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Nitrogen GATA Factors Participate in Transcriptional Regulation of Vacuolar Protease Genes in *Saccharomyces cerevisiae*

JONATHAN A. COFFMAN AND TERRANCE G. COOPER*

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

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The expression of most nitrogen catabolic genes in *Saccharomyces cerevisiae* is regulated at the level of transcription in response to the quality of nitrogen source available. This regulation is accomplished through four GATA-family transcription factors: two positively acting factors capable of transcriptional activation (Gln3p and Gat1p) and two negatively acting factors capable of down-regulating Gln3p- and/or Gat1p-dependent transcription (Dal80p and Deh1p). Current understanding of nitrogen-responsive transcriptional regulation is the result of extensive analysis of genes required for the catabolism of small molecules, e.g., amino acids, allantoin, or ammonia. However, cells contain another, equally important source of nitrogen, intracellular protein, which undergoes rapid turnover during special circumstances such as entry into stationary phase, and during sporulation. Here we show that the expression of some (*CPS1*, *PEP4*, *PRB1*, and *LAP4*) but not all (*PRC1*) vacuolar protease genes is nitrogen catabolite repression sensitive and is regulated by the GATA-family proteins Gln3p, Gat1p, and Dal80p. These observations extend the global participation of GATA-family transcription factors to include not only well-studied genes associated with the catabolism of small nitrogenous compounds but also genes whose products are responsible for the turnover of intracellular macromolecules. They also point to the usefulness of considering control of the nitrogen-responsive GATA factors when studying the regulation of the protein turnover machinery.

Identifying regulatory-circuit components and interactions that integrate the control of cellular processes is a highly active field of research. The developing picture of molecular events controlling the cell division cycle represents one of the more impressive successes in our quest to understand such complex regulatory networks. Transcriptional regulation of the genes whose products catalyze transport and degradation of small nitrogenous compounds (e.g., amino acids, allantoin, and ammonia, etc.) in yeast and fungi is also being used as a model to investigate eucaryotic regulatory networks and their responses to environmental signals. In this case, nitrogen catabolic gene expression responds to the presence of preferred nitrogen sources (e.g., glutamine or asparagine) by decreasing expression of genes associated with the transport and degradation of poor nitrogen sources (e.g., proline, allantoin, and γ-amino butyrate, etc.); this physiological control is designated nitrogen catabolite repression (NCR) (1, 3, 4, 7, 10–22, 24–28, 36–38, 43–45, 50–52, 54–56).

NCR-sensitive gene expression in *Saccharomyces cerevisiae* is mediated by four GATA-family transcription factors, each containing a GATA-binding zinc finger motif that has been conserved in organisms from yeast to metazoans (2, 29, 31, 33, 41, 42, 53). The yeast GATA factors may be grouped on the basis of related functions: two act positively (Gln3p and Gat1p/Nil1p) while the other two act negatively (Dal80p/Uga43p and Deh1p/Gzf3p/Nil2p/YJL110c) (1, 10–15, 18–20, 24–28, 32, 36–38, 45, 46, 51, 54, 56). Both Gln3p and Gat1p are required for maximal NCR-sensitive gene expression and support transcriptional activation when recruited upstream of a core promoter that is devoid of upstream activation sequence (UAS) elements (4, 7, 11–13, 18, 22, 26, 27, 37, 38, 51). Both Dal80p and Deh1p down-regulate genes dependent on Gln3p and Gat1p, as indicated by the fact that transcription of these genes increases markedly when either *DAL80* or *DEH1* is deleted (1, 10, 12, 13, 15, 17, 20, 24–26, 28, 52, 54). The positively and negatively acting GATA factors appear to function in opposition to one another to regulate NCR-sensitive gene expression (1, 15, 28). The fact that Dal80p and Deh1p have been shown to bind to the same DNA sequences that also bind Gln3p has led to the further suggestion that they may down-regulate Gln3p- and Gat1p-dependent transcription by competing with these activator proteins for binding to their target promoter elements (4, 13, 15, 24–28, 36).

In addition to the above four GATA-family transcription factors, nitrogen catabolic gene expression is negatively regulated by Ure2p and one or more unidentified proteins which appear to function analogously (1, 11–13, 21, 22, 55, 56). These negative regulators, which are not GATA related, down-regulate Gln3p and Gat1p operation through a biochemical mechanism(s) that is not currently understood. Only the C-terminal domain of Ure2p is required for Gln3p and Gat1p control (55); the N-terminal domain of Ure2p possesses the property of giving rise to a prion form, Ure3p (55).

