H4 mRNA levels were used to determine loading and transfer efficiencies.

which is not surprising given that Ure2p has been shown to exert its negative control of transcription through Gln3p (18). Together, the above data demonstrate the existence of NCR-sensitive *GAP1* expression that is independent of the major global *trans*-acting factors reported to participate in the regulation of nitrogen catabolic gene expression. This expression is most unambiguously observed in the triple mutant. It also argues that Ure2p is not an exclusive regulator of Gln3p-independent, NCR-sensitive gene expression.

Expression of *CAN1* in nitrogen-regulatory mutants. To assess whether the Gln3p-, Ure2p-, and Dal80p-independent, NCR-sensitive expression observed for *GAP1* extended to other NCR-sensitive genes, we performed similar assays with a *CAN1* hybridization probe. As shown in Fig. 4, significant *CAN1* mRNA remained in a *gln3*Δ mutant strain (RR91) growing with proline as the nitrogen source compared with that in wild-type strain TCY1 (lanes A and B), and the level of *CAN1* mRNA increased in the *gln3*Δ *dal80*::*hisG* double mutant strain (RR92) provided with proline as the nitrogen source over the level observed in *gln3*Δ strain RR91 (lanes B and C; note standard H4 hybridization levels). *CAN1* mRNA decreased to nearly undetectable levels when asparagine replaced proline as the nitrogen source for the double mutant (Fig. 4, compare lanes C and D). Two *CAN1*-specific mRNA species with different mobilities were observed when asparagine was provided in the medium (Fig. 4, lane D, faint bands designated by the pair of arrows). These two RNA species are more easily observed in an RNA preparation derived from wild-type cells (strain TCY1) growing in glucose-asparagine medium (Fig. 4, lanes F and G). The more rapidly migrating species is observed with a variety of nonrepressive nitrogen sources, such as proline and GABA. The more slowly migrating species, on the other hand, is produced only when a repressive nitrogen source (e.g., asparagine or glutamine) is provided in the culture medium. We have not determined the sequence of these two RNA species and hence cannot comment on their origins, i.e., whether they represent two different mRNAs derived from one gene or two RNAs from different genes. The size difference favors the latter possibility. We have not carried this interesting observation further in this work because *CAN1* is not its primary focus. To determine the effect of Ure2p on *CAN1* mRNA synthesized in the *gln3*Δ *dal80*::*hisG* double mutant, we compared *CAN1* mRNAs found in double mutant strain RR92 and *gln3*Δ *dal80*::*hisG ure2*Δ triple mutant strain JCY48. As shown in Fig. 4, lanes H and I, deletion of *URE2* resulted in a modest increase in *CAN1* mRNA when proline was the nitrogen source. In contrast to expectations emanating from the current model of nitrogen regulation, this increase was independent of Gln3p. A similar result was found for *GAP1* (Fig. 3, lanes I and J).

To assess the effects of *ure2* mutations, alone and in combination with mutations in the other nitrogen-regulatory protein genes, we grew the *ure2*Δ mutant JCY125 and the *ure2*Δ *dal80*::*hisG* double mutant JCY23 in glucose-proline or glucose-glutamine medium. The *ure2*Δ mutant produced similar levels of *CAN1*-specific mRNA in both glucose-proline and glucose-glutamine medium (Fig. 5, lanes A and B). When the *ure2*Δ *dal80*::*hisG* double mutant was assayed under these conditions, *CAN1*-specific mRNA was produced at higher levels than in the single *ure2*Δ mutant. Moreover, *CAN1* mRNA was produced regardless of the nitrogen source. This is an expected result if Dal80p and Gln3p are competing for the same *cis*-acting sites upstream of the gene and deletion of *URE2* results

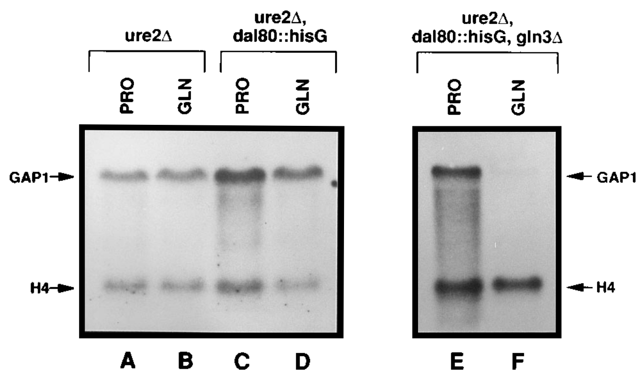

FIG. 3. Steady-state *GAP1* mRNA levels in various mutant strains provided with nonrepressive (proline [PRO]; lanes A, C, and E) or repressive (glutamine [GLN]; lanes B, D, and F) nitrogen sources. *ure2*Δ strain JCY125 (lanes A and B), *ure2*Δ *dal80*::*hisG* strain JCY23 (lanes C and D), and *ure2*Δ *dal80*::*hisG gln3*Δ strain JCY48 (lanes E and F) were grown as described in Materials and Methods. Poly(A)$^+$ RNA immobilized on a nylon membrane (10 μg per lane) was hybridized with radioactive *GAP1* DNA (see Fig. 1 legend). Histone H4 mRNA levels were used to determine loading and transfer efficiencies.

