ELSEVIER

Gene 290 (2002) 1–18

GENE
AN INTERNATIONAL JOURNAL ON GENES AND GENOMES

www.elsevier.com/locate/gene

Review

Nitrogen regulation in *Saccharomyces cerevisiae*

Boris Magasanik*, Chris A. Kaiser

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Received 21 November 2001; received in revised form 19 February 2002; accepted 11 March 2002
Received by A. Van Wijnen

Abstract

Yeast cells can respond to growth on relatively poor nitrogen sources by increasing expression of the enzymes for the synthesis of glutamate and glutamine and by increasing the activities of permeases responsible for the uptake of amino acids for use as a source of nitrogen. These general responses to the quality of nitrogen source in the growth medium are collectively termed nitrogen regulation. In this review, we discuss the historical foundations of the study of nitrogen regulation as well as the current understanding of the regulatory networks that underlie nitrogen regulation. One focus of the review is the array of four GATA type transcription factors which are responsible for the regulation the expression of nitrogen-regulated genes. They are the activators Gln3p and Nil1p and their antagonists Nil2p and Dal80p. Our discussion includes consideration of the DNA elements which are the targets of the transcription factors and of the regulated translocation of Gln3p and Nil1p from the cytoplasm to the nucleus. A second focus of the review is the nitrogen regulation of the general amino acid permease, Gap1p, and the proline permease, Put4p, by ubiquitin mediated intracellular protein sorting in the secretory and endosomal pathways.

Keywords: *Saccharomyces cerevisiae*; Nitrogen regulation; Signal transduction; GATA factors; GATA sites; Permease sorting

1. Introduction

Many microorganisms have the ability to use a variety of nitrogen-containing compounds as the sole source of all cellular nitrogen. This ability requires permeases for the transport of these compounds and enzymes for the generation of ammonia by their metabolism. Once inside the cell ammonia can react with α-ketoglutarate, provided by the metabolism of the carbon source of the growth medium, to produce glutamate, and can react with glutamate to produce glutamine. The amino nitrogen of glutamate serves as the source of 85% of the total cellular nitrogen and the amide group of glutamine is the source of the remaining 15% (Cooper, 1982).

Nitrogen regulation (nitrogen catabolite repression) is the mechanism designed to prevent or reduce the unnecessary divergence of the cells' synthetic capacity to the formation of enzymes and permeases for the utilization of compounds that are non-preferred sources of glutamate and glutamine when a preferred nitrogen source is available (Magasanik, 1992). In this review we will discuss nitrogen regulation in the yeast *Saccharomyces cerevisiae* with an emphasis on our view of important emerging principles and key unanswered questions. This review is not meant to be comprehensive and we will focus on the particular question of how the activity of yeast genes required for nitrogen utilization can be controlled by the quality of the nitrogen source available in the growth medium, without discussing the many other ways that genes involved in metabolism of nitrogen-containing compounds can be regulated. Some of the other regulatory mechanisms that we will not discuss in this review but that have been described elsewhere are the activation of arginine and proline utilization pathways by their substrates (Dubois and Messenguy, 1997; des Etages et al., 1996), regulation of genes involved in general control of amino acid biosynthesis (Hinnebusch, 1992), and transcriptional regulation of certain amino acid permeases by the *SSY1* pathway for sensing amino acids in the medium (Forsberg and Ljungdahl, 2001; Bernard and André, 2001). An overview of all systems subject to nitrogen regulation can be found in two recent publications (Hofman-Bang, 1999; ter Schure et al., 2000).

The preferred sources of nitrogen for *S. cerevisiae* used in the laboratory are glutamine, asparagine, and the mixture of amino acids and peptides in commercial Bactopeptone. The non-preferred source of nitrogen used in most studies of

Abbreviations: CIP, calf intestine alkaline phosphatase; ER, endoplasmic reticulum; PVC, prevacuolar compartment; TOR, target of rapamycin

* Corresponding author. Department of Biology, Room 68-625, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, MA 02139, USA. Tel.: +1-617-253-2637; fax: +1-617-252-1875.
E-mail address: bmag@mit.edu (B. Magasanik).

0378-1119/02/$ - see front matter
PII: S0378-1119(02)00558-9

nitrogen regulation is proline; other non-preferred sources are ornithine, γ-aminobutyrate, allantoin, and urea. There are two different criteria that can be used to judge the quality of a particular nitrogen source. The growth rate that can be supported by a source of nitrogen would seem to be the simplest criterion for quality. Although the preferred nitrogen source, glutamine, does support faster growth than all non-preferred sources of nitrogen, the differences in growth rate supported by very different nitrogen sources are often surprisingly small and it is therefore difficult, in practice, to use growth rate as a way to make clear distinctions between the qualities of different nitrogen sources. A second criterion, which we will use in this review, is based on the level to which systems for use of alternative nitrogen sources are derepressed during growth on a particular nitrogen source. Thus, nitrogen sources that do not derepress the pathways for utilization of alternative nitrogen sources are generally considered to be preferred nitrogen sources, whereas nitrogen sources that do lead to derepression of the alternative pathways are considered to be non-preferred. This criterion has the advantage that different nitrogen sources often cause easily observable differences in the expression of the pathways for alternative nitrogen sources. Moreover, a criterion based on the expression patterns of nitrogen-regulated pathways lends itself to whole-genome transcriptional profiling experiments, which are increasingly becoming the standard way used to monitor the metabolic state of a yeast cell.

Most nitrogen sources can be classified unambiguously as being either preferred or non-preferred, if either the growth rate or induction of pathways for alternative nitrogen sources is used to judge the quality of the nitrogen source. However, there are some exceptional cases where a clear classification of nitrogen source quality has not been possible. For example, strains of the Σ1278b genetic background, which was used for many of the early experiments on nitrogen regulation, display a very different response to growth on ammonia as a nitrogen source than strains of the S288C genetic background, which is used for most modern molecular genetic studies of *S. cerevisiae*. Although strains of both genetic backgrounds grow well on ammonia as a source of nitrogen, when strains of the two genetic backgrounds grown on ammonia are compared on the basis of whether pathways for alternative sources of nitrogen are derepressed, ammonia appears to be a preferred nitrogen source for Σ1278b strains but not for S288C strains. This difference was first demonstrated by the observation that ammonia interfered with amino acid transport by the general amino acid permease (Gap1p) in strain Σ1278b, but not in strain S288C, while glutamine and glutamate blocked this transport in either strain. Heterozygous diploids produced by mating Σ1278b to S288C strains exhibit repression of Gap1p activity by ammonia demonstrating that the Σ1278b trait is genetically dominant (Rytka, 1975). Analysis of tetrads from these diploids reveals that multiple genes specify the differences in response to ammonia. Two of these genes were identified as two homologous genes (*MPR1* and *MPR2*) found near telomeres in Σ1278b that are not present in the genomic sequence of S288C (Takagi et al., 2000). In addition to *MPR1* and *MPR2* we have found that at least two other loci that have a significant effect on the response to ammonia segregate in crosses of Σ1278b to S288C (C. Kaiser, unpublished data). Because of these complex differences in response to the nitrogen source, it has often been difficult to compare experiments conducted with the Σ1278b and S288C genetic backgrounds when ammonia is used as a nitrogen source.

Even in strain S288C, ammonia cannot be simply classified as a non-preferred source of nitrogen: we shall discuss in a later section of this review, that in contrast to proline, ammonia can play a role in preventing the expression of some nitrogen-regulated genes in S288C. Similarly, glutamate cannot be classified as either a clearly preferred or non-preferred source of nitrogen, but like ammonia occupies an intermediate position.

2. The core pathway for nitrogen assimilation

All of the pathways for the utilization of non-preferred sources of nitrogen feed into a common set of reactions for the production of glutamate and glutamine. These reactions outlined in Fig. 1 allow the synthesis of glutamate from α-ketoglutarate and ammonia and for the synthesis of glutamine from glutamate and ammonia. By using different sets of enzymes, these core reactions also allow glutamine as the sole source of nitrogen to be converted into glutamate, and for glutamate as the sole source of nitrogen to be converted into glutamine.

When cells have an abundant source of ammonia, either by conversion of a non-preferred nitrogen source to ammonia or by growth on ammonia itself, the $NADP^+$-linked glutamate dehydrogenase, the product of *GDH1*, is responsible for the synthesis of glutamate by combining ammonia with the citric acid cycle intermediate α-ketoglutarate (Grenson et al., 1974).

(1) $\alpha\text{-Ketoglutarate} + NH_4^+ + NADPH + H^+ \leftrightarrow$
$\text{glutamate} + NADP^+$

Glutamate can then combine with ammonia in a reaction catalyzed by glutamine synthetase, the product of *GLN1* (Mitchell and Magasanik, 1983; Mitchell, 1985).

(2) $\text{Glutamate} + NH_4^+ + ATP \rightarrow \text{glutamine} + ADP + P_i$

The entry of ammonia into the cell is facilitated by three permeases, the products of *MEP1*, *MEP2*, and *MEP3*. The high affinity permeases *MEP1* and *MEP2* also appear to play a role in the intracellular retention of ammonia generated by the degradation of arginine and urea (Marini et al., 1997).

When glutamate is the sole source of nitrogen, the NAD^+-linked glutamate dehydrogenase, the product of

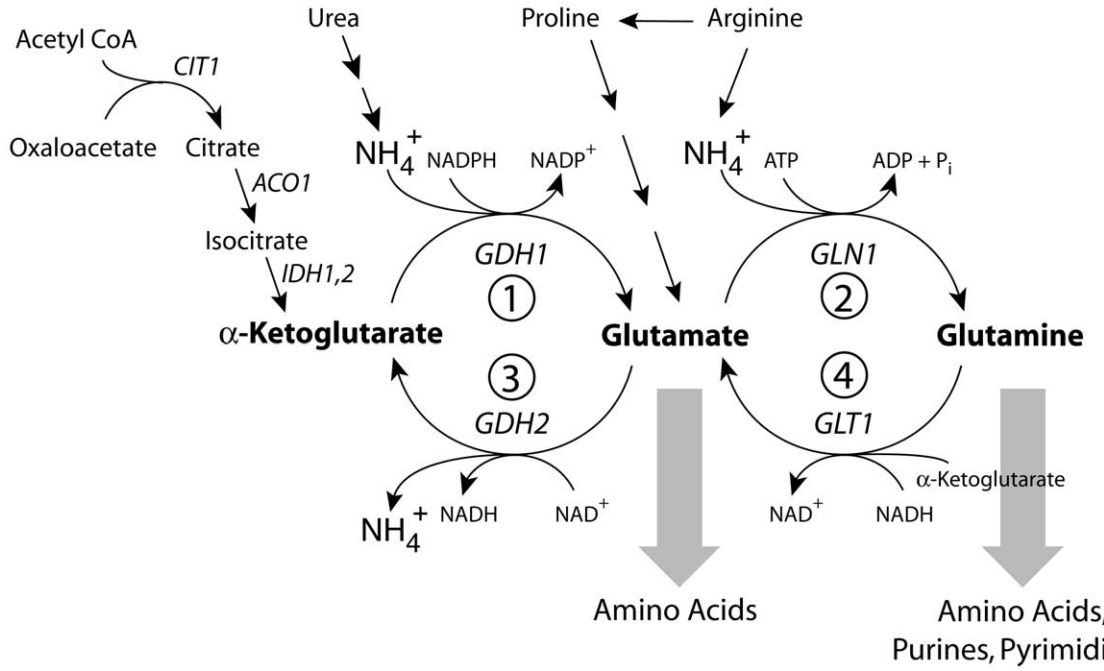

Fig. 1. Central pathways for nitrogen metabolism. The nitrogenous compounds in the cell are synthesized from either glutamate or glutamine. The major pathway for glutamate synthesis is through combination of ammonia with α-ketoglutarate, which is synthesized from acetyl CoA and oxaloacetate through the early steps of the citric acid cycle. Glutamine is synthesized by the combination of ammonia with glutamate. The pathways for utilization of a variety of nitrogen sources, including urea, proline and arginine, are shown. The *S. cerevisiae* gene for each of the enzymatic steps is designated in italics.

GDH2, is responsible for the release of ammonia required for the synthesis of glutamine from glutamate (Miller and Magasanik, 1990).

(3) Glutamate + $NAD^+ \leftrightarrow NH_4^+$ + NADH + H^+ + α-ketoglutarate

Glutamine synthetase is the only means by which glutamine can be synthesized and cells lacking this enzyme require glutamine for growth. On the other hand, the loss of *GDH1* only reduces the rate of growth on ammonia by one half (Wiame et al., 1985). The ability of *gdh1* mutants to grow on ammonia is due to the existence of the enzyme glutamate synthase, the product of *GLT1*, which catalyzes the following reaction (Miller and Magasanik, 1990; Cogoni et al., 1995).

