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Role of GATA Factor Nil2p in Nitrogen Regulation of Gene Expression in *Saccharomyces cerevisiae*

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We have identified the product of the *NIL2* gene of *Saccharomyces cerevisiae* which contains a zinc finger region highly homologous to those of the GATA factors Gln3p and Nil1p as an antagonist of Nil1p and to a lesser extent of Gln3p. The expression of many nitrogen-regulated genes of *Saccharomyces cerevisiae* requires activation by GATA factor Gln3p or Nil1p and is prevented by the presence of glutamine in the growth medium. Disruption of *NIL2* results in a great increase in the expression of *NIL1* and of *GAP1*, the structural gene for the general amino acid permease, in glutamine-grown cells in response to activation by Nil1p. The primary effect of the elimination of Nil2p appears to be an increase in the intracellular level of Nil1p, which in turn is responsible for increased expression of *GAP1*. Experiments using an artificial UAS (upstream activating site) consisting of three GATAAGATAAG sites revealed that Nil2p exerts its effect by competing primarily with Nil1p and less effectively with Gln3p for these sites. Apparently, the principal role of Nil2p is to prevent activation of transcription by Nil1p unless Nil1p has been converted to a more active state by the absence of glutamine and glutamate.

It is now well established that the expression of many genes of *Saccharomyces cerevisiae* whose products are responsible for the utilization of different nitrogen compounds as sources of nitrogen is activated by two zinc finger proteins that recognize the sequence GATAAG located upstream of these genes (4, 5, 16, 21, 23, 25). One of these activators is the product of the *GLN3* gene, and its ability to activate transcription is opposed by the product of the *URE2* gene in response to an increase in the intracellular level of glutamine (2, 6, 7, 13, 19, 21). The other activator, the product of *NIL1* (also called *GAT1*) has a zinc finger highly homologous to that of Gln3p and is capable of activating some of the same promoters as Gln3p, but its activity appears to be antagonized by an as yet unknown protein in response to the rise in the intracellular concentration of glutamate (5, 25). As a result, transcription of a susceptible gene such as *GAP1*, coding for the general amino acid permease, is activated almost exclusively by Gln3p during growth with glutamate as the source of nitrogen and almost exclusively by Nil1p during growth with ammonia or urea as the source of nitrogen and is not activated at all during growth in a medium containing glutamine (25).

In addition to possessing homologous zinc fingers, Gln3p and Nil1p also resemble one another by possessing highly acidic amino-terminal domains, characteristic of many activators (25). Two other proteins also possess zinc fingers with high homology to those of Gln3p and Nil1p, but they lack the acidic amino-terminal portions (8, 25). One of these proteins, the product of *DAL80*, has been identified as an antagonist of Gln3p in the case of some, but not all, Gln3p-dependent genes (10, 12). Apparently, Dal80p requires two GATAAG sequences located not more than 20 bp apart to be effective (9). In the case of these genes, deletion of *DAL80* results in greatly increased Gln3p-dependent expression in a medium with proline as the source of nitrogen, but not in a medium containing asparagine in which Ure2p keeps Gln3p in an inactive form (12).

We are now describing the effects of eliminating the fourth of the homologous zinc finger proteins, Nil2p, on the expression of nitrogen-regulated genes. Our results show that Nil2p primarily antagonizes Nil1p and that a major effect of Nil2p is to prevent the autogenous activation of *NIL1* expression by Nil1p. Consequently, the level of Nil1p in cells lacking Nil2p grown with glutamine as the source of nitrogen is sufficiently high to allow the activation of transcription of *GAP1* and of other Nil1p-responsive genes.

MATERIALS AND METHODS

Yeast strains. The strains of *S. cerevisiae* used in this study are listed in Table 1 and are all derived from strain PM38. Disruption of *NIL2* was accomplished by integrative transformation with the plasmid pCXΔBB (25) after digestion with *Sac*I and *Xba*I. The *URA3* gene was lost by selection on 5-fluoroorotic acid. Disruption was confirmed by PCR of genomic DNA.

Yeast media and methods. Yeast synthetic minimal medium (SD) was composed of 0.17% yeast nitrogen base without amino acids and ammonium sulfate, 2% glucose, and either 0.1% glutamine, 0.128% monosodium glutamate, or 0.2% ammonium sulfate as the nitrogen source. Standard methods were used for the growth and manipulation of yeast cells (1, 22).

Construction of the *NIL1-lacZ* and *NIL2-lacZ* gene fusion plasmids. The *NIL1-lacZ* gene fusion was constructed by digestion of the *NIL1* clone pZF24-3 with *Xho*I, rendering the ends blunt, and digestion with *Sal*I. A 0.86-kb fragment from pZF24-3 from bp −672 to +186 was ligated into plasmid pSLFΔ178K (14) after digestion with *Bam*HI, filling in, and digestion with *Xho*I to create pRE10. Plasmid pSLFΔ178K contains a 2 μm yeast replication origin and an *URA3* marker, in addition to the *lacZ* gene.