Much information has recently been reported about the interrelationships of the four yeast GATA factors and their integrated participation in the regulation of nitrogen metabolism in yeast (1, 13–15, 24, 26, 28, 51, 52). There is, however, a deficiency in our overall understanding of nitrogen catabolism in *S. cerevisiae*. Our understanding of NCR and its global regulation of nitrogen catabolism in this organism has been derived exclusively from the investigation of gene and enzyme systems responsible for the accumulation and catabolism of small nitrogenous compounds. These studies have ignored protein as a potentially significant source of intracellular nitrogen.

The study of protease biochemistry, processing, cellular compartmentation, and targeting in yeast is highly advanced (5, 6, 9, 23, 30, 34–35, 40, 47, 49), as is the study of the role of

* Corresponding author. Phone: (901) 448-6175. Fax: (901) 448-8462. E-mail: tcooper@utmem1.utmem.edu.

protein turnover in metabolic inactivation and sporulation. There is preliminary evidence that *CPS1*, encoding carboxypeptidase S, responds to nitrogen source availability and to nitrogen starvation (5, 6, 12). However, no data address whether or not protease gene expression, including that of *CPS1*, is regulated by the global NCR network responsible for the regulation of nitrogen catabolism in yeast cells. Here we present evidence that some, but not all, of the vacuolar protease genes are regulated by the GATA-family transcription activators responsible for global nitrogen regulation in *S. cerevisiae*. This information will be useful to researchers studying the detailed regulation of these gene and enzyme systems. One of the results reported here has also been independently reported by Naik et al. (40).

All strains used in this work have been described earlier: *gln3*Δ strain RR91 (13, 28), *gat1*Δ strain RJ71 (13), *dal80*::*hisG* strain TCY17 (24–26), *ure2*Δ strain JCY125 (11), *gln3*Δ *ure2*Δ strain JCY37 (12), *gln3*Δ *DAL80*::*hisG* strain RR92 (12), and *gln3*Δ *gat1*Δ strain RJ72 (13). Strains were grown in Wickerham's minimal medium with glucose and a nitrogen source at concentrations of 0.6 and 0.1%, respectively. Uracil (20 mg/liter), L-glutamine (30 mg/liter), and L-lysine (40 mg/liter) were added as auxotrophic requirements when needed. Cultures were grown to mid-log phase, and RNA was prepared and analyzed as described earlier (8, 10–15).

Regulation of the *CPS1* gene, encoding vacuolar carboxypeptidase S. The first vacuolar protease gene whose regulation we investigated was *CPS1*. This was done by assaying the steady-state levels of *CPS1* mRNA in GATA-factor mutant strains possessing defects in the control of nitrogen catabolic gene expression by Northern blot analysis. Steady-state levels of *CPS1* mRNA decreased in a *gln3* deletion mutant, indicating a partial dependence on Gln3p (Fig. 1, lanes A, B, F, and G). However, residual Gln3p-independent *CPS1* expression remained NCR sensitive (lanes B and C), i.e., lower *CPS1* mRNA levels were observed with glutamine as the nitrogen source than with proline. To further characterize nitrogen-responsive *CPS1* expression, we assayed the effects of deleting the negative regulator of Gln3p operation, Ure2p. Although deletion of *URE2* resulted in increased *CPS1* expression under repressive conditions when Gln3p was present (see Fig. 3, lanes F, H, I, and K, in reference 11), this increase (i.e., derepression or NCR insensitivity) was not observed in a *gln3* deletion background (Fig. 1, lanes D and E). In addition, NCR-sensitive, Gln3p- and Ure2p-independent *CPS1* expression was observed (Fig. 1, lanes D and E). These characteristics are indicative of a gene whose expression requires the participation of both positively acting GATA factors, Gln3p and Gat1p. To directly test this possibility, we determined the levels of *CPS1* expression in wild-type strains, *gln3* and *gat1* single-deletion mutant strains, and *gln3 gat1* double-deletion mutant strains (TCY1, RR91, RJ71, and RJ72, respectively) (Fig. 1, lanes F to I). *CPS1* expression was partially dependent upon both Gln3p and Gat1p; barely detectable levels of *CPS1* mRNA were observed in the *gln3 gat1* double-deletion mutant (lane H). The Gln3p requirement was greater, however, than that for Gat1p.

