Ammonia-specific Regulation of Gln3 Localization in Saccharomyces cerevisiae by Protein Kinase Npr1*

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Events directly regulating Gln3 intracellular localization and nitrogen catabolite repression (NCR)-sensitive transcription in Saccharomyces cerevisiae are interconnected with many cellular processes that influence the utilization of environmental metabolites. Among them are intracellular trafficking of the permeases that transport nitrogenous compounds and their control by the Tor1,2 signal transduction pathway. Npr1 is a kinase that phosphorylates and thereby stabilizes NCR-sensitive permeases, e.g. Gap1 and Mep2. It is also a phosphoprotein for which phosphorylation and kinase activity are regulated by Tor1,2 via Tap42 and Sit4. Npr1 has been reported to negatively regulate nuclear localization of Gln3 in SD (ammonia)-grown cells. Thus we sought to distinguish whether Npr1: (i) functions directly as a component of NCR control; or (ii) influences Gln3 localization indirectly, possibly as a consequence of participating in protein trafficking. If Npr1 functions directly, then the ability of all good nitrogen sources to restrict Gln3 to the cytoplasm should be lost in an npr1Δ just as occurs when URE2 (encoding this well studied negative Gln3 regulator) is deleted. We show that nuclear localization of Gln3-Myc13 in an npr1Δ occurred only with ammonia as the nitrogen source. Other good nitrogen sources, e.g. glutamine, serine, or asparagine, restricted Gln3-Myc13 to the cytoplasm of both wild type and npr1Δ cells. In other words, the npr1Δ did not possess the uniform phenotype for all repressive nitrogen sources characteristic of ure2Δ. This suggests that the connection between Gln3 localization and Npr1 is indirect, arising from the influence of Npr1 on the ability of cells to utilize ammonia as a repressive nitrogen source.

The increasing use of rapamycin derivatives in the clinical treatment of tissue rejection and neoplastic disease has motivated intense research into the mechanisms through which their target, mTor (mammalian target of rapamycin), regulates the many processes it directly and indirectly influences (1–4). Saccharomyces cerevisiae has been a highly informative model organism in this research, proving especially useful for identifying proteins that interact with Tor and elucidating their functions (5, 6).

In this yeast, there are two Tor proteins, Tor1 and Tor2, that possess serine/threonine kinase activity and are specifically inhibited by a complex composed of rapamycin and prolyl isomerase (7). These kinases possess partially redundant functions, with only Tor2 being essential for viability (8). A major cellular response to rapamycin treatment is global changes in gene expression (9–11). Tor1 and Tor2 are thought to affect these changes by controlling the phosphorylation of specific transcription factors, which in turn regulates their intracellular localization and thus their ability to mediate transcription. Studies of the GATA family transcription activator Gln3, and the response of its intracellular localization to rapamycin, were instrumental in developing our current view of transcription factor regulation by Tor1,2 (9–12). Those studies demonstrated that treating cells with rapamycin induced three important effects: (i) dephosphorylation of Gln3, (ii) nuclear localization of Gln3, and (iii) increased transcription of genes whose expression depended on Gln3 (9–12). All of these effects occurred under growth conditions in which the GATA transcription factors would normally have been localized to the cytoplasm of untreated cells and hence unable to function.

Gln3 and Gat1/Nil1 are GATA family proteins that activate nitrogen catabolite repression (NCR)2-sensitive transcription, which allows S. cerevisiae to selectively utilize good nitrogen sources in preference to poor ones. Physiological control by NCR occurs in the following manner. Gln3 and Gat1 mediate high level expression of genes encoding the permeases and enzymes needed to transport and degrade poor nitrogen sources (e.g. proline) when good nitrogen sources (e.g. glutamine and in some strains ammonia) are not available or are present in limited amounts. On the other hand, when good nitrogen sources are present in sufficient quantities, Gln3 and Gat1 do not bind to their target GATA sequences upstream of NCR-sensitive genes, are localized to the cytoplasm, and the expression of these genes is repressed (13–15). Ure2, the first negative regulator of NCR-sensitive gene expression to be identified (16, 17), is thought to be responsible for cytoplasmic sequestration of Gln3, because a Gln3-Ure2 complex can be isolated from cells in which NCR-sensitive transcription is repressed (11, 12, 18). A similar Gat1-Ure2 complex has not yet been reported.

Reported connections between NCR-sensitive gene expression and rapamycin inhibition of Tor1,2 are shown in abbreviated form in Fig. 1A. According to this model, a rich nitrogen supply generates high intracellular levels of glutamine (or a metabolite of it), which is sensed by and activates the Tor1,2 protein kinases (19). Activated Tor1,2 phosphorylate Tap42

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2 The abbreviations used are: NCR, nitrogen catabolite repression; E3, ubiquitin-protein isopeptide ligase; YNB, yeast nitrogen base.
Npr1 Regulation of Gln3 Localization in S. cerevisiae

**FIGURE 1. Abbreviated diagram of proposed Tor1,2 regulation of Gln3 intracellular localization.** Major components of this diagram were derived from the summary figures in the work of Grenson (22), Galan et al. (26), Beck and Hall (12), Schmidt et al. (26), Jacinto et al. (21), and Crespo et al. (19, 22). We have not diagramed the alternative views and detailed events associated with Tap42, Tip41, and the SIT4-associated proteins or Saps. Therefore, significantly greater complexity exists in these portions of the pathway than is shown. Arrows and bars indicate positive and negative regulation, respectively. Circled P indicates the protein is phosphorylated. *Gap1*, the steps associated with phosphorylation and degradation were elucidated in studies of Gap1 (23–39). However, npr1 mutants were shown to similarly affect the ammonia, proline, and ureidosuccinate permeases (23, 24, 30–32).