(4) Glutamine + α-ketoglutarate + NADH + $H^+ \rightarrow$ 2 glutamate + NAD^+

Reactions (2) and (4) together catalyze the net synthesis of glutamate from α-ketoglutarate and ammonia. Reaction (3) is not required in cells growing on ammonia, but is essential for the synthesis of glutamine in cells using glutamate, or nitrogen compounds which are precursors of glutamate such as proline or γ-aminobutyrate as a source of nitrogen (Miller and Magasanik, 1990).

Reactions (1) to (4) catalyze the final steps in the conversion of non-preferred sources of nitrogen to glutamate and glutamine, which in turn serve as the sources of all cellular nitrogen. Consequently, an increase in the level of these enzymes is required to compensate for the low intracellular concentrations of their substrates in cells growing on non-preferred sources of nitrogen.

In the following sections of this review we shall present the evidence that the expression of nitrogen-regulated genes is activated by two transcription factors, Gln3p and Nil1p, and that glutamine and glutamate serve as the intracellular signals to prevent this activation.

In some cases, the enzymes responsible for the utilization of a non-preferred source of nitrogen are induced by the presence of the particular nitrogen source in the growth medium. For example, expression of the genes coding for the enzymes needed for the utilization of arginine and proline as sources of nitrogen are induced by the presence in the growth medium of arginine and proline, respectively. Strong activation by arginine depends on the activators Arg80p and Arg81p, whereas strong activation by proline depends on Put3p. Although the genes coding for the enzymes needed for the utilization of arginine and proline can be activated independently of Gln3p and Nil1p, these transcription factors do have an effect on the degree to which these enzymes are expressed because of inducer exclusion. The expression and activity of the permeases for the uptake of arginine (Gap1p) or proline (Put4p) are subject to nitrogen regulation (Wiame et al., 1985; Lasko and Brandriss, 1981; Stanbrough and Magasanik, 1995; Roberg et al., 1997a) and the presence of a preferred source of nitrogen can lead to decreased activity of the permeases and as a consequence the induction of the specific nitrogen

utilization pathways will be decreased (Courchesne and Magasanik, 1983; Brandriss and Magasanik, 1979; Cooper et al., 1992).

3. The elements of nitrogen regulation

3.1. Discovery

A brief discussion of the observations that led to the discovery of the elements of nitrogen regulation provides a useful guide to the understanding of their relationships and of the roles they play in this complex process. Note that most of these early experiments were performed in genetic backgrounds in which ammonia behaves as a preferred nitrogen source.

H. Holzer and his coworkers made the initial observations leading to the concept of nitrogen regulation in *S. cerevisiae* (Hierholzer and Holzer, 1963; Kolhaw et al., 1965). They showed that the intracellular levels of the NAD^+-linked glutamate dehydrogenase and of glutamine synthetase were much lower in cells grown with ammonia, glutamine, or asparagine than in those grown with glutamate or aspartate as sources of nitrogen. Subsequently, Grenson, Hou and Crabeel reported the discovery of a general amino acid permease (GAP) present in cells grown with proline, but not in those grown with ammonia as a source of nitrogen (Grenson et al., 1970).

A common genetic basis for nitrogen regulation was suggested by the discovery in 1972 of a recessive mutation known as *ure2*, which prevented the inhibition by ammonia of the uptake of ureidosuccinate (Drillien and Lacroute, 1972), a pyrimidine precursor that enters the cell through the nitrogen-regulated permease for allantoate (Turoscy and Cooper, 1987). The *ure2* mutation was pleiotropic, as shown by the fact that it also resulted in high levels of NAD^+-linked glutamate dehydrogenase, of allophanate hydrolase, an enzyme in the pathway of urea degradation, of glutamine synthetase and of asparaginase II in cells grown with ammonia or glutamine as a source of nitrogen (Wiame et al., 1985). In addition, the *ure2* mutation prevented the inactivation of glutamine synthetase, when glutamine was added to wild type cells grown with glutamate as a source of nitrogen (Legrain et al., 1982; Coschigano and Magasanik, 1991).

The *GLN3* gene was identified by a mutation that resulted in a phenotype opposite to that of *ure2*. By starting with a *gln1* mutant that could grow slowly in the absence of glutamine, a *gln3 gln1* double mutant was selected by its total inability to grow in the absence of glutamine (Mitchell and Magasanik, 1984). When combined with a functional structural gene for glutamine synthetase, the *gln3* mutant grew in the absence of glutamine, but was unable to produce glutamine synthetase at a high level when grown with glutamate as a source of nitrogen. The *gln3* mutation was pleiotropic: the *gln3* mutant failed to produce NAD^+-linked glutamate dehydrogenase and four other proteins, identified by two-dimensional gel analysis, at a high level when grown with glutamate as a source of nitrogen (Mitchell and Magasanik, 1984).

Evaluation of the phenotype of *gln3 ure2* double mutants revealed an inability to increase the level of glutamine synthetase and of the NAD^+-linked glutamate dehydrogenase in response to growth with glutamate as a source of nitrogen. Taken together, all of these data suggested that the product of *GLN3* activated the formation of these enzymes and that the product of the *URE2* gene blocked this activation in response to glutamine (Courchesne and Magasanik, 1988; Coschigano and Magasanik, 1991).

The role of Gln3p is the activation of the initiation of transcription at the promoters of nitrogen-regulated genes. This was demonstrated in the case of *GLN1* by the rapid rise in the level of *GLN1* specific mRNA in wild type but not in *gln3* mutant cells upon the shift of the cells from a medium containing glutamine to one containing glutamate as a source of nitrogen; conversely, addition of glutamine to the wild type cells grown on glutamate resulted in the rapid disappearance of *GLN1* specific mRNA whose half-life is approximately 4 min (Benjamin et al., 1989). The role of Gln3p as the agent responsible for the activation of the expression of nitrogen-regulated genes was confirmed by the finding that the transcription of the genes for allantoin utilization (*DUR1*, *DUR2*, *DAL5*, and *DAL7*) did not occur in cells grown with asparagine as a source of nitrogen, and required a functional *GLN3* gene in cells grown with proline as a source of nitrogen (Cooper et al., 1990).

The analysis of the DNA sequence located upstream from the *DAL5* gene suggested that the site (UAS_N) responsible for nitrogen regulation of the transcription of this gene had the sequence GATAAG (Rai et al., 1989). The isolation and sequencing of the *GLN3* gene revealed Gln3p to possess a zinc finger region, with high homology to that of the GATA transcription factors of higher organisms, which bind to both GATA containing sequences and to the products of *NIT2* and *AREA*, the nitrogen regulatory transcription factors of *Neurospora crassa* and *Aspergillus nidulans*. Immunoprecipitation experiments with Gln3p polyclonal antibody indicated that Gln3p binds to a site located upstream of *GLN1* (Minehart and Magasanik, 1991), and immunoprecipitation experiments also showed that Gln3p could bind to the regulatory factor Ure2p (Blinder et al., 1996).

Another protein with a zinc finger closely related to that of Gln3p was discovered by its inhibitory effect on the expression of some, but not of all, genes responsive to nitrogen regulation: the loss of the product of the *DAL80 (UGA43)* gene was found to result in greatly enhanced expression of the genes coding for some of the permeases and enzymes responsible for the degradation of urea and allantoin (*DAL*) and for those responsible for the degradation of γ-amino butyrate (*UGA*) in cells grown with proline, but not in those grown with asparagine or ammonia as a source of nitrogen. The expression of the *DAL80* gene itself was found to be subject to nitrogen regulation (Chisholm and Cooper, 1982; Vissers et al., 1990; Cunningham and Cooper, 1991).

The study of the expression of the *GAP1* gene, coding for the general amino acid permease, revealed the existence of a gene responsible for the activation of *GAP1* expression in cells grown with urea or ammonia, but not in cells grown with glutamate as a source of nitrogen. This gene, *NIL1 (GAT1)*, has a zinc finger highly homologous to that of Gln3p and Dal80p. In contrast to *GLN3*, which is expressed constitutively regardless of the nitrogen source, the *NIL1* gene is subject to nitrogen regulation and requires Gln3p or Nil1p for its expression (Stanbrough, 1993; Stanbrough et al., 1995; Coffman et al., 1996; Rowen et al., 1997).

A search for additional genes encoding proteins with zinc fingers highly homologous to those of Gln3p, Dal80p, and Nil1p resulted in the discovery of the *NIL2 (GZF3*, *DEH1)* gene. Deletion of this gene results in increased activation by Nil1p of the expression of nitrogen-regulated genes during the growth of the cells with glutamine, asparagine or ammonia as sources of nitrogen (Stanbrough, 1993; Stanbrough et al., 1995; Soussi-Boudekou et al., 1997; Coffman et al., 1997; Rowen et al., 1997).

3.2. Transcription factors and their targets

The four transcription factors share a characteristic zinc finger region which apparently permits them to bind to a region of DNA, UAS_N, located several hundred base pairs upstream from the genes subject to nitrogen regulation and to activate (in the case of Gln3p and Nil1p) or to block (in the case of Dal80p and Nil2p) the initiation of transcription.

The greatest homology, 80% based on the identity of amino acid residues, is found in the region of 52 amino acid residues overlapping the zinc fingers of Nil1p, Nil2p and Dal80p; the corresponding region of Gln3p is 65% homologous to that of the other three GATA factors (Stanbrough et al., 1995). The only other zinc finger region with some homology to that of the GATA factors is found in Ash1p, a protein that plays a role in the repression of the HO endonuclease gene in daughter cells after cell division (Bobola et al., 1996; Sil and Herskowitz, 1996). The zinc finger region of Ash1p differs from that of the GATA factors by the presence of three additional amino acid residues in the region separating the two zinc fingers and is only 35% homologous to the GATA factors. It seems therefore that Gln3p, Nil1p, Nil2p, and Dal80p are the only transcription factors able to recognize the GATA sequences of the nitrogen-regulated promoters.

The two positive transcription factors, Gln3p and Nil1p, differ from the negative transcription factors, Nil2p and Dal80p, by having a homologous region, located approximately 200 amino acid residues from the amino-terminus that is exceptionally rich in asparagine residues (Stanbrough et al., 1995). On the other hand, Nil2p and Dal80p share a carboxyl terminal domain containing a leucine zipper, which is not found in Gln3p or Nil1p, and may be responsible for the ability of these proteins to dimerize (Cunningham and Cooper, 1991; Coffman et al., 1997; Soussi-Boudekou et al., 1997).

The evidence for specific binding to its target is strongest for Gln3p. This protein, overproduced in cells of *S. cerevisiae* and purified as a tetramer of 500 kDa, was shown to retard the gel migration of DNA preparations containing the 5′-GATAAGATAAG-3′ and 5′-GATTAGATTAG-3′ sequences located upstream of the *GLN1* and *GDH2* genes, respectively, and to protect the latter from digestion by DNAase (Blinder and Magasanik, 1995). Expression of epitope-tagged Gln3p in *Escherichia coli* yielded an extract that contained several proteins that carried the epitope fused to Gln3p, but were all smaller than the Gln3p and thus appeared to be breakdown products of Gln3p. This extract of *E. coli* cells was able to retard the gel migration of DNA preparations containing GATA sequences located upstream from a number of nitrogen-regulated genes, presumably because one or more of the degradation products of Gln3p contains the zinc finger region present in the four response regulators. The results of these experiments, though not proving the specific affinity of the sites as targets of Gln3p, makes it possible to discriminate between the presumed and actual targets of the GATA factors. Accordingly, the sequence 5′-GATAA-3′ found upstream from most nitrogen-regulated genes, usually as 5′-GATAAG-3′, is a strong binding site, the sequence 5′-GATTAGATTAG-3′ located upstream from *GDH2* is a weaker binding site, and the sequences 5′-GATGAT-3′ and 5′-GATAGT-3′ fail to serve as binding sites. Although many of the DNA preparations used in these experiments contained more than one GATAA sequence, the presence of a single GATAAG, GATAAGATAAG or GATTAGATTAG sequence was adequate for binding the degradation product(s) of Gln3p (Cunningham et al., 1996).

A different result was obtained with the product of the *DAL80* gene, which had been identified as an antagonist of nitrogen-regulated transcription activation. Expression of the *DAL80* coding sequence in *E. coli* gave rise to an apparently full-length protein in extracts. This extract had very little ability to retard the gel mobility of DNA preparations carrying a single GATAAG sequence, but effectively retarded the gel migration of two GATAAG sequences separated by 15–30 bp. This observation is in good accord with genetic evidence that the antagonistic effect of Dal80p on the expression of nitrogen-regulated genes is restricted to those having multiple GATAA sequences upstream from their transcriptional start sites (Cunningham and Cooper, 1993).

Although no experimental evidence directly demonstrating the binding of Nil1p to its target has been published, the fact that it carries a zinc finger highly homologous to those of Gln3p, Dal80p and Nil2p and that it can activate the transcription of nitrogen-regulated genes with a GATAAG sequence located upstream from their transcriptional start site strongly indicates that Nil1p has the ability to bind to DNA containing a GATAA site.