The *NIL2-lacZ* fusion was created by PCR amplification of a fragment of *NIL2* from bp −854 to +13 from genomic DNA with sites for *Kpn*I and *Bam*HI added to the 5′ and 3′ ends, respectively. After digestion with *Kpn*I and *Bam*HI, the PCR product was cloned into *Kpn*I- and *Bam*HI-digested pSLFΔ178K to create plasmid pRE22.

β-Galactosidase assays. Fresh transformants were used to inoculate synthetic minimal cultures with the various nitrogen sources. The cultures were grown overnight, and cells were harvested by centrifugation when the optical density at 600 nm (OD_{600}) was between 0.4 and 0.8. The cells were washed in 0.1 M sodium phosphate buffer, pH 7.0, and the cell pellet was stored at −70°C. For assay, the cells were resuspended in assay buffer, and β-galactosidase activity was determined as previously described with the units normalized to the OD_{600} (1, 17). The β-galactosidase activity was measured from three independent transformants and agreed within 10%.

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TABLE 1. Yeast strains and plasmids used in this study

Strain or plasmid	Genotype or description[a]	Source
Strains		
YDR10	*MATα ura3-52*	This study
YNE12	*MATα ura3-52 nil2ΔBB::hisG*	This study
PM71	*MATα ura3-52 leu2-3,112 gln3Δ5::LEU2*	19
YNE15	*MATα ura3-52 leu2-3,112 gln3Δ5::LEU2 nil2ΔBB::hisG*	This study
MS221	*MATα ura3-52 nil1::hisG*	25
YNE17	*MATα ura3-52 nil1::hisG nil2ΔBB::hisG*	This study
YDR21	*MATα ura3-52 leu2-3,112 gln3Δ5::LEU2 nil1::hisG*	25
YNE19	*MATα ura3-52 leu2-3,112 gln3Δ5::LEU2 nil1::hisG nil2ΔBB::hisG*	This study
Plasmids		
pCXΔBB	*nil2::hisG::URA3::hisG*	25
pRE10	*NIL1* (−672 to +186 bp)-*lacZ* in pSLFΔ178K	This study
pRE22	*NIL2* (−854 to +13 bp)-*lacZ*	This study
pPM70	*GLN1* (−659 to +49 bp)-*lacZ*	20
pL77	*GDH2* (−856 to −125 bp)-*CYC1-lacZ*	18
pSE	*GAP1* (−749 to −204 bp)-*CYC1-lacZ*	24
pNA	*GAP1* (−652 to −510 bp)-*CYC1-lacZ*	24
pRA	*GAP1* (−584 to −510 bp)-*CYC1-lacZ*	24
pAX	*GAP1* (−510 to −378 bp)-*CYC1-lacZ*	24
pXE	*GAP1* (−378 to −204 bp)-*CYC1-lacZ*	24

[a] All base pair numbering is relative to the translational start site of each gene.

For the experiments in which cells were shifted from media containing glutamine as the nitrogen source to ammonia, the cultures were grown to an OD_{600} of about 0.3 in SD-glutamine, and the cells were collected by filtration, washed with water, and resuspended in prewarmed SD-ammonia medium. Aliquots were removed at timed intervals for β-galactosidase assay and cell density measurement in Klett units.

RESULTS

Effect of Nil2p elimination. We initially observed that cells with an insertion in *NIL2* differed from the wild-type cells by their ability to activate transcription of the *GAP1* gene during growth with glutamine as the source of nitrogen. We subsequently investigated the effect of the elimination of Nil2p on the expression of a number of genes which are potential targets for nitrogen regulation because of the presence of one or more GATAAG elements in the DNA located upstream of their promoters. To this end, we used plasmids carrying fusions of these regions to *lacZ* in wild-type cells and cells lacking Nil2p and measured the levels of β-galactosidase during growth with glutamine, glutamate, or ammonia as the source of nitrogen.

We found that the expression of the *GLN3* and *NIL2* genes was not affected by the nitrogen source or by the absence of Nil2p and that the expression of *GLN1* (which largely depends on Gln3p) and *GDH2* (which depends entirely on Gln3p) were, as previously shown, subject to nitrogen regulation (6, 18, 19, 21) but were not affected by the lack of Nil2p (data not shown). On the other hand, the expression of *GAP1*, of a fusion of *lacZ* to the GATAAGATAAG element which was repeated three times (20), and of *NIL1* were greatly increased by the elimination of Nil2p when cells carrying the respective plasmids were grown with glutamine as the source of nitrogen (Table 2).