Npr1 was first discovered as a mutant whose phenotype was a pleiotropic loss of general amino acid, ammonia, proline, and ureidosuccinate permease activities (23, 24). Subsequently, Npr1 was shown to be required for the stability of the NCR-sensitive general amino acid permease (Gap1) protein itself (Fig. 1) (23). The mechanism by which Npr1 mediates NCR-sensitive permease stabilization has since been studied in significant detail (23–39). In the absence of Npr1-mediated phosphorylation, Gap1 is ubiquitinated, which targets it for endocytosis and degradation in a process that requires Ubi1 (23–25), Ubi1, also designated RSP5, encodes the E3 ubiquitin ligase (24, 27, 28, 33, 34), and Bul1 and -2 are components of the Rsp5-E3 ubiquitin ligase complex (Fig. 1) (38, 39). Although not as thoroughly studied as Gap1, ammonia permeases encoded by the MEP genes are reported to be regulated similarly (23–25, 30–32).

Insights into the mechanism through which Npr1 itself is regulated were achieved with the following findings. (i) Npr1 is phosphorylated in ammonia-grown cells and dephosphorylated following rapamycin treatment or transfer to nitrogen-free or proline-containing medium (26). (ii) Rapamycin-induced Npr1 dephosphorylation does not occur in sit4 mutants (Fig. 1) (21). (iii) Active Npr1 positively correlates with its dephosphorylated form (Fig. 1) (26).

Although many of the regulatory steps depicted in Fig. 1 are soundly supported by experimental data, an increasing number of observations have led to the suggestion that Tor1,2 regulation of Gln3 phosphorylation and intracellular localization may be more complicated than or somewhat different from the model described above (40–42). Among these observations are the following. (i) Association with Tap42 is required for Sit4 phosphatase activity, making Tap42 a positive rather than a negative regulator of Sit4 (43–45). (ii) Cells provided with glutamine, ammonia (good nitrogen sources), or proline (a poor nitrogen source) exhibit nearly indistinguishable levels of detectable Gln3 phosphorylation; in fact, Gln3 is sometimes slightly more phosphorylated in proline-grown cells than in those provided with glutamine (46). (iii) Detectable Gln3 phosphorylation correlates with its nuclear localization at short, but not long, times after rapamycin treatment (46). (iv) Detectable dephosphorylation of Gln3 does not correlate with nitrogen-starvation (46). (v) Msx and rapamycin treatment both induce nuclear localization of Gln3 but have opposite effects on Gln3 phosphorylation (47). (vi) Rapamycin-induced Gat1 dephosphorylation and degradation in a process that requires Ubi1 phosphorylation, Gap1 is ubiquitinated, which targets it for the cytoplasm (23–39). Therefore, in excess nitrogen, Gln3 is highly phosphorylated and restricted to the cytoplasm (11, 12). Whether Gln3 phosphorylation causes it to associate with Ure2, thereby preventing nuclear entry, or alternatively Ure2 stabilizes the phosphorylated form of Gln3, achieving the same outcome, remains unclear (11, 12).

In Msx- or rapamycin-treated cells, Tor1 and Tor2 are inactivated, in the first instance because glutamine production, and hence the posited signal of nitrogen excess, is inhibited and does not reach Tor1,2 (19), and in the second because of direct inhibition of the kinases (12) (Fig. 1). As a result, Tap42 and/or Tip41 are not phosphorylated, Sit4 is freed from its association with Tap42 and is then able to dephosphorylate Gln3 (11, 12). Rapamycin-induced dephosphorylation of Gln3 correlates with its nuclear localization and increased NCR-sensitive transcription.

A search for additional factors controlling Gln3 identified Npr1 as a negative regulator of Gln3 nuclear localization. Gln3 was nuclear in an npr1Δ growing under conditions (in SD medium, which contains ammonia as the nitrogen source) that restrict it to the cytoplasm of wild type cells (22). Nuclear Gln3 localization in SD-grown npr1Δ cells additionally requires Rsp5 and Bul1/2, leading to their being designated as positive regulators of Gln3 nuclear localization (22). The finding that rsp5 and bul1/2 mutations were epistatic to npr1 suggested that these proteins functioned downstream of Npr1 in the regulation of Gln3 (Fig. 1) (22). Npr1-mediated negative regulation of Gln3 was concluded to be independent of Sit4, because the NCR-sensitive reporter gene, MEP2-lacZ, was expressed at the same levels in npr1 single and npr1sit4 double mutants (22).

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Npr1 Regulation of Gln3 Localization in S. cerevisiae

phorylation could not be demonstrated in cells where it clearly occurred with Gln3, even though both Gln3 and Gat1 phosphorylation levels were demonstrated to increase in parallel following carbon starvation (48).

