Alterations in the Ure2 αCap Domain Elicit Different GATA Factor Responses to Rapamycin Treatment and Nitrogen Limitation*

Received for publication, May 24, 2012, and in revised form, November 21, 2012. Published, JBC Papers in Press, November 26, 2012, DOI 10.1074/jbc.M112.385054

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* This work was supported, in whole or in part, by National Institutes of Health Grant GM-35642-23A1 (to J. J. T., R. R. A., and T. G. C.). This work was also supported by Commission Communautaire Française (COCOF) and the Fonds de la Recherche Fondamentale Collective (FRFC 2.4547.11) (to I. G. and E. D.).

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‡ The abbreviations used are: NCR, nitrogen-catabolite repression; Msx, methionine sulfoximine; Rap, rapamycin; Am, ammonia.

Ure2 is a phosphoprotein and central negative regulator of nitrogen-responsive Gln3/Gat1 localization and their ability to activate transcription. This negative regulation is achieved by the formation of Ure2-Gln3 and Gln3-Gat1 complexes that are thought to sequester these GATA factors in the cytoplasm of cells cultured in excess nitrogen. Ure2 itself is a dimer, the monomers of which consist of two core domains and a flexible protruding αcap. Here, we show that alterations in this αcap abolish rapamycin-elicited nuclear Gln3 and, to a more limited extent, Gat1 localization. In contrast, these alterations have little demonstrable effect on the Gln3 and Gat1 responses to nitrogen limitation. Using two-dimensional PAGE we resolved eight rather than the two previously reported Ure2 isoforms and demonstrated Ure2 dephosphorylation to be stimulus-specific, occurring after rapamycin treatment but only minimally if at all in nitrogen-limited cells. Alteration of the αcap significantly diminished the response of Ure2 dephosphorylation to the TorC1 inhibitor, rapamycin. Furthermore, in contrast to Gln3, rapamycin-elicited Ure2 dephosphorylation occurred independently of Sit4 and Pph21/22 (PP2A) as well as Sln14, Ptc1, and Ppz1. Together, our data suggest that distinct regions of Ure2 are associated with the receipt and/or implementation of signals calling for cessation of GATA factor sequestration in the cytoplasm. This in turn is more consistent with the existence of distinct pathways for TorC1- and nitrogen limitation-dependent control than it is with these stimuli representing sequential steps in a single regulatory pathway.

Ure2, a pre-prion protein in Saccharomyces cerevisiae, is a negative regulator of nitrogen catabolite repression (NCR)2.

Results:
Nitrogen catabolite repression-sensitive control of Gln3/Gat1 is normal in ure2 αcap mutants that no longer respond to TorC1 inhibitor, rapamycin.

Conclusion:
Different regions of Ure2 are associated with the Gln3/Gat1 response to TorC1 inhibition and nitrogen availability.

Significance:
Gln3, Gat1, and Ure2 respond to TorC1 and nitrogen availability via distinct regulatory pathways.

Background:
TorC1, excess nitrogen, and Ure2 negatively regulate Gln3 and Gat1.
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The second objective of the work was to use the mutants we generated to evaluate two views of GATA factor and Ure2 regulation. The second more recent view has developed from the work of multiple laboratories (26–45) and posits that Gln3 and Gat1 respond to two distinct regulatory pathways, one associated with rapamycin treatment and the other with nitrogen limitation or Msx addition (45). It is supported by the observations that nuclear Gln3 and Gat1 localization in response to limiting nitrogen (NCR) and rapamycin inhibition of TorC1 exhibit different Sit4 and PP2A phosphatase requirements (34, 38, 40, 41, 45).

Finally, we investigated a question that derives from the finding that, like Gln3, the electrophoretic mobility of Ure2 increases after rapamycin treatment, leading to the suggestion that Ure2 and Gln3 are regulated in parallel (17). Therefore, we analyzed the requirements of Ure2 dephosphorylation and conditions associated with its increased mobility and then compared them with those of Gln3.

We find that substitution of five serine/threonine residues at the end of the flexible Ure2 αcap (23, 24) abrogates rapamycin-elicted nuclear Gln3 localization observed in the absence of these changes but has little effect on NCR-sensitive regulation. We further demonstrate that rapamycin treatment alters gross Ure2 phosphorylation, whereas limiting nitrogen elicits only a modest effect. These differences are not expected if nitrogen limitation and rapamycin treatment represent sequential steps in a linear regulatory pathway. In contrast with Gln3, whose dephosphorylation in nitrogen replete medium is highly Sit4- and PP2A-dependent, alterations in the Ure2 phosphorylation profile are independent of these and other phosphatases. Altogether, our data demonstrate (i) αcap participation in Gln3 regulation, (ii) suggest that control of Ure2 phosphorylation does not parallel that of Gln3, and (iii) support a two regulatory pathway model for nitrogen-responsive Gln3 regulation more than they do a single linear pathway.

