Nitrogen-responsive Regulation of GATA Protein Family Activators Gln3 and Gat1 Occurs by Two Distinct Pathways, One Inhibited by Rapamycin and the Other by Methionine Sulfoximine*

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Background: Nitrogen availability and TorC1 regulate the localization and function of transcription factors Gln3 and Gat1.

Results: Gln3 and Gat1 responses to rapamycin and methionine sulfoximine differ markedly and exhibit different phosphatase requirements.

Conclusion: Gln3 and Gat1 localization and function are regulated by two distinct pathways, one inhibited by rapamycin and the other by methionine sulfoximine.

Significance: Glutamine starvation regulates Gln3 via a TorC1-independent pathway.

Nitrogen availability regulates the transcription of genes required to degrade non-preferentially utilized nitrogen sources by governing the localization and function of transcription activators, Gln3 and Gat1. TorC1 inhibitor, rapamycin (Rap), and glutamine synthetase inhibitor, methionine sulfoximine (Msx), elicit responses grossly similar to those of limiting nitrogen, implicating both glutamine synthesis and TorC1 in the regulation of Gln3 and Gat1. To better understand this regulation, we compared Msx- versus Rap-elicited Gln3 and Gat1 localization, their DNA binding, nitrogen catabolite repression-sensitive gene expression, and the TorC1 pathway phosphatase requirements for these responses. Using this information we queried whether Rap and Msx inhibit sequential steps in a single, linear cascade connecting glutamine availability to Gln3 and Gat1 control as currently accepted or alternatively inhibit steps in two distinct parallel pathways. We find that Rap most strongly elicits nuclear Gln3 localization and expression of genes whose transcription is most Gat1-dependent. Msx, on the other hand, elicits nuclear Gln3 but not Gat1 localization and expression of genes that are most Gln3-dependent. Importantly, Rap-elicited nuclear Gln3 localization is absolutely Sit4-dependent, but that elicited by Msx is not. PP2A, although not always required for nuclear GATA factor localization, is highly required for GATA factor binding to nitrogen-responsive promoters and subsequent transcription irrespective of the gene GATA factor specificities. Collectively, our data support the existence of two different nitrogen-responsive regulatory pathways, one inhibited by Msx and the other by rapamycin.

Saccharomyces cerevisiae cells live in a boom and bust nutritional environment. At times nitrogen sources are available in abundance, whereas at others they are not, and scavenging for whatever non-preferentially utilized/poor nitrogen sources can be found is imperative to survive (1). The finely tuned responses of yeast to these contrasting environments are controlled by the nitrogen-responsive localizations and functions of the GATA family transcriptional activators Gln3 and Gat1 (2–10).

Initial characterization (before 1999) of nitrogen-responsive transcriptional regulation, designated nitrogen catabolite repression (NCR)² (1, 11) (Fig. 1, Pathway I), demonstrated that Gln3 and Gat1 bind to UASGATA elements in the promoters of NCR-sensitive genes. These two GATA factors are the activators responsible for NCR-sensitive transcription when cells are provided with a poor nitrogen source, e.g. proline, with a limiting amount of a preferentially utilized/good nitrogen source or when nitrogen assimilation pathways are impaired, e.g. in gdh1 or gln1 mutants (1, 12–15). Repression of Gln3- and Gat1-dependent, NCR-sensitive transcription depends upon the Ure2 ability to negatively regulate them in nitrogen-rich conditions (16–19). The isolation of a Gln3-Ure2 complex provided a more concrete biochemical function for Ure2 in this regulation and the beginnings of a molecular basis for NCR (7, 8, 20–24).

This rather primitive view of GATA factor control dramatically expanded when several correlations strongly implicated Tor complex 1 kinase (TorC1), Sit4, and glutamine synthetase as primary components of nitrogen-responsive transcription factor localization and transcriptional regulation (Refs. 7–10; see Ref. 5 for a comprehensive review of Tor). Treating cells with the TorC1-specific inhibitor, rapamycin, or the glutamine synthetase inhibitor, methionine sulfoximine (Msx), mimicked

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2 The abbreviations used are: NCR, nitrogen catabolite repression; TorC1, Tor complex 1 kinase; Msx, methionine sulfoximine; IP, immunoprecipitation; IN, input; Rap, rapamycin; YNB, yeast nitrogen base; Am, ammonia.
the effects of nitrogen limitation, i.e. nuclear Gln3 localization and high level NCR-sensitive gene expression (7–10, 25). Identification and assembly of these correlations by multiple laboratories led to the following molecular model.

In repressive, nitrogen-rich conditions, glutamine (or a glutamine metabolite) is thought to activate the TorC1 kinase (Fig. 1, Pathway 2) (25). TorC1, in turn, phosphorylates Tap42, which facilitates complex formation between Tap42, the Sit4 or PP2A phosphatases, and TorC1 (26–30). When in ternary complexes with TorC1, Tap42-Sit4 and Tap42-PP2A binary complexes are inactive (29). Thus inhibited, these phosphatases are unable to dephosphorylate Gln3 and thereby facilitate its dissociation from the Ure2-Gln3 complex, the latter being required for its nuclear localization (8). As a result, the UAS-GATA elements of NCR-sensitive genes remain unoccupied, and NCR-sensitive transcription is repressed (31, 32).

On the other hand, during nitrogen starvation, limitation, or inhibition of glutamine production by Msx, TorC1 activation, needed for Sit4 and PP2A inactivation, does not occur. This situation can also be mimicked by rapamycin treatment, which causes release of Tap42-Sit4 or Tap42-PP2A binary complexes from TorC1, concomitantly activating Sit4 and PP2A (29). Activated Sit4-Tap42 complexes dephosphorylate Gln3, permitting it to dissociate from Ure2, enter the nucleus, bind to UAS-GATA elements, and activate transcription. The active Tap42-Sit4 and Tap42-PP2A complexes subsequently dissociate, yielding inactive Sit4 or PP2A (29). Although analyzed in much less detail, Gat1 was reported to be regulated similarly to Gln3 (8).