The other antagonist of the expression of nitrogen-regulated genes, Nil2p, when produced in *E. coli* cells can retard the gel migration of DNA preparations containing GATAAG sequences; in contrast to Dal80p, Nil2p can antagonize the initiation of transcription by Nil1p on a *lacZ* reporter plasmid containing a single GATAAG site (Coffman et al., 1997; Rowen et al., 1997).

3.3. The unit of regulation

A single GATA sequence is essential, but not adequate for nitrogen regulation. Activation of transcription by Gln3p or Nil1p requires in addition a second such site, or the sequence TTGT/GT, presumably the binding site for an as yet unidentified auxiliary activator or a binding site for Abf1p, or perhaps Rap1p, which in other cases serve as auxiliary activators of gene expression. Nevertheless, a single GATAAGATAAG site without auxiliary sites was adequate for the activation of transcription by Gln3p in a *ure2* mutant strain. Apparently, the lack of Ure2p can compensate for the lack of auxiliary activation (Minehart and Magasanik, 1992; Miller and Magasanik, 1991; Stanbrough and Magasanik, 1996).

The discriminatory role of auxiliary promoter sequences in activation was demonstrated in experiments using segments of the DNA sequence located upstream from the *GAP1* gene with a single GATAAG sequence and either a single TTGGT sequence or single Abf1p binding site fused to *lacZ* on a plasmid carried in wild type, *gln3* or *nil1* mutant cells. The Abf1p binding site supported activation of transcription by either Gln3p or Nil1p, but the TTGGT sequence supported activation of transcription by Gln3p, but not by Nil1p (Rowen et al., 1997). This difference may explain why the transcription of *GDH2*, the structural gene for the NAD^+-linked glutamate dehydrogenase, can only be activated by Gln3p and not by Nil1p. This gene differs from all other known nitrogen-regulated genes because it contains only a single nitrogen-responsive site with the sequence GATTAGATTAG. The expression of *GDH2* in response to a shift from a medium with glutamine to one with glutamate as the source of nitrogen depends on the presence of a TTGGT site, approximately 50 bp further downstream (Miller and Magasanik, 1991). The expression of the *lacZ* gene fused to a segment of DNA containing these two sites could be activated by Gln3p in cells growing with glutamate as a source of nitrogen, but was not activated by Nil1p in cells growing with urea as a source of nitrogen. On the other hand, a DNA segment located upstream from the *GLN1* gene that contains a binding site for Abf1p and the sequence TTTGTT in addition to a single GATAAGATAAG site can be activated in cells growing with glutamate as a source of nitrogen by Gln3p, and in cells growing with urea as a source of nitrogen by Nil1p (Stanbrough et al., 1995).

Although auxiliary activation sites enhance the potential of a single GATA site to respond to activation by Gln3p or Nil1p, they are no more effective than additional GATA sites. Experiments using plasmids carrying synthetic oligonucleotides fused to a *lacZ* reporter have demonstrated that a series of three GATAAG or GATAAGATAAG sites is adequate for strong activation of gene expression by Gln3p or Nil1p. The effect of the sites is synergistic and not additive. The ratio of the β-galactosidase levels in cell carrying plasmids with one, two, or three GATAAGATAAG sequences growing with glutamate as a source of nitrogen was 1:5:37. Increasing the number of GATAAGATAAG sequences to seven did not further increase the level of β-galactosidase (Minehart and Magasanik, 1992).

Another element affecting the expression of some nitrogen-regulated genes is the transcription factor Dal80p. Its ability to bind to two GATA sites 15–30 bp apart allows it to interfere with the use of these sites for activation by Nil1p. This antagonistic role of Dal80p is well documented by experiments with plasmids carrying the DNA region upstream from the *UGA4* gene coding for the permease for γ-aminobutyrate. This region contains four GATA sites capable of binding Dal80p, and the level of β-galactosidase in cells growing with proline as a source of nitrogen carrying plasmids in which this DNA region is fused to *lacZ* is increased more than ten-fold by the deletion of the *DAL80* gene (André et al., 1995; Coffman et al., 1997). Inactivation of one of these GATAAG sequences by site directed mutagenesis reduced their sensitivity to activation, but to an even greater extent their sensitivity to inactivation by Dal80p (Cunningham et al., 1994).

4. Molecular basis of nitrogen regulation

4.1. Stimuli and signals

Regulation of gene expression occurs in response to a change in the environment of the cells, the stimulus, causing the generation of an intracytoplasmic signal, which is transduced by one or more signal transducers to the transcription factor responsible for the activation of gene expression. In the case of nitrogen regulation the stimulus responsible for the inability of Gln3p and Nil1p to activate gene expression is the presence of a preferred source of nitrogen in the growth medium (Mitchell and Magasanik, 1984; Stanbrough et al., 1995; Coffman et al., 1996). Furthermore, the presence of glutamate in the growth medium is the stimulus resulting in the inability of Nil1p, but not of Gln3p, to activate gene expression (Stanbrough and Magasanik, 1995; Stanbrough et al., 1995; Rowen et al., 1997).

The expression of genes coding for the enzymes catalyzing the individual steps of the pathways leading to amino acids is regulated by the feedback exerted by the final products of these pathways (Hinnebusch, 1992). As discussed previously, glutamine and glutamate are the final products of the pathways for the utilization of non-preferred sources of nitrogen. We may therefore expect that increases in the intracytoplasmic concentrations of

glutamine and glutamate would serve as the signals to arrest the expression of nitrogen-regulated genes.

The fact that glutamine, asparagine, and commercial Bactopeptone are the stimuli for nitrogen regulation is in good accord with this view. Glutamine and asparagine are efficiently transported into the cell by two permeases, the products of *GNP1* and *AGP1* (Schreve et al., 1998; Zhu et al., 1996). Glutamine inside of the cell can be converted to glutamate by glutamate synthase, by either of two glutaminases, or by glutamine transaminase (Cogoni et al., 1995; Soberon and Gonzalez, 1987a,b). An intracellular asparaginase is responsible for the hydrolysis of asparagine to a mixture of ammonium and aspartate, an excellent source of glutamate (Warner and Bonthron, 1994). The combination of glutamate and ammonium provides an excellent source of glutamine as shown by the observation that a mixture of glutamate and ammonium blocked transcription of GAP1 as efficiently as glutamine (Stanbrough and Magasanik, 1995). Finally, the protein hydrolyzate in Bactopeptone serves as an excellent source of both glutamine and glutamate.

The intracytoplasmic signal generated by the presence of the preferred nitrogen source in the growth medium is transduced to Gln3p by Ure2p, as shown by the observation that the deletion of *URE2* results in full expression of genes subject to activation by Gln3p in media containing a preferred source of nitrogen (Courchesne and Magasanik, 1988). This signal is apparently an increase in the intracytoplasmic concentration of glutamine as shown by the study of the expression of *GDH2*, the structural gene for the NAD^+-linked glutamate dehydrogenase. The level of this enzyme is high in cells of *ure2* mutants, irrespective of the nitrogen source of the growth medium: six-fold higher than in wild type cells grown with glutamate as a source of nitrogen and more than 100-fold higher than in wild type cells grown with ammonia or glutamine as a source of nitrogen (Courchesne and Magasanik, 1988; Coschigano and Magasanik, 1991; Mitchell and Magasanik, 1984). It was possible to lower the intracytoplasmic concentration of glutamine by using a strain with a leaky *gln1* mutation; this mutant grew slowly when ammonia or glutamate, but rapidly when glutamine served as the source of nitrogen. The level of the NAD^+-linked glutamate dehydrogenase in cells of this mutant grown with ammonia or glutamate as a source of nitrogen was as high as the level of this enzyme in cells of the *ure2* mutant, but very low in cells grown with glutamine as a source of nitrogen (Mitchell and Magasanik, 1984). These observations provide strong evidence that the signal responsible for blocking the ability of Gln3p to activate gene expression is an increase in the intracytoplasmic concentration of glutamine. Apparently, the activity of Gln3p is inversely proportional to the intracytoplasmic concentration of glutamine which is highest in cells growing in a medium containing glutamine, lower in one containing ammonia, and still lower in one containing glutamate.

The expression of *GAP1*, *NIL1* and of a *lacZ* reporter fused to an element consisting of three GATAAGATAAG sequences (3 × GATA) can be activated by either Gln3p or Nil1p in a medium containing proline as a source of nitrogen. Substitution of glutamate for proline as a source of nitrogen blocks the activation of the expression of these genes by Nil1p but not by Gln3p, while substitution of ammonia for proline as a source of nitrogen blocks the activation of the expression of these genes by Gln3p, but not by Nil1p. Neither Gln3p nor Nil1p is able to activate the expression of these genes when glutamine serves as the source of nitrogen (Rowen et al., 1997). The results of these experiments provide strong evidence for the view that different intracytoplasmic signals are responsible for blocking the abilities of Gln3p and of Nil1p to activate transcription. Additional evidence for this view is the fact that the deletion of *URE2* does not enable Nil1p to activate gene expression significantly in cells grown with glutamine as a source of nitrogen (Stanbrough and Magasanik, 1996; Coffman et al., 1996).

It is possible on the basis of these experiments to reach the tentative conclusion that the signal blocking the activation of gene expression by Nil1p is an increase in the intracytoplasmic concentration of glutamate. We may expect that the presence of either glutamate or of glutamine, an excellent source of glutamate, in the growth medium will raise the intracytoplasmic concentration of glutamate. The fact that the cells grow slightly more slowly in ammonia than in glutamine medium (Cooper, 1982) is compatible with the view that the $NADP^+$-linked glutamate dehydrogenase catalyzes the growth rate limiting step, and that the resulting glutamate can be rapidly converted to glutamine in the reaction catalyzed by glutamine synthetase. Consequently, in cells using ammonia as a source of nitrogen the intracytoplasmic concentration of glutamine would be sufficiently high to interfere with the activation of gene expression by Gln3p, but that of glutamate would not be high enough to block the activation of the expression of the same set of genes by Nil1p.

In conclusion, we may consider that the role of the transcription factor Gln3p is the activation of gene expression in response to a deficiency of glutamine, and that of Nil1p, the activation of gene expression in response to a deficiency of glutamate.

4.2. Signal transduction

It has recently been shown that the TOR kinases have an essential role in preventing the expression of nitrogen-regulated genes in cells using Bactopeptone or glutamine as a source of nitrogen. This conclusion is based on the results of experiments using rapamycin to inactivate the TOR kinases; this treatment results in the activation of the expression of nitrogen-regulated genes, but in contrast to growth on proline as a source of nitrogen, also affects many other cellular activities (Hardwick et al., 1999; Cardenas et al.,

1999; Beck and Hall, 1999). The TOR kinases are central controllers of cell growth and their inactivation results in physiological changes leading to the cellular state characteristic of starvation and finally the total arrest of growth (Schmelzle and Hall, 2000). It is therefore not likely that the signals resulting in the activation of nitrogen-regulated genes are transduced to the transcription factors only by way of the TOR kinases; nevertheless these studies have revealed the important role of the TOR kinases in preventing the expression of nitrogen-regulated genes and have led to a better understanding of the role of Ure2p. In cells growing with Bactopeptone or glutamine as a source of nitrogen, Gln3p is present in the cytoplasm in a phosphorylated form complexed with Ure2p. Treatment of these cells with rapamycin or the shift to a medium with proline as a source of nitrogen results in the release of Gln3p phosphate from Ure2p, its dephosphorylation and translocation to the nucleus (Blinder et al., 1996; Beck and Hall, 1999; Bertram et al., 2000) (Fig. 2). It is likely that the agent responsible for the phosphorylation of Gln3p is a TOR kinase, as shown by the observation that immunoprecipitated Tor1p, but not kinase-inactivated Tor1p is able to phosphorylate bacterially produced Gln3p (Bertram et al., 2000). The inactivation of the TOR proteins by rapamycin would therefore prevent the phosphorylation of Gln3p. In addition, this inactivation of TOR proteins results in the activation of the Sit4p phosphatase, which may be the agent responsible for the dephosphorylation of phosphorylated Gln3p. This assumption is supported by the observation that in mutants in which this phosphatase is inactive or absent, rapamycin treatment does not result in the translocation of Gln3p to the nucleus (Beck and Hall, 1999). The dephosphorylation of phosphorylated Gln3p appears to be essential for its translocation from the cytoplasm to the nucleus, since the agent responsible for this translocation through the nuclear pore, the karyopherin Srp1p, is capable of binding non-phosphorylated but not phosphorylated Gln3p (Carvalho et al., 2001).