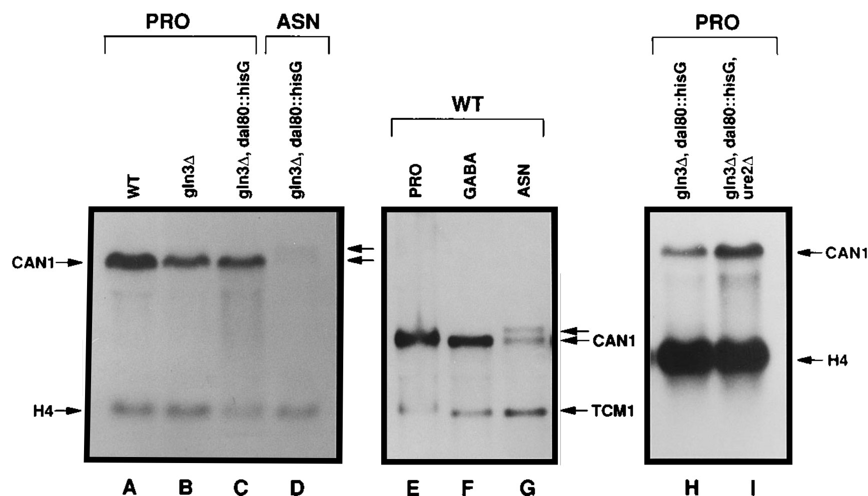

FIG. 4. Steady-state *CAN1* mRNA levels in the wild-type (WT) and various mutant strains provided with nonrepressive (proline [PRO], lanes A, B, C, E, H, and I; or GABA, lane F) or repressive (asparagine [ASN], lane G) nitrogen sources. Strains and experimental conditions were as described in the legend to Fig. 2.

in NCR-insensitive Gln3p operation. To test this, we repeated the experiment with a strain (JCY48) in which the *GLN3* locus was also deleted. In this triple mutant, *CAN1* mRNA was produced with proline but not glutamine as the nitrogen source (Fig. 5, lanes E and F). In sum, the *CAN1* data argue that there is NCR-sensitive, Gln3p-independent expression of *CAN1*. In addition, the most striking characteristic of the *gln3*Δ *dal80*::*hisG* versus *gln3 dal80*::*hisG ure2*Δ mutant phenotypes was that loss of Ure2p in a *gln3*Δ *dal80*::*hisG* double mutant results in increased levels of *CAN1* mRNA (Fig. 4, lanes H and I). This is surprising, because the negative effects of both Dal80p and Ure2p on NCR-sensitive gene expression are thought to occur through mechanisms associated with Gln3p (2, 7, 10, 13, 14, 16, 17). This was also observed for *GAP1* (Fig. 3, lanes I and J). These data argue that Ure2p functions in some way not previously recognized or reported.

Expression of *DAL5* in nitrogen-regulatory mutants. In contrast to the loci discussed above, *DAL5* gene expression is drastically reduced by the loss of *GLN3* (18). As shown in Fig. 6, lanes A and B, the *DAL5* message was barely detectable in

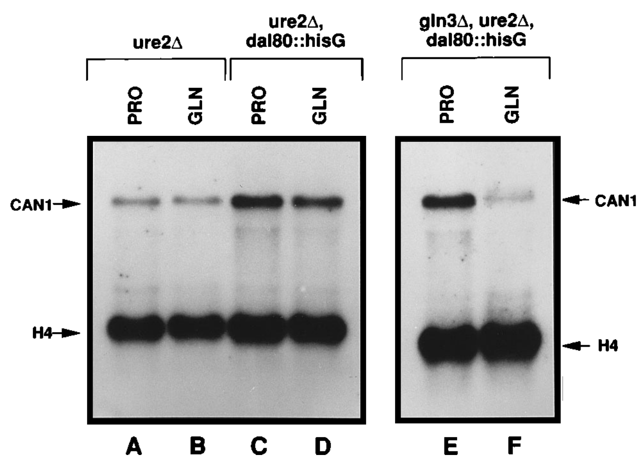

FIG. 5. Steady-state *CAN1* mRNA levels in various mutant strains provided with non-repressive (PRO; lanes A, C, and E) or repressive (GLN; lanes B, D, and F) nitrogen sources. Strains and experimental conditions were as described in the legend to Fig. 3.