Despite the elegance of the TorC1 regulation model described above (Fig. 1, Pathway 2), accumulating evidence suggests the characteristics of nitrogen-responsive GATA factor regulation are not always congruent with the expectations it predicts (26–30, 32–45). A major unanswered question critical to our understanding of the mechanisms of nitrogen-responsive GATA factor regulation is whether glutamine and TorC1 control Gln3 and Gat1 localization and/or function via a single linear pathway (Pathway 2) or two distinct pathways acting in parallel (Pathways 1 and 2). This is tantamount to asking (i) whether Msx functions at the top of the TorC1 regulatory cascade (Pathway 2 in Fig. 1) or alternatively functions independently of TorC1 (Pathway 1 in Fig. 1) or (ii) whether there is one input signal directing Gln3 and Gat1 to relocate into the nucleus or two input signals.

Two observations raised the possibility that rapamycin and Msx may not function in the same cascade; Msx elicits nuclear Gln3 but not Gat1 localization (42), and Msx addition leads to increased Gln3 phosphorylation, whereas rapamycin treatment decreases it (40). Whether additional differences pertinent to this question exist between Msx- and rapamycin-elicited responses is unknown because, in contrast with rapamycin, Msx-elicited responses remain largely uncharacterized.

The purpose of this work has been to investigate the requirements of Msx-elicited GATA factor regulation at the levels of localization, DNA binding, and transcription using Gln3- versus Gat1-specific genes and to compare them with those elicited by rapamycin treatment. Because one versus two pathway GATA factor regulation predicts distinct experimental outcomes, the data we obtained specifically address this question.

### MATERIALS AND METHODS

**Strains and Culture Conditions**—*S. cerevisiae* strains used in this work are listed in Table 1. Deletion strains involving insertion of kanMX or natMX cassettes were constructed using the long flanking homology strategy of Wach (46) using primers described previously (43). Growth conditions were identical to those described in Tate et al. (40, 41). Cultures (50 ml) were grown to mid-log phase (A600 nm = 0.5–0.55) in YNB minimal ammonia (ammonia, 0.1% final concentration) medium. Appropriate supplements (120 μg/ml leucine, 20 μg/ml uracil, histidine, tryptophan, and arginine) were added to the medium as necessary to cover auxotrophic requirements. For live cell real time fluorescence microscopy, strains were transformed with CEN-based plasmids pRS416-Gat1-GFP and pRS416-Gln3-GFP whose construction and detailed validation for normal regulation have appeared (44, 47, 48). Only freshly prepared transformants were assayed. Where indicated, cells were treated with 200 ng/ml rapamycin or 2 mM methionine sulfoxi-
mine (final concentrations). Fixed treatment times were 20 min for rapamycin and 30 min for Msx.

Quantitative RT-PCR—RNA isolation and cDNA synthesis were conducted as described in Georis et al. (49). Quantification of specific cDNA targets was measured by real-time PCR performed on a StepOnePlus device (Applied Biosystems) using primers GDH2-O1 (5’-CGGACGATTTGATCGAACA-3’) and GDH2-O2 (5’-TAGCCTTGCTGAATTCCGT-3’) or primers that have been described previously (DAL5; GAT1, DAL80, 49). The values reported represent the averages of at least two experiments from independent cultures; error bars indicate S.E.

Chromatin Immunoprecipitation—Cell extracts and immunoprecipitations were conducted as described in Georis et al. (32, 49). Concentrations of specific DNA targets in immunoprecipitation (IP) and input (IN) samples were measured by real-time PCR performed on a StepOnePlus device (Applied Biosystems) using primers GDH2-P1 (5’-GGCCATATGGCGTGGTTTCAAGCGGACTTGGTT-3’), GDH2-P2 (5’-GAG1, DAL80, and DAL5 in response to Msx and rapamycin. Total RNA was isolated from wild type (TB50), gln3Δ (FV005), and gat1Δ (FV006) cells grown in YNB-ammonia medium and treated with rapamycin (+Rap) or methionine sulfoximine (+Msx). Control cells were similarly grown but untreated (Am.). mRNA levels were quantified by quantitative RT-PCR as described under “Materials and Methods.” GDH2 (panel A), GAT1 (panel B), DAL80 (panel C), and DAL5 (panel D) values were normalized with TBP1.

FIGURE 1. Simplified diagrammatic representation, based on data in the literature, of the two possible pathways responsible for nitrogen-responsive GATA factor regulation. This poses the question, Where does Msx function as an inhibitor, at the top of the TorC1 pathway (Pathway 2) or in a separate pathway (Pathway 1)? Green colors in arrows or molecule designations are used to indicate that a molecule is active or a reaction is facilitated. Red colors, on the other hand, indicate inactive molecules or inhibition of a reaction. Phosphorylation of GATA factors and Ure2 are those associated with Pathway 2. No specific molecular models related to Msx-elicited phosphorylation have been proposed.