The paradoxical observations cited above, and the regulatory function of Npr1 in the turnover of NCR-sensitive permeases, prompted us to query whether or not the apparent negative regulation of Gln3 nuclear localization by Npr1 occurred indirectly, deriving from the fact that Npr1 is required for ammonia to serve as an effective repressive nitrogen source. The experiments described below show that loss of Gln3 restriction to the cytoplasm in npr1 mutants is specific to the use of ammonia as nitrogen source. Gln3 nuclear localization and NCR-sensitive gene expression in wild type and npr1Δ mutants are similarly regulated when glutamine instead of ammonia is provided as sole nitrogen source. Cytoplasmic restriction of Gln3 in npr1Δ cells also occurs with serine, asparagine, or YPD as the nitrogen source. These observations indicate that apparent Npr1-mediated negative regulation of Gln3 derives indirectly from defects in the ability of ammonia to serve as a repressive nitrogen source in npr1Δ mutants rather than from direct control of Gln3 localization itself.

MATERIALS AND METHODS

Strains and Culture Conditions—S. cerevisiae strains used in this work were TCY36 (Mata lys2 ura3 trp1::hisG leu2::hisG), RR140 (Mata lys2 ura3 trp1::hisG leu2::hisG npr1Δ::TRP1), TB123 (MATa leu2–3,112, ura3–52, rme1, trp1, his4, GALΔ+, HMLa, GLN3-Myc13[KanMX]), TB138-1a (MATa leu2–3,112, ura3–52, rme1, his4, GALΔ+, HMLa, GLN3-Myc13[KanMX], ura2::URA3), and RR143 (MATa leu2–3,112, ura3–52, rme1, trp1, his4, GALΔ+, HMLa, GLN3-Myc13[KanMX], npr1Δ::TRP1). TCY36 and RR140 are derivatives of Σ1278b, and TB123, TB138-1a, and RR143 are derivatives of JK9-3da. Strains were grown at 30 °C to mid-log phase (A600 nm = 0.5) in YPD (yeast extract, 10 g; peptone, 20 g; dextrose, 20 g/liter or in Difco yeast nitrogen base (YNB); without amino acids and ammonium sulfate) medium containing 2% glucose, the required auxotrophic supplements (120 μg/ml leucine, 20 μg/ml uracil, 20 μg/ml histidine, 40 μg/ml lysine, 20 μg/ml tryptophan, 20 μg/ml arginine), and the nitrogen source indicated (0.1% final concentration unless otherwise indicated). Note that this is a different medium than the one used in early studies of NPR1. Rapamycin (Sigma-Aldrich) (dissolved in 10% Tween 20 + 90% ethanol) was added to the cultures, where indicated, to a final concentration of 0.2 μg/ml for 30 min prior to cell harvest for either RNA isolation or indirect immunofluorescence microscopy.

Experiments in which yeast were transferred from one medium to another were performed as follows. A sample of the exponentially growing cells (A600 nm = 0.4–0.5) was harvested from the initial medium for indirect immunofluorescence imaging. The remaining volume of the culture (or half of it in the case of a split culture) was quickly collected by filtration and resuspended in an equal volume of the new medium; this process required 35–45 s. Additional samples were then collected and processed at the indicated times.

Strain Construction—A DNA fragment containing wild type NPR1 from TCY36 was generated using PCR and primers (positions −368 to −338 and +2532 to +2499) to which had been added recognition sites for Xhol and EcoRI. The PCR product was cloned into pBS (KS+) and digested with Xhol and EcoRI to yield pRR490. This plasmid was digested with EcoRI, and a 2.1-kb fragment from the interior of npr1Δ was deleted and replaced with a 0.8-kb EcoRI fragment containing TRP1 (~94 to +726 in which the native StuI site was replaced with EcoRI by PCR methods) to yield pRR494. The 1.6-kb Xhol-EagI fragment containing the npr1Δ and TRP1 marker was isolated from pRR494, and the linear DNA was used to transform TCY36 and TB123. Transformants containing the deletion RR140 and RR143, respectively, were selected on solid YNB medium.

Southern blot analyses were used to confirm the structure of the deletions in RR140 and RR143.

Northern Blot Analyses—Total RNA was isolated and Northern blots performed as described previously (47). Hybridization probes other than that for NPR1 were described previously (50, 51). The NPR1 probe was prepared by PCR using primers 5′-CTTCAACCTCATGCCAGAAA-3′ and 5′-AAGACAAAAGAGGAGCTTGTGC-3′. Radiolabeled hybridization probes were prepared using the Invitrogen RadPrime™ labeling system.

Indirect Immunofluorescence Microscopy—Cell preparation and assay of Gln3-Myc13 by indirect immunofluorescence was performed using a modified form of a method described earlier (47). Although the earlier method performed well for wild type strains, it was found to be inadequate for analysis of Gln3-Myc13 intracellular distribution in some mutants. The modifications were: (i) fixation in the growth medium for 60 min, (ii) the addition of β-mercaptoethanol (10 mM final concentration) to the zymolase digestion mixture, and (iii) adjustment of the digestion times depending on the strain and growth conditions used. The optimum digestion time was determined for each strain and condition.