an RNA preparation isolated from a *gln3*Δ strain (RR91). A similarly low level of *DAL5* mRNA was observed in a *gln3*Δ *dal80*::*hisG* double mutant (strain RR92) (lane C); i.e., loss of Dal80p did not demonstrably affect *DAL5* expression in a $Gln3^{-}$ background. When *DAL5* mRNA was assayed in a *gln3*Δ *dal80*::*hisG ure2*Δ triple mutant strain (JCY48), however, a clear signal was present (Fig. 6, lanes B to D and G). The data in Fig. 6 (lanes M and N), along with those in lanes C and D, indicate that the increased level of *DAL5* mRNA (lane D versus lane C) required the loss of both Ure2p and Dal80p. The significance of this observation derives from the current view that Ure2p is thought to function in the NCR regulatory network by negatively regulating Gln3p (7, 13, 14). Figure 6, lanes C and D, demonstrated that loss of Ure2p resulted in an increase in *DAL5* mRNA in cells growing in proline medium even though the strain used for this experiment lacked Gln3p, the reported target of Ure2p regulation (14). In other words, Ure2p was apparently affecting a previously unrecognized part of the nitrogen control network distinct from Gln3p. Similar observations were made when *GAP1* mRNA levels were determined (Fig. 2, lanes I and J). The *DAL5* expression observed in the triple mutant was, however, NCR sensitive, as demonstrated by the loss of the signal when glutamine was provided as the nitrogen source (Fig. 6, lanes G and H). This effect was the same as that observed with *CAN1* and *GAP1* above. A significant increase in *DAL5* mRNA production was observed upon loss of the Dal80p if the strain contained Gln3p (Fig. 6, compare lanes J and L). The *ure2*Δ *dal80*::*hisG* versus *ure2*Δ phenotypes probably derive from at least a twofold effect, one part of which is analogous to that shown in Fig. 6, lanes C and D, and the other part of which may be derived from the cessation of Dal80p competition with Gln3p for binding to the *DAL5* UAS_{NTR} sequences (Fig. 6, lanes J and L) (17). In this regard, disruption of *DAL80* alone results in a two- to threefold increase in *DAL5* mRNA level compared with that in the wild type (15, 16, 30a).

Expression of *UGA1* in nitrogen-regulatory mutants. The data in Fig. 1 indicated that *UGA1* expression could be detected in a *gln3*Δ mutant grown in proline medium. As shown in Fig. 7, *UGA1* expression was also observed in a *gln3*Δ *dal80*::*hisG* double mutant (strain RR92) and was NCR sensitive, as indicated by loss of the *UGA1* mRNA when glutamine was provided as the nitrogen source (lanes B versus C). Results

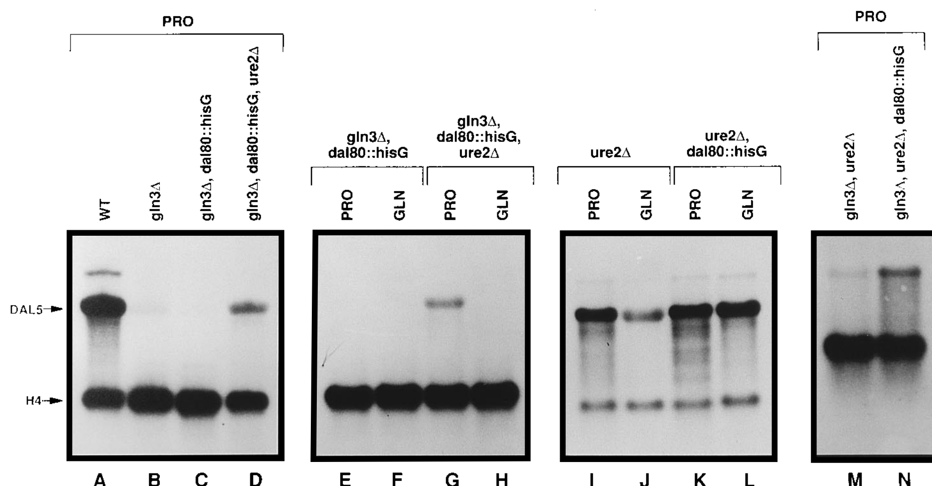

FIG. 6. Steady-state *DAL5* mRNA levels in the wild-type (WT) and various mutant strains provided with nonrepressive (PRO; lanes A, B, C, D, E, G, I, and K) or repressive (GLN; lanes F, H, J, and L) nitrogen sources. Strains and experimental conditions were as described in the legends to Fig. 2 and 3. Randomly primed plasmid pRR20 (29, 30) was used as a radioactive hybridization probe for *DAL5* mRNA.

somewhat similar to those in Fig. 6, lanes B and C, were observed with the *gln3*Δ *dal80*::*hisG ure2*Δ triple mutant (strain JCY48); *UGA1* mRNA was produced at low levels when proline was provided as the nitrogen source and decreased a bit further when glutamine was provided in place of proline (Fig. 7, lanes E and F).