FIGURE 2. Transcription of NCR-sensitive Genes GDH2, GAT1, DAL80, and DAL5 in response to Msx and rapamycin. Total RNA was isolated from wild type (TB50), gln3Δ (FV005), and gat1Δ (FV006) cells grown in YNB-ammonia medium and treated with rapamycin (+Rap) or methionine sulfoximine (+Msx). Control cells were similarly grown but untreated (Am.). mRNA levels were quantified by quantitative RT-PCR as described under “Materials and Methods.” GDH2 (panel A), GAT1 (panel B), DAL80 (panel C), and DAL5 (panel D) values were normalized with TBP1.
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(A) W.T.  
pph21Δpph22Δ  

sit4Δ

Gln3-Myc13

DAPI

(B)  

Cytoplasmic  Nucl.-Cyto.  Nuclear

Percentage of Cells

Am.  + Msx  + Rap

W.T.  
pph21Δpph22Δ  

sit4Δ

(C) W.T.  
pph21Δpph22Δ  

sit4Δ

Gal1-Myc13

DAPI

(D)  

Cytoplasmic  Nucl.-Cyto.  Nuclear

Percentage of Cells

Am.  + Msx  + Rap

W.T.  
pph21Δpph22Δ  

sit4Δ
Intracellular Gln3 and Gat1 Localization—Two methods were used to determine Gln3 and Gat1 intracellular localization, indirect immunofluorescence of Gln3-Myc13 and Gat1-Myc13, and real time Gln3-GFP and Gat1-GFP fluorescence for the kinetics of intracellular Gln3 and Gat1 movement. Comprehensive descriptions of both procedures appear in Tate et al. (41, 43, 44).

Images (subsequently designated, primary images) were collected at room temperature using a Zeiss Axioplan 2 microscope equipped with a 100×/1.40 (Myc13 fluorescence) or 63×/1.40 (live cell GFP assay) Plan-APOCHROMAT oil objective, a Zeiss Axio camera, and Zeiss Axiovision 4.1 software. Only primary, unaltered .zvi file images were used for scoring intracellular Gln-Myc13, Gat1-Myc13, Gln3-GFP, and Gat1-GFP distributions as described below. For the microscopic images presented, primary .zvi files were converted to .tif files and processed for publication using Adobe Photoshop and Illustrator software. Gamma settings of microscopic images presented in this work were unaltered or only minimally altered where necessary to avoid loss of detail relative to what was observed in the microscope. Changes were applied uniformly to the image presented and as similarly as possible from one image to another.

Scoring of Intracellular GATA Factor Localization—We manually scored Gln3-Myc13 and Gat1-Myc13 localization in 200 or more cells from multiple, randomly chosen fields from which each immunofluorescence image was taken. All of the cells in the two images collected from each Gln3-GFP or Gat1-GFP culture sample (average number of cells scored per sample was 91). In all cases, scoring was performed using primary, unaltered .zvi file images and Zeiss AxioVision 4.6.3 software.

Cells were classified into one of three categories: cytoplasmic (cytoplasmic fluorescence only), nuclear-cytoplasmic (fluorescence in the cytoplasm and the nucleus), or nuclear (fluorescence exclusively restricted to the nucleus). These categories are color-coded as red, yellow, and green bars, respectively, in the histograms. Criteria applied in the determination of GATA factor localization, including panels of standard images demonstrating the differences between the categories for both immunofluorescence and GFP fluorescence measurements appear in Fig. 2 of Tate et al. (43) and Fig. 1 of Tate et al. (44), as has detailed evaluations of the precision of the scoring procedures (41, 44). Precision for both methods ranges from ±4% when GATA factors in ≥80% of the cells are situated in a single cellular compartment to ±10% when GATA factors are more or less equally distributed in two or three cellular compartments. Further evaluation of scoring precision can also be obtained by comparing the similar experiments within and between this and previous publications from our laboratory (32, 41, 43, 44, 50, 51). All experiments were performed two or more times with similar results.

As a result of the low concentration of Gln3 in the cell and high background fluorescence, it is not possible to confidently distinguish cells in which Gln3-GFP is nuclear from those where it is cytosolic (51). Therefore, only two-category scoring was performed for Gln3-GFP, i.e. it was scored only as completely cytoplasmic or nuclear-cytoplasmic (the latter being the sum of nuclear plus nuclear-cytoplasmic values when data are compared with those obtained using indirect immunofluorescence of Gln3-Myc13).

RESULTS

The experiments described below were designed to address two specific questions. (i) Do Msx and rapamycin inhibit sequential steps in a single linear cascade controlling Gln3 and Gat1 (depicted as Pathway 2; Fig. 1) or, alternatively, individual steps in two distinct parallel pathways (depicted as Pathways 1 + 2 with Msx inhibiting only Pathway 1, Fig. 1)? (ii) Do Msx- and rapamycin-inhibited reactions impact equally on both Gln3 and Gat1 regulation, or do the inhibitors exhibit GATA factor specificity? Alternative answers to each question generated clearly testable predictions. If Msx and rapamycin inhibit sequential steps of a single, TorC1-dependent regulatory pathway (Pathway 2), then we would expect them to elicit uniform effects on downstream processes, i.e. GATA factor localization, NCR-sensitive transcription, GATA factor DNA binding, and possess common Tor pathway phosphatase requirements for the effects. On the other hand, if Msx and rapamycin are situated in distinct pathways, one inhibited by rapamycin but not Msx (Pathway 2) and the other inhibited by Msx but not rapamycin (Pathway 1), one might expect differing inhibitor-specific responses on these downstream processes and their phosphatase requirements. Stating the question in another way, does Msx function in TorC1-dependent Pathway 2 or TorC1-independent Pathway 1?

To address these questions, we systematically compared Msx- and rapamycin-elicted responses and GATA factor requirements for well established NCR-sensitive catabolic genes: GDH2, encoding the catabolic NAD-glutamate dehydrogenase responsible for glutamate degradation and ammonia production (37, 52, 53, 55); GAT1, encoding a GATA-family transcriptional activator (56–58); DAL80, encoding a GATA-family repressor that down-regulates expression of some but not all NCR-sensitive genes (49, 59–62); DAL5, encoding allantoate permease (63, 64). The rationale for choosing these particular genes was that the respective Gln3 and Gat1 contributions to their overall transcriptional activation are known to differ.