MATERIALS AND METHODS

Strains and Culture Conditions—The S. cerevisiae strains used in this work appear in Table 1. Note that two different strain backgrounds were used. The growth conditions were identical to those described earlier (37). Cultures were grown to mid-log phase (A_{600 nm} = 0.4 – 0.5) in YPD-rich medium or YNB minimal medium (without ammonium sulfate or amino acids) containing glutamine, ammonium sulfate, or proline at a final concentration of 0.1%. Appropriate supplements (120 μg/ml leucine and 20 μg/ml each of uracil, histidine, and tryptophan) were added to the medium as necessary to cover auxotrophic requirements. Where indicated, cells were treated with 200 ng/ml rapamycin (Rap) or 2 mM Msx (final concentrations) for the times indicated in the figure legends.
### TABLE 1

| Strain   | Pertinent genotype               | Parent | Complete genotype                                                                 | Reference |
|----------|----------------------------------|--------|-----------------------------------------------------------------------------------|-----------|
| 25T0b    | WT                               | FY     | MATa, ura3, his3, trp1                                                             | 60        |
| TB50     | MATa, leu2-3, rra3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [KanMX] |         |                                                                                   | 12        |
| TB123    | WT GLN3-MYC3                     | TB     | MATa, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [KanMX]                           | 12        |
| TB138–1a | ure2Δ GLN3-MYC3                  | TR     | MATa, ura3-112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [KanMX]                 | 12        |
| 03752b   | ppp22Δ                            | 25T0b  | MATa, ppp22-KanMX                                                                   | This work |
| 03823c   | ppp21Δpph22Δ                      | FB057 × 03752b | ura3, his3, trp1, ppp21-KanMX, pph22-kanMX                                              | 60        |
| FV077    | sitΔ                              | 25T0b  | MATa, ura3, his3, trp1, sIT14-KanMX                                                | This work |
| FV088    | sitΔ                              | FB063  | MATa, ura3-112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [HIS3], ura3-2:MATa     | 38        |
| FV237    | ure2Δ                             | TRB0   | MATa, ura3-3, ura3-52, trp1, his3, rme1, HMLa, ura3-2:MATa                           | This work |
| FV250    | WT GLN3-MYC3                     | TB     | MATa, ura3-112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [HIS3]                   | 42        |
| FV275    | ure2Δ GLN3-MYC3                  | FB063  | MATa, ura3-112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [HIS3], ura3-2:MATa     | This work |
| FV279    | ure2Δ GAT1-MYC3                  | FB063  | MATa, ura3-112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [HIS3], ura3-2:MATa     | This work |
| FV285    | sitΔ14 URE2-FA3                   | FV077  | MATa, ura3, his3, trp1, URE2-HA [TRP1], sitΔ14-KanMX                               | This work |
| FV286    | [TRP1], ppz1                      | FV077  | MATa, ura3, his3, trp1, URE2-HA [TRP1], ppz1-KanMX                                 | This work |
| FV287    | sitΔ URE2-FA3                     | FV077  | MATa, ura3, his3, trp1, URE2-HA [TRP1], sitΔ14-KanMX                               | This work |
| FV289    | sitΔ URE2-FA3                     | FV027  | MATa, ura3, his3, trp1, sIT14-KanMX                                                 | This work |
| FV290    | ppp21Δpph22Δ URE2-FA3             | 03823c | ura3, his3, trp1, ppp21-KanMX, pph22-KanMX, URE2-HA [TRP1]                         | This work |
| FV419    | WT URE2-HA3                       | FV237  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, ura3-2:MATa, pppp-LAF317        | This work |
| FV420    | ure2Δ [TTSS-AAAA]-HA3             | FV237  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, ura3-2:MATa, pppp-LAF324        | This work |
| FV421    | Ure2Δ [TTSS-DDDD]-HA3             | FV237  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, ura3-2:MATa, pppp-LAF325        | This work |
| FV422    | URE2-FA3 GLN3-MYC3                | FV275  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [HIS3], ura3-2:MATa | This work |
| FV423    | ure2Δ [TTSS-AAA]-HA3 GLN3-MYC3    | FV275  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [HIS3], ura3-2:MATa | This work |
| FV424    | Ure2Δ [TTSS-DDDD]-HA3 GLN3-MYC3   | FV275  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [HIS3], ura3-2:MATa | This work |
| FV425    | URE2-FA3 GAT1-MYC3                | FV279  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GAT1-MYC3 [HIS3], ura3-2:MATa | This work |
| FV426    | ure2Δ [TTSS-AAA]-HA3 GAT1-MYC3    | FV279  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GAT1-MYC3 [HIS3], ura3-2:MATa | This work |
| FV427    | ure2Δ [TTSS-DDDD]-HA3 GAT1-MYC3   | FV279  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GAT1-MYC3 [HIS3], ura3-2:MATa | This work |
| FV494    | ure2Δ [TRP1]-HA3                  | FV237  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, ura3-2:MATa, pppp-LAF318        | This work |
| FV495    | ure2Δ [TRP1]-HA3 GLN3-MYC3        | FV275  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [HIS3], ura3-2:MATa | This work |
| FV496    | ure2Δ [TRP1]-HA3 GAT1-MYC3        | FV279  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GAT1-MYC3 [HIS3], ura3-2:MATa | This work |
| FV497    | ure2Δ [TRP1]-HA3                  | FV237  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, ura3-2:MATa, pppp-LAF318        | This work |
| FV498    | ure2Δ [TRP1]-HA3 GLN3-MYC3        | FV275  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GLN3-MYC3 [HIS3], ura3-2:MATa | This work |
| FV499    | ure2Δ [TRP1]-HA3 GAT1-MYC3        | FV279  | MATa, leu2-3, 112, ura3-52, trp1, his3, rme1, HMLa, GAT1-MYC3 [HIS3], ura3-2:MATa | This work |