The *UGA1* expression observed in *ure2*Δ and *ure2*Δ *dal80*::*hisG* mutants exhibited the same pattern of regulation as observed with *CAN1* (Fig. 5). As before, we observed an increase in *UGA1* mRNA levels in response to loss of the Dal80p (Fig. 7, compare lanes G and I). Finally, in contrast to observations discussed above, reasonable *UGA1* expression persisted even when cells were provided with glutamine as the nitrogen source. Although the transcription factors mediating this expression remain to be identified, the NCR-insensitive Uga3p may be a good candidate.

Expression of *PUT1* in nitrogen-regulatory mutants. Figure 8 depicts results from an abbreviated analysis of *PUT1* expression similar to those discussed above. *PUT1* expression responded to mutation of the transcription factors in a manner similar to *UGA1*. High-level *PUT1* expression, as previously reported (7, 18, 31), was highly Gln3p dependent (Fig. 8, compare lanes A and B). This is clearly a different response than that observed in the strain background recently assayed by Xu et al. (42); in that case, expression of a plasmid-borne *PUT1-lacZ* fusion did not respond to deletion of *GLN3*. *PUT1* expression in the *gln3*Δ *dal80*::*hisG* and *gln3*Δ *dal80*::*hisG ure2*Δ mutants differed by only twofold or less (Fig. 8, lanes B to D). This response, although smaller, parallels those observed when this comparison was made for *CAN1*, *GAP1*, and *DAL5* above. There was also only a small increase in *PUT1* expression when *DAL80* was disrupted in a *ure2*Δ background, i.e., in the presence of Gln3p (Fig. 8, lanes E to H). This is consistent with the small effect reported for the *dal80*::*hisG* mutation alone on *PUT1* expression (18).

Expression of *GAP1*, *CAN1*, *DAL5*, *UGA1*, and *PUT1* in a *gln3 ure2* double mutant. The response of gene expression for all combinations of mutations of the three known global nitrogen regulators except the combination *gln3 ure2* has been discussed above. As shown in Fig. 9, all of the genes were expressed when the *gln3*Δ *ure2*Δ double mutant strain was provided with proline as the nitrogen source. Expression of all of the genes except *UGA1* was lost when glutamine was substituted for proline. Here, as above, we observed NCR-sensitive expression in the absence of the Ure2p-Gln3p regulatory pathway, arguing either that the functions previously ascribed

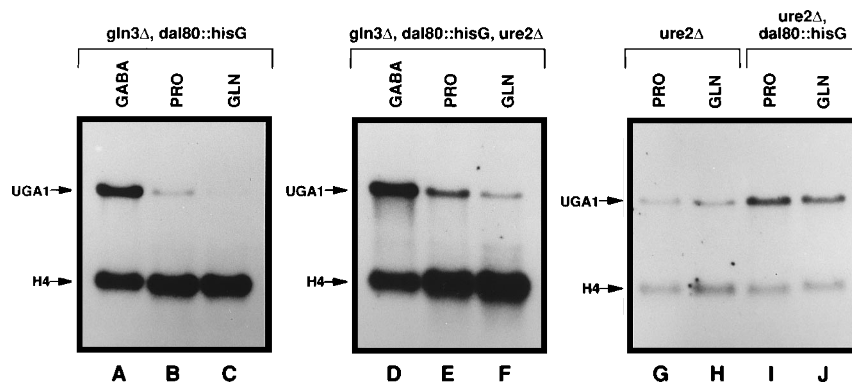

FIG. 7. Steady-state *UGA1* mRNA levels in various mutant strains provided with inducing (GABA; lanes A and D), nonrepressive (PRO; lanes B, E, G, and I), or repressive (GLN; lanes C, F, H, and J) nitrogen sources. Strains and experimental conditions were as described in the legends to Fig. 2 and 3. See Fig. 1 legend for the *UGA1* hybridization probe.