NCR-sensitive Genes, GDH2, GAT1, DAL80, and DAL5 Respond Differently to Msx and Rapamycin—We initiated our investigation by assessing the transcription profiles of GDH2,
GAT1, DAL80, and DAL5 in ammonia-grown cells treated with either Msx or rapamycin. Msx, but not rapamycin, elicited high level GDH2 transcription. This transcription was dependent only on Gln3 as shown by the fact that Msx-induced transcription was eliminated in a gln3Δ/H9004 but unaffected in a gat1Δ/H9004 (Fig. 2A). A similar result was obtained for GAT1 transcription; it was highly induced by Msx in a Gln3-dependent manner, with no transcription being elicited by rapamycin addition (Fig. 2B).

In contrast with these two genes, which exhibited a response to only one inhibitor and one GATA family activator, DAL80 transcription increased after the addition of either Msx or rapamycin, stimulation being 5-fold greater with rapamycin than with Msx (Fig. 2C). However, the two inhibitors exhibited GATA factor specificities. Msx-induced DAL80 transcription was completely Gat1- and Gln3-dependent, whereas rapamycin-induced DAL80 transcription was absolutely Gat1-dependent but only partially required Gln3. Finally, DAL5 transcription responded about equally to both Msx or rapamycin addition, and both transcription factors were required, but the specificity was less obvious (Fig. 2D). The Msx response was absolutely Gln3-dependent and strongly Gat1-dependent (there was a small response to Msx in the gat1Δ/H9004). Gat1 and Gln3 were equally required for rapamycin-induced DAL5 transcription. Overall, the GDH2, GAT1, DAL80, and DAL5 transcriptional responses elicited by Msx and rapamycin treatments were not uniform. They were gene-, GATA family activator-,

Response of Intracellular Gln3 and Gat1 Localization to Msx and Rapamycin Addition—The above data demonstrated clear differences in GATA factor-specific transcriptional responses to Msx and rapamycin. Our next objective was to determine the extent to which these observations correlated with Msx- and
rapamycin-induced GATA factor localization. Both Msx and rapamycin relocalized Gln3-Myc\textsuperscript{13} from the cytoplasm to the nuclei of ammonia-grown wild type cells (Fig. 3, A and B). However, the effect was significantly stronger with Msx than rapamycin, i.e., Msx treatment elicited complete nuclear Gln3-Myc\textsuperscript{13} localization, whereas with rapamycin more cells exhibited nuclear-cytoplasmic than nuclear localization (see “Materials and Methods” for distinction, scoring criteria, and assay precision). Just the reverse occurred with Gat1-Myc\textsuperscript{13} (Fig. 3, C and D). In contrast to Gln3-Myc\textsuperscript{13}, treating wild type cells with Msx had a minimal effect on Gat1-Myc\textsuperscript{13} localization. Rapamycin treatment, on the other hand, induced complete relocalization of Gat1-Myc\textsuperscript{13} to the nucleus. Therefore, as observed with transcription, the responses to Msx and rapamycin treatments were markedly different and largely GATA factor-specific, the Msx response correlating with Gln3-Myc\textsuperscript{13} localization, whereas the rapamycin response was more predominant with Gat1-Myc\textsuperscript{13} localization.

Gln3 and Gat1 Binding to the GDH2 and DAL5 Promoters

Gln3-GFP and Gat1-GFP in wild type and PP2A (pph21\textsuperscript{15}/pph22\textsuperscript{15}) mutant strains in response to Msx.

Wild type (FV063; panels A, B, E, and F) or pph21\textsuperscript{15}/pph22\textsuperscript{15} (03879c; panels C, D, G, and H) mutant strains containing a chromosomal copy of Gat1-Myc\textsuperscript{13} were transformed with CEN-plasmids pRS416-Gln3-GFP or pRS416-Gat1-GFP, and transformants were grown in YNB-ammonia medium. Six samples from each culture were successively collected for real time imaging as described under “Materials and Methods.” Msx was then added, and sample collection was continued thereafter as indicated. Microscopic images in panels A, C, E, and G were derived from images used to determine intracellular Gat1-GFP localization in the histograms of panels B, D, F, and H. Times that appear on the abscissa are the averages of the two times at which the pairs of images generated from each sample were completed after the addition of methionine sulfoximine to the cultures. For each histogram, cells were scored for intracellular Gat1-GFP localization using the three category method (cytoplasmic, red bars; nuclear-cytoplasmic, yellow bars; nuclear, green bars) as described under “Materials and Methods.” Due to weak signals and the high background fluorescence, it is not possible to confidently distinguish cells in which Gln3-GFP is nuclear from those where it is nuclear-cytoplasmic (51). Therefore, only two category scoring was performed; Gln3-GFP was scored as completely cytoplasmic or nuclear-cytoplasmic. Error bars in histograms derived from untreated cells designate 1 S.D. relative to the average of the data collected. Error bars do not appear in the case of data obtained from treated cells because the response time courses modestly vary (plus or minus a few minutes) from day to day. Normalization of the shapes of the curves, which we are unable to do, would be required to present statistical analysis of the data. It is the qualitative responses of the treatments and their overall time courses that we feel is most important.