Activation by Gln3p and Nil1p in the presence or absence of Nil2p. We placed plasmids carrying fusions of the upstream regions of genes shown to be responsive to Nil2p into wild-type, *gln3*, *nil1*, and *gln3 nil1* cells which either possessed or lacked a functional *NIL2* gene and measured the levels of β-galactosidase in these cells, which were grown with glutamine, glutamate, or ammonia as the source of nitrogen. The results of these experiments are presented in Table 2, where the β-galactosidase activity of glutamate-grown cells is set at 100 to allow comparison. Considering first the regulation of *GAP1* expression, it can be seen that, as previously reported, the expression of this gene requires either Gln3p or Nil1p (5, 25) and that it is largely dependent on Gln3p in cells growing with glutamate as the source of nitrogen (column 2, compare rows 1 and 3) and largely dependent on Nil1p in cells growing with ammonia as the source of nitrogen (column 3, compare rows 1 and 5) (25). Elimination of Gln3p enhanced the Nil1p-dependent expression in ammonia-grown cells (column 3, compare rows 1 and 3). Elimination of Nil2p had no effect on Gln3p-activated *GAP1* expression, as shown by the observation that in *nil1* mutant cells, the loss of Nil2p did not allow Gln3p to activate the expression of *GAP1* when these cells were grown with glutamine or ammonia as the source of nitrogen (columns 1 and 3, compare rows 5 and 6). On the other hand, the elimination of Nil2p increased expression in wild-type cells grown with glutamine as the source of nitrogen (column 1, compare rows 1 and 2) and in *gln3* mutant cells grown with either glutamine or glutamate as the source of nitrogen (columns 1 and 2, compare rows 3 and 4); apparently, the absence of Nil2p enabled Nil1p to activate expression of *GAP1* during growth on these sources of nitrogen. It is likely that Nil2p, whose zinc finger is homologous to that of Nil1p, competes with Nil1p for the GATAAG sites required for the activation.

The homology of the zinc fingers of Gln3p and Nil2p raises the question whether Nil2p can also interfere in the same manner with the expression activated by Gln3p at GATAAG sites other than those upstream of *GAP1*. The experiments with an artificial activating region consisting of three GATAA GATAAG sites separated from one another by 21 bp (3GATA) (20) demonstrate that this is indeed the case. As shown in Table 2, the expression of 3GATA requires either Gln3p or Nil1p; there is no expression in a strain lacking both Gln3p and Nil1p (unpublished observation). In this case, elimination of Nil2p resulted in significant increases in the level of

TABLE 2. Effects of activation by Gln3p and Nil1p in the presence or absence of Nil2p on the levels of β-galactosidase in cells grown on different nitrogen sources

Bacterial strain genotype[a]	Relative β-galactosidase sp act[b]								
	GAP1-lacZ			3GATA-*lacZ*			*NIL1-lacZ*		
	Gln	Glt	NH_3	Gln	Glt	NH_3	Gln	Glt	NH_3
Wild type	2	100	36	0.2	100	11	18	100	94
nil2	46	109	67	87	223	164	58	114	104
gln3	0.2	13	82	0.2	11	75	3	7	31
gln3 nil2	21	30	73	39	155	157	27	25	29
nil1	0.4	66	8	1	247	34	9	66	39
nil1 nil2	0.3	84	11	5	475	97	11	67	34
gln3 nil1	0.01	0.01	0.01	ND	ND	ND	3	2	3
gln3 nil1 nil2	0.01	0.01	0.01	ND	ND	ND	5	2	2

[a] The wild-type bacterial strain was YDR10, the *nil2* strain was YNE12, the *gln3* strain was PM71, the *gln3 nil2* strain was YNE15, the *nil1* strain was MS221, the *nil1 nil2* strain was YNE17, the *gln3 nil1* strain was YDR21, and the *gln3 nil1 nil2* strain was YNE19.

[b] Plasmids carrying fusions of different regions were placed in bacterial strains. Plasmid pRE10 carried the *NIL1-CYC1-lacZ* fusion, pSE carried the *GAP1-CYC1-lacZ* fusion, and pB135 carried the 3GATA-*GAP1-CYC1-lacZ* fusion. The β-galactosidase levels, measured as units per OD_{600}, of cells growing with glutamate as the source of nitrogen (197 for *NIL1-lacZ*, 802 for *GAP1-lacZ*, and 87 for 3GATA-*lacZ*) were set at 100. Glutamine (Gln), Glutamate (Glt), and ammonia (NH_3) were the sources of nitrogen. ND, not determined.