**Strain Construction**—Deletion strains involving insertion of KanMX or natMX cassettes were constructed as described earlier (34) using the long flanking homology strategy of Wach (47) with primers described by Georges et al. (38), Tate et al. (40), or with primers targeting PTC1 (5′, −419 to −393 and −18 to −1; 3′, 847 to 866 and 1165 to 1188), SIW14 (5′, −492 to −472 and −38 to −1; 3′, 847 to 887 and 1397 to 1417), or PPZ1 (5′, −489 to −469 and −36 to −1; 3′, 2079 to 2112 and 2445 to 2465). Chromosomal GLN3 and GAT1 were Myc3-tagged as described previously (34, 38). Chromosomal URE2 was tagged at the C terminus with three copies of the HA epitope (HA3), as described by Longtine et al. (48) using primers 5′-GATGAG-
AAGACCCGCGGTATACGAAGGATGCTTGTGAACGAGTTGCCCCTTTGTAATTTAA-3' and 5'-TTTCTTCTCCTTTTTTCTGTTTTTTTAAGCAGCCTGAATTC-

Mutant Ure2-HA3 Strain Construction—Plasmid pHAF317 was derived from the insertion of a PCR fragment, generated with Ure2-HA3-specific primers (Table 2) amplified on FV077 genomic DNA into the BamHI restriction site of integrative URA3 vector pFL34 (49). Plasmids pHAF318, pHAF323, pHAF324, and pHAF325 were generated from pHAF317 by QuikChange Lightning Multisite-directed Mutagenesis kit (Agilent) according to manufacturer’s protocol using the corresponding primers described in Table 2. Mutant Ure2-HA3 strain constructions were performed by polymerizing the plasmids described above using XhoI restriction enzyme followed by integration into the URE2 promoter of the ure2Δ locus in the indicated strains (Table 1).

Preparation of Immunoprecipitated Proteins for Two-dimensional Gel Electrophoresis—Total protein extracts were obtained from 50-ml cultures. Cells were harvested at an A660 nm = 0.5, washed with cold water, and resuspended in 300 μl of buffer U7 (7 M urea, 2 M thiourea, 2% CHAPS) containing 1% DTT, and complete protease inhibitor mixture tablets (Roche Applied Science) and phosphatase inhibitor mixture tablets (Roche Applied Science) according to the manufacturer’s protocols. Lysis was performed by vortexing with 425–600 ml of buffer U7. Lysis was performed by vortexing with 425–600 ml of buffer U7. (Roche Applied Science) and phosphatase inhibitor mixture tablets (Roche Applied Science) and phosphatase inhibitor mixture tablets (Roche Applied Science) according to the manufacturer’s protocols. Lysis was performed by vortexing with 425–600 ml of buffer U7. (Roche Applied Science) and phosphatase inhibitor mixture tablets (Roche Applied Science) and phosphatase inhibitor mixture tablets (Roche Applied Science) according to the manufacturer’s protocols. Lysis was performed by vortexing with 425–600 ml of buffer U7.

A total of 7000 V/h was reached. Strips were then equilibrated for 20 min in 5 ml of 75 mM Tris-HCl (pH 8.8), 6 mM urea, 30% glycerol, 2% SDS, 50 mg of DTT, and 0.002% bromphenol blue followed by an additional 20 min of incubation with the same solution lacking DTT and supplemented with 125 mg of iodoacetamide. Second-dimension electrophoresis was performed on an SDS 4–12% NuPAGE polyacrylamide resolving gel (Invitrogen). Proteins were detected by immunoblotting using anti-HA antibodies, and were spots visualized using a chemiluminescence camera (Chemi-Smart from Vilbert-Lourmat). Spot intensities were quantified using the Bio-1D algorithm.

Quantitative RT-PCR—RNA isolation and cDNA synthesis were conducted as described in Georis et al. (45) using primers that have been described previously (38, 45). The values reported represent the averages of at least two experiments from independent cultures; error bars indicate S.E.

Indirect Immunofluorescence Microscopy—Cell collection and fixation for indirect immunofluorescence microscopy were performed using successive modifications of the method of Schwartz et al. (50). In the current protocol cells were fixed at 30 °C for 80 min after the addition of 0.55 ml of 1 M potassium phosphate buffer (pH 6.5) and 0.5 ml of 37% formaldehyde to a 5-ml aliquot of the desired culture. After fixation, the samples were washed and resuspended in 0.1 M potassium phosphate buffer (pH 6.5) containing 1.2 M sorbitol. Zymolyase digestion of cells was performed as described in Tate et al. (51). Cells were then plated onto 0.1% poly-lysine coated slides (poly-L-lysine}