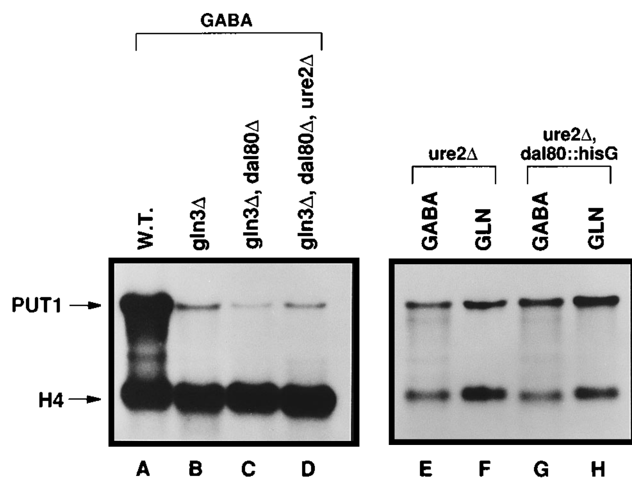

FIG. 8. Steady-state *PUT1* mRNA levels in the wild-type (W.T.) and various mutant strains provided with nonrepressive (GABA; lanes A, B, C, D, E, and G) or repressive (GLN; lanes F and H) nitrogen sources. Strains and experimental conditions were as described in the legends to Fig. 2 and 3. A synthetic oligonucleotide complementary to *PUT1* positions 1541 to 1590 (38) was used as the radioactive hybridization probe.

to these proteins are duplicated in another regulatory pathway or that the models for Ure2p-Gln3p functions and relationships are in need of revision.

DISCUSSION

The first indication of an additional NCR-sensitive control pathway participating in the regulation of nitrogen catabolic gene expression was the report of Daugherty et al. documenting the lack of correlation between a requirement for Gln3p for expression of particular genes and their sensitivity to NCR (18). This incongruity broadened when Coffman et al. observed wide variation in the extent to which deletion of *URE2* resulted in loss of NCR sensitivity (7). Xu et al. presented further support for these conclusions, reporting that *PUT1* and *PUT2* expression was independent of Gln3p in the particular strain background that they assayed (42); it was not the MB1000, i.e., Σ1278b, background that is broadly used in studies of nitrogen regulation.

Here we have demonstrated NCR-sensitive expression of several genes in the absence of three global nitrogen regulatory proteins, Gln3p, Dal80p, and Ure2p. The fact that we see the reported pattern of regulation broadly represented is consistent with the suggestion that it reflects global regulation by NCR rather than an esoteric characteristic of a specific gene. These results therefore demonstrate the existence of a new transcription factor whose production and/or operation depends upon the quality of the nitrogen source available. The existence of this factor is evident when one or more of the nitrogen-regulatory proteins is eliminated from the cell. That we could not see the influence of this factor(s) in assays of wild-type cells probably derives from the fact that the physiological situations in which the putative new regulatory pathway predominates are at present unknown. This conclusion is most clearly supported in the cases of *GAP1* and *CAN1* expression. In the triple mutant, expression of these genes was observed when proline was provided as the nitrogen source and dropped to low levels when glutamine or asparagine was used instead. A similar result was observed for *DAL5* gene expression, although the effects were less dramatic than those observed with *GAP1* and *CAN1*. A related but not congruent set of results was observed for the *UGA1* and *PUT1* genes, i.e., Gln3p- and Ure2p-independent, NCR-sensitive expression. The results for *UGA1* and *PUT1* were less clear because the expression of both genes contained an NCR-insensitive component. This NCR-insensitive expression makes it more difficult to unambiguously visualize a second NCR-sensitive transcription system.

Possible sources of the NCR-insensitive transcription observed with *UGA1* and *PUT1* are the inducer-dependent transcription activator proteins Uga3p (1) and Put3p (26) associated with the two pathways, respectively. Although our observations were made in the absence of the pathway inducer, we still suggest that these proteins are the most likely sources. This suggestion is based on the result that Put3p is able to activate transcription in the absence of inducer (Fig. 5 and 6 in reference 31). This activation is both insensitive to NCR and independent of Gln3p (31).

Three possible characteristics of the putative new transcription factor can be suggested if it is assumed that its direct influence was seen in our present assays. First, the qualitatively different levels of *GAP1* and *CAN1* expression in a strain lacking three nitrogen regulator protein genes (compared with that of *DAL5* or *GLN1*) suggest that the putative NCR-sensitive transcription factor does not participate in the expression of all genes to the same extent. Second, NCR-sensitive expression of the *GAP1*, *CAN1*, *DAL5*, *UGA1*, and *PUT1* genes in the *gln3*Δ *ure2*Δ strain suggests that the NCR responsiveness of the new transcription factor is not predominantly mediated through negative control by Ure2p. Finally, the only *cis*-acting element known to mediate NCR-sensitive gene expression is