Regulation of the *PRB1* and *PRC1* genes, encoding vacuolar endoproteinase B and carboxypeptidase Y. The NCR sensitivity of *CPS1* prompted us to evaluate the transcriptional regulation of the genes that encode vacuolar endoproteinase B (*PRB1*) and carboxypeptidase Y (*PRC1*) (34, 39). Steady-state *PRB1* mRNA levels were lower in minimal glucose-asparagine medium than they were in glucose-proline medium, indicating the NCR sensitivity of this gene's expression (Fig. 2, lanes F and G). Recently, a similar observation has also been reported

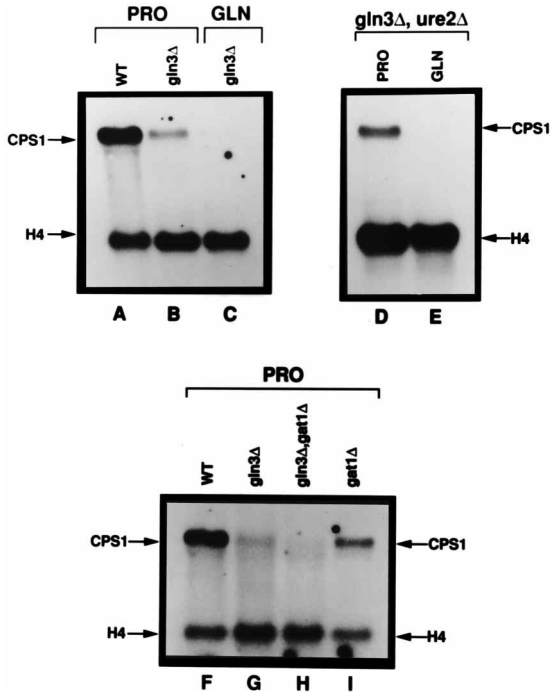

FIG. 1. Northern analysis demonstrating the effects of regulatory mutations and NCR on the transcription of *CPS1*. Steady-state mRNAs were obtained from wild-type (WT; strain TCY1) (lanes A and F), *gln3*Δ (strain RR91) (lanes B, C, and G), *gln3*Δ *ure2*Δ (strain JCY37, lanes D and E), *gln3*Δ *gat1*Δ (strain RJ72) (lane H), and *gat1*Δ (strain RJ71) (lane I) strains following growth with the depicted nitrogen source (proline [PRO] or glutamine [GLN]). Poly(A)$^+$ RNAs (10 μg per lane) immobilized to a nylon membrane were visualized by hybridization with end-labeled synthetic oligonucleotides complementary to the *CPS1* (positions 162 to 112) and histone H4 genes (5, 6, 48). Levels of histone H4 mRNA were assayed as a control to determine loading and transfer efficiencies.

(40). In addition, an increase in *PRB1* mRNA levels in a *dal80* disruption mutant indicated negative regulation by Dal80p (Fig. 2, lanes D and E). Consistent with these findings was the observation that *PRB1* expression was partially Gln3p dependent (Fig. 2, lanes A and B). Deletion of *URE2* had little if any demonstrable effect on *PRB1* steady-state mRNA levels, suggesting that Ure2p does not play a significant role in the NCR sensitivity of this gene (Fig. 2, lanes F to I). The *ure2* mutant was assayed both with proline and with glutamine as the nitrogen source because a *ure2* phenotype can, under some circum-

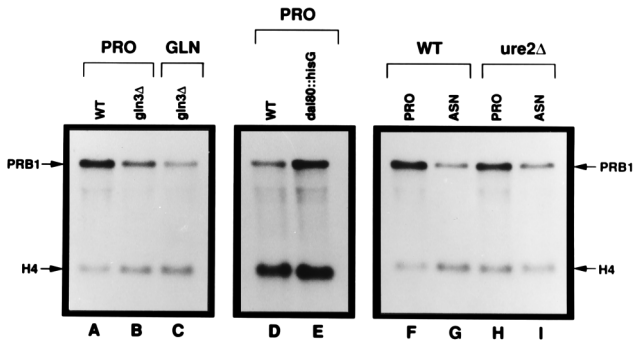

FIG. 2. Northern analysis demonstrating the effects of regulatory mutations and NCR on the transcription of *PRB1*. Strains and experimental procedures were as described for Fig. 1. The hybridization probe for *PRB1* mRNA was an end-labeled oligonucleotide covering positions 806 to 756 (39). The *ure2*Δ and *dal80*Δ strains were JCY125 and TCY17, respectively.