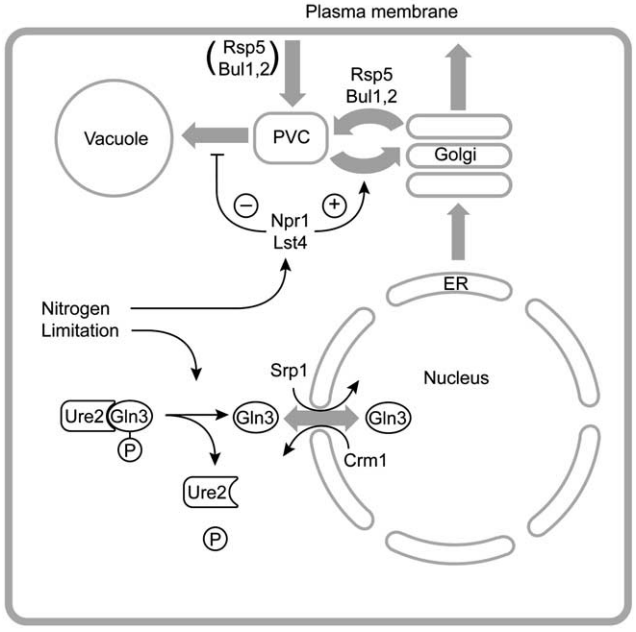

Fig. 2. The role of intracellular trafficking pathways in nitrogen regulation. Conditions of nitrogen limitation can trigger dephosphorylation of cytoplasmic Gln3p and release from Ure2p. With the aid of the nuclear import factor Srp1, Gln3p can then enter the nucleus where it can activate transcription of nitrogen-regulated genes. The nitrogen-regulated permease Gap1p is transported through the secretory pathway to the Golgi complex. In the Golgi, ubiquitination of Gap1p by the E3 ubiquitin ligase complex consisting of Rsp5p, Bul1p and Bul2p causes Gap1p to be diverted to the prevacuolar compartment (PVC) and the vacuole. Conditions of nitrogen limitation allow Gap1p to be recycled to the Golgi and to the plasma membrane. Recycling may involve the action of the Npr1p kinase and the membrane protein Lst4p.

Not only treatment with rapamycin, but also nitrogen starvation, activates Srp1p to translocate Gln3p from the cytoplasm to the nucleus, implying that Gln3p must be present in its non-phosphorylated form in *ure2* mutants because it can activate the expression of nitrogen-regulated genes regardless of the source of nitrogen. It therefore seems likely that Ure2p can protect phosphorylated Gln3p against dephosphorylation by phosphatases. It has not yet been determined whether the phosphatase responsible for the dephosphorylation of phosphorylated Gln3p is Sit4p, or one of the other phosphatases present in yeast cells. If Sit4p were solely responsible, then mutants lacking active Sit4p should be unable to translocate Gln3p to the nucleus even when lacking Ure2p, a possibility that has not been tested. On the other hand, the observation that the calf intestine alkaline phosphatase (CIP) is able to dephosphorylate Gln3p and that Ure2p can protect Gln3p against dephosphorylation by CIP makes it more likely that yeast phosphatases other than Sit4p are responsible for the dephosphorylation of phosphorylated Gln3p in the absence of Ure2p (Bertram et al., 2000). Sit4p may differ from other phosphatases by its ability to bring about this dephosphorylation even when its association with Ure2p protects the phosphorylated Gln3p.

The intracytoplasmic glutamine appears to be the agent responsible for the ability of Ure2p to bind phosphorylated Gln3p. According to this view, a drop in the intracytoplasmic concentration of glutamine would result in a conformational change of the Gln3p-Ure2p complex leading to release of phosphorylated Gln3p, which would then be dephosphorylated in the cytoplasm, allowing it to bind to Srp1p and to be translocated to the nucleus. There is some circumstantial evidence for the view that glutamine interacts with Ure2p, an interaction which may be responsible for increasing the affinity of Ure2p for Gln3p: it has been shown that treatment of cells with glutamine results in the reversible inactivation of glutamine synthetase and that this inactivation requires Ure2p, but not Gln3p (Coschigano and Magasanik, 1991).

The entire process of the passage of Gln3p from its association with Ure2p in the cytoplasm to its association with GATA sites on the DNA in the nucleus is reversible. As discussed previously, the addition of glutamine to cells

growing with glutamate as the source of nitrogen resulted in the almost immediate arrest of the Gln3p activated expression of the *GLN1* gene (Benjamin et al., 1989). The transposition of Gln3p from the nucleus to the cytoplasm requires the export factor Crm1p, as shown by the observation that in *crm1* mutants grown with Bactopeptone as a source of nitrogen, Gln3p is located in the nucleus and activates the expression of the nitrogen-regulated *GAP1* gene (Carvalho et al., 2001). Apparently, the Gln3p in the nucleus and the phosphorylated Gln3p bound to Ure2p in the cytoplasm are in equilibrium and an increase in the intracellular concentration of glutamine favors the translocation of Gln3p from the nucleus to the cytoplasm and its phosphorylation by increasing the affinity of Ure2p for Gln3p.

The experiments using rapamycin to inactivate the TOR kinases have also shown that this treatment results in the translocation of Nil1p from the cytoplasm to the nucleus (Beck and Hall, 1999). Consequently, it is likely that just as in the case of Gln3p, a cytoplasmic protein is responsible for retaining Nil1p in the cytoplasm in cells grown with Bactopeptone or glutamine as a source of nitrogen. The overproduction of Ure2p can prevent the activation of the expression of *DAL80* by Nil1p and cause the retention of Nil1p in the cytoplasm (Cunningham et al., 2000a). Furthermore, the expression of *DAL5* in cells grown with proline as a source of nitrogen requires both Gln3p and Nil1p, but the deletion of *URE2* enables Nil1p alone to activate *DAL5* expression in cells grown with proline, but not in cells grown with glutamine as a source of nitrogen (Coffman et al., 1996). Similarly, Nil1p is able to activate the expression of *GAP1* and of *NIL1* in *gln3 ure2* mutant cells grown with proline, but not with glutamine as a source of nitrogen (Stanbrough and Magasanik, 1996; Coffman et al., 1996). Finally, deletion of *URE2* results in a slight increase in the expression of *PUT1* in a *gln3* mutant grown with asparagine as the source of nitrogen (Xu et al., 1995). Thus, it appears that Ure2p has some ability to bind Nil1p, but that an as yet unidentified protein is responsible for preventing the translocation of Nil1p from the cytoplasm to the nucleus in cells grown with glutamate or glutamine as a source of nitrogen.

The intracellular concentration of the signals glutamate and glutamine depends not only on the nature of the source of nitrogen but also on the availability of α-ketoglutarate. In all studies of nitrogen regulation, glucose has been used as the source of carbon and energy. In glucose-grown cells, enzymes of the citric acid cycle are used exclusively for the synthesis of α-ketoglutarate, and not for energy generation. Thus, only the enzymes required for the synthesis of α-ketoglutarate from pyruvate and acetyl CoA, and not the enzymes required for the conversion of α-ketoglutarate to oxaloacetate and CO_2 are present in these cells (Liu and Butow, 1999; Komeili et al., 2000). The expression of the genes coding for the enzymes required for the synthesis of α-ketoglutarate is greatly reduced by the presence of glutamate or glutamine in the growth medium, in good accord with their role of providing the carbon skeleton of these amino acids.

A system composed of four proteins is responsible for the regulation by glutamate of the enzymes responsible for the synthesis of α-ketoglutarate. The products of *RTG1* and *RTG3* together constitute the transcription factor responsible for the expression of the genes that encode enzymes of the early steps of the citric acid cycle such as *PYC1*, *CIT1*, *ACO1*, *IDH1* and *IDH2*. In cells grown with glutamine or glutamate as a source of nitrogen, Rtg1p and Rtg3p are located in the cytoplasm, but are translocated to the nucleus by the shift of the cells to a medium with ammonia or urea as a source of nitrogen, or by exposure of the cells to rapamycin. Either treatment also results in the immediate expression of *ACO1*, *IDH1*, and *IDH2* (Liu and Butow, 1999; Komeili et al., 2000). The agent responsible for the retention of Rtg1p and Rtg3p in the cytoplasm is apparently the product of *MKS1*. The evidence for this view is the observation that overexpression of *MKS1* greatly reduces the growth rate of the cells when ammonia, but not when glutamate serves as a source of nitrogen and that the deletion of *MKS1* results in a five-fold increase in the level of citrate synthase, the product of *CIT1* and of greatly increased accumulation of α-ketoglutarate and glutamate in the cytoplasm of the cells using ammonia as a source of nitrogen (Edskes et al., 1999; Feller et al., 1997). The *RTG2* gene encodes a forth element of this regulatory system. Similar to mutations in *RTG1* or *RTG3*, mutations in *RTG2* prevent the expression of the genes coding for the enzymes required for the synthesis of α-ketoglutarate and result in the inability to grow in the absence of glutamate (Liu and Butow, 1999; Komeili et al., 2000). However, this effect of an *rtg2* mutation can be reversed by mutation of *mks1* and an *rtg2 mks1* double mutation leads to constitutive expression of the enzymes for the synthesis of α-ketoglutarate (Dilova et al., 2002; Sekito et al., 2002). This epistasis relationship suggests that when intracellular glutamate levels are low Rtg2p may act to inhibit Mks1p leading to activation of Rtg1p and Rtg3p.

The regulation of α-ketoglutarate synthesis, in turn, affects nitrogen regulation. This relationship became evident when it was found that the overexpression of *MKS1* resulted in a phenotype characteristic of a *ure2* mutant: activation of the expression of the nitrogen-regulated gene *DAL5* in cells grown with ammonia as a source of nitrogen (Edskes et al., 1999). Apparently, the overexpression of *MKS1* prevents the translocation of Rtg1p and Rtg3p into the nucleus and consequently reduces the synthesis of α-ketoglutarate. The resulting low levels of α-ketoglutarate may lead to decreased cytoplasmic concentrations of glutamine, which could cause the release of Gln3p from its association with Ure2p.

4.3. Negative regulation

The parsimonious explanation of the function of the tran-

scription factors Dal80p and Nil2p is to antagonize the activation of transcription by Nil1p and to a lesser extent by Gln3p. As described previously, the zinc finger regions of Dal80p and Nil2p are highly homologous to that of Nil1p, and somewhat less homologous to that of Gln3p, but Dal80p and Nil2p lack the domains presumably responsible for the ability of Gln3p and Nil1p to activate gene expression (Stanbrough and Magasanik, 1995). We may therefore assume that Dal80p and Nil2p exert their effect by competing with Nil1p and to a lesser extent with Gln3p for the GATA sites located upstream from the transcriptional start sites of nitrogen-regulated genes. There is no evidence that the translocation of Dal80p and Nil2p from the cytoplasm to the nucleus is regulated in response to the nitrogen source of the medium and it has recently been shown that Dal80p is located in the nucleus in cells grown with either glutamine or proline as a source of nitrogen (Distler et al., 2001).

The intranuclear concentration of each transcription factor depends on the rate of its synthesis and, in the case of Gln3p and Nil1p, on its nitrogen-regulated translocation to the nucleus. The expression of *GLN3* is not subject to nitrogen regulation and, similarly, the expression of *NIL2* is not significantly affected by the nitrogen source of the growth medium (Rowen et al., 1997; Soussi-Boudekou et al., 1997). On the other hand, the expression of *NIL1* and *DAL80* is subject to nitrogen regulation: the expression of *NIL1* can be activated by either Gln3p or Nil1p and the expression of *DAL80* in cells grown with proline as a source of nitrogen requires Gln3p and is stimulated four-fold by Nil1p (Rowen et al., 1997; Coffman et al., 1997).

The expression of *DAL80* is subject to nitrogen regulation, and as a consequence the intracellular concentration of Dal80p is very low in cells grown with glutamine or asparagine as a source of nitrogen. Therefore, when cells grown with glutamine or asparagine as a source of nitrogen are shifted to a medium with a poor source of nitrogen such as proline, Dal80p does not initially interfere with the expression of nitrogen-regulated genes. However, the subsequent activation of *DAL80* expression by Gln3p and Nil1p by growth on proline results in an increase in the intracellular concentrations of Dal80p sufficient to limit the expression of the genes subject to this regulation (Cunningham et al., 2000b). Thus, Dal80p appears to provide a negative feedback loop that will set an appropriate maximum level of nitrogen-regulated gene expression (Fig. 3A).