FIGURE 6. Localization of Gln3-GFP and Gat1-GFP in wild type and PP2A (pph21\textsuperscript{15}/pph22\textsuperscript{15}) mutant strains in response to Msx. Wild type (FV063; panels A, B, E, and F) or pph21\textsuperscript{15}/pph22\textsuperscript{15} (03879c; panels C, D, G, and H) mutant strains containing a chromosomal copy of Gat1-Myc\textsuperscript{13} were transformed with CEN-plasmids pRS416-Gln3-GFP or pRS416-Gat1-GFP, and transformants were grown in YNB-ammonia medium. Six samples from each culture were successively collected for real time imaging as described under “Materials and Methods.” Msx was then added, and sample collection was continued thereafter as indicated. Microscopic images in panels A, C, E, and G were derived from images used to determine intracellular Gat1-GFP localization in the histograms of panels B, D, F, and H. Times that appear on the abscissa are the averages of the two times at which the pairs of images generated from each sample were completed after the addition of methionine sulfoximine to the cultures. For each histogram, cells were scored for intracellular Gat1-GFP localization using the three category method (cytoplasmic, red bars; nuclear-cytoplasmic, yellow bars; nuclear, green bars) as described under “Materials and Methods.” Due to weak signals and the high background fluorescence, it is not possible to confidently distinguish cells in which Gln3-GFP is nuclear from those where it is nuclear-cytoplasmic (51). Therefore, only two category scoring was performed; Gln3-GFP was scored as completely cytoplasmic or nuclear-cytoplasmic. Error bars in histograms derived from untreated cells designate 1 S.D. relative to the average of the data collected. Error bars do not appear in the case of data obtained from treated cells because the response time courses modestly vary (plus or minus a few minutes) from day to day. Normalization of the shapes of the curves, which we are unable to do, would be required to present statistical analysis of the data. It is the qualitative responses of the treatments and their overall time courses that we feel is most important.
a gln3Δ mutant. The fact that Msx induced as much Gat1-Myc13 binding to GDH2 as did rapamycin was somewhat surprising given the nearly complete lack of effect of Msx on Gat1-Myc13 localization (Fig. 3, C and D). A possible explanation of this observation is that there is a substantial amount of nuclear Gat1-Myc13 in untreated, ammonia-grown cells. If Gln3 facilitated Gat1 binding, as Gat1 does for Gln3 binding (32), then Msx addition, which greatly increases nuclear Gln3-Myc13 localization, would generate enhanced binding of the limited amount of Gat1-Myc13 already in the nucleus. Consistent with this explanation is the fact that Msx-induced Gat1-Myc13 binding to GDH2 was Gln3-dependent, whereas rapamycin-induced Gat1-Myc13 binding was not.

The overall pattern of inhibitor-induced Gln3-Myc13 binding to the DAL5 promoter was reversed relative to binding to GDH2. Gat1-dependent Gln3-Myc13 binding at the DAL5 promoter was induced by both Msx and rapamycin, but rapamycin was 7-fold more effective than Msx (Fig. 4B, panels A and B). The response of Gln3-Myc13 localization to Msx addition in the pph21Δpph22Δ mutant was indistinguishable from that in the wild type except that 9–10 additional min elapsed between Msx addition and Gln3-GFP movement (Fig. 6, C and D). At higher scoring resolution, using the Myc13-based indirect fluorescence assay, a measurable Pph21/22 requirement could be detected (Fig. 3, A and B). Irrespective of the assay, however, Msx relocalized a major portion of Gln3 to the nucleus in a largely Pph21/22-independent manner.

Gat1-Myc13 behaved quite differently from Gln3-GFP. First, Gat1-GFP was more nuclear than Gln3-GFP in untreated, ammonia-grown, wild type cells, a reflection of its lower sensitivity to NCR regulation (Fig. 6, E and F). Foremost, however, Msx had only a small effect on Gat1-GFP localization (Fig. 6, E and F). In an untreated pph21Δpph22Δ mutant, nuclear-cytoplasmic Gat1-GFP localization completely disappeared and was not affected by the addition of Msx (Fig. 6, G and H). In contrast, using the Myc13-based indirect fluorescence assay, Gat1-Myc13 was nuclear-cytoplasmic in ∼20% Msx-treated pph21Δpph22Δ cells (Fig. 3, C and D).

The loss of Sit4 elicited a somewhat greater effect on Msx-induced Gln3-GFP localization than did that of Pph21/22 (Fig. 7, C and D). Nuclear-cytoplasmic plus nuclear Gln3-GFP decreased from around 90% that of wild type cells to about 60–70% that in the sit4Δ, indicating that Msx-induced nuclear Gln3-GFP localization was only slightly Sit4-dependent. This modest Sit4 requirement was less obvious using the Gln3-Myc13 indirect fluorescent assay (Fig. 3, A and B) in that Msx-treated sit4Δ cells with nuclear Gln3-Myc13 localization decreased by only about 10–15%. The greatest difference occurred in the kinetics of the response. Loss of Pph21/22 resulted in ∼9–10 min lag before the mutant cells responded to Msx (compare Fig. 6, B with D). In contrast, the loss of Sit4 doubled the lag to ∼19–20 min. The transition of Gln3-GFP...
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from the cytoplasm in wild type cells was nearly complete before it ever started in the sit4Δ (Compare Fig. 7, B and D).

For reasons that we cannot explain, two opposite results have been reported for characterizations of the effects of Msx on Gln3-Myc13 localization. In the initial report describing Msx-elicited nuclear Gln-Myc13 localization, Sit4 was required for the effect (Fig. 2A of Ref. 25). As seen in this work (Fig. 3, A and B), however, we did not observe a Sit4 requirement for Msx-elicited nuclear Gln3-Myc13 localization. Although the same strains were assayed, there were minor differences in the media used in the present and earlier experiments. Therefore, we repeated the Gln3-Myc13 localization experiments using conditions identical to those initially reported but were still unable to obtain a demonstrable Sit4 requirement (data not shown). We did observe a modest effect of Sit4 loss when the kinetics of Msx-elicited Gln3-GFP relocation were followed (compare Fig. 7, A and B with C and D), but it was minor when compared with that reported earlier. A possible explanation for the differing observations might be that Gln3-Myc13 localization was quantitatively determined in the present work, whereas only a single image was reported earlier. However, this reasoning would not explain that other equally important differences were observed between the initial and subsequent reports of Msx-elicited Gln3-Myc13 phosphorylation (25, 40).