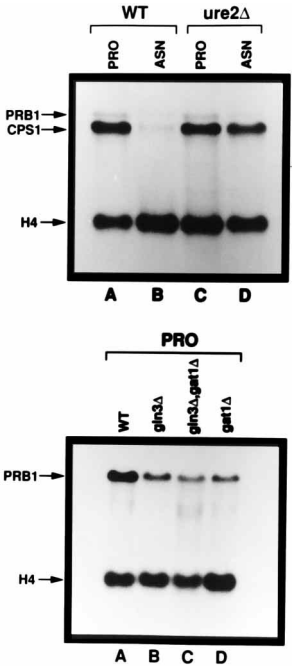

FIG. 3. (Top) Northern blot analysis demonstrating differences in the response of *CPS1* and *PRB1* expression to the deletion of *URE2*. (Bottom) Northern blot analysis demonstrating the effects of deleting the nitrogen-regulatory GATA-factor genes, *GLN3* and *GAT1*, on the steady-state mRNA levels of *PRB1*. Strains and experimental procedures were as described for Fig. 1 and 2.

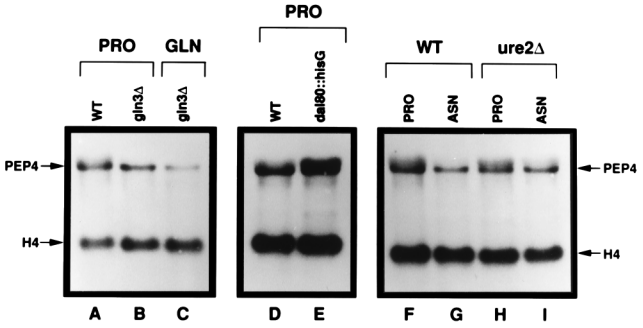

FIG. 4. Northern analysis demonstrating the effects of regulatory mutations and NCR on *PEP4* mRNA levels. Strains and experimental procedures were as described for Fig. 1 and 2. The blots were hybridized with end-labeled oligonucleotides complementary to *PEP4* (positions 372 to 322) and H4 (34a–35).

stances, be observed with nonrepressing as well as repressing nitrogen sources (12). To confirm that *PRB1*'s lack of response to the deletion of *URE2* was not an artifact, we rehybridized the same blot with a *CPS1*-specific hybridization probe; *CPS1* expression served as an internal positive control (Fig. 3, upper panel). In agreement with previous observations, *CPS1* steady-state mRNA levels increased in the *ure2* deletion strain relative to the wild-type strain, i.e., expression became less NCR sensitive (Fig. 3, upper panel, lanes B and D). There was significant Gln3p-independent *PRB1* expression (Fig. 2, lanes A and B), raising the possibility that *PRB1* expression was also Gat1p dependent. *PRB1* expression was about equally dependent upon both Gln3p and Gat1p, as indicated in Fig. 3 by the similar decreases in *PRB1* mRNA found in *gln3* and *gat1* deletion mutant strains relative to the wild-type strain (Fig. 3, lower panel, lanes A, B, and D). In contrast to the situation with *CPS1*, detectable amounts of *PRB1* mRNA were present in the *gln3 gat1* double-deletion mutant, indicating the existence of transcription that was independent of the nitrogen-regulatory factors (Fig. 3, lower panel, lane C).

To determine the regulatory characteristics of the carboxypeptidase Y gene, *PRC1*, we made measurements similar to those described for Fig. 1 to 3. Steady-state levels of *PRC1* mRNA did not respond to the nature of the nitrogen source provided or to mutation of nitrogen-regulatory genes (data not shown). Consistent with these data is the correlation that the *PRC1* promoter does not contain UAS_{NTR}-homologous GATA sequences.

Regulation of *PEP4*, encoding vacuolar proteinase A. Proteinase A is responsible for posttranslational modification and activation of multiple vacuolar proteases as well as of alkaline phosphatase (34–35). We assayed expression of its cognate gene (*PEP4*) in the presence of different-quality nitrogen sources. *PEP4* expression was higher in cells growing in glucose-proline medium than in glucose-asparagine, indicating NCR sensitivity (Fig. 4, lanes F and G). This NCR sensitivity did not, however, involve Ure2p, as indicated by the fact that the wild-type strain and *ure2* deletion mutant possessed similar levels of *PEP4* mRNA (Fig. 4, lanes F to I). *PEP4* expression was greater in a *dal80* disruption mutant than in the wild type, indicating the participation of this negatively acting regulatory element in the regulation of *PEP4* expression (Fig. 4, lanes D and E). Finally, *PEP4* expression was partially Gln3p dependent (Fig. 4, lanes A and B), and the expression observed in the *gln3* deletion mutant was modestly NCR sensitive (Fig. 4; compare lanes B and C).