The activation of gene expression by Nil1p is particularly sensitive to the antagonism exerted by Dal80p because Dal80p can both antagonize the activation of the expression of nitrogen-regulated genes by Nil1p and antagonize the ability of Nil1p to auto-activate its own expression. This effect of Dal80p is apparent in a study of the expression of a *lacZ* fusion to four GATA sites located upstream of *UGA4*. This expression requires Gln3p and is strongly stimulated by Nil1p. Dal80p can prevent the stimulation of *UGA4* expression by Nil1p, but does not interfere with

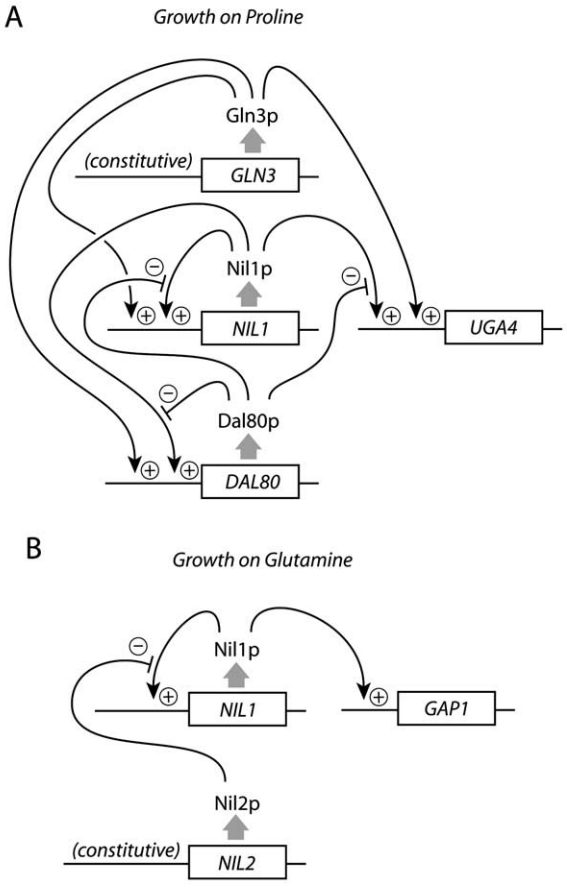

Fig. 3. Regulatory circuits involving the inhibitory action of GATA type transcription factors. (A) Dal80p can antagonize the transcriptional activator Nil1p. When cells are grown on proline, the presence of Dal80p antagonizes activation of the target gene UGA4 and auto-activation of *NIL1*. This negative feedback loop limits the maximum activation by Nil1p on proline medium. (B) Nil2p is expressed at a low level and can antagonize auto-activation of *NIL1*. During growth on glutamine, inhibition by Nil2p blocks a positive feedback loop that would allow spontaneous auto-activation of Nil1p.

the activation of its expression by Gln3p as shown by the fact that the deletion of *DAL80* resulted in a ten-fold increase in the expression of *UGA4* in the *GLN3 NIL1* strain but did not increase its expression in the *GLN3 nil1* strain (Coffman et al., 1997). On the other hand, a study of a *lacZ* fusion to *DAL3* showed that in this case Dal80p was also able to antagonize activation by Gln3p: the deletion of *NIL1* resulted in a three-fold increase of *DAL3-lacZ* expression, presumably because this deletion had resulted in a four-fold reduction of *DAL80* expression and therefore diminished the interference of Dal80p with the activation of the expression of *DAL3* by Gln3p. The fact that the deletion of *DAL80* caused a higher level of *DAL3* expression in cells with a functional *NIL1* gene than in a *nil1* mutant indicates that Nil1p can stimulate the expression of *DAL3*, but is strongly antagonized by Dal80p (Coffman et al., 1997).

So far the expression of *DAL80* and the effect of Dal80p on the activation of gene expression have only been studied

in cells grown with proline as a source of nitrogen. However, there is some indirect evidence that Dal80p can also be effective in cells grown with ammonia as a source of nitrogen. The expression of *GAP1*, which is activated equally well by Gln3p and by Nil1p, was increased from two- to three-fold in cells grown with ammonia as a source of nitrogen by the deletion of *GLN3* (Stanbrough et al., 1995; Rowen et al., 1997). Similarly, the expression of a *lacZ* reporter fused to an element consisting of three GATAAGATAAG sequences (3× GATA) was increased ten-fold by the deletion of *GLN3* (Rowen et al., 1997). The apparent reason for the increased ability of Nil1p to activate gene expression in these cells is their inability to produce Dal80p, due to the complete dependence of *DAL80* expression on Gln3p.

The deletion of *NIL2* enabled Nil1p, but not Gln3p to activate the expression of *NIL1*, *GAP1*, *UGA4*, and of 3× GATA in cells grown with glutamine as a source of nitrogen (Soussi-Boudekou et al., 1997; Coffman et al., 1997; Rowen et al., 1997). The apparent role of Nil2p is to oppose the autogenous activation of *NIL1* expression by Nil1p, which is responsible for raising the intracellular concentration of Nil1p to the level required for the activation of *GAP1* and other nitrogen-regulated genes.

The great sensitivity of *NIL1* expression to autogenous activation by Nil1p was demonstrated by an experiment in which a *gln3* mutant carrying a plasmid with a *NIL1-lacZ* fusion was grown with glutamine as a source of nitrogen and shifted to a medium with ammonia as a source of nitrogen. The initial β-galactosidase activity of these cells was very low, but increased rapidly upon the shift of the cells to medium with ammonia as a source of nitrogen at a rate proportional to the increase in cell mass. Apparently, the intracellular concentration of Nil1p in cells grown with glutamine as a source of nitrogen was adequate for the full auto-activation of *NIL1* gene expression when the Nil1p present in the cytoplasm of the glutamine-grown cells was translocated into the nucleus upon shifting to ammonia medium. On the other hand, cells harboring a plasmid with a *GAP1-lacZ* reporter when subjected to the same experimental protocol displayed a relatively slow rate of β-galactosidase synthesis that was proportional to the intracellular concentration of Nil1p, as estimated from the concentration of β-galactosidase produced by the *NIL1-lacZ* plasmid (Rowen et al., 1997).

These observations provide a good rationale for the role of Nil2p as a damper of the positive feedback loop afforded by the ability of Nil1p to activate its own expression (Fig. 3B). A small fluctuation in the intranuclear concentration of Nil1p in cells growing with glutamine or glutamate as the source of nitrogen could result in the autogenous activation of *NIL1* expression and raise its cytoplasmic concentration to a level beyond the capacity of the agent responsible for its retention in the cytoplasm, resulting in the irreversible activation of the expression of *NIL1* and of the other genes subject to activation by Nil1p. In fact, deletion of the *NIL2* gene does result in inappropriate activation of nitrogen-responsive genes during growth on glutamine or glutamate. Nil2p appears to prevent this inappropriate activation of *NIL1* expression in cells by blocking the access of Nil1p to the GATA sites located upstream of the *NIL1* gene. On the other hand, when growth on ammonia or proline permits the unhindered translocation of Nil1p to the nucleus, it can successfully compete with Nil2p for these GATA sites. The outcome of this competition in favor of Nil1p is due to the fact that *NIL2* is expressed constitutively at a low level and is not induced by growth on ammonia or proline.

In summary, the two negative transcription factors play very different roles in the activation of gene expression by Nil1p. Nil2p antagonizes the autogenous activation of *NIL1* expression in cells grown with glutamine or glutamate as a source of nitrogen. Dal80p limits the activation of gene expression in cells grown on a non-preferred source of nitrogen.

5. Nitrogen-regulated intracellular sorting of amino acid permeases

Saccharomyces cerevisiae encodes 19 amino acid permeases identifiable as members of a diverse family of transporters for amino acids, polyamines and choline (APC) found in bacteria, fungi and mammalian cells (Nelissen et al., 1997). The amino acid permeases are integral membrane proteins with 12 predicted transmembrane domains which are delivered by the secretory pathway to the plasma membrane where they function to take up amino acids for protein synthesis and for use as sources of nitrogen (André, 1995; Regenberg et al., 1999). These permeases can be divided into two classes according to their regulation and function (Sophianopoulou and Diallinas, 1995). The nitrogen-regulated permeases include Gap1p, which transports all naturally occurring amino acids (Jauniaux and Grenson, 1990), and Put4p, which transports only proline (Lasko and Brandriss, 1981; Vandenbol et al., 1989). Both of these permeases are coordinately derepressed during growth on poor nitrogen sources, implying that their function is to provide the cell with amino acids to be used as a source of nitrogen (Wiame et al., 1985; Courchesne and Magasanik, 1983). Members of the other class of permeases are expressed even when cells are grown on a preferred source of nitrogen. Most of these permeases are specific for particular amino acids, or chemically related sets of amino acids, such as the histidine permease, Hip1p (Tanaka and Fink, 1985), the basic amino acid permease, Can1p (Hoffmann, 1985), and Tat2p, a tryptophan permease (Schmidt et al., 1994). This permease class is thought to transport amino acids that are available in the growth medium for use in protein synthesis.

The earliest experiments on *GAP1* regulation followed the Gap1p activity after transfer of a yeast culture growing on proline as a nitrogen source to a medium containing

ammonia. The Gap1p activity as measured by cellular uptake of radio-labeled citrulline is high in cells grown on proline medium. Because the genetic background used in these experiments was Σ1278b, transfer to ammonia brought about a rapid decline in Gap1p activity, an effect known as ammonia inactivation (Grenson, 1983a). Some of the decline in Gap1p activity could be attributed to a cessation in *GAP1* transcription brought about by the inactivation of transcriptional activators Gln3p and Nil1p by the presence of ammonia. However, a *ure2* mutant which transcribes *GAP1* constitutively also exhibited full inactivation by ammonia (in these early studies, the *ure2* mutation was designated *gdhCR*). The important implication of this finding was that much of ammonia inactivation occurs by a process that is independent of transcriptional regulation of *GAP1* (Grenson, 1983a).

For S288C strains, ammonia is not a repressing nitrogen source and as a consequence an effect of ammonia inactivation is not observed in this genetic background. Nevertheless, strains of this genetic background have also been shown to exhibit post-transcriptional regulation of *GAP1*. As described previously, *GAP1* is transcribed in S288C strains grown on ammonia, proline, urea, or glutamate, whereas transcription is off in cells grown on glutamine as a nitrogen source. Post-transcriptional inactivation of Gap1p is revealed by the fact that Gap1p permease activity is very low in cells grown on glutamate despite the high rate of *GAP1* transcription in glutamate medium (Stanbrough and Magasanik, 1995; Roberg et al., 1997a). When *GAP1* is expressed from a heterologous constitutive promoter, which does not respond to the nitrogen source, the amount of Gap1p permease activity nevertheless registers the full response to growth on glutamate or glutamine as a nitrogen source, showing that much of the observed nitrogen regulation of Gap1p transport activity can be attributed to post-transcriptional regulation (Chen and Kaiser, unpublished data).

Experiments following the intracellular location of Gap1 protein showed that the mechanism of post-transcriptional regulation of Gap1p was a consequence of regulated protein sorting in the late secretory pathway. Fractionation of cell membranes carried out in parallel with the activity measurements demonstrated that in cells grown on glutamate, Gap1p is located in ER and Golgi compartments but *not* in the plasma membrane. Under these conditions Gap1p is transported to the vacuole without ever being delivered to the plasma membrane. When cells grown on glutamate are transferred to urea medium a dramatic increase in Gap1p activity is accompanied by a redistribution of Gap1p protein to the plasma membrane (Roberg et al., 1997a). Redistribution of Gap1p to the plasma membrane depends on the function of secretion genes such as *SEC6*, which is required for fusion of post-Golgi secretory vesicles with the plasma membrane. Thus, the activity of Gap1p permease appears to be regulated largely by the amount of Gap1p that is located in the plasma membrane and the key process that controls delivery of Gap1p to the plasma membrane is the nitrogen-dependent sorting of Gap1p in the intracellular Golgi and prevacuolar (PVC) compartments.

The early experiments on ammonia inactivation of Gap1p in the Σ1278b genetic background were extended by experiments to follow the fate of Gap1 protein after transfer of cells grown on proline to ammonia. The observation that the decline in Gap1p activity after transfer to ammonia is accompanied by degradation of Gap1p protein in the vacuole suggested that the endocytosis of Gap1p is largely responsible for ammonia inactivation (Hein and André, 1997). It was further proposed that ammonia inactivation is the result of a greatly increased rate of Gap1p endocytosis in response to ammonia (Springael and André, 1998). In this case, we could hypothesize that the quality of the nitrogen source controls both an intracellular sorting event that governs the rate of delivery of Gap1p to the plasma membrane and an endocytic sorting process which determines the rate at which Gap1p is removed from the plasma membrane. An alternative possibility, that is consistent with all of the available data on the regulation of Gap1p sorting, is that Gap1p is endocytosed continuously under all conditions and that only the intracellular sorting which determines the rate of delivery to the plasma membrane is regulated by nitrogen. According to this idea, ammonia inactivation would be the consequence of constitutive endocytosis causing Gap1p to be removed from the plasma membrane after a cessation of new Gap1p delivery to the plasma membrane caused by the effect of ammonia on Golgi sorting of Gap1p. Resolution of the question of whether or not Gap1p endocytosis is regulated by nitrogen must await the development of specific assays that will allow measurement of the rate of Gap1p endocytosis to be measured independently of the rate of delivery to the plasma membrane.