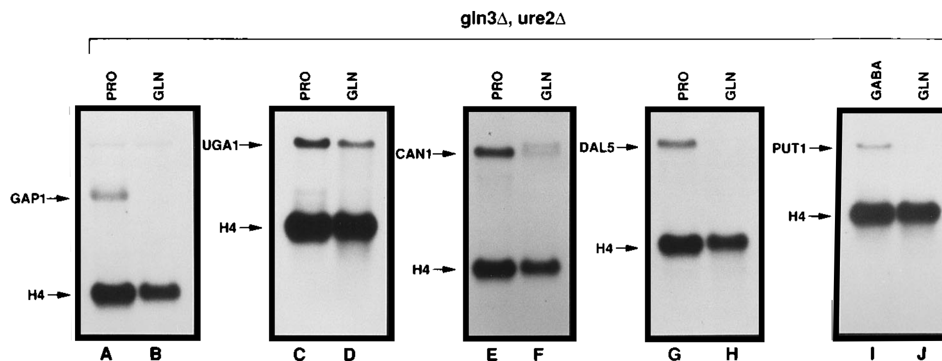

FIG. 9. Steady-state mRNA levels for various genes in a *gln3*Δ *ure2*Δ double mutant strain (JCY37) provided with a nonrepressive (PRO, proline) or repressive (GLN, glutamine) nitrogen source. The experimental and assay conditions were as described in the legend to Fig. 2.

the GATAA-containing UAS_{NTR} (11). It is also an element present upstream of all genes studied in this work. When this element was characterized by saturation mutagenesis, a surprisingly large number of mutations in the element seemed to be tolerated (4). It is therefore possible that both Gln3p-dependent and -independent, NCR-sensitive transcription is mediated by UAS_{NTR}-related elements and that the putative new transcription factor may be structurally related to Gln3p and Dal80p (12, 15, 28).

Little if anything is known about the biochemical function of Ure2p, but two potentially useful observations for future studies derive from this work. First, regulation by Ure2p in response to the quality of nitrogen source provided is not restricted to Gln3p. The data in Fig. 2 (lanes I and J), 4 (lanes H and I), and 6 (lanes C and D and E and G) indicate that deletion of the *URE2* locus increases gene expression even though the strain lacks Gln3p and Dal80p. This effect, however, is only observed when proline is the nitrogen source. These observations notwithstanding, data obtained with the triple mutant demonstrate that Ure2p, like Gln3p, does not appear to be necessary for NCR regulation to be exerted on the newly identified transcription system, because the triple mutant strain lacks both proteins. Second, Ure2p functions irrespective of the nitrogen source provided, i.e., downregulation is exerted by Ure2p when cells are grown in proline, glutamine, or asparagine. That Ure2p functions under conditions of NCR is not surprising; it was an aberration in response to this condition by which mutations of the locus were first identified (19, 20). It is more surprising to observe it functioning under conditions of minimal NCR, i.e., when proline is provided as the nitrogen source.

ACKNOWLEDGMENTS

We thank members of the UT Yeast Group who read this manuscript and offered suggestions for its improvement. Oligonucleotides used in these studies were prepared by the UT Molecular Resource Center.

This work was supported by Public Health Service grant GM-35642 from the National Institute of General Medical Sciences.

ADDENDUM IN PROOF

The transcription factor whose existence is predicted in this work has been isolated and characterized. It is Gat1p, a transcriptional activator of the GATA family of DNA-binding proteins (accession no. U27344). Gat1p is required for transcription of NCR-sensitive genes. *GAT1* expression is Gln3p dependent, NCR sensitive, and Dal80p regulated (J. Coffman, R. Rai, T. Cunningham, V. Svetlov, and T. G. Cooper. GAT1, a GATA-type DNA-binding protein that participates in nitrogen-regulated transcriptional activation in *Saccharomyces cerevisiae.* Submitted for publication).