### Table 2: Primers used in this work

| Plasmids     | Relevant Ure2 Mutation | Starting plasmids | Codon replacement | Primers                                      |
|--------------|------------------------|-------------------|-------------------|----------------------------------------------|
| pHAF317      | WT                     | pFL34             |                   | GAAGATCTagggctttacaaagccgagtcac                       |
| pHAF318      | S283A                  | pHAF317           | S283A             | GAAGatctATATTACTGATCTGCTTACCGG                    |
| pHAF323      | S283D                  | pHAF317           | S283D             | ggaasatgcccctgcatacGTgctgtacacacacactatg           |
| pHAF324      | TTSS-AAAA              | pHAF317           | T286A/S287A/S290A/S292A | GGCGCTGATCATCTACGTGCTTACGATCTGCTGTTACCGG         |
| pHAF325      | TTSS-DDDD              | pHAF317           | T286D/S287D/S290D/S292D | GCCTTGCAATTTACGTGCTGATTGCTTTTTAAC-3'            |
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hydrobromide, Sigma) and incubated overnight at 4 °C in phosphate buffer (pH 7.5) containing 0.5% bovine serum albumin (Sigma) and 0.5% Tween 20. Antibody incubations and subsequent washes were performed using this phosphate buffer. Primary antibody labeling of Gln3-Myc13 was performed using 9E10 (c-myc) monoclonal antibody (Covance MMS-150P, at a dilution of 1:1000) followed by secondary labeling with Alexa Fluor 594 goat anti-mouse IgG antibody (Invitrogen Molecular Probes, dilution of 1:200). Antibody labeling for Gat1-Myc13 was performed as described above with the exception of a 1:4000 dilution for the 9E10 (c-myc) monoclonal antibody.

Primary images were collected at room temperature using a Zeiss Axioplan 2 microscope with a 100×/1.40 Plan-Apochromat oil objective, Zeiss Axio camera, and Zeiss Axiovision 4.8.1 software. Only primary .zvi images were used for scoring of intracellular localization of either Gln3-Myc13 or Gat1-Myc13. For the images presented for publication the .zvi files were converted to .tif files and processed using Adobe Photoshop and Illustrator programs. Gamma settings for the images presented in this publication were unaltered or minimally altered to retain clarity as observed in the microscope. These changes were applied uniformly for the images presented.

**Scoring Intracellular Localization of Gln3-Myc13 and Gat1-Myc13**—Intracellular scoring of both Gln3-Myc13 and Gat1-Myc13 were performed as described in Georis et al. (45). Three-category scoring, cytoplasmic (cytoplasmic fluorescence only), nuclear-cytoplasmic (cytoplasmic fluorescence and fluorescence co-localizing with DAPI positive material), nuclear (fluorescence colocalizing with DAPI positive material only), was employed as described (34, 41). Extensive examples showing Gln3-Myc13 situated in each of the above three categories appears in Fig. 2 of Ref. 34. The precision of the scoring procedures has also been extensively evaluated, and the results documented in Tate et al. (34, 40, 41).

**Western Blot Analyses**— Cultures were grown as indicated above and harvested by the filtration method of Tate et al. (51). Before inhibitor addition, the cultures were divided into two equal parts; the untreated portion was collected immediately, and the remainder was treated with rapamycin for 20 min before harvesting. Crude protein was extracted, and samples were processed for electrophoresis (SDS-PAGE, 6% polyacrylamide) using the combined methods of Tate et al. (52) and Liu et al. (53). Protein detection after transfer to nitrocellulose membranes was carried out as previously described (33), and results are recorded on Kodak BioMax XAR film. Multiple exposures were collected for each experiment. Gamma settings were changed uniformly for all of the published images to ensure that no bands were lost (gray background visible) and that the final images are represented as closely as possible the data visualized in the x-ray films.

**RESULTS**

Because the experiments described below depended on the analysis of ure2 mutants, we first constructed a HA3-tagged version of Ure2 (expressed from its natural genomic locus under the native URE2 promoter) as the parent strain. This permitted us to monitor its behavior via two-dimensional gel electrophoresis; antibodies against untagged Ure2 available to us were not sufficiently sensitive to be useful. Validation experiments were then performed that compared the abilities of the HA3-tagged and untagged versions of Ure2 to negatively regulate Gln3 and Gat1. Untagged Ure2 and Ure2-HA3 functioned indistinguishably to (i) sequester Gln3 and Gat1 in the cytoplasm of cells cultured with excess nitrogen (glutamine), (ii) permit their nuclear localization during nitrogen limitation (proline) and after rapamycin addition (Fig. 2, A–D), and (iii) support largely equivalent control of GDH2 and DAL5 transcription (Fig. 2, E and F). Furthermore, rapamycin addition increased Ure2-HA3 mobility as previously reported for untagged Ure2 (Fig. 2G) (14, 17). In light of these validation data, two Ure2-HA3 substitution mutants were prepared and analyzed.

While editing the manuscript describing these analyses, we performed an unplanned, additional experiment for the sake of completeness and much to our surprise discovered that untagged and HA3-tagged Ure2 did not yield identical Gln3-Myc13 gross phosphorylation profiles in SDS gels (Fig. 2H). Adding rapamycin to cells containing Ure2-HA3 increased Gln3-Myc13 mobility just as in cells with untagged Ure2 (compare Fig. 2H, lanes 1 and 2, with lanes 4 and 3). However, the entire Gln3-Myc13 profile was shifted up in the gel such that Gln3-Myc13 in untreated Ure2-HA3 cells migrated similarly to Gln3-Myc13 in an ure2Δ (Fig. 2H, lanes 3 and 5). Consequently, even though Ure2-HA3 behaved the same as untagged wild type Ure2 in all but one of assays described above, it had to be considered a mutant protein. This raised two critical questions. (i) Would data obtained with Ure2-HA3 substitution mutants effectively assess whether the Ure2 ocap participated in Gln3 regulation and unambiguously distinguish the one versus two pathway alternatives we queried? (ii) What limitations had to be imposed to prudently interpret the data obtained? For our experiments, the nature of alterations in the Ure2 molecule was not paramountly important, because the Ure2-HA3 molecule was a constant in each test; rapamycin addition and nitrogen limitation or the presence or absence of substitutions in the external surface of the ocap were the variables. Therefore, prudent limitations were the same ones associated with any protein containing multiple alterations. From this reasoning we concluded that Ure2-HA3 and substitution mutants of this parent could be used effectively to answer the questions we posed.