In the S288C genetic background, Gap1p has an unusual intracellular distribution for a plasma membrane protein. Gap1p is delivered to the cell surface relatively slowly; even when cells are grown under conditions that give maximum Gap1p activity, the steady state distribution of Gap1p reveals that less than half of the protein is in the plasma membrane and the majority is located in intracellular compartments that probably correspond to the Golgi and PVC (Roberg et al., 1997a; Helliwell et al., 2001). This distribution differs markedly from that of other well-studied plasma membrane proteins such as Pma1p and Gas1p, which are found almost exclusively at the plasma membrane in steady state (Roberg et al., 1999). An attractive possibility is that Gap1p engages a recycling loop between the *trans*-Golgi and the PVC. Such recycling could account for the abundant intracellular pool of Gap1p and could also explain why eventual plasma membrane delivery of Gap1p takes so long. Gap1p engaged in cycling between the Golgi and PVC could be thought of as an uncommitted, internal storage form of Gap1p ready to be directed either to the cell surface or the vacuole according to the quality of the nitrogen source. Cells that have been grown on glutamate, which

have an intracellular pool of Gap1p but none at the cell surface, will rapidly redistribute active Gap1p to the cell surface when transferred to urea medium (Roberg et al., 1997a). The physiological significance of the intracellular pool of Gap1p may be to provide cells with the means to rapidly adjust Gap1p activity at the cell surface in response to changing availability of nitrogen.

In its natural habitat, the yeast cell will often utilize breakdown products of proteins containing glutamate, proline, and arginine as their source of nitrogen. These cells would initially use the glutamate and avoid the unnecessary synthesis of Gap1p, Put4p and the enzymes for the utilization of proline and arginine as sources of nitrogen. The eventual exhaustion of glutamate would then allow the permeases to be expressed at the plasma membrane and allow the entry of proline and arginine, which in turn would induce the enzymes required for their utilization as sources of nitrogen.

6. Genes that control intracellular sorting of Gap1 permease

A different set of genes appears to be responsible for nitrogen regulation of the intracellular sorting of *GAP1* gene product than the genes required for nitrogen regulation of *GAP1* transcription. As described previously, a *ure2* mutation allows constitutive transcription of *GAP1* regardless of the nitrogen source, but a *ure2* mutation has little effect on ammonia inactivation of *GAP1* (Grenson, 1983a). Conversely, it has been possible to isolate mutations in the Σ1278b genetic background that completely block the early phase of ammonia inactivation but have little effect on the ability of ammonia to block transcription. The first mutations of this type were originally called *mut2* and *mut4*, subsequently renamed *npi1* and *npi2* (Grenson, 1983a; Jauniaux and Grenson, 1990). Isolation of the *NPI1* gene revealed that it is the same as *RSP5*, a gene previously shown to encode an E3 ubiquitin ligase which catalyses the addition of a ubiquitin moiety to lysine residue(s) in target proteins (Huibregtse et al., 1995; Hein et al., 1995). Isolation of the *NPI2* gene revealed that it was the same as the *DOA4* gene, which encodes a ubiquitin isopeptidase needed to maintain levels of free ubiquitin in the cell for use by the protein degradation machinery (Papa and Hochstrasser, 1993; Springael et al., 1999). The identities of both genes are consistent with an essential role for ubiquitination in the inactivation and vacuolar degradation of Gap1p. The hypothesis that Gap1p itself is the target of ubiquitination was confirmed by experiments demonstrating the existence of ubiquitinated forms of Gap1p that increased in abundance on exposure of cells to ammonia (Springael and André, 1998). Importantly, some of the mutations within the *GAP1* gene itself that rendered the altered Gap1p insensitive to ammonia inactivation were shown to decrease the amount of Gap1p ubiquitination. The most refined mutational studies have identified lysine residues at positions 9 and 16 of the amino-terminal cytosolic domain of Gap1p as the residues that are modified by ubiquitination (Soetens et al., 2002).

Two additional redundant gene products necessary for the ubiquitination and proper intracellular sorting of Gap1p were identified in a screen for genes that influence the intracellular sorting of Gap1p in the S288C genetic background. Overexpression of either Bul1p or Bul2p, two closely related proteins first identified by their interaction with the Rsp5p ubiquitin ligase complex, causes Gap1p to be sorted to the vacuole regardless of the nitrogen source (Helliwell et al., 2001). The double mutant, *bul1 bul2*, has the inverse phenotype causing Gap1p to be delivered to the plasma membrane more efficiently than in wild type cells. Bul1p and Bul2p appear to exert their specific influence on Gap1p sorting through their interaction with Rsp5p because a point mutation in *BUL1* that specifically abolishes interaction with Rsp5p completely abrogates the ability of the mutant Bul1p to influence Gap1p sorting (Yashiroda et al., 1998; Helliwell et al., 2001). Despite the fact that Rsp5p, Bul1p and Bul2p are components of the same ubiquitin ligase complex, they appear to have different effects on Gap1p ubiquitination. The *bul1 bul2* double mutant blocks the formation of polyubiquitinated Gap1p but concomitantly increases the amount of mono-ubiquitinated forms of Gap1p. The *rsp5-1* mutation prevents all Gap1p ubiquitination, consistent with the presumptive role of Rsp5p as the catalytic subunit of the complex. Since the *bul1 bul2* double mutant has the same effect on Gap1p sorting as *rsp5-1*, it seems that polyubiquitination is the key determinant for Gap1p trafficking from the Golgi to the vacuole.

Multiple observations indicate that *BUL1* and *BUL2* exert their control on Gap1p localization by their influence on the intracellular sorting of Gap1p. The effect of overexpression of either *BUL1* or *BUL2* in reducing delivery of Gap1p to the plasma membrane can be partially suppressed by *pep12* or *vps45* mutants, which are blocked in vesicular transport from Golgi to PVC. Conversely, a *bul1 bul2* double mutant exhibits more efficient delivery of Gap1p to the plasma membrane than the wild type (Helliwell et al., 2001). Finally, a *bul1 bul2* double mutation can override the effect of mutations that completely block Gap1p sorting to the plasma membrane (see below).

A second general class of mutants has the opposite effect of the mutations that affect Gap1p ubiquitination; these mutants cause Gap1p and Put4p permeases to be sorted to the vacuole regardless of the nitrogen source but they have little or no effect on the activity of other permeases such as Hip1p and Can1p (Table 1). The first mutant of this class to be isolated was *npr1*, which was found to affect a protein with homology to Ser/Thr protein kinases (Grenson, 1983b; Vandenbol et al., 1987, 1990). Although Gap1p is a phospho-protein (Stanbrough and Magasanik, 1995), an *npr1* mutation does not prevent the formation of phospho-Gap1p, indicating that the target of the Npr1p kinase may

Table 1
Genes that control Gap1p permease sorting

Gene	Mutant phenotype	Essential	Features	Specificity	References
RSP5/NPI1	Gap1p in PM	Yes	E3 ubiquitin ligase	Many targets including membrane proteins	Hein et al., 1995
DOA4/NPI2	Gap1p in PM	No	Deubiquitinating enzyme	General factor for recycling ubiquitin	Papa and Hochstrasser, 1993; Springael et al., 1999
BUL1, *BUL2*	Gap1p in PM	No	Binds to Rsp5p	Specific for nitrogen-responsive permeases	Helliwell et al., 2001
NPR1	Gap1p to vacuole	No	Ser/Thr kinase	Specific for nitrogen-responsive permeases	Grenson, 1983b; Vandenbol et al., 1990
SEC13	Gap1p to vacuole	Yes	COPII subunit	Some alleles specific for *GAP1* and *PUT4*	Roberg et al., 1997b
LST4	Gap1p to vacuole	No	Integral membrane protein	Specific for nitrogen-responsive permeases	Roberg et al., 1997b
LST7	Gap1p to vacuole	No	Hydrophilic protein	Specific for nitrogen-responsive permeases	Roberg et al., 1997b
LST8	Gap1p to vacuole	Yes	Peripheral membrane protein	Affects *GAP1* and *PUT4* and other permeases	Roberg et al., 1997b

not be Gap1p itself (De Craene et al., 2001). Mutations in *NPR1* cause Gap1p to be transported from the Golgi to the vacuole without ever reaching the plasma membrane indicating that Npr1p controls intracellular sorting of Gap1p, probably in either the Golgi or PVC compartment (De Craene et al., 2001).

Tat2p, a permease for tryptophan and other aromatic amino acids, is regulated differently than Gap1p in response to the nitrogen source but nevertheless appears to be under control of some of the same processes that regulate sorting of Gap1p. When yeast cells are either starved for nitrogen or are treated with rapamycin, conditions under which Gap1p permease is stable, Tat2p permease is degraded in the vacuole (Beck et al., 1999). Similar to Gap1p, vacuolar degradation of Tat2p depends on ubiquitination of Tat2p and newly synthesized Tat2p can be transported directly to the vacuole without reaching the plasma membrane (Beck et al., 1999). The Npr1p kinase is itself a phosphoprotein and treatment of cells with rapamycin causes dephosphorylation of Npr1p, suggesting that Npr1p may be responsible for transmitting a signal generated by rapamycin inhibition of TOR function to bring about changes in Tat2p sorting (Schmidt et al., 1998). Consistent with this idea, increased dosage of *NPR1* will cause a corresponding decrease in Tat2p activity, but this effect is only observed in cells treated with rapamycin that carry a *TOR2-1* mutation, which is resistant to rapamycin but in which Tor1p is still a target for the drug (Schmidt et al., 1998). This result is particularly interesting in its implication that *NPR1* is a negative regulator of Tat2p activity, in contrast to the positive effect *NPR1* has on Gap1p activity. However, the simple prediction that an *npr1* mutation should render Tat2p resistant to the effects of rapamycin has not yet been tested and indirect effects of the loss of Gap1p and Put4p activity on Tat2p regulation could explain the observed dosage effects of *NPR1* on tryptophan uptake.

In the S288C genetic background, a collection of mutants have been isolated that cause Gap1p and Put4p to be transported to the vacuole regardless of the nitrogen source. The first such mutant was *sec13-1*, an allele of one of the subunits of the COPII coat required for vesicular transport from the ER to the Golgi (Roberg et al., 1997a). A mutational analysis of *SEC13* reveals that the function of this gene in ER to Golgi transport can be separated from its effect on sorting of nitrogen-regulated permeases in the late secretory pathway (Roberg et al., 1997b). Additional mutations that cause Gap1p to be constitutively sorted to the vacuole were isolated on the basis of their synthetic lethal genetic interactions with *sec13-1*. These mutations define the integral membrane protein Lst4p and two apparently soluble proteins Lst7p and Lst8p (Roberg et al., 1997b). The part that each of these genes has in either sensing of the quality of the nitrogen source or in controlling the membrane trafficking of Gap1p has not been elucidated.

Epistasis tests have been designed to uncover the functional relationship between mutants that cause constitutive sorting of Gap1p to the plasma membrane and mutants that cause constitutive sorting of Gap1p to the vacuole. Combinations of mutations that have been examined include *npr1 rsp5* double mutants (Grenson, 1983b), *npr1 bul1 bul2* triple mutants (Soetens et al., 2002), and *lst4 bul1 bul2* triple mutants (Helliwell et al., 2001). In all three cases, the combined mutants cause Gap1p to be always sorted to the plasma membrane. One explanation for this result is that genes such as *NPR1* and *LST4*, which are required for Gap1p sorting to the plasma membrane, are negative regulators of the E3 ubiquitin ligase complex encoded by the *RSP5*, *BUL1*, and *BUL2* genes. According to this hypothesis, *npr1* or *lst4* mutations should give rise to an increase in the amount of ubiquitinated Gap1p, an effect that has not yet been documented. An alternative possibility, represented in Fig. 2, is that the overall partitioning of newly synthesized Gap1p between the plasma membrane and the vacuole is controlled at two stages: by sorting at the *trans*-Golgi as

controlled by Gap1p ubiquitination and by recycling from the PVC to the Golgi, which in turn would depend on Npr1p and Lst4p. According to this idea, a mutation in the E3 ubiquitin ligase complex would prevent Gap1p from ever entering the compartment where Npr1p and Lst4p can influence the sorting decision. A resolution of these two possible general schemes to explain the relationship between the genes that control Gap1p sorting will require more refined assays for Gap1p ubiquitination and further dissection of the intracellular compartments in which sorting of Gap1p takes place.

7. Outlook

Although we now understand many of the molecular details of how transcription of nitrogen-regulated genes is controlled, we know relatively little about the nature and origin of the intracellular signals that govern the activity of the transcription factors. Although there is strong circumstantial evidence that a high cytoplasmic concentration of glutamine is the signal that controls the activity of the Gln3p transcriptional activator, the precise role of glutamine has not yet been elucidated. Glutamine could, for example, bind to Ure2p and thus enable Ure2p to retain Gln3p in the cytoplasm. However, it has not been shown that glutamine can interact directly with Ure2p to enable it to bind Gln3p phosphate. An alternative possibility is that glutamine may act on Gln3p to stimulate its phosphorylation, which in turn could be responsible for its binding to Ure2p.