Immunofluorescence staining was carried out as described by Cox et al. (46). Gln3-Myc13 was visualized using 9E10(c-myc) monoclonal antibody (Covance MMS-150P, at a dilution of 1:1000) as the primary antibody and Alexa Fluor 594 goat anti-mouse IgG antibody (Molecular Probes, at a dilution of 1:200) as secondary antibody.

Cells were imaged using a Zeiss Axiplan 2 imaging microscope with a ×100 Plan-Apochromat 1.40 oil objective. Images were acquired using a Zeiss Axio camera and AxioVision 3.0 software (Zeiss).

Determination of Intracellular Gln3-Myc13 Distribution—To determine the intracellular distribution of Gln3-Myc13 generated under the experimental conditions used, 200 or more cells from randomly chosen fields of each experimental condition were scored in a double blind manner. Experiments were repeated independently from two to seven times. Cells were classified into three categories, those in which Gln3-Myc13 was cytoplasmic (cytoplasmic fluorescence only), nuclear–cytoplasmic (fluorescence could be clearly seen in both the cytoplasm and co-localizing with 4,6-diamidino-2-phenylindole (DAPI)-positive material), or nuclear (co-localizing only with DAPI-positive material). Recognizing that boundaries between the middle category and those flanking it would be unavoidably subjective, we performed two types of control experiments to
determine the precision with which we placed cells into two adjacent categories. First, we categorized Gln3-Myc\textsuperscript{13} as being cytoplasmic or nuclear-cytoplasmic in cells from one microscopic image and then repeated the count a second time using the same image. Next, we repeated this procedure using a second, randomly selected image derived from the same slide as the first image. The cell sample used in this experiment superficially appeared to contain approximately equal numbers of cells in which Gln3-Myc\textsuperscript{13} was categorized as cytoplasmic and nuclear-cytoplasmic, respectively. The two counts yielded four sets of values, two from each image used above. Values from the four counts varied from one another by plus or minus 2–3%.

Finally, we performed an analogous experiment using two images of a cell sample that appeared to contain approximately equal numbers of cells in which Gln3-Myc\textsuperscript{13} was categorized as being nuclear-cytoplasmic and nuclear, respectively. Similar variation (±2–3%) was observed in this second experiment as well. These results argued that we could categorize Gln3-Myc\textsuperscript{13} localization with acceptable precision. The patterns of Gln3-Myc\textsuperscript{13} distribution between cell compartments in response to various experimental conditions (strains, nitrogen source, inhibitor treatment) were also reproducible, as was the distribution of Gln3-Myc\textsuperscript{13} among the three intracellular compartments in replicate images from a single experimental sample. Experiment-to-experiment variation was 2–10%, except when proline was used as the nitrogen source. Here, experiment-to-experiment values occasionally varied into the 20% range for isolated samples. We also noticed that sample-to-sample and experiment-to-experiment variation was greater when a group of samples were not counted in one or a number of closely spaced settings. All samples from a given experiment were counted at the same time (within a day when possible or two consecutive days for the largest experiments). Our best efforts to maximize quantitative accuracy and precision notwithstanding, we recommend attributing less significance to the precise percentages of Gln3-Myc\textsuperscript{13} distribution among the three possible intracellular categories than to the patterns of change observed in the distribution of Gln3-Myc\textsuperscript{13} when comparing one experimental condition with another, i.e. presence versus absence of inhibitor, nitrogen source identity, and wild type versus mutant strains.

RESULTS

**NCR-sensitive Gene Expression in Σ1278b-derived Wild Type and npr1Δ Strains**—The control of NCR-sensitive transcription has been most thoroughly studied and is best understood in Σ1278b-derived strains. This was also the genetic background in which npr1 mutants were discovered and the function of Npr1 has been most studied (23, 24). As a result, conducting analyses in this genetic background have facilitated the unraveling and rectifying of some of the more challenging paradoxical observations concerning the control of nitrogen catabolism and its interface with carbon catabolism (49–51). With these considerations in mind, we constructed an npr1Δ and investigated its influence on the control of NCR-sensitive transcription first in a Σ1278b genetic background.

Nitrogen source-dependent expression profiles of two representative NCR-sensitive genes (DAL5 and DAL80) are shown in Fig. 2, A and B, lanes A–H. Expression of both genes was high in nonrepressive proline medium and were repressed to undetectable levels when ammonia or glutamine was provided as nitrogen source (Fig. 2, A and B, lanes A, C, E, and G). Expression dramatically increased when ammonia-grown wild type cells were treated with rapamycin (Fig. 2, A and B, lanes C–F). Similar results were obtained irrespective of whether the medium contained 0.1 or 0.5% ammonia. The responses in proline- or glutamine-grown cells to rapamycin treatment, however, were more diverse. Only DAL80 expression detectably increased in rapamycin-treated, proline-grown cells, although variation in loading accounted for at least a portion of the observed difference (Fig 2B, lanes A and B). With glutamine, the response to rapamycin treatment was minimal with both genes (Fig. 2, A and B, lanes G and H). These responses indicated that results obtained following rapamycin treatment were nitrogen source-dependent, in agreement with earlier reports (49–51).
Expression profiles of the npr1Δ mutant were also nitrogen source-dependent. In 0.1 and 0.5% ammonia medium, DAL5 and DAL80 expression was uniformly high, in concurrence with the observation made when Npr1 was concluded to negatively regulate intracellular localization of Gln3 and NCR-sensitive transcription (Fig. 2, A and B, lanes K–N) (22). However, constitutive expression in the npr1Δ decreased to nearly undetectable levels when glutamine replaced ammonia as the nitrogen source (Fig. 2, A and B, lane O). In other words, Npr1 appeared to be a negative regulator of NCR-sensitive transcription when ammonia, but not glutamine, was provided as the repressing nitrogen source.