**Selection of the ocaps Residues To Be Substituted**—The main objectives of the work argued that the greatest amount of information would be gained if we selected residues at the tip of the Ure2 ocap because this was the portion of the protein most likely to interact with any partner molecules. Second, because one of the Ure2 characteristics we planned to investigate was its phosphorylation/dephosphorylation, serine and threonine residues would be preferable targets. To this end we employed the NetPhosYeast 1.0 prediction server to query which Ure2 residues were the most likely targets for phosphorylation (54). Fortunately, five serine/threonine residues in the ocaps fulfilled both criteria: Ure2 residues Ser-283, Thr-286, Thr-287, Ser-290 and Ser-292. These are the yellow residues in Fig. 1, B and C. Views from opposite sides of the Ure2 molecule are shown so that one can more precisely see where all of the substitutions are located. Using this information, we constructed four mutant strains. The first two mutants were
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alanine or aspartate substitutions of four clustered residues Ure2T286A,T287A,S290A,S292A and Ure2T286D,T287D,S290D,S292D (referred to as Ure2TTSSAAA-HA or Ure2TTSSDDDD-HA). The last two mutants were alanine or aspartate substitutions of a single serine residue (Ure2S283A or Ure2S283D).

Gln3-Myc13 and Gat1-Myc13 Localization Respond Oppositely to Rapamycin and Limiting Nitrogen in Ure2-HA3 Ser/Thr Substitutions Mutants—We first analyzed Gln3-Myc13 and Gat1-Myc13 localization in Ure2-HA3, and the acap Ure2TTSSDDD and Ure2TTSSAAAA substitution mutants asking whether they affected GATA factor localization. Gln3-Myc13 localization remained unchanged, exclusively (100%) sequestered in the cytoplasm of the rapamycin-treated Ure2TTSSDDD mutant (Fig. 3, C and D), and it was nearly as unresponsive (~80%) in the Ure2TTSSAAAA mutant (Fig. 3, E and F). In sharp contrast, Gln3-Myc13 localization in ammonia-grown Ure2TTSSAAA and Ure2TTSSDDD cells with or without Msx or in proline-grown cells were similar to those seen in the unaltered Ure2-HA3 strain (Fig. 3, C–F, compared with Fig. 3, A and B). Gat1-Myc13 localization responded similarly, but less strongly, in both rapamycin-treated and ure2 mutants, i.e. there was a moderate cytoplasmic shift relative to wild type (Fig. 4). The modest response of Gat1 may not be too surprising given that Gat1 localization is less responsive than that of Gln3 to NCR and negative regulation by Ure2 (38). So far, the reasons for differences between Gln3 and Gat1 sensitivities to NCR and Ure2-mediated regulation is not known but has been characterized in some detail (41, 45).

The single Ure2S283D substitution exhibited a phenotype much like that of Ure2TTSSDDD cells, i.e. the Gln3-Myc13

![FIGURE 2. Validation of the Ure2-HA3 strain relative to the untagged, wild type with respect GATA factor localization, NCR-sensitive transcription, and Ure2 phosphorylation. Panel A and B, Gln3-Myc13 localization is shown. Wild type Gln3-Myc13 (TB123), Gln3-Myc13 Ure2-HA3 (FV422), and Gln3-Myc13 ure2Δ (TB136–1a) cells were cultured in YNB glutamine or proline (Pro) medium in the presence (+ Rap, 20 min) or absence (Gln) of rapamycin. Samples were collected for indirect immunofluorescence microscopy (images presented in panel A) as described under "Materials and Methods" and Gln3-Myc13 localization scored in three categories: cytoplasmic (completely cytoplasmic fluorescence only), nuclear-cytoplasmic (fluorescence in the cytoplasm as well as co-localizing with DAPI-positive material), or nuclear (co-localization only with DAPI-positive material) (histograms above each panel). See "Materials and Methods" for criteria and precision of scoring. Panels C and D, Gat1-Myc13 localization is shown. Wild type Gat1-Myc13 (FV303), Gat1-Myc13 Ure2-HA3 (FV425), and Gat1-Myc13 ure2Δ (FV088) cells were prepared and analyzed as in panels A and B. Panels E and F, DALS and GDH2 expression is shown. Total RNA was isolated from cells grown in YNB-glutamine with or without rapamycin treatment (Gln + Rap, Gln, respectively), or YNB-ammonia with or without 20 min rapamycin (Am. + Rap, Am., respectively) or methionine sulfoximine (Am. + Mxs) treatment. DALS (panel E) or GDH2 (panel F) expression was measured in wild type (TB50), Ure2-HA3 (FV419), and ure2Δ (FV237) cells. mRNA levels were quantified by quantitative RT-PCR as described under "Materials and Methods." Values were normalized with TBP1. Panel G, shown is Ure2-HA3 mobility in response to rapamycin. A wild type Ure2-HA3 (FV419) was grown to mid-log phase in YPD (lanes 1 and 2) or YNB glutamine (Gln; lanes 3 and 4) medium at 30 °C. One-half of each culture was immediately collected for Western blot analysis (see "Materials and Methods"), and the remainder was collected after treatment with rapamycin for 20 min (+ Rap). The absence (−) or presence (+) of rapamycin is indicated above each lane. Mobility of Ure2-HA3, prepared from crude protein extracts, was assessed for each strain and condition. Panel H, Gln3-Myc13 mobility is shown in response to rapamycin. Wild type (TB123), wild type Ure2-HA3 (FV422), and Ure2-HA3 ure2Δ (TB136–1a) strains were grown in YNB glutamine medium at 30 °C and processed as in panel G. The absence (−) or presence (+) of rapamycin is indicated above each lane. Strains used are indicated above each panel, and each lane has been numbered.
response to rapamycin treatment was lost (Fig. 5, C and D). In contrast, Gln3-Myc\(^{13}\) largely relocated to the nuclei of proline-grown cells, indicating a wild type response to nitrogen limitation. On the other hand, the Ure2S283A substitution responded differently in that its phenotype was largely wild type (Fig. 5, E and F). The muted phenotypes of the Ure2S283 substitutions compared with those of Ure2TTSS carried through to Gat1-Myc\(^{13}\) localization with only a small loss of its rapamycin responsiveness (Fig. 6). However, the limited decrease in nuclear Gat1-Myc\(^{13}\) localization was observed in both Ure2S283D and Ure2S283A substitutions (Fig. 6, C–F). As with Gln3-Myc\(^{13}\), Gat1-Myc\(^{13}\) localization was minimally affected by nitrogen limitation.