Our information on how the presence of glutamine or glutamate in the growth medium prevents the activation of gene expression by Nil1p is even scantier. Although it is likely that the signal is a high intracytoplasmic concentration of glutamate, the signal transducer responsible for preventing the transfer of Nil1p from the cytoplasm to the nucleus in cells grown with glutamine or glutamate as a source of nitrogen has not yet been identified.

Another unanswered question concerns the possible role of the TOR kinases in nitrogen regulation. The experiments using rapamycin to inactivate the TOR kinases have provided clear evidence that their activity is required for preventing the transfer of Gln3p and Nil1p from the cytoplasm to the nucleus in cells growing on a preferred source of nitrogen. However, it has not yet been established that changes in the composition of the growth medium can affect the activities of the TOR kinases and the possibility remains that treatment of cells with rapamycin produces a non-physiological state that does not exactly correspond to a regulatory state produced by nitrogen limitation.

Finally, although we are beginning to understand the factors that govern the intracellular sorting decisions that control the activities of permeases such as Gap1p, we do not yet understand the signals that control these sorting decisions. In the future it will be important to determine at the molecular level why growth on glutamate generates a signal for trafficking of Gap1p to the vacuole whereas growth on urea or proline generates a signal for trafficking to the plasma membrane.

In overview, we can see that nitrogen metabolism in *S. cerevisiae* can be understood to be the result of three distinct elements. These are (i) the enzymes responsible for the synthesis and interconversion of nitrogenous compounds, (ii) the permeases for uptake of nitrogenous compounds, and (iii) the transcription factors and membrane trafficking proteins that regulate the activity of the enzymes and permeases. In considering how a yeast cell responds to growth on different nitrogen-containing media the activities of each element of nitrogen metabolism must be considered, but it must also be appreciated that the elements are interlinked such that each one can exert powerful effects on the other two. For example, the activity of transcription factors governs the relative activities of the enzymes responsible for the interconversion of α-ketoglutarate, glutamate, and glutamine, thus setting the concentrations of these key compounds. Changes in intracellular glutamate and glutamine concentrations can, in turn, produce signals that will alter the activity of permeases leading to changes in the rate of uptake of the precursors to glutamate and glutamine. The existence of the many possible feedback loops, setting the activity of transcription factors, small molecule concentrations and activities of permeases, has made it difficult to understand nitrogen regulation as a whole. Much of the progress in working out the key causal relationships presented in this review has been achieved by studying discrete components of the system in isolation. Now, as we know a great deal about the individual elements of nitrogen regulation it is appropriate to begin to reconstruct how these elements function together.

References

André, B., 1995. An overview of membrane transport proteins in *Saccharomyces cerevisiae*. Yeast 11, 1575–1611.

André, B., Talibi, D., Soussi-Boudekou, S., Hein, C., Vissers, S., Coornaert, D., 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.

Beck, T., Hall, M.N., 1999. The TOR signaling pathway controls nuclear localization of nutrient-regulated transcription factors. Nature 402, 689–692.

Beck, T., Schmidt, A., Hall, M.N., 1999. Starvation induces vacuolar targeting and degradation of the tryptophan permease in yeast. J. Cell Biol. 146, 1227–1238.

Benjamin, P.M., Wu, J.I., Mitchell, A.P., Magasanik, B., 1989. Three regulatory systems control expression of glutamine synthetase in *Saccharomyces cerevisiae* at the level of transcription. Mol. Gen. Genet. 217, 370–377.

Bernard, F., André, B., 2001. Genetic analysis of the signalling pathway activated by external amino acids in *Saccharomyces cerevisiae*. Mol. Microbiol. 41, 489–502.

Bertram, P.G., Choi, J.H., Carvalho, J., Ai, W., Zeng, C., Chan, T.F., Zheng, X.F., 2000. Tripartite regulation of Gln3p by TOR, Ure2p, and phosphatases. J. Biol. Chem. 275, 35727–35733.

Blinder, D., Magasanik, B., 1995. Recognition of nitrogen-responsive

upstream activation sequences of *Saccharomyces cerevisiae* by the product of the *GLN3* gene. J. Bacteriol. 177, 4190–4193.

Blinder, D., Coschigano, P.W., Magasanik, B., 1996. Interaction of the GATA factor Gln3p with the nitrogen regulator Ure2p in *Saccharomyces cerevisiae*. J. Bacteriol. 178, 4734–4736.

Bobola, N., Jansen, R.P., Ho Shin, T., Nasmyth, K., 1996. Asymmetric accumulation of Ash1p in postanaphase nuclei depends on a myosin and restricts yeast making type switching to mother cells. Cell 84, 699–709.

Brandriss, M.C., Magasanik, B., 1979. Genetics and physiology of proline utilization in *Saccharomyces cerevisiae*: mutation causing constitutive enzyme expression. J. Bacteriol. 140, 504–507.

Cardenas, M.E., Cutler, N.S., Lorenz, M.C., DiComo, C.J., Heitman, J., 1999. The TOR signaling cascade regulates gene expression in response to nutrients. Genes Dev. 13, 3271–3279.

Carvalho, J., Bertram, P.G., Wente, S., Zheng, X.F., 2001. Phosphorylation regulates the interaction between Gln3p and the nuclear import factor Srp1p. J. Biol. Chem. 276, 25359–25365.

Chisholm, G., Cooper, T.G., 1982. Isolation and characterization of mutants that produce the allantoin-degrading enzymes constitutively in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 2, 1088–1095.

Coffman, J.A., Rai, R., Cunningham, T., Svetlov, V., Cooper, T.G., 1996. Gat1p, a GATA family protein whose production is sensitive to nitrogen catabolite repression, participates in transcriptional activation of nitrogen-catabolic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 16, 847–858.

Coffman, J.A., Rai, R., Loprete, D.M., Cunningham, T., Svetlov, V., Cooper, T.G., 1997. Cross regulation of four GATA factors that control nitrogen catabolic gene expression in *Saccharomyces cerevisiae*. J. Bacteriol. 179, 3416–3429.

Cogoni, C., Valenzuela, L., Gonzalez-Halphen, D., Olivera, H., Macino, G., Ballario, P., Gonzalez, A., 1995. *Saccharomyces cerevisiae* has a single glutamate synthase gene coding for a plant-like high-molecular-weight polypeptide. J. Bacteriol. 177, 792–798.

Cooper, T.G., 1982. Nitrogen metabolism in *Saccharomyces cerevisiae*. In: Strathern, J.N., Jones, E.W., Broach, J.R. (Eds.). The Molecular Biology of the Yeast *Saccharomyces*: Metabolism and Gene Expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 39–99.

Cooper, T.G., Ferguson, D., Rai, R., Bysani, N., 1990. The GLN3 gene product is required for transcriptional activation of allantoin system gene expression in *Saccharomyces cerevisiae*. J. Bacteriol. 172, 1014–1018.

Cooper, T.G., Kovari, L., Sumrada, R.A., Park, H.D., Luche, R.M., Kovari, I., 1992. Nitrogen catabolite repression of arginase (*CAR1*) expression in *Saccharomyces cerevisiae* is derived from regulated inducer exclusion. J. Bacteriol. 174, 48–55.

Coschigano, P.W., Magasanik, B., 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-transferases. Mol. Cell. Biol. 11, 822–832.

Courchesne, W.E., Magasanik, B., 1983. Ammonia regulation of amino acid permeases in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 3, 672–683.

Courchesne, W.E., Magasanik, B., 1988. Regulation of nitrogen assimilation in *Saccharomyces cerevisiae*: roles of the *URE2* and *GLN3* genes. J. Bacteriol. 170, 708–713.

Cunningham, T.S., Cooper, T.G., 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.

Cunningham, T.S., Cooper, T.G., 1993. The *Saccharomyces cerevisiae DAL80* repressor protein binds to multiple copies of GATAA-containing sequences (URSGATA). J. Bacteriol. 175, 5851–5861.

Cunningham, T.S., Dorrington, R.A., Cooper, T.G., 1994. The *UGA4* UASNTR site required for *GLN3*-dependent transcriptional activation also mediates *DAL80*-responsive regulation and *DAL80* protein binding in *Saccharomyces cerevisiae*. J. Bacteriol. 176, 4718–4725.

Cunningham, T.S., Svetlov, V.V., Rai, R., Smart, W., Cooper, T.G., 1996. G1n3p is capable of binding to UAS(NTR) elements and activating transcription in *Saccharomyces cerevisiae*. J. Bacteriol. 178, 3470–3479.

Cunningham, T.S., Andhare, R., Cooper, T.G., 2000a. Nitrogen catabolite repression of *DAL80* expression depends on the relative levels of Gat1p and Ure2p production in *Saccharomyces cerevisiae*. J. Biol. Chem. 275, 14408–14414.

Cunningham, T.S., Rai, R., Cooper, T.G., 2000b. The level of *DAL80* expression down-regulates GATA factor-mediated transcription in *Saccharomyces cerevisiae*. J. Bacteriol. 182, 6584–6591.

De Craene, J.-O., Soetens, O., André, B., 2001. The Npr1 kinase controls biosynthetic and endocytic sorting of the yeast Gap1 permease. J. Biol. Chem. 276, 43939–43948.

des Etages, S.A., Falvey, D.A., Reece, R.J., Brandriss, M.C., 1996. Functional analysis of the *PUT3* transcriptional activator of the proline utilization pathway in *Saccharomyces cerevisiae*. Genetics 142, 1069–1082.

Dilova, I, Chen, C., Powers, T., 2002. Mks1 in concert with TOR signaling negatively regulates RTG target gene expression in *S. cerevisiae*. Curr. Biol. 12, 289–395.

Distler, M., Kukarni, A., Rai, R., Cooper, T.G., 2001. Green fluorescent protein-Dal80p illuminates up to 16 distinct foci that colocalize with and exhibit the same behavior as chromosomal DNA proceeding through the cell cycle of *Saccharomyces cerevisiae*. J. Bacteriol. 183, 4636–4642.

Drillien, R., Lacroute, F., 1972. Ureidosuccinic acid uptake in yeast and some aspects of its regulation. J. Bacteriol. 109, 203–208.

Dubois, E., Messenguy, F., 1997. Integration of the multiple controls regulating the expression of the arginase gene *CAR1* of *Saccharomyces cerevisiae* in response to different nitrogen signals: role of Gln3p, ArgRp-Mcm1p, and Ume6p. Mol. Gen. Genet. 253, 568–580.

Edskes, H.K., Hanover, J.A., Wickner, R.B., 1999. Mks1p is a regulator of nitrogen catabolism upstream of Ure2p in *Saccharomyces cerevisiae*. Genetics 153, 585–594.

Feller, A., Ramos, F., Pierard, A., Dubois, E., 1997. Lys80p of *Saccharomyces cerevisiae*, previously proposed as a specific repressor of *LYS* genes, is a pleiotropic regulatory factor identical to Mks1p. Yeast 13, 1337–1344.

Forsberg, H., Ljungdahl, P.O., 2001. Genetic and biochemical analysis of the yeast plasma membrane Ssy1p-Ptr3p-Ssy5p sensor of extracellular amino acids. Mol. Cell. Biol. 21, 814–826.

Grenson, M., 1983a. Inactivation-reactivation process and repression of permease formation regulate several ammonia-sensitive permeases in the yeast *Saccharomyces cerevisiae*. Eur. J. Biochem. 133, 135–139.

Grenson, M., 1983b. Study of the positive control of the general amino-acid permease and other ammonia-sensitive uptake systems by the product of the NPR1 gene in the yeast *Saccharomyces cerevisiae*. Eur. J. Biochem. 133, 141–144.

Grenson, M., Hou, C., Crabeel, M., 1970. Multiplicity of the amino acid permeases in *Saccharomyces cerevisiae*. IV. Evidence for a general amino acid permease. J. Bacteriol. 103, 770–777.

Grenson, M., Dubois, E., Piotrowska, M., Drillien, R., Aigle, M., 1974. Ammonia assimilation in *Saccharomyces cerevisiae* as mediated by the two glutamate dehydrogenases. Evidence for the *gdhA* locus being a structural gene for the NADP-dependent glutamate dehydrogenase. Mol. Gen. Genet. 128, 73–85.

Hardwick, J.S., Kuruvilla, F.G., Tong, J.K., Shamji, A.F., Schreiber, S.L., 1999. Rapamycin-modulated transcription defines the subset of nutrient-sensitive signaling pathways directly controlled by the Tor proteins. Proc. Natl. Acad. Sci. USA 96, 14866–14870.

Hein, C., André, B., 1997. A C-terminal di-leucine motif and nearby sequences are required for NH4(+)-induced inactivation and degradation of the general amino acid permease, Gap1p, of *Saccharomyces cerevisiae*. Mol. Microbiol. 24, 607–616.