These observations led us to suspect that loss of sensitivity to NCR in an ammonia-grown npr1Δ more likely derived from indirect effects of the deletion on ammonia transport and/or catabolism than from Npr1 acting as a direct negative regulator of Gln3 localization. If Npr1 was a direct Gln3 regulator, DAL5 and DAL80 expression should have been similarly high with glutamine and ammonia in the npr1Δ, i.e. the npr1Δ should have possessed the same pleiotropic phenotype as an ure2Δ.

If Npr1 was necessary for the full activity and/or stability of the ammonia permeases, then its loss would diminish the ability of ammonia to serve as a repressive nitrogen source. Glutamine, on the other hand, the uptake of which is mediated to a significant degree by the Ssy1-responsive, NCR-insensitive Gnp1 permease (50, 51), would not a priori be expected to be so adversely affected by an npr1Δ (26) and hence would retain its ability to repress NCR-sensitive transcription. This, in fact, was what we observed experimentally (Fig. 2, A and B, lanes G and O).

Unfortunately, we were unable to test the above explanation directly. The lack of radioactive ammonia and the insensitivity of the techniques used to detect the stable isotopes of nitrogen prevented us from directly measuring and comparing initial rates of ammonia transport in wild type and npr1Δ strains at the concentrations of unlabeled ammonia used in the media of our experiments and those of Crespo et al. (7.5–76 mM) (22).

Therefore, to determine whether differences between the abilities of glutamine to repress NCR-sensitive gene expression in an npr1Δ derived from a defect in ammonia uptake or ammonia assimilation into amino acids, we provided cultures with an alternative source of ammonia used in the media of our experiments and those of Crespo et al. (7.5–76 mM) (22). However, we were unable to test the above explanation directly. The lack of radioactive ammonia and the insensitivity of the techniques used to detect the stable isotopes of nitrogen prevented us from directly measuring and comparing initial rates of ammonia transport in wild type and npr1Δ strains at the concentrations of unlabeled ammonia used in the media of our experiments and those of Crespo et al. (7.5–76 mM) (22). In other words, Npr1 appeared to be a negative regulator of NCR-sensitive transcription when ammonia, but not glutamine, was provided as the repressing nitrogen source.

Serine-mediated repression decreased the likelihood that a defect in ammonia assimilation into amino acids was responsible for the loss of nitrogen repression in the npr1Δ. This, and the well documented requirement of Npr1 in NCR-sensitive Gap1 and Mep activity and stability, pointed to the ammonia defect likely being associated with ammonia uptake, which would be expected to occur at a lower rate in the npr1Δ.

NCR-sensitive Gene Expression in J9-3da-derived Wild Type and npr1Δ Strains—With a more detailed characterization of the relationship between the npr1Δ, the nitrogen source provided in the medium, and NCR, we turned to the less well characterized wild type strain (TB123) originally used to establish the connections among rapamycin, Tor1,2, Sit4, and the regulation of Gln3 (12). Although ammonia did repress DAL5 expression (Fig. 3A, lanes C and E), we had previously observed TB123 to be less NCR-sensitive than the Σ1278b-derived strains (46).3 The diminished NCR sensitivity of TB123 was more clearly observed when various concentrations of serine and glutamine were provided as nitrogen source (Fig. 3, B and C, lanes A–F). In Σ1278b-derived, wild type TCY36, DAL5, and DAL80 expression was barely visible at the lowest serine con-

3 A. Kulkarni, K. H. Cox, J. J. Tate, and T. G. Cooper, unpublished observations.
Npr1 Regulation of Gln3 Localization in S. cerevisiae

FIGURE 4. NPR1 expression in Ξ1278b and JK9-3da-derived wild type (W.T.) and npr1Δ cells provided with various nitrogen sources. Wild type TCY36 and npr1Δ (RR140) of the Ξ1278b genetic background were analyzed in lanes A–C and D–F, respectively. Wild type TB123 and npr1Δ (RR143) in the JK9-3da background were analyzed in lanes G and H, and I and J, respectively. Other experimental details were as described in the legend for Fig. 2.

centration used for growth and was undetectable when provided at the higher concentrations (Fig. 2, C and D, lanes B–D). In contrast, in TB123 cells cultured with 0.4% serine, residual DAL5 and DAL80 expression remained (Fig. 3, B and C, lanes B–D). This residual DAL5 and DAL80 expression in TB123 was, however, all but eliminated when 0.1 and 0.2% glutamine were provided (Fig. 3, B and C, lanes E and F). These data indicated that although serine was a repressive nitrogen source in TB123, it was not nearly as effective in this strain background as in Ξ1278b-derived Tcy36 and RR140. On the other hand, glutamine similarly repressed NCR-sensitive transcription in both genetic backgrounds, especially when the expression of a highly NCR-sensitive gene such as DAL5 was assayed.