Together, the localization data demonstrated the necessity of an unaltered Ure2 αcap to respond normally to rapamycin treatment. In contrast, the response of GATA factor localization to nitrogen limitation was immune to alteration of the αcap. Because mutations in the Ure2 αcap impact differently on rapamycin and nitrogen limitation response, we concluded that these conditions exerted their effects on Ure2 through two distinct pathways.

**Rapamycin Retains Its Ability to Elicit Gln3-Myc\(^{13}\) Dephosphorylation in Ure2-HA\(^{3}\) TTSS Mutants**—Because rapamycin treatment was unable to relocate Gln3-Myc\(^{13}\) from the cytoplasm to the nuclei of glutamine-grown cells in Ure2\(_{TTSS}^{AAAA}\)-HA\(^{3}\) and Ure2\(_{TTSS}^{DDDD}\)-HA\(^{3}\) mutants, we queried whether its ability to dephosphorylate Gln3-Myc\(^{13}\) had also been lost in parallel. To this end, we analyzed the phosphorylation state of Gln3-Myc\(^{13}\) in Ure2-HA\(^{3}\), Ure2\(_{TTSS}^{AAAA}\)-HA\(^{3}\) and Ure2\(_{TTSS}^{DDDD}\)-HA\(^{3}\) mutants. We analyzed whether its ability to dephosphorylate Gln3-Myc\(^{13}\) had also been lost in parallel. To this end, we analyzed the phosphorylation state of Gln3-Myc\(^{13}\) in Ure2-HA\(^{3}\), Ure2\(_{TTSS}^{AAAA}\)-HA\(^{3}\) and Ure2\(_{TTSS}^{DDDD}\)-HA\(^{3}\) mutants. In both mutants, rapamycin continued to elicit gross Gln3-Myc\(^{13}\) dephosphorylation (Fig. 7, A and B). In the case of the Ure2\(_{TTSS}^{DDDD}\) mutant, the response was indistinguishable from wild type Ure2-HA\(^{3}\) (Fig. 7B). The Ure2\(_{TTSS}^{AAAA}\) mutant also responded like Ure2-HA\(^{3}\) except that rapamycin treatment elicited an additional Gln3-Myc\(^{13}\) species with a higher mobility than those observed in similarly treated wild type cells (Fig. 7A, lane 3, black dot between lanes 2 and 3). Therefore, rapamycin-elicited gross
Gln3-Myc<sup>13</sup> dephosphorylation and Gln3-Myc<sup>13</sup> localization did not correlate with one another as observed earlier in PP2A mutants (40).

**Effects of Serine/Threonine Residue Substitutions on GATA Factor-mediated Transcription**—Next we assayed the ultimate goal of Gln3 and Gat1 localization, the regulation of their abilities to activate NCR-sensitive transcription. To this end we assayed DAL5 and GDH2 expression in wild type, Ure2<sub>TTSS</sub>, and Ure2<sub>S283</sub> mutants. In our strain background (TB), GDH2 gene expression is Gln3-dependent, Gat1-independent, strongly responsive to Msx addition, more moderately responsive to growth in proline, and fails to respond to rapamycin treatment, whereas DAL5 expression requires both GATA factors and does respond to rapamycin (45).

The most striking effect of both Ure2<sub>TTSS→AAAA</sub> and Ure2<sub>TTSS→DDDD</sub> mutations was that in both glutamine- and ammonia-grown cells, DAL5 expression exhibited a much reduced rapamycin response (Fig. 8A) compared with Ure2-HA<sup>3</sup> cells. Furthermore, the Ure2<sub>TTSS→AAAA</sub> and Ure2<sub>TTSS→DDDD</sub> substitutions had little effect on DAL5 expression in Msx-treated cells and in proline-grown cells. Like DAL5, GDH2 expression in the Ure2<sub>TTSS</sub> mutants was largely unaffected (Fig. 8B). These observations positively correlated with GATA factor localization. Inexplicably, however, DAL5 and GDH2 levels modestly increased in ammonia-grown Ure2<sub>TTSS→DDDD</sub> cells despite the fact that both GATA activators were cytosolic (Figs. 4, C and D, and 5, C and D).