In untreated and Msx-treated sit4Δ cells (Fig. 7, G and H, as in pph21/22Δ cells (Fig. 6, G and H), nuclear-cytoplasmic Gat1-GFP localization was eliminated. In sit4Δ mutant cells, Msx had no effect whatever on Gat1-GFP localization (Fig. 7, G and H), unlike the small amount of Msx elicited relocalization in the wild type (Fig. 7, E and F). However, as in pph21/22Δ cells, nuclear-cytoplasmic Gat1-GFP localization was eliminated in untreated and Msx-treated sit4Δ cells. In contrast with the Gat1-GFP assay, the loss of Sit4 had little effect on nuclear-cytoplasmic or nuclear Gln3-Myc13 localization that occurred in ~20–30% of untreated or Msx-treated cells (Fig. 3, C and D). Potential explanations for this difference will be discussed later.

Overall, the effects of Msx on GATA factor localization in ammonia-grown cells were almost exclusively restricted to Gln3. Msx effects on GATA localization were minimal. Although there was detectable deterioration in the extent of nuclear Gln3 localization when Pph21/22 (Fig. 3, A and B) or Sit4 (Fig. 7, C and D) activities were eliminated, the effects were minimal to modest at best, i.e. nuclear Gln3 localization in response to Msx addition was largely Pph21/22- and Sit4-independent.

Effects of Eliminating Pph21/22 and Sit4 Activities on Rapamycin-influenced Gln3-GFP and Gat1-GFP Localization—The kinetics of rapamycin-induced Gln3-GFP movement in wild type cells differed from those of Msx. The response to rapamycin was faster, being largely complete in 5–7 min, whereas by this time after Msx addition, Gln3-GFP movement into the nucleus was just getting well under way (compare Fig. 8, A and B, with Fig. 6, A and B). Furthermore, the loss of Pph21/22, which minimally affected Msx-induced Gln3-GFP movement, resulted in a moderate decrease in the degree to which Gln3-GFP moved from the cytoplasm into the nuclear-cytoplasmic and nuclear scoring categories (Fig. 8, C and D). Additionally, the response in the pph21Δpph22Δ became transitory with Gln3-GFP remaining maximally nuclear for only ~10–12 min.

In contrast with the lack of a response to Msx, Gat1-GFP rapidly relocated to the nucleus after rapamycin addition (Fig. 8, E and F). Although there was some evidence that Gat1-GFP began to leave the nuclei of rapamycin-treated cells, the effect was relatively modest (Fig. 8, E and F). Gat1-GFP relocation from the cytoplasm to the nuclei of pph21Δpph22Δ mutant cells, on the other hand, was markedly muted and clearly transient (Fig. 8, G and H). There was also noticeable similarity in the kinetics of Gat1-GFP and Gln3-GFP responses to rapamycin in the pph21Δpph22Δ mutant (compare Fig. 8, C and D with G and H).

The effect of deleting SIT4 on rapamycin-elicited nuclear localization of Gln3-GFP was far stronger than that of Pph21/22 (compare Fig. 9, C and D, with Fig. 8, C and D) or that for Msx responses in either pph21Δpph22Δ or sit4Δ (compare Fig. 9, C and D, with Fig. 7, C and D, and Fig. 8, C and D). The Sit4 requirement for rapamycin-elicited nuclear localization of Gat1-GFP was more modest than was that of Pph21/22 (compare Fig. 9, G and H with Fig. 8, G and H) or that of Sit4 for Gln3-GFP (Fig. 9, G and H with Fig. 8, C and D).

One additional parenthetic result emanates from Figs. 6 and 7. Occasions arise when double-tagged strains are required, raising the question of whether the dual presence of Gat1-Myc13 and Gln3-GFP or Gat1-GFP influences the outcomes observed. To comprehensively assess this possibility, all of the experiments in Figs. 6–9 were performed both in strains that did or did not contain Gat1-Myc13. The presence of Gat1-Myc13 had no convincing effect on either wild type or mutant outcomes. This conclusion can be directly assessed by comparing Gln3-GFP and Gat1-GFP results observed in the wild type strains in Figs. 6 and 7.

Effects of Eliminating Pph21/22 and Sit4 Activities on Msx and Rapamycin-influenced Gln3-Myc13 and Gat1-Myc13 Binding to the GDH2 and DAL5 Promoters—To ascertain the Pph21/22 and Sit4 requirements for Msx- and rapamycin-induced GATA factor binding to the GDH2 and DAL5 promoters, we performed ChIP assays in wild type and mutant strains. Msx induced high levels Gln3-Myc13 binding to the GDH2 promoter that was substantially Sit4- and Pph21/22-independent (Fig. 10A). These observations correlated well with Gln3 localization as in all three instances there was substantial nuclear Gln3. Surprisingly, despite its reduced nuclear localization in the phosphatase mutants, rapamycin-induced Gln3-Myc13 binding to GDH2 was largely Sit4- and partially Pph21/22-independent (Fig. 10A).

Msx-induced Gat1-Myc13 binding was partially Sit4-independent, whereas binding elicited by rapamycin treatment was fully Sit4-independent (Fig. 10B). In the pph21Δpph22Δ, Gat1-Myc13 binding to GDH2, whether triggered by Msx or rapamycin, was minimally over background levels, demonstrating a
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Gln3-GFP - Wild Type

Minutes of Rapamycin Treatment

Percentage of Cells

Cytosolic, Nucleo-Cytoplasmic, Nuclear

Gln3-GFP - s(l4Δ)

Minutes of Rapamycin Treatment

Percentage of Cells

Cytosolic, Nucleo-Cytoplasmic, Nuclear

Gat1-GFP - Wild Type

Minutes of Rapamycin Treatment

Percentage of Cells

Cytosolic, Nucleo-Cytoplasmic, Nuclear

Gat1-GFP - s(l4Δ)