β-galactosidase irrespective of the source of nitrogen (columns 4, 5, and 6, compare rows 1 and 2). The level of 3GATA expression in ammonia-grown cells was increased either by the elimination of Gln3p or by that of Nil2p, indicating that either one of these proteins can interfere with the activation by Nil1p (column 6, compare rows 1, 2, and 3). The fact that the elimination of Nil1p also increased 3GATA expression in ammonia-grown cells, though to a lesser degree than the elimination of Gln3p, indicates interference by Nil1p with Gln3p-activated expression (column 6, compare rows 1 and 5). Similarly, the expression of 3GATA in glutamate-grown cells was decreased by the elimination of Gln3p and increased either by elimination of Nil1p or by that of Nil2p, indicating that either one of these proteins can interfere with activation by Gln3p (column 5, compare rows 1, 2, 3, and 5); consequently, the highest expression of 3GATA was observed in glutamate-grown cells lacking both Nil1p and Nil2p (column 5, row 6).

It has previously been reported that in cells grown with proline as the source of nitrogen, the expression of *NIL1* was stimulated by Gln3p (5). We find now that as shown in Table 2, in glutamate-grown cells, the expression of this gene is almost totally dependent on Gln3p and that maximal expression requires both Gln3p and Nil1p (column 8, compare rows 1, 3, 5, and 7). Similarly, maximal expression in ammonia-grown cells requires both Gln3p and Nil1p (column 9, compare rows 1, 3, 5, and 7). It can also be seen that the elimination of Nil2p resulted in a significant increase of *NIL1* expression in glutamine-grown cells (column 7, compare rows 1 and 2); this increase was mediated solely by Nil1p, since the elimination of Nil2p did not alter the levels of β-galactosidase in *nil1* mutant cells (column 7, compare rows 5 and 6), but elimination of Nil2p remarkably augmented the levels of β-galactosidase in *gln3* mutant cells (column 7, compare rows 3 and 4). The elimination of Nil2p also increased the expression of *NIL1-lacZ* in *gln3* mutant cells, but not in *nil1* mutant cells, grown on glutamate (column 8, compare rows 3, 4, 5, and 6). Expression of *NIL1* in *gln3* mutant cells growing on ammonia was not affected by the lack of Nil2p; apparently, in these cells the activation of *NIL1* expression by Nil1p is maximal and cannot be further raised by the elimination of Nil2p (column 9, rows 3 and 4).

These results are in good accord with the view that the increased intracellular concentration of Nil1p resulting from the increased transcription of *NIL1* in mutants lacking Nil2p, is primarily responsible for the increased transcription of *GAP1*. In this connection, it is of some interest that the level of *NIL1* expression in the *gln3 nil2* double mutant is the same in cells grown on glutamine, glutamate, or ammonia but that the level of *GAP1* expression in this double mutant is lower in glutamine- and glutamate-grown cells than in ammonia-grown cells (Table 2, compare the values in row 4 for columns 1, 2, and 3 with those for columns 7, 8, and 9); apparently the loss of Nil2p has increased the Nil1p-dependent expression of *GAP1* by a mechanism other than rendering Nil1p insensitive to inactivation by glutamine or glutamate. On the other hand, in the *gln3* mutant grown on ammonia, the loss of Nil2p has increased the expression of 3GATA, even though the level of *NIL1* expression was the same in the absence or presence of Nil2p (column 6, compare rows 3 and 4). Consequently, it appears that Nil2p can also interfere directly with the activation of 3GATA by Nil1p.

Expression of *NIL1* and *GAP1* after a shift from glutamine to ammonia as the source of nitrogen. We attempted to correlate the expression of *GAP1* with that of *NIL1* by growing cells lacking Gln3p carrying either the plasmid with the fusion of the region upstream from *NIL1* to *lacZ* (pRE10) or the fusion of the region upstream of *GAP1* to *lacZ* (pSE) with glutamine as the source of nitrogen. The cells were harvested in the exponential phase of growth and were used to inoculate a medium with ammonia as the source of nitrogen to the same optical density measured as Klett units. The cultures grew after a short lag at the same rate, measured by the increase in Klett units. The expressions of *NIL1* and of *GAP1* were measured as units of β-galactosidase activity.