DAL5 and GDH2 expression was also characterized in Ure2<sub>S283</sub> mutants (Fig. 8, A and B). Msx-elicited DAL5 expression increased 2–3-fold in ammonia-grown Ure2<sub>S283A</sub> but not in the Ure2<sub>S283D</sub> mutant. DAL5 expression was also reduced in rapamycin-treated, glutamine-grown Ure2<sub>S283D</sub> cells, correlating with the loss of nuclear Gln3 localization under this condition. GDH2 expression, on the other hand, was derepressed in proline-, ammonia-grown, and Msx-treated, ammonia-grown Ure2<sub>S283</sub> cells. The similarities between A and D substitution mutants strongly suggested that the phenotypes observed resulted from structural changes rather than an alteration of a phosphorylated site whose modification was critical for Ure2 regulation.

*Ure2-HA<sup>3</sup> Exists as at Least Eight Different Species, Some of Which Are Phosphorylated*—To follow potential phosphorylation changes resulting from the Ure2-HA<sup>3</sup> substitution
mutants, we wanted an assay with greater resolution than could be achieved with standard one-dimensional Western blots (Fig. 2G). Attempts to use mass spectral analyses were abandoned due to the unfortunate location of the protease sites in Ure2, the limited Ure2 success achieved in others’ reported mass spectral analyses, and our inability to obtain significant results using that approach. The assay we developed was two-dimensional gel electrophoresis of immunoprecipitated Ure2-HA3 and Western blot analyses. In YPD-grown wild type cells this method yielded eight species of Ure2-HA3 with isoelectric points between pH 5 and 6 (Fig. 9A, left panel) compared with the two species normally observed using one-dimensional Western analyses of wild type cell extracts (Fig. 2G) (14, 15, 17). A corresponding histogram quantifies the intensity of each species’ signal normalized to the sum of intensities of all of the species in that blot (Fig. 9A, right panel).

Treating wild type extracts with FastAP phosphatase markedly shifted the profile to more basic species (Fig. 9B). However, more than one isoform remained after phosphatase treatment, suggesting that these isoforms are not phosphoforms or that some phosphorylated residues are not accessible to the phosphatase. This basic shift was completely eliminated if a phosphatase inhibitor mixture was added along with the phosphatase (Fig. 9C). Adding rapamycin to cells before extraction yielded the same Ure2 isoelectric point profile as that seen after treating the extract with phosphatase (Fig. 9D). The fact that Ure2-HA3 profiles in Fig. 9, D and F, were nearly identical to the one observed with the phosphatase-treated wild type extract (Fig. 9B) suggested rapamycin treatment resulted in Ure2-HA3 dephosphorylation in agreement with previous reports (14, 15, 17). It is significant that phosphatase treatment did not alter the Ure2 isoelectric point profile beyond that elicited by rapamycin treatment alone (Fig. 9E); i.e. the effects were not additive. These data indicated that some but not all Ure2-HA3 isoforms were phosphorylated and that rapamycin treatment affected the phosphorylated species. It was not possible from these data, however, to identify which of the Ure2 isoforms were phosphorylated.

Substitutions of Ser/Thr Residues Affect the Ure2-HA3 Isoelectric Point Profiles—To determine whether the residues targeted in our mutants affected Ure2 phosphorylation, we assayed the isoelectric point profiles of the mutant proteins. Alanine and aspartate substitutions yielded strikingly consistent results. The
isoelectric point profiles of the Ure2TTSS3AAAA mutant exhibited a strong basic shift compared with that of untreated wild type cells as expected if phosphorylation was prevented (Fig. 10, A and C). In contrast, substitution of phosphomimetic aspartate for the serine/threonine residues elicited an equally strong acidic shift in the Ure2 isoelectric point profile (Fig. 10, A and E). Adding rapamycin to the alanine substitution mutant caused a slight additional basic shift compared with untreated cultures (Fig. 10, C and D), i.e. the Ure2 alanine substitution mutant protein remained responsive to rapamycin but just barely so.

The single Ure2S283A substitution produced a basic shift in the isoelectric point profile weaker than the one observed in the Ure2TTSS3AAAA mutant (Fig. 10, G and H). The acidic shift observed with the Ure2S283D substitution was less strong than that seen with the Ure2TTSS3DDDD mutant (Fig. 10, I and J). In both mutants rapamycin treatment elicited a strong basic shift that was significantly greater than occurred with the Ure2TTSS mutants (compare Fig. 10, G–J, with Fig. 10, C–F).