Experiments similar to those described above with Ξ1278b-derived RR140 (Fig. 2, A and B) were performed next with the ammonia-grown TB123-derived npr1Δ, RR143 (Fig. 3A, lanes I–P). Here, as with RR140, DAL5 expression in an ammonia-grown npr1Δ (RR143) was much greater than in the wild type (Fig. 3A, lanes C, E, K, and M). This concurred with the observations of Crespo et al. (22). However, with glutamine as the nitrogen source, DAL5 expression was as highly repressed as we had observed in the Ξ1278b genetic background (Fig. 3, A, lanes G and O). In fact, decreased DAL5 expression was observed even after the addition of rapamycin to glutamine-grown RR143 cultures (Fig. 3A, lanes O and P). As expected from the serine results obtained with wild type TB123 (Fig. 3, B and C, lanes B–D), this amino acid was unable to overcome the effects of the npr1Δ in RR143 (Fig. 3, B and C, lanes H–J). Together, however, these data argued that overall NCR control of transcription remained intact in the npr1Δ irrespective of the genetic background.

NPR1 Expression Is Not Detectably NCR-sensitive—To evaluate whether changes in Npr1 production itself had contributed to the above results, we measured NPR1 expression in cells provided with nitrogen sources ranging from good (glutamine) to poor (proline). NPR1 expression increased slightly as the growth conditions progressed from good to poor nitrogen sources (Fig. 4, lanes A–C). As expected, there was no NPR1 expression in the npr1Δ (Fig. 4, lanes D–F, I, and J). The changes we observed in steady-state levels of NPR1 mRNA were insufficient to account for the NCR-sensitive transcription profiles in the npr1Δ.

Gln3 Intracellular Localization in Isogenic Wild Type TB123 and npr1Δ Strains—Transcription of even highly NCR-sensitive genes derives from the combined actions of transcription factors bound to all of the functional cis-acting regulatory elements in a given promoter. This includes transcription mediated by Gln3 and Gat1, which are known to be regulated differently. The NCR-sensitive gene CAR1 is a good example. Fourteen cis-acting elements have been shown to activate and repress CAR1 transcription, only two of which are directly associated with NCR-sensitivity (55). This fact, in addition to the rate of nitrogen source transport, accounts for some of the gene- and strain-specific variations observed in the expression of NCR-sensitive genes (50) and likely contributes to the differences seen above when comparing strains TB123 and Tcy36. This limitation in measurements of NCR-sensitive transcription prompted us to evaluate the effects of deleting NPR1, more directly by following intracellular Gln3-Myc13 localization using indirect immunofluorescence microscopy.

In wild type TB123, Gln3-Myc13 was localized largely to the nuclei of cells grown in proline medium (~80%) (Fig. 5, left histogram, green bar). It was cytoplasmic in a few cells (red bar) and was present in both compartments (nuclear-cytoplasmic) in about 20% of them (yellow bar). Providing ammonia in place of proline as the nitrogen source resulted in Gln3-Myc13 being cytoplasmic in about 80% of wild type (TB123) cells. With glutamine, serine, asparagine, or in rich (YPD) medium, Gln3-Myc13 was completely cytoplasmic (Fig. 5, left histogram). In the isogenic npr1Δ, Gln3-Myc13 was nuclear or nuclear-cytoplasmic in both proline and ammonia medium (Fig. 5, right histogram). However, in sharp contrast, Gln3-Myc13 was similarly restricted to the cytoplasm of both wild type and npr1Δ cells provided with glutamine, serine, asparagine, or YPD as nitrogen source (Fig. 5, right histogram; representative microscopic images of the npr1Δ provided with the various nitrogen sources appear above the histograms). In other words, ammonia was the only normally repressive nitrogen source in which cytoplasmic localization of Gln3-Myc13 was abrogated in npr1Δ cells. This behavior contrasts with that of a ure2 mutant in which Gln3-Myc13 was similarly nuclear in glutamine, ammonia, and proline media (Fig. 5; images below the histograms).

Rapid Responses of Gln3 Intracellular Localization to Changes in the Nitrogen Source—Assays used in Fig. 5 demonstrated long-term differences in the responses of npr1Δ cells to various good nitrogen sources, because they were conducted during steady-state growth. As such, it was possible that those results represented the sum of regulatory contributions operating over different time scales or after compensatory mechanisms had taken over control of intracellular Gln3-Myc13 localization. By this reasoning, similar nuclear Gln3-Myc13 localization might have been observed with all good nitrogen sources if we had assayed the response in an npr1Δ over a much shorter time scale. To assess this possibility, we assayed the intracellular distribution
of Gln3-Myc\textsuperscript{13} in wild type and \textit{npr1}\textDelta cultures immediately following transfer from proline to glutamine medium. Gln3-Myc\textsuperscript{13} was completely redistributed from nuclei to the cytoplasm of both cell types in less than 5 min (Fig. 6A). The same result was also observed when wild type cells were transferred from proline to ammonia medium (Fig. 6B, left histogram). In contrast, Gln3-Myc\textsuperscript{13} remained largely nuclear in an \textit{npr1}\textDelta transferred from proline to ammonia medium (Fig. 6B, right histogram). There was a small shift from nuclear to nuclear-cytoplasmic Gln3-Myc\textsuperscript{13} localization. This is likely related to the much stronger shift in Gln3-Myc\textsuperscript{13} localization that occurs when proline-grown cells are transferred to fresh proline medium (compare data in Fig. 6B, right histogram, with those in Ref. 56, Fig. 1B therein).