Hein, C., Springael, J.Y., Volland, C., Haguenauer-Tsapis, R., André, B., 1995. NPl1, an essential yeast gene involved in induced degradation of Gap1 and Fur4 permeases, encodes the Rsp5 ubiquitin-protein ligase. Mol. Microbiol. 18, 77–87.

Helliwell, S.B., Losko, S., Kaiser, C.A., 2001. Components of a ubiquitin ligase complex specify polyubiquitination and intracellular trafficking of the general amino acid permease. J. Cell Biol. 153, 649–662.

Hierholzer, G., Holzer, H., 1963. Repression der Synthese von DPN-abhänginer Glutamin-Säure Dehydrogenase in *Saccharomyces cerevisiae* durch Ammonium Ionen. Biochem. Z. 339, 175–185.

Hinnebusch, A.G., 1992. General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*. In: Jones, E.W., Pringle, J.R., Broach, J.R. (Eds.). The Molecular and Cellular Biology of the Yeast *Saccharomyces cerevisiae*: Gene Expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 319–414.

Hoffmann, 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.

Hofman-Bang, J., 1999. Nitrogen catabolite repression in *Saccharomyces cerevisiae*. Mol. Biotechnol. 12, 35–73.

Huibregtse, J.M., Scheffner, M., Beaudenon, S., Howley, P.M., 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase. Proc. Natl. Acad. Sci. USA 92, 2563–2567.

Jauniaux, J.C., Grenson, M., 1990. *GAP1*, the general amino acid permease gene of *Saccharomyces cerevisiae*. Nucleotide sequence, protein similarity with the other bakers yeast amino acid permeases, and nitrogen catabolite repression. Eur. J. Biochem. 190, 39–44.

Kolhaw, G., Drägert, W., Holzer, H., 1965. Parallel-Repression der Synthese von Glutamin-Synthetase und DPN-abhänginger Glutamat-Dehydrogenase in Hefe. Biochem. Z. 341, 224–238.

Komeili, A., Wedaman, K.P., O'Shea, E.K., Powers, T., 2000. Mechanism of metabolic control. Target of rapamycin signaling links nitrogen quality to the activity of the Rtg1 and Rtg3 transcription factors. J. Cell Biol. 151, 863–878.

Lasko, P.F., Brandriss, M.C., 1981. Proline transport in *Saccharomyces cerevisiae*. J. Bacteriol. 148, 241–247.

Legrain, C., Vissers, S., Dubois, E., Legrain, M., Wiame, J.M., 1982. Regulation of glutamine synthetase from *Saccharomyces cerevisiae* by repression, inactivation and proteolysis. Eur. J. Biochem. 123, 611–616.

Liu, Z., Butow, R.A., 1999. A transcriptional switch in the expression of yeast tricarboxylic acid cycle genes in response to a reduction or loss of respiratory function. Mol. Cell. Biol. 19, 6720–6728.

Magasanik, B., 1992. Regulation of nitrogen utilization. In: Strathern, J.N., Jones, E.W., Broach, J.R. (Eds.). The Molecular Biology of the Yeast *Saccharomyces cerevisiae*: Metabolism and Gene Expression, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp. 283–317.

Marini, A.M., Soussi-Boudekou, S., Vissers, S., André, B., 1997. A family of ammonium transporters in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 17, 4282–4293.

Miller, S.M., Magasanik, B., 1990. Role of NAD-linked glutamate dehydrogenase in nitrogen metabolism in *Saccharomyces cerevisiae*. J. Bacteriol. 172, 4927–4935.

Miller, S.M., Magasanik, B., 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.

Minehart, P.L., Magasanik, B., 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.

Minehart, P.L., Magasanik, B., 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.

Mitchell, A.P., 1985. The *GLN1* locus of *Saccharomyces cerevisiae* encodes glutamine synthetase. Genetics 111, 243–258.

Mitchell, A.P., Magasanik, B., 1983. Purification and properties of glutamine synthetase from *Saccharomyces cerevisiae*. J. Biol. Chem. 258, 119–124.

Mitchell, A.P., Magasanik, B., 1984. Regulation of glutamine-repressible gene products by the *GLN3* function in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 4, 2758–2766.

Nelissen, B., De Wachter, R., Goffeau, A., 1997. Classification of all putative permeases and other membrane plurispanners of the major facilitator superfamily encoded by the complete genome of *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 21, 113–134.

Papa, F.R., Hochstrasser, M., 1993. The yeast *DOA4* gene encodes a deubiquitinating enzyme related to a product of the human tre-2 oncogene. Nature 366, 313–319.

Rai, R., Genbauffe, F.S., Sumrada, R.A., Cooper, T.G., 1989. Identification of sequences responsible for transcriptional activation of the allantoate permease gene in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 9, 602–608.

Regenberg, B., During-Olsen, L., Kielland-Brandt, M.C., Holmberg, S., 1999. Substrate specificity and gene expression of the amino-acid permeases in *Saccharomyces cerevisiae*. Curr. Genet. 36, 317–328.

Roberg, K.J., Rowley, N., Kaiser, C.A., 1997a. Physiological regulation of membrane protein sorting late in the secretory pathway of *Saccharomyces cerevisiae*. J. Cell Biol. 137, 1469–1482.

Roberg, K.J., Bickel, S., Rowley, N., Kaiser, C.A., 1997b. Control of amino acid permease sorting in the late secretory pathway of *Saccharomyces cerevisiae* by *SEC13*, *LST4*, *LST7* and *LST8*. Genetics 147, 1569–1584.

Roberg, K.J., Crotwell, M., Espenshade, P., Gimeno, R., Kaiser, C.A., 1999. *LST1* is a *SEC24* homologue used for selective export of the plasma membrane ATPase from the endoplasmic reticulum. J. Cell Biol. 145, 659–672.

Rowen, D.W., Esiobu, N., Magasanik, B., 1997. Role of GATA factor Nil2p in nitrogen regulation of gene expression in *Saccharomyces cerevisiae*. J. Bacteriol. 179, 3761–3766.

Rytka, J., 1975. Positive selection of general amino acid permease mutants in *Saccharomyces cerevisiae*. J. Bacteriol. 121, 562–570.

Schmelzle, T., Hall, M.N., 2000. TOR, a central controller of cell growth. Cell 103, 253–262.

Schmidt, A., Hall, M.N., Koller, A., 1994. Two FK506 resistance-conferring genes in *Saccharomyces cerevisiae*, *TAT1* and *TAT2*, encode amino acid permeases mediating tyrosine and tryptophan uptake. Mol. Cell. Biol. 14, 6597–6606.

Schmidt, A., Beck, T., Koller, A., Kunz, J., Hall, M.N., 1998. The TOR nutrient signalling pathway phosphorylates NPR1 and inhibits turnover of the tryptophan permease. EMBO J. 17, 6924–6931.

Schreve, J.L., Sin, J.K., Garrett, J.M., 1998. The *Saccharomyces cerevisiae* YCC5(YCL025c) gene encodes an amino acid permease, AgP1, which transports asparagine and glutamine. J. Bacteriol. 180, 2556–2559.

Sekito, T., Liu, Z., Thornton, J., Butow, R.A., 2002. RTG-dependent mitochondria-to-nucleus signaling is regulated by *MKS1i* and is linked to formation of yeast prion [*URE3*]. Mol. Biol. Cell 13, 795–804.

Sil, A., Herskowitz, I., 1996. Identification of an asymmetrically localized determinant, Ash1p, required for lineage-specific transcription of the yeast HO gene. Cell 84, 711–722.

Soberon, M., Gonzalez, A., 1987a. Physiological role of glutaminase activity in *Saccharomyces cerevisiae*. J. Gen. Microbiol. 133, 1–8.

Soberon, M., Gonzalez, A., 1987b. Glutamine degradation through the ω-amidase pathway in *Saccharomyces cerevisiae*. J. Gen. Microbiol. 133, 9–14.

Soetens, O., De Craene, J.-O., André, B., 2002. Ubiquitin is required for sorting to the vacuole of the yeast Gap1 permease. J. Biol. Chem. in press.

Sophianopoulou, V., Diallinas, G., 1995. Amino acid transporters of lower eukaryotes: regulation, structure and topogenesis. FEMS Microbiol. Rev. 16, 53–75.

Soussi-Boudekou, S., Vissers, S., Urrestarazu, A., Jauniaux, J.C., André, B., 1997. Gzf3p, a fourth GATA factor involved in nitrogen-regulated

transcription in *Saccharomyces cerevisiae*. Mol. Microbiol. 23, 1157–1168.

Springael, J.Y., André, B., 1998. Nitrogen-regulated ubiquitination of the Gap1 permease of *Saccharomyces cerevisiae*. Mol. Biol. Cell 9, 1253–1263.

Springael, J.Y., Galan, J.M., Haguenauer-Tsapis, R., André, B., 1999. NH_4^+-induced down-regulation of the *Saccharomyces cerevisiae* Gap1p permease involves its ubiquitination with lysine-63-linked chains. J. Cell Sci. 112, 1375–1383.

Stanbrough, M., 1993. Transcriptional and Post-Transcription Regulation of the General Amino Acid Permease of *Saccharomyces cerevisiae*, MIT Press, Cambridge, MA.

Stanbrough, M., Magasanik, B., 1995. Transcriptional and posttranslational regulation of the general amino acid permease of *Saccharomyces cerevisiae*. J. Bacteriol. 174, 94–102.

Stanbrough, M., Magasanik, B., 1996. Two transcription factors, Gln3p and Nil1p, use the same GATAAG sites to activate the expression of *GAP1* of *Saccharomyces cerevisiae*. J. Bacteriol. 178, 2465–2468.

Stanbrough, M., Rowen, D.W., Magasanik, B., 1995. Role of the GATA factors Gln3p and Nil1p of *Saccharomyces cerevisiae* in the expression of nitrogen-regulated genes. Proc. Natl. Acad. Sci. USA 92, 9450–9454.

Takagi, H., Shichiri, M., Takemura, M., Mohri, M., Nakamori, S., 2000. *Saccharomyces cerevisiae* sigma 1278b has novel genes of the N-acetyltransferase gene superfamily required for L-proline analogue resistance. J. Bacteriol. 182, 4249–4256.

Tanaka, J., Fink, G.R., 1985. The histidine permease gene (HIP1) of *Saccharomyces cerevisiae*. Gene 38, 205–214.

ter Schure, E.G., van Riel, N.A.W., Verrips, C.T., 2000. The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. FEMS Microbiol. Rev. 24, 67–83.

Turoscy, V., Cooper, T.G., 1987. Ureido-succinate is transported by the allantoate transport system in *Saccharomyces cerevisiae*. J. Bacteriol. 169, 2598–2600.

Vandenbol, M., Jauniaux, J.C., Vissers, S., Grenson, M., 1987. Isolation of the *NPR1* gene responsible for the reactivation of ammonia-sensitive amino-acid permeases in *Saccharomyces cerevisiae*. RNA analysis and gene dosage effects. Eur. J. Biochem. 164, 607–612.

Vandenbol, M., Jauniaux, J.C., Grenson, M., 1989. Nucleotide sequence of the *Saccharomyces cerevisiae PUT4* proline-permease-encoding gene: similarities between *CAN1*, *HIP1* and *PUT4* permeases. Gene 83, 153–159.

Vandenbol, M., Jauniaux, J.C., Grenson, M., 1990. The *Saccharomyces cerevisiae NPR1* gene required for the activity of ammonia-sensitive amino acid permeases encodes a protein kinase homologue. Mol. Gen. Genet. 222, 393–399.

Vissers, S., André, B., Muyldermans, F., Grenson, M., 1990. Isolation and characterization of mutants that produce the allantoin-degrading enzymes constitutively in *Saccharomyces cerevisiae*. Eur. J. Biochem. 187, 611–616.

Warner, S.K., Bonthron, D.T., 1994. The ASP1 gene of *Saccharomyces cerevisiae* encoding the intracellular isozyme of L-asparaginase. Gene 144, 37–43.

Wiame, J.M., Grenson, M., Arst Jr., H.N., 1985. Nitrogen catabolite repression in yeasts and filamentous fungi. Adv. Microbiol. Physiol. 26, 1–88.

Xu, S., Falvey, D.A., Brandriss, M.C., 1995. Roles of *URE2* and *GLN3* in the proline utilization pathway of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 15, 2321–2330.

Yashiroda, H., Kaida, D., Toh-e, A., Kikuchi, Y., 1998. The PY-motif of Bul1 protein is essential for growth of *Saccharomyces cerevisiae* under various stress conditions. Gene 225, 39–46.

Zhu, X., Garrett, J., Schreve, J., Michaeli, T., 1996. *GNP1*, the high affinity glutamine permease of *S. cerevisiae*. Curr. Genet. 30, 107–114.