REFERENCES

1. **Andre, B.** 1990. The *UGA3* gene regulating the GABA catabolic pathway in *Saccharomyces cerevisiae* codes for a putative zinc-finger protein acting on RNA amount. Mol. Gen. Genet. **220:**269–276.
2. **Andre, B., D. Talibi, S. S. Boudekou, C. Hein, S. Vissers, and D. Coornaert.** 1995. Two mutually exclusive regulatory systems inhibit UAS_{GATA}, a cluster of 5′GAT(A/T)A-3′ upstream from the *UGA4* gene of *Saccharomyces cerevisiae*. Nucleic Acids Res. **23:**558–564.
3. **Brandriss, M. C., and B. Magasanik.** 1979. Genetics and physiology of proline utilization in *Saccharomyces cerevisiae*: mutation causing constitutive enzyme expression. J. Bacteriol. **140:**504–507.
4. **Bysani, N., J. R. Daugherty, and T. G. Cooper.** 1991. Saturation mutagenesis of the UAS_{NTR} (GATAA) responsible for nitrogen catabolite repression-sensitive transcriptional activation of the allantoin pathway genes in *Saccharomyces cerevisiae*. J. Bacteriol. **173:**4977–4982.
5. **Carlson, M., and D. Botstein.** 1982. Two differentially regulated mRNAs with different 5′ ends encode secretory and intracellular forms of yeast invertase. Cell **28:**145–154.
6. **Chisholm, G., and T. G. Cooper.** 1982. Isolation and characterization of mutations that produce the allantoin-degrading enzymes constitutively in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **2:**1088–1095.
7. **Coffman, J. A., H. M. El Berry, and T. G. Cooper.** 1994. The URE2 protein regulates nitrogen catabolic gene expression through the GATAA-containing UAS_{NTR} element in *Saccharomyces cerevisiae*. J. Bacteriol. **176:**7476–7483.
8. **Cooper, T. G.** 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*, p. 39–99. *In* J. N. Strathern, E. W. Jones, and J. Broach (ed.), The molecular biology of the yeast *Saccharomyces*: metabolism and gene expression. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
9. **Cooper, T. G.** 1994. Regulation of allantoin catabolism in *Saccharomyces cerevisiae*, p. 139–169. *In* G. Marzluf and R. Bambrl (ed.), The mycota. III. Biochemistry and molecular biology. Springer-Verlag, Berlin.
10. **Cooper, T. G., D. Ferguson, R. Rai, and N. Bysani.** 1990. The *GLN3* gene product is required for transcriptional activation of allantoin system gene expression in *Saccharomyces cerevisiae*. J. Bacteriol. **172:**1014–1018.
11. **Cooper, T. G., R. Rai, and H. S. Yoo.** 1989. Requirement of upstream activation sequences for nitrogen catabolite repression of the allantoin system genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**5440–5444.
12. **Coornaert, D., S. Vissers, B. Andre, and M. Grenson.** 1992. The *UGA43* negative regulatory gene of *Saccharomyces cerevisiae* contains both a GATA-1 type zinc finger and a putative leucine zipper. Curr. Genet. **21:**301–307.
13. **Coshigano, P. W., and B. Magasanik.** 1991. The *URE2* gene product of *Saccharomyces cerevisiae* plays an important role in the cellular response to the nitrogen source and has homology to glutathione-*S*-transferase. Mol. Cell. Biol. **11:**822–832.
14. **Courchesne, W. E., and B. Magasanik.** 1988. Regulation of nitrogen assimilation in *Saccharomyces cerevisiae*: roles of the *URE2* and *GLN3* genes. J. Bacteriol. **170:**708–713.
15. **Cunningham, T. S., and T. G. Cooper.** 1991. Expression of the *DAL80* gene, whose product is homologous to the GATA factors and is a negative regulator of multiple nitrogen catabolic genes in *Saccharomyces cerevisiae*, is sensitive to nitrogen catabolite repression. Mol. Cell. Biol. **11:**6205–6215.
16. **Cunningham, T. S., and T. G. Cooper.** 1993. The *Saccharomyces cerevisiae DAL80* repressor protein binds to multiple copies of GATAA-containing sequences (URS_{GATA}). J. Bacteriol. **175:**5851–5861.
17. **Cunningham, T. S., R. A. Dorrington, and T. G. Cooper.** 1994. The *UGA4* UAS_{NTR} site required for *GLN3*-dependent transcriptional activation also mediates *DAL80*-responsive regulation and DAL80 protein binding in *Saccharomyces cerevisiae*. J. Bacteriol. **176:**4718–4725.
18. **Daugherty, J. R., R. Rai, H. M. El Berry, and T. G. Cooper.** 1993. Regulatory circuit for responses of nitrogen catabolic gene expression to the GLN3 and DAL80 proteins and nitrogen catabolite repression in *Saccharomyces cerevisiae*. J. Bacteriol. **175:**64–73.
19. **Drillen, R., M. Aigle, and F. Lacroute.** 1973. Yeast mutants pleiotropically impaired in the regulation of two glutamate dehydrogenases. Biochem. Biophys. Res. Commun. **53:**367–372.
20. **Drillen, R., and F. Lacroute.** 1972. Ureidosuccinic acid uptake in yeast and some aspects of its regulation. J. Bacteriol. **109:**203–208.
21. **Grenson, M., E. Dubois, M. Piotrowska, R. Drillen, and M. Aigle.** 1974. Ammonia assimilation in *Saccharomyces cerevisiae* as mediated by the two glutamate dehydrogenases. Mol. Gen. Genet. **128:**73–85.
22. **Hoffman, W.** 1985. Molecular characterization of the *CAN1* locus in *Saccharomyces cerevisiae*: a transmembrane protein without N-terminal hydrophobic signal sequence. J. Biol. Chem. **260:**11831–11837.
23. **Ito, H., Y. Fukuda, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
24. **Jauniaux, J.-C., and M. Grenson.** 1990. *GAP1*, the general amino acid permease gene of *Saccharomyces cerevisiae*: nucleotide sequence, protein similarity with other baker's yeast amino acid permeases, and nitrogen catabolite repression. Eur. J. Biochem. **190:**39–44.
25. **Ljungdahl, P. O., J. Gimeno, C. A. Styles, and G. R. Fink.** 1992. *SHR3*: a novel component of the secretory pathway specifically required for localization of amino acid permeases in yeast. Cell **71:**463–478.
26. **Marczak, J. E., and M. C. Brandriss.** 1991. Analysis of constitutive and noninducible mutations of the *PUT3* transcriptional activator. Mol. Cell. Biol. **11:**2609–2619.
27. **Miller, S. M., and B. Magasanik.** 1991. Role of the complex upstream region of the *GDH2* gene in nitrogen regulation of the NAD-linked glutamate dehydrogenase in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**6229–6247.
28. **Minehart, P. L., and B. Magasanik.** 1991. Sequence and expression of *GLN3*, a positive nitrogen regulatory gene of *Saccharomyces cerevisiae* encoding a protein with a putative zinc finger DNA-binding domain. Mol. Cell. Biol. **11:**6216–6228.
29. **Minehart, P. L., and B. Magasanik.** 1992. Sequence of the *GLN1* gene of *Saccharomyces cerevisiae*: role of the upstream region in regulation of glutamine synthetase expression. J. Bacteriol. **174:**1828–1836.