The above experiment prompted another comparison between wild type and \textit{npr1}\textDelta strains, i.e. intracellular Gln3-Myc\textsuperscript{13} distribution following transfer of cells from glutamine to proline or ammonia medium. If Npr1 was required for ammonia uptake and/or utilization, then its loss would result in ammonia behaving like a poor nitrogen source in the mutant strain but not the wild type. This, indeed, was the result observed. When wild type cells were transferred from glutamine to proline medium, Gln3-Myc\textsuperscript{13} began relocating from the cytoplasm to the nucleus within 5 min and was largely nuclear within 30–60 min (Fig. 7A, left histogram). The speed of the relocation was not as rapid as when the transfer occurred in the opposite direction. This was probably because more time was required to exhaust the larger amino acid pools in glutamine-grown cells. In contrast, the percentage of wild type cells in which Gln3-Myc\textsuperscript{13} was cytoplasmic transiently decreased for about 30 min following transfer from glutamine to ammonia medium and increased again thereafter. In reciprocal fashion, the percentage of wild type cells in which Gln3-Myc\textsuperscript{13} was nuclear-cytoplasmic transiently increased and was followed shortly by a similar transient increase in the percentage of cells with nuclear Gln3-Myc\textsuperscript{13} (Fig. 7B, left histogram).

The intracellular distribution of Gln3-Myc\textsuperscript{13} in an \textit{npr1}\textDelta following transfer from glutamine to ammonia medium was nearly identical to that following transfer from glutamine to proline (Fig. 7, A and B, right histograms). The only difference was that relocation of Gln3-Myc\textsuperscript{13} from cytoplasmic to nuclear-cytoplasmic to nuclear was slightly slower with ammonia than with proline. Overall ammonia appeared to behave like a poor nitrogen source.

Finally, we queried whether deleting \textit{NPR1} affected rapamycin-induced NCR-sensitive transcription and Gln3-Myc\textsuperscript{13} localization as expected from the loss of a component in the Tor1,2 signal transduction pathway. This expectation was reinforced by the observation that Npr1 is dephosphorylated in response to rapamycin-inhibition of Tor1,2 (26). As shown in Figs. 2, A and B, and 3A, \textit{lames} \textit{O} and \textit{P}, \textit{DAL5} and \textit{DAL80} expression increased in rapamycin-treated \textit{npr1}\textDelta cells. Furthermore, rapamycin-induced nuclear localization of Gln3-Myc\textsuperscript{13} occurred similarly in wild type and \textit{npr1}\textDelta cultures provided with all of the nitrogen sources except glutamine, where nuclear localization was modestly greater in the mutant than wild type (Fig. 8).

**DISCUSSION**

The data presented in this work demonstrate that nuclear Gln3-Myc\textsuperscript{13} localization in SD- or YNB-ammonia-grown \textit{npr1}\textDelta cells is specific to ammonia and is not observed with other good nitrogen sources such as glutamine, asparagine, serine, or YPD. Similar ammonia-specific responses were observed in both long-term (cells growing in steady state; Fig. 5) and short-term (cells assayed 5 min after transfer from one
medium to another; Figs. 6 and 7) measurements. We note, however, that changes occurring on very short time scales, i.e. seconds or less, would not have been detected. This suggests that the effects of deleting NPR1, on intracellular Gl3 localization and NCR-sensitive transcription in ammonia-grown cells, most likely are the indirect consequences of the well characterized role of this kinase in the processing, activity, and turnover of membrane proteins.

A good or repressive nitrogen source that can be assimilated in sufficient amounts is required for NCR to be exerted on GATA factor-mediated transcription. In some strains ammonia is such a repressive nitrogen source, in contrast to a poor or nonrepressive one like proline. Any environmental condition or mutation that diminishes the rate of uptake or utilization of a good nitrogen source can result in its becoming nonrepressive. The loss of Npr1, required for proper processing and stability of the ammonia permeases (Mep1–3), could then result in ammonia, normally repressive, becoming nonrepressive. Consistent with these expectations, transferring an npr1\(\Delta\)/H9004 from glutamine to ammonia yielded the same kinetics on Gln3-Myc\(^{13}\) relocalization from the cytoplasm to the nucleus as a transfer to the commonly used nonrepressive nitrogen source, proline (Fig. 7).