Regulation of *LAP4*/*APE1*, encoding vacuolar aminopeptidase I. The last vacuolar protease gene we analyzed was *LAP4*/*APE1*, encoding aminopeptidase I (9, 23, 34). *LAP4* expression was only modestly NCR sensitive (Fig. 5, upper panel, lanes D and E), and this sensitivity was not decreased (mRNA levels did not increase) upon deletion of *URE2* (Fig. 5, upper panel, lanes F and G). Expression of *LAP4* exhibited a significant requirement for Gln3p, as evidenced by a decrease in steady-state *LAP4* mRNA in a *gln3* deletion mutant (Fig. 5, upper

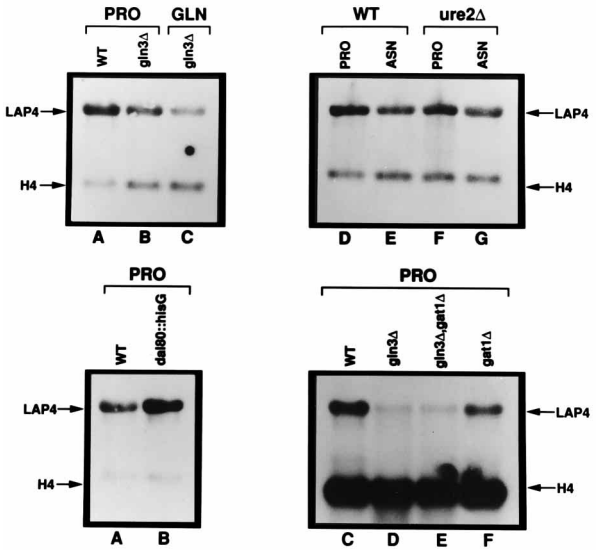

FIG. 5. Northern analysis demonstrating the effects of regulatory mutations and NCR on the transcription of *LAP4*. Strains and experimental procedures were as described for Fig. 1 and 2. The hybridization probe for *LAP4* was an end-labeled oligonucleotide covering positions 468 to 418 (9, 23).

panel, lanes A and B, and lower panel, lanes C and D). At first glance, the data in the upper panel of Fig. 5, lanes A and B, appear to demonstrate a smaller requirement than is observed in lanes C and D of the lower panel. This derives from the poor uniformity of loading in upper-panel lanes A and B, i.e., lane A is underloaded. Although *LAP4* expression was Gln3p dependent, loss of Gat1p reduced expression less than two- or threefold (Fig. 5, lower panel, lanes C and F). A similarly small effect (increase in expression) was observed in *LAP4* expression upon deletion of *DAL80* (Fig. 5, lower panel, lanes A and B).

Finally, the regulation of vacuolar protease genes by GATA-family transcriptional regulators prompted us to investigate preliminarily whether such control was observed for other components of the protein turnover system (30). Although preliminary experiments detected small nitrogen-related effects on expression of the *TOR1*, *UBR1*, and *UBC4* genes, we were not able to obtain reproducible evidence that convincingly demonstrated GATA-factor control of genes encoding components of the ubiquitin-associated pathway of protein degradation (10a).

This work demonstrates that expression of several, but not all, genes encoding vacuolar proteases is NCR sensitive and is regulated by the GATA-family proteins Gln3p, Gat1p, and Dal80p. The degree of regulation we observed is highly gene specific and ranges from high NCR sensitivity and major regulation of *CPS1* expression to very modest regulation of *PEP4* and the lack of such regulation in the case of *PRC1*. These data suggest that expression of genes whose products participate in the catabolism of macromolecules such as proteins is subject, in some cases, to the same mechanisms of regulation that are imposed on pathways responsible for the degradation of small nitrogenous compounds available externally or mobilized from the cell vacuole. The sharing of these global transcription-regulatory mechanisms further integrates utilization of all nitrogen sources, large and small, available to the cell both internally and externally.

While common regulation can be demonstrated for some protease genes and those associated with small-molecule nitrogen sources, it is important to point out that NCR-sensitive control represents only part of the overall regulation exerted on protease gene expression. A clear example of additional, nitrogen-independent regulation of protease gene transcription has recently been characterized and reported for *PRB1* (40). Finally, although our results point out the pertinence of nitrogen-responsive transcriptional regulation to researchers investigating the genes responsible for protein turnover who might not otherwise consider this form of control on the genes they study, they do not provide insight into the question of why some protease genes but not others are regulated in response to the quality of nitrogen source available. These insights are likely to surface only when we have a more complete view of the details of transcriptional regulation imposed on the large number of cellular components that participate in macromolecular turnover.

We greatly appreciated the opportunity to exchange our experimental data set with that derived from the nitrogen regulation experiments of R. R. Naik, V. Nebes, and E. W. Jones before either set was submitted for publication. We also thank members of the University of Tennessee Yeast Group who read this manuscript and offered suggestions for improvement. Oligonucleotides were prepared by the University of Tennessee Molecular Resource Center.

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