30. **Mitchell, A. P., and B. Magasanik.** 1984. Regulation of glutamine-repressible gene products by the *GLN3* function in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:**2758–2766.
30a. **Rai, R., T. S. Cunningham, and T. G. Cooper.** Unpublished data.
31. **Rai, R., J. R. Daugherty, and T. G. Cooper.** 1995. UAS_{NTR} functioning in combination with other UAS elements underlies exceptional patterns of nitrogen regulation in *Saccharomyces cerevisiae*. Yeast **11:**247–260.
32. **Rai, R., F. S. Genbauffe, and T. G. Cooper.** 1987. Transcriptional regulation of the *DAL5* gene in *Saccharomyces cerevisiae*. J. Bacteriol. **169:**3521–3524.
33. **Rai, R., F. S. Genbauffe, and T. G. Cooper.** 1987. Structure and transcription of the allantoate permease gene (*DAL5*) from *Saccharomyces cerevisiae*. J. Bacteriol. **170:**266–271.
34. **Rai, R., F. S. Genbauffe, R. A. Sumrada, and T. G. Cooper.** 1989. Identification of sequences responsible for transcriptional activation of the allantoate permease gene in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **9:**602–608.
35. **Smith, M. M., and O. S. Anderson.** 1983. DNA sequences of yeast H3 and H4 histone genes from two non-allelic gene sets encode identical H3 and H4 proteins. J. Mol. Biol. **169:**663–690.
36. **Svetlov, V., T. S. Cunningham, R. Rai, and T. G. Cooper.** *S. cerevisiae* Gln3p binds to UAS_{NTR} elements and activates transcription of nitrogen catabolite repression-sensitive genes. Submitted for publication.
37. **Vissers, S., B. Andre, F. Muyldermans, and M. Grenson.** 1989. Positive and negative regulatory elements control the expression of the *UGA* gene coding for the inducible 4-aminobutyric-acid-specific permease in *Saccharomyces cerevisiae*. Eur. J. Biochem. **181:**357–361.
38. **Wang, S. S., and M. C. Brandriss.** 1987. Proline utilization in *Saccharomyces cerevisiae*: sequence, regulation, and mitochondrial location of the *PUT1* gene product. Mol. Cell. Biol. **7:**4431–4440.
39. **Wiame, J.-M., M. Grenson, and H. Arst.** 1985. Nitrogen catabolite repression in yeasts and filamentous fungi. Adv. Microb. Physiol. **26:**1–87.
40. **Wickerham, L. J.** 1946. A critical evaluation of the nitrogen assimilation tests commonly used in the classification of yeasts. J. Bacteriol. **52:**293–301.
41. **Wickner, R. B.** 1994. [*URE3*] as an altered URE2 protein: evidence for a prion analog in *Saccharomyces cerevisiae*. Science **264:**566–569.
42. **Xu, S., D. A. Falvey, and M. C. Brandriss.** 1995. Roles of *URE2* and *GLN3* in the proline utilization pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **15:**2321–2330.