This interpretation not only explains the npr1 phenotype that we, in this study, and Crespo et al. (22) observed, but it is consistent with and explains the observation that Ubi1/Rsp5 and Bul1,2 were required for NCR-sensitive gene expression in the npr1 mutant but not the wild type. Loss of Ubi1/Rsp5 and/or Bul1,2 would not affect ammonia uptake and hence its ability to repress NCR-sensitive transcription as long as Npr1 remained active and phosphorylated Mep1–3. Therefore, ubi1/ rsp5 and bul1,2 mutations had no effect on the low level of MEP2-lacZ expression in an otherwise wild type strain (22). In the npr1 mutant, however, the ammonia permeases would be

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**FIGURE 6.** Time-dependent changes in intracellular distribution of Gln3-Myc\(^{13}\) in wild type (W.T.) (TB123) and npr1\(\Delta\) (RR143) cells following transfer from poor to good nitrogen sources. Cultures were grown overnight in YNB-proline medium. Each culture was then split into two portions, and one portion was transferred to YNB-glutamine medium (A) and the other to YNB-ammonia medium (B). Samples were harvested from each of the four cultures at the times indicated.

**FIGURE 7.** Time-dependent changes in intracellular distribution of Gln3-Myc\(^{13}\) in wild type (W.T.) (TB123) and npr1\(\Delta\) (RR143) cells following transfer from good to poorer nitrogen sources. Cultures were grown overnight in YNB-glutamine medium. Each culture was then split into two portions, and one portion was transferred to YNB-proline medium (A) and the other to YNB-ammonia medium (B). Samples were harvested from each of the four cultures at the times indicated.

**FIGURE 8.** Intracellular distribution of Gln3-Myc\(^{13}\) in TB123 (W.T.) and RR143 (npr1\(\Delta\)) following rapamycin treatment. Strains were grown and processed for indirect immunofluorescence microscopy as described under "Materials and Methods." Gln3-Myc\(^{13}\) localization was categorized as described in the legend for Fig. 5. Where indicated, cultures were treated with rapamycin (Rap +) for 30 min prior to being harvested.
subject to endocytosis and degradation but only if ubiquitination were intact, as shown in studies with Gap1 (23, 24, 27, 28, 39). Ubii/Rsp5 and Bul1,2 are required for this ubiquitination and hence Mep1–3 degradation. Loss of Mep1–3 activities and the accompanying adverse effects on ammonia uptake and utilization, in turn, are required to abrogate repression of MEP2-lacZ expression. This accounts for the positive requirement of RSP5 and BUL1,2 for NCR-sensitive expression in a npr1 mutant. It also explains the epistasis of ubii/rsp5, bul1, and bul2 mutations to those at npr1 observed by Crespo et al. (22).

Nitrogen sources such as glutamine, whose uptake also occurs by transport systems that are not nitrogen-regulated, like that of the ammonia permeases, retain their ability to elicit NCR and cytoplasmic localization of Gln3-Myc13, as seen in Fig. 5. Furthermore, because Npr1 does not transmit the signal from Tor1,2 to Gln3, rapamycin-induced NCR-sensitive transcription occurs in npr1Δ cells provided with these nitrogen sources, albeit less well than in wild type (Fig. 8). Decreased rapamycin-induced NCR-sensitive transcription is the opposite of the phenotype expected (see Fig. 1) because of loss of the putative negative regulator, Npr1.

These data are consistent with and support earlier observations demonstrating that the ability of rapamycin to induce both NCR-sensitive and retrograde gene expression is nitrogen source-dependent, i.e. responses to the nitrogen source provided and its subsequent utilization are “dissociable” from and can override Tor1,2 influence on these metabolic pathways (Refs. 50 and 51 and this work). The recently reported “separability” in the response of retrograde gene expression to rapamycin treatment and mitochondrial dysfunction supports those earlier conclusions (57).

We observed a curious characteristic when comparing the results obtained following transfer of wild type cells from glutamine to proline or ammonia medium (Fig. 7, A and B, left histograms). As expected, Gln3-Myc13 relocated from the cytoplasm to the nucleus when cells were transferred from glutamine to proline medium, i.e. a good to poor nitrogen source. Curiously, a similar but far more modest and transient relocation in the same direction occurred when cells were transferred from glutamine to ammonia. Why did Gln3-Myc13 transiently leave the cytoplasm, since both glutamine and ammonia are good nitrogen sources? We suggest that this behavior derives from the fact that glutamine is a better nitrogen source than ammonia. Therefore, transfer from glutamine to ammonia medium represents a “downshift” in nitrogen metabolism to which Gln3-Myc13 temporarily responds. Once the change has been accommodated, Gln3-Myc13 begins moving back into the cytoplasm. If this explanation is correct, it points to the high sensitivity with which Gln3-Myc13 intracellular distribution responds to the usable nitrogen supply of the cell.

Finally, these data once again emphasize the high impact of genetic backgrounds, and the complications they impose, on the interpretation of data used to construct models describing the regulation of even a seemingly simple cellular process such as nitrogen metabolism. Although such complications make arriving at unified models more challenging, they truly reflect the impressive flexibility of the cells and their multilayered responses as they achieve and maintain homeostasis in the face of both changing environmental conditions and the varied genetically imposed capabilities for dealing with them. In this regard, it is important to point out that culture doubling times are not always a reliable indicator of the ability of a nitrogen source to elicit NCR. For example, although strains S288c and Σ1278b exhibit the same doubling times in minimal ammonia medium (58), the former strain is largely insensitive to NCR with this nitrogen source, whereas the latter is highly sensitive.

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Npr1 Regulation of Gln3 Localization in S. cerevisiae

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