Minutes of Rapamycin Treatment

Percentage of Cells

Cytosolic, Nucleo-Cytoplasmic, Nuclear
DISCUSSION

The evidence presented above suggests that Msx and rapamycin inhibit distinct nitrogen-responsive regulatory pathways rather than sequential steps in a single linear cascade. Thus, the Msx-inhibited glutamine synthetase reaction is not situated at the top of the TorC1 pathway (Pathway 2, Fig. 1) as currently accepted (25), but is situated in a separate pathway (Location 1, in Fig. 1). This conclusion implies the existence of at least two distinct nitrogen-responsive inputs regulating Gln3 and Gat1. In addition to answering the question of one pathway or two, the data presented demonstrate that Gln3 and Gat1 each exhibit remarkably strong sensitivity to regulation by one of the two pathways relative to the other. These conclusions are based on GATA factor responses to Msx and rapamycin treatment measured at three different levels: GATA factor localization, binding to nitrogen-responsive promoters, and GATA factor-mediated transcription.

At the level of GATA factor localization in wild type cells, the responses were both GATA factor- and inhibitor-specific. Msx relocated Gln3 from the cytoplasm to the nuclei of essentially all ammonia-grown cells but minimally if at all affected Gat1 localization. In contrast, rapamycin relocalized Gat1 to the nuclei of essentially all cells but had only a moderate comparative effect on Gln3 localization, i.e. Gln3 was completely nuclear in less than half the cells.

Transcriptional responses as well as the GATA factor requirements for these responses were inhibitor-specific. In addition, the responses and GATA factor requirements were also gene-specific, reflecting the contribution of promoter structure to the overall end response. GDH2 transcription was strongly induced by Msx treatment in a highly Gln3-dependent and Gat1-independent manner. Rapamycin, on the other hand, was strikingly ineffective; no response whatever was observed. GAT1 transcription behaved similarly. DAL80 transcription was intermediate in the spectrum of responses. Rapamycin elicited a much greater response than did Msx, that response being partially Gln3-dependent. Not surprisingly given Gln3 participation, Msx also elicited moderate DAL80 transcription that was absolutely Gln3-dependent. At the other end of the spectrum, Msx and rapamycin elicited equal levels of DAL5 transcription that was largely dependent on both GATA factors. Thus at transcription, as with localization, Msx and rapamycin elicited responses correlated best with Gln3 and Gat1, respectively. Importantly, these results emphasize the inadequacy of using nitrogen catabolic gene expression as a reporter of nitrogen-responsive regulation in the absence of
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detailed information concerning the specific GATA factor requirements of that transcription under the growth conditions being used (54). If this detailed information is ignored, the conclusions obtained can easily be dictated by the gene assayed or growth conditions employed thus making broad generalizations risky.

GATA factor binding was perhaps the most difficult to straightforwardly correlate because not only did it display inhibitor and gene specificity, but one also sees the influence that binding of one GATA factor has on the other. The Gat1 requirement for Gln3 binding was gene- but not inhibitor-specific, whereas the Gln3 requirement for Gat1 binding was inhibitor- but not gene-specific.

Keeping in mind that overall GDH2 transcription relies on Gln3, that DAL80 is Gat1-dependent, and that DAL5 expression requires both Gln3 and Gat1, we can draw reasonable connections between the inhibitor added to the culture and the final outcome, gene transcription. For example, Msx treatment leads to nuclear Gln3 localization, which correlates well with increased Gln3 binding to the GDH2 promoter and transcription. In contrast, Gat1 is largely if not completely immune to Msx treatment, resulting in an absence of Gat1-dependent GDH2 expression. Rapamycin treatment, on the other hand, elicits low nuclear Gln3 localization correlating with low GDH2 binding and transcription. Rapamycin treatment also results in high nuclear Gat1 localization correlating with elevated Gat1 binding to the DAL5 promoter and with high, Gat1-dependent DAL5 and DAL80 transcription. Inhibitor-induced DAL5 activation requires both Gat1 and Gln3. Lower Gln3 binding suffices to support some transcriptional activation in the absence of Gat1. In contrast, even elevated Gat1 binding is not able to activate DAL5 to wild type levels in the absence of Gln3 (rapamycin-treated gln3Δ cells), suggesting that, in ammonia-grown cells, Gln3 is more “competent” than Gat1 to support DAL5 transcriptional activation when the GATA factors are assayed separately.

Although compelling positive correlations exist among the three different levels of inhibitor responses (localization, binding, expression), there are also negative correlations, especially with respect to GATA factor binding, some of which can be explained and others that at present cannot. Such negative correlations emphasize the limits that must be placed on our interpretations, conceding that much still remains to be learned about these regulatory pathways. For example, Gln3 binding to the DAL5 promoter in response to Msx and rapamycin treatment does not correlate with Gln3 localization but may be explained by the Gat1 requirement for Gln3 binding irrespective of the inhibitor used. Among the negative correlations that cannot be explained are the following three examples. (i) Rapamycin failed to elicit GDH2 transcription despite the fact that rapamycin elicited nuclear localization of both GATA factors as well as binding of the GATA factors in wild type, gln3Δ, and gat1Δ strains. At face value, this observation raises the speculative possibility that a rapamycin-inhibited event plays a necessary intranuclear role with respect to GDH2 transcription. (ii) Msx-elicited GDH2 transcription did not decrease in a gat1Δ mutant despite reduced Gln3-Myc13 binding to the promoter. (iii) Equivalent levels of Msx- and rapamycin-elicited DAL5 transcription occur despite much lower binding of both GATA factors in the Msx-treated cells. These yet unexplained negative correlations suggest that more than just GATA factor localization and binding account for the regulated transcriptional outputs.