Figure 1A shows the correlation of the increase in cell density with the increase in β-galactosidase activity per milliliter of culture. It is apparent that the increase in the expression of *NIL1* is proportional to the increase in cell density from the very beginning; since the expression of *NIL1* depends on Nil1p in these *gln3* mutant cells, we can therefore conclude that the level of Nil1p in the glutamine-grown cells is adequate for full expression of *NIL1* during growth with ammonia as the source of nitrogen. It can be seen in Fig. 1A that in the case of the *NIL1-lacZ* plasmid the increase in β-galactosidase activity per Klett unit is approximately 2.6 U. On the other hand, the glutamine-grown cells used as inoculum contained 0.26 U of β-galactosidase per Klett unit. Apparently, the replacement of glutamine by ammonia as the source of nitrogen has resulted in a 10-fold increase in the ability of Nil1p to activate the expression of *NIL1.*

A different picture emerges when we correlate the increase of *GAP1* expression with the increase of cell mass: in Fig. 1A it can be seen that this increase accelerates during growth of the culture. Taking into account that in these *gln3* mutant cells the expression of *GAP1* depends on Nil1p, this observation indicates that the rate of *GAP1* expression reflects the Nil1p level of the cells. In that case we would expect the rate of *GAP1* expression to increase with the square of the rate of *NIL1* expression. We therefore plotted the specific activity of β-galactosidase generated by the *GAP1-lacZ* plasmid against the corresponding specific activity of the *NIL1-lacZ* plasmid on log-log paper, as illustrated in Fig. 1B. It should be pointed out that the highest point of the graph was not determined in the experiment shown in Fig. 1A but is taken from the β-galactosidase levels in *gln3* mutant cells that had grown with ammonia as the source of nitrogen recorded in Table 2 for *GAP1* and *NIL1*. It can be seen that the results reasonably approximate a straight line with a slope of close to 2.

In summary, these results suggest that *NIL1* is more sensitive to Nil1p-activated expression than *GAP1*: it can be fully activated by the low level of Nil1p in glutamine-grown cells. On the other hand, the expression of *GAP1* appears to depend on the intracellular concentration of Nil1p.

Effects of Gln3p, Nil1p, and Nil2p on activation from a single GATAAG element. The upstream regions of the promoters used in the experiments presented in Table 2 contained more than one GATAAG site and, in the case of *NIL1* and of *GAP1*, also contained auxiliary sites (5, 24). Therefore, we examined the expression from the region upstream of *GAP1* containing a single GATAAG element and either one or both of two elements previously identified as playing important auxiliary roles. One of these elements is a binding site for Abf1p, and the other is the sequence TTG(G/T)T (24). We determined the effects of the elimination of Gln3p and of Nil2p on the levels of β-galactosidase in cells growing on either glutamine, glutamate, or ammonia. The results of these experiments are summarized in Table 3. It can be seen that the presence of the Abf1p-binding site is essential for activation of expression by Nil1p, but not by Gln3p. Plasmid pAX which contains a TTGTT site, but not an Abf1p-binding site, responds well to activation by Gln3p when the cells are grown on glutamate but cannot be activated in ammonia-grown cells,

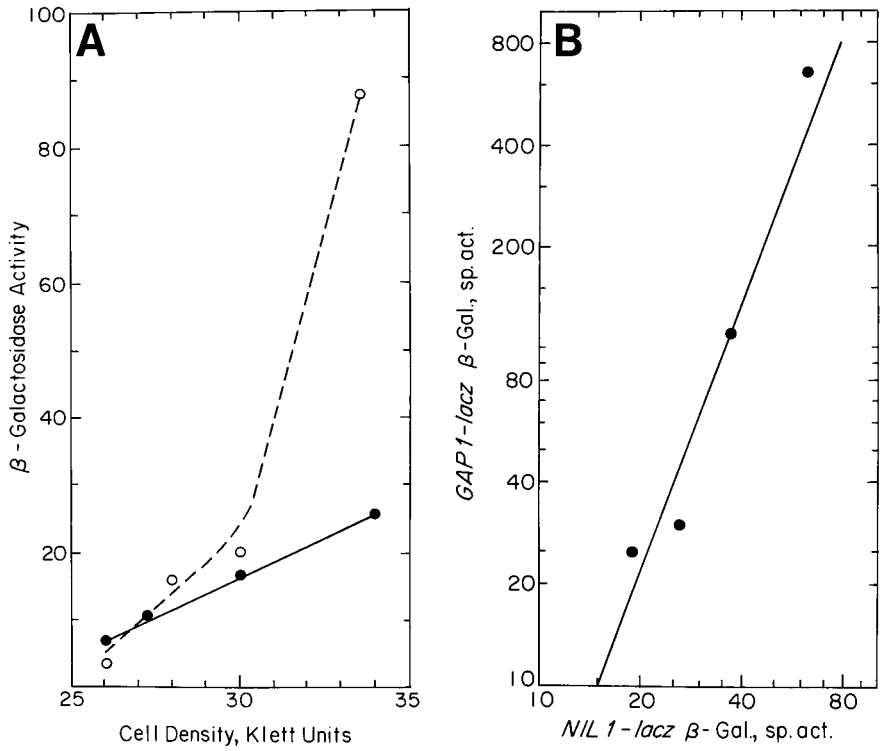

FIG. 1. Correlation of *NIL1* and *GAP1* expression in cells shifted from growth on glutamine to growth on ammonia. (A) Differential rates of *NIL1* (●) and *GAP1* (○) expression. The increase in β-galactosidase activity is per milliliter of culture plotted against the increase in cell mass measured in Klett units. (B) Log-log plot of the specific activity of β-galactosidase generated by the *GAP1-lacZ* fusion against the specific activity of β-galactosidase generated by the *NIL1-lacZ* fusion. The highest point is not from the shift experiments but from the specific activities of ammonia-grown cells of the *gln3 NIL1 NIL2* strain in Table 2. β-Gal., β-galactosidase; sp. act., specific activity.