Differing Msx- and Rapamycin-elicited Responses in the Phosphatase Mutants—Comparing the phosphatase requirements for the Msx and rapamycin responses provided an important insight into the question of whether one or two regulatory pathways are operative. If Msx and rapamycin inhibit sequential steps in a linear pathway, then the phosphatase requirements for the most downstream inhibitor, rapamycin, should be observed for both Msx and rapamycin. Msx-elicited responses might be expected to exhibit additional requirements not required for a rapamycin response, but the requirements for a rapamycin response should always be present whether cells are treated with rapamycin or Msx. The reasoning here is similar to that of the epistasis relationships expected of two loss of function mutations situated sequentially in a linear cascade. Only the Gln3 response can be evaluated in this manner because Gat1 localization does not respond sufficiently to Msx addition. Msx-elicited Gln3 localization is nearly Sit4-independent, whereas the rapamycin-elicited response is absolutely Sit4-dependent. In other words, the rapamycin and Msx responses exhibit opposite Sit4 requirements. Although more complex schemes can be envisioned, the Sit4 independence of Msx-elicited nuclear Gln3 localization is the expected result if Msx and rapamycin are inhibiting steps in two different regulatory pathways or a branched pathway. They are not consistent with the inhibitors acting on sequential steps of a single linear cascade. Taken together, the data presented in this work also demonstrate the GATA factor and inhibitor specificity of the phosphatase requirements.

At transcription, the phosphatase requirements were more uniform, PP2A (Pph21/22) being absolutely required irrespective of the gene and the inhibitor added, whereas the Sit4 requirement was only partial. The uniformity mainly reflects the intranuclear influence of one GATA factor effects on the binding of the other as noted below.

At promoter binding, the phosphatase requirements became gene- and inhibitor-specific, reflecting the interdependence of one GATA factor binding on the other. Msx- and rapamycin-induced Gln3 binding to the GDH2 promoter was partially PP2A- and Sit4-dependent, whereas binding to the DAL5 promoter exhibited absolute requirements of both phosphatases. Rapamycin- and Msx-elicited Gat1 binding to the GDH2 and DAL5 promoters exhibited a much greater PP2A than Sit4 requirement.

There were both positive and negative correlations with respect to phosphate requirements. In rapamycin-treated ammonia grown sit4Δ cells, low Gln3 binding to the DAL5 promoter correlated with minimal nuclear Gln3 localization. On the other hand, rapamycin elicited substantial nuclear Gat1 localization in the sit4Δ, which again correlated with binding to DAL5 and GDH2. Furthermore, rapamycin-induced DAL5 transcription was reduced in the sit4Δ, correlating with wild type Gat1 binding but low Gln3 binding. In a rapamycin-treated pph21Δpph22Δ, nuclear Gln3 and Gat1 localizations are
reduced, positively correlating with reduced binding at GDH2 and DAL5 and abolished transcription in DAL5.

Unexplained negative correlations, however, suggested that phosphatases could be responsible for regulated GATA factor functions beyond localization and DNA binding. For example, there is no Msx-elicited GDH2 transcription in a pph21 Δpph22Δ despite nearly wild type levels of Gln3 binding to the GDH2 promoter.

**Kinetics of GATA Factor Movement Are Inhibitor-specific** —The responses of GATA factor localizations to the inhibitors followed quite different time courses. In Msx-treated wild type, pph21 Δpph22Δ, and sitΔΔ cells, Gln3 relocated to the nucleus and remained there, although the onset of relocation was modestly and more dramatically delayed in the pph21 Δpph22Δ and sitΔΔ strains, respectively. In the case of rapamycin, transient responses were the norm. For Gln3, a transient response was more marked than in the wild type.

One other observation merits noting. This is the first time that Myc13 and GFP-tagged GATA factor localization could be compared in phosphatase mutants. With one significant exception, the results obtained with Myc13- and GFP-tagged GATA factors were consistent with one another when considering that it was possible to employ three category scoring of Gln3–Myc13 but only two category scoring of Gln3–GFP (comparison requires combining cytoplasmic and nuclear values of the Myc13 results). The exception occurred with the responses of Gat1–Myc13 and Gat1–GFP localizations in untreated, ammonia-grown sitΔΔ mutant cells and in Msx-treated pph21 Δpph22Δ cells (compare Fig. 3, C and D with Fig. 6, G and H, and Fig. 7, G and H). Using the Myc13 tag, Gat1 is nuclear-cytoplasmic in about 15–20% of the cells, whereas with the GFP tag it is absolutely cytoplasmic. Although the two assays differ both in the tag and the use of formalin fixation when the Myc13 tag is used (51), we cannot explain with confidence the differing results. It is, however, important to keep in mind that such differences can occur.

We end where we began, with the overall goal of the work, i.e. to decide whether rapamycin and Msx inhibited two steps in a linear cascade linking nitrogen availability with GATA factor responses or the inhibitors participated in separate regulatory pathways. The preponderance of data supports the conclusion that Msx and rapamycin are not inhibiting tandem steps of a linear pathway. The differences in the inhibitor responses and requirements at all levels are just too great. On the other hand, one cannot rigorously conclude that the pathways are totally separate unless we ignore the negative correlations.

If, as the data suggest, there are two regulatory pathways, it prompts the question of whether or not they are redundant. Gene expression data in Fig. 1 argue that the two GATA factors and two putative regulatory pathways are not merely redundant as clear differential effects of both the inhibitors and GATA factors (gln3Δ and gat1Δ) are observed. The question that present data will not answer is whether both regulatory systems are required for growth on a poorly utilized nitrogen source, e.g. proline, or a single functional pathway would suffice. Although a comprehensive explanation of GATA factor regulation will undoubtedly require integrating the responses to individual inputs and outputs of both pathways, specific GATA factor mutations eliminating responses to one or the other of the regulatory pathways, should effectively help to solve that issue. Furthermore, additional factors and regulatory pathway requirements involved in localization, DNA binding, and transcription will almost certainly emerge from such studies. It is toward this end that future work will be directed.

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