and the elimination of Gln3p prevents activation, but the loss of Nil1p has almost no effect (columns 8 and 9, compare rows 1, 3, and 5). Nevertheless, the fact that elimination of Nil2p has no effect on the expression of this fusion in the *nil1* mutant but slightly raises the level of β-galactosidase in *GLN3 NIL1* and *gln3 NIL1* cells grown with glutamine as the source of nitrogen suggests some ability of Nil1p to activate transcription (column 7, compare rows 1, 2, 3, and 4). On the other hand, Nil1p and Gln3p are both effective activators when the plasmids carry an Abf1-binding site in addition to the GATAAG site; the effect of the TTGTT site is discernible by the overall higher β-galactosidase levels in cells carrying plasmid pNA over those of cells carrying plasmid pRA (see footnote *b* in Table 3). It can be seen that expression in glutamate-grown cells is almost totally dependent on Gln3p (columns 2 and 5, compare rows 1 and 3), that elimination of Gln3p raises the expression in ammonia-grown cells (columns 3 and 6, compare rows 1 and 3), and that elimination of Nil2p raises the β-galactosidase level in glutamine-grown cells irrespective of the presence of Gln3p (columns 1 and 4, compare rows 1, 2, 3, and 4). In conclusion, the expression of a fusion containing a single GATAAG site and a single Abf1-binding site responds to Gln3p, Nil1p, and Nil2p in the same manner as a fusion to the entire region upstream of *GAP1* which contains two functional GATAAG sites, two TTG (G/T)T sites and a single Abf1p-binding site (24).

We have reported previously that the three GATAAG sites located between the effective sites and the transcriptional start site do not support the activation of transcription (24). We considered the possibility that Nil2p renders these sites ineffective; however, plasmid pXE (204 to 378 bp upstream from the translational start site), which carries only this portion of the upstream region of *GAP1* was unable to produce β-galactosidase in any one of the three growth media, irrespective of the presence or absence of Nil2p (not shown).

DISCUSSION

The experimental results of this study show that the previously identified GATA factor Nil2p exerts its effect on the expression of some nitrogen-regulated genes by antagonizing

TABLE 3. Effects of elimination of Gln3p and Nil2p on the levels of β-galactosidase in cells grown on different nitrogen sources

Bacterial strain genotype[a]	Relative β-galactosidase sp act[b]								
	pNA (GATA, Abf, TG)			pRA (GATA, Abf)			pAX (GATA, TG)		
	Gln	Glt	NH_3	Gln	Glt	NH_3	Gln	Glt	NH_3
Wild type	10	100	62	9	100	59	0.2	100	0.7
nil2	40	110	201	50	121	137	9	108	0.7
gln3	6	4	197	6	9	162	0.1	3	3
gln3 nil2	29	13	159	25	22	141	4	10	2
nil1	1	ND	ND	ND	ND	ND	0.2	81	ND
nil1 nil2	ND	ND	ND	ND	ND	ND	0.2	74	ND

[a] The strains are described in Table 1 and in footnote *a* of Table 2.

[b] Plasmids carrying different elements or sites were placed in the bacterial strains. Plasmid pNA possesses the DNA region 510 to 652 bp upstream from the *GAP1* translational start site and contains one GATAAG, one TTGTT, and one Abf1p site; plasmid pRA, from 510 to 584 bp upstream from *GAP1*, contains one GATAAG and one Abf1p site; plasmid PAX, from 378 to 510 bp upstream from *GAP1*, contains one GATAAG and one TTGGT site. The β-galactosidase levels (units per OD_{600}) of the cells growing on glutamate (90 for pNA, 32 for pRA, and 152 for pAX) were set at 100. Glutamine (Gln), Glutamate (Glt), and ammonia (NH_3) were the sources of nitrogen. ND, not determined.

the activation of the expression of these genes by the GATA factor Nil1p. The zinc finger regions and the adjacent C-terminal regions of Gln3p, Nil1p, Nil2p, and Dal80p are highly homologous (25). The ability of Gln3p produced in *S. cerevisiae* to bind to the GATA regions of *GLN1* and *GDH2* has been demonstrated (3, 19). In addition, it has been shown that Dal80p as well as fragments of Gln3p (11) and of the corresponding Nit2p of *Neurospora crassa* (15), carrying the zinc finger regions of these proteins produced in *Escherichia coli* can bind to GATA sites. Although we have not as yet demonstrated the ability of Nil2p to recognize these sites, the high homology of the zinc finger and adjacent region of these proteins leaves little doubt that Nil2p can in fact bind to GATA sites.

Our initial observation showed that the elimination of Nil2p resulted in the expression of *GAP1* in cells growing with glutamine as the source of nitrogen. We had previously shown that the expression of this gene requires either one of two activators, Gln3p or Nil1p (16, 23, 25). These observations were subsequently confirmed by Coffman et al. who refer to the *NIL1* gene as *GAT1* (4, 5). Neither activator is capable of activating the expression in cells grown with glutamine or asparagine as the source of nitrogen. Elimination of Nil2p allowed Nil1p, but not Gln3p, to activate the transcription of *GAP1* in glutamine-grown cells, as shown by the observation of an increased β-galactosidase level in glutamine-grown *NIL1 GLN3* and *NIL1 gln3* cells, but not in *nil1 GLN3* cells (Table 2).

These observations suggest an antagonistic role for Nil2p in activation by Nil1p corresponding to that of Ure2p in activation by Gln3p (2). However, whereas the elimination of Ure2p results in an almost equally effective response to Gln3p in glutamine- and glutamate-grown cells (4, 23), it can be seen that the Nil1p-activated expression of *GAP1* in *gln3 nil2* cells is more than threefold lower in glutamine-grown cells than in ammonia-grown cells (Table 2). A similar effect of glutamine is observed when a fragment of the upstream region of *GAP1*, containing a single GATAAG, is used (Table 3). We would therefore propose that Nil2p exerts its effect by competing with Nil1p for the occupation of a GATAAG site and that an unidentified protein is responsible for the inactivation of Nil1p by glutamate or glutamine.

A major effect of Nil2p is to antagonize the expression of *NIL1*. It has previously been shown that growth with asparagine as the source of nitrogen reduces the expression of *NIL1*; a similar reduction results from the elimination of Gln3p in proline-grown cells (5). Our present observations confirm these results by the demonstration that the expression of *NIL1* is lower in glutamine-grown cells than in glutamate-grown cells and that in the absence of Gln3p the expression of *NIL1* by these cells is almost completely eliminated, indicating the important role of Gln3p in the expression of *NIL1*. However, the expression of *NIL1* in ammonia-grown cells is only diminished, not prevented by the lack of Gln3p, but it is entirely prevented in a strain incapable of producing Nil1p as well as Gln3p. The ability of Nil1p but not of Gln3p to activate the expression of *NIL1* in glutamine- and glutamate-grown cells is increased by the loss of Nil2p to the level found in ammonia-grown cells. We can account for these observations by assuming that Nil1p, even if kept in a less-active form by the presence of glutamate or glutamine is capable of full autogenous activation of *NIL1* expression in the absence of Nil2p. On the other hand, the fact that Nil1p is unable to activate *NIL1* expression in *NIL2* cells growing with glutamate or glutamine as the source of nitrogen suggests that to overcome the blockage by Nil2p, Nil1p must be kept in an active form by the absence of glutamate or glutamine.

The great sensitivity of *NIL1* expression to activation by Nil1p is illustrated in Fig. 1A. There it can be seen that upon shifting *gln3* mutant cells from a medium containing glutamine to one containing ammonia as the source of nitrogen, the expression of *NIL1* is fully activated: the differential rate of *NIL1* expression, measured as the increase of β-galactosidase in cells carrying the *NIL1-lacZ* fusion relative to the increase in cell mass, is constant from the beginning. Assuming that the level of Nil1p in the cells depends on the expression of *NIL1*, it appears that the intracellular level of Nil1p in glutamine-grown cells is sufficient for full expression of *NIL1* once Nil1p has been activated by the removal of glutamine. On the other hand, since the expression of *GAP1* in response to Nil1p appears to be proportional to the level of active Nil1p in ammonia-grown cells and is highest in cells grown with ammonia as the source of nitrogen which contain the maximal level of Nil1p (Fig. 1b and Table 1), one can conclude from these results that Nil2p affects *GAP1* expression indirectly by controlling the level of Nil1p and not directly by competing with Nil1p for GATAAG sites located upstream of *GAP1*. Nevertheless, experiments using a *lac* fusion to a synthetic UAS (upstream activating site) region consisting of three GATAA GATAAG elements indicate that Nil2p has the ability to block directly activation by Nil1p and to a lesser degree by Gln3p at these GATA sites, as expected from the homology of the zinc finger regions of these GATA factors.

The upstream region of 3GATA differs from that of *GAP1* by lacking the auxiliary elements, a TTG(G/T)T site and an Abf1p-binding site, which have been shown to play an important role in the expression of *GAP1* (24); it also differs from the upstream region of *NIL1* which contains in addition to the GATAAG sites three TTG(G/T)T sites (at positions −309 to −305, −263 to −259, and −252 to −248) and though it lacks an Abf1p-binding site, contains a *RAP1* site (at positions −318 to −330) which in many cases serves as an auxiliary site for gene expression (5). We find that in the case of cells with functional *GLN3* and *NIL1* genes carrying the 3GATA fusion, elimination of Nil2p increases expression in all three media (Table 2). Furthermore, it can be seen that the β-galactosidase level is increased in *gln3* mutant cells growing with ammonia as the source of nitrogen by the elimination of Nil2p (Table 2). In view of the fact that the expression of *NIL1* in such cells is the same in the presence or absence of Nil2p, this observation suggests that the increased expression of 3GATA in these cells results from the elimination of interference by Nil2p with activation by Nil1p at the 3GATA element. It can also be seen that in the case of 3GATA in *nil1* mutant cells growing on glutamate, elimination of Nil2p increases expression. Apparently in this case Nil2p interferes with the activation by Gln3p.

In summary, the results of our experiments indicate that the major role of Nil2p is to antagonize activation by Nil1p, and in particular to block the Nil1p-activated expression of *NIL1* in cells growing with glutamine or glutamate as the source of nitrogen. Nil2p differs from the other negative regulator, GATA factor Dal80p, in two important ways: (i) The lack of Dal80p does not enable Gln3p to activate transcription in asparagine-grown cells and presumably in glutamine-grown cells (12) but only serves to increase Gln3p-activated gene expression in proline- and presumably glutamate-grown cells. (ii) Regulation by Nil2p requires, as shown in Table 3, only a single GATAAG site, while regulation by Dal80p, requires two GATAAG sites separated by approximately 20 bp (9). The differences between the two negative regulators may be ex-

plained by considering their regulation and that of the activators Gln3p and Nil1p.

The failure of the removal of Dal80p to enable Gln3p to activate gene expression in glutamine-grown cells may be due to the fact that Gln3p is converted by Ure2p in the presence of glutamine to a form almost totally lacking in the ability to activate transcription; on the other hand, Nil1p, even when kept in a relatively inactive form by glutamate may be able to activate the autogenous expression of *NIL1*, as long as Nil2p is not present to compete with Nil1p for the complex array of GATAAG sites upstream of *NIL1* (5). The absence of glutamate would result in the conversion of Nil1p to a more-active form, capable of initiating *NIL1* expression even in the presence of Nil2p. The increase in the intracellular concentration of Nil1p, resulting from the increased expression of *NIL1*, which is not matched by a corresponding increase in *NIL2* expression, would ensure continued production of Nil1p and would overcome any direct blockage by Nil2p of other genes, such as *GAP1*, whose expression is activated by Nil1p.

On the other hand, it may be important that *DAL80* expression is activated by Gln3p in media lacking glutamine, without a corresponding increase in the expression of *GLN3* (8). As a result, during the switch from growth with glutamine to growth with glutamate or proline as the source of nitrogen, Gln3p will be increasingly antagonized by Dal80p. The fact that the expression of Nil2p is not increased by the absence of glutamine may explain why Nil2p is generally not an effective antagonist of Gln3p. We need assume only that Gln3p has greater affinity for the appropriate GATAAG sites than Nil2p, so that these sites are preferentially occupied by Gln3p; on the other hand, the fact that in glutamine or glutamate-grown cells Nil2p effectively antagonizes activation by Nil1p suggests that unless Nil1p is activated by the lack of glutamate, Nil2p competes effectively with Nil1p for the occupation of the GATAAG sites. The observation that the lack of Gln3p greatly stimulates expression of *GAP1* and 3GATA by Nil1p in ammonia-grown cells (25) (Tables 2 and 3), is in good accord with the higher affinity of Gln3p for GATAAG sites. Apparently, even when kept in a largely inactive form by Ure2p, Gln3p can occupy these sites to exclude Nil1p.

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ADDENDUM IN PROOF

After submission of this paper, the results of a study of the effects of the deletion of the *NIL2* gene (there called *GZF3*) were published, likewise showing the antagonistic role of Nil2p in the activation of transcription of *GAP1* by Nil1p (S. Soussi-Boudekou, S. Vissers, A. Urrestarazu, J.-C. Jauniaux, and B. André, Mol. Microbiol. **23:**1157–1168, 1997).

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