In sum, the data suggested that rapamycin caused a basic shift in the isoelectric point profiles of all four Ure2 alanine substitution mutants. However, as with Gln3, the effects of rapamycin treatment were not reflected in a corresponding effect on Gln3-Myc13 and Gat1-Myc13 localization (Figs. 3–6).
Effect of Nitrogen Supply on the Isoelectric Point Distribution of Ure2-HA3 Species—We next determined whether the cell nitrogen supply resulted in changes to Ure2-HA3 isoelectric profiles that correlated with those brought about by rapamycin. The largest number of Ure2-HA3 species occurred in repressive YPD, YNB-glutamine, and YNB-ammonia media (Fig. 11, A, C, and E). Here, rapamycin addition also led to dramatic basic shifts in the isoelectric profiles (Fig. 11, B and D). In contrast, only modest basic shifts occurred in extracts from ammonia-grown, Msx-treated, and proline-grown cells (Figs. 11, F and G) even though these derepressive conditions elicited much greater nuclear Gln3-Myc13 localization than rapamycin (Fig. 8).
Myc13 phosphorylation profiles observed in cells (TB123 genetic background) (40). (ii) Gat1 exhibits partial phosphatases. (i) Sit4 and PP2A are absolutely required for a phosphorylation levels are highly dependent on multiple protein requirements, i.e. single deletions exhibit strong phenotypes. In more diverse genetic backgrounds, up to five phosphatases have been reported to participate in the regulation of Gln3 and/or Gat1 localization: Sit4, PP2A, Siw14, Ptc1, and Ppz1 (13, 16, 34, 36, 38, 40-42, 55-58). Therefore, we investigated the phosphatase requirements for rapamycin-elicited changes in the charge distribution of Ure2-HA3 species. Existing models predict them to parallel those of Gln3 (14-17). To this end extracts were prepared from untreated and rapamycin-treated, YPD-grown wild type, sit4Δ, pph21Δpph22Δ, ptc1Δ, siw14Δ, and ppz1Δ cells. Quite surprisingly, the strong basic shift in the Ure2-HA3 isoelectric point profiles of rapamycin-treated cells were very similar in the wild type and all of the phosphatase mutant strains (Fig. 12, A-L). This is not the expected result if, as occurs with Gln3, one or more of the above phosphatases were central to Ure2 dephosphorylation associated with Gln3 regulation. However, it would be expected if, unlike the case with Gln3 and Gat1, any of the phosphatases were functionally redundant with respect to Ure2.

**DISCUSSION**

Importance of the Flexible ocap to Ure2 Function—Ure2 has long been known to negatively regulate nuclear Gln3 and Gat1 localization and their ability to activate transcription. Additionally, it is generally felt that cytoplasmic GATA factor sequestration is achieved through Gln3- and presumably Gat1-Ure2 complexes (11-19). However, beyond the early work of Kulkarni et al. (18), Wickner and co-workers (20, 59), and Carvalho and Zheng (19), the particular regions of Ure2 associated with abrogation of its nitrogen regulatory functions have not been addressed. Here, we demonstrate the importance of the flexible Ure2 ocap (Ure2TTSS) in the response of Gln3 and Gat1 localization/function to rapamycin addition but not nitrogen limitation. Substitution of a single serine (Ure2S283A/D) or four clustered serine/threonine (Ure2Thr-286, Thr-287, Ser-290, Ser-292) residues in the ocap eliminated rapamycin-elicited nuclear Gln3-Myc13 and to a lesser extent, Gat1-Myc13 localization, whereas nuclear localization that occurred when cells were cultured in poor nitrogen sources or after Msx addition remained unaffected. Correlating with these localization results were parallel observations that NCR-sensitive gene expression was diminished in rapamycin-treated Ure2TSS mutant cells, whereas the response to nitrogen limitation was largely unaffected. The Ure2TSS substitutions clearly distinguished the responses of Gln3 and Gat1 to rapamycin versus growth with limiting nitrogen (proline or ammonia +Msx), suggesting that genetically separable, response-specific regions of Ure2 are associated with the relaxation of its negative Gln3 and Gat1 regulation. As such, they also support our recent proposals that GATA factor regulation is likely imposed through distinct TorC1- and NCR-dependent mechanisms.

These conclusions are, however, subject to several caveats. First, it is unlikely that the losses of normal Ure2 function and regulation observed in our ure2 mutants derive from specific phosphorylation or dephosphorylation of the altered residues because alanine and aspartate substitutions generated the same phenotypes. If phosphorylation/dephosphorylation of these particular residues were the determinants of Ure2 function we would have expected constitutively nuclear cells for one mutant and constitutively cytoplasmic ones for the other. Second, it is pertinent that we have no way of knowing whether serine/threonine substitutions in the ocap destroy or only cripple its nor-
mal operation. This would be an important consideration if the responses to rapamycin versus limiting nitrogen differed quantitatively rather than qualitatively. In such a situation the data would argue that the rapamycin response is more sensitive to the alterations than is the response to nitrogen limitation. This, however, does not greatly affect the test of the one versus two regulatory hypothesis because if both stimuli are situated as sequential steps in a single pathway, they should have generated only one outcome, that of rapamycin addition, because it is viewed as the step in the pathway most proximal to GATA factor localization.

Present data also illuminate another important question, i.e. does Ure2 function only with respect to NCR or does it possess a specific function in TorC1-dependent regulation of GATA factor localization as well? It is difficult to convincingly interpret previous data related to this question because only ure2 loss-of-function mutations were employed (11–19). Therefore, although these early experiments demonstrated a clear Ure2 requirement for cytoplasmic retention of the GATA factors, they were unable to distinguish whether nuclear Gln3-Myc\(^{13}\) localization and increased transcription of NCR-sensitive genes observed in an ure2\(^{-}\) mutant derived from the loss of NCR regulation, TorC1-dependent control, or both. In the above experiments, URE2 substitution mutations were able to distinguish regulation imposed by the two pathways. Because Ure2 function in NCR-mediated regulation of Gln3-Myc\(^{13}\) localization remained intact in the ure2\(_{\text{TTSS}}\) substitution mutants, loss of rapamycin-elicited nuclear Gln3-Myc\(^{13}\) localization observed in them must have derived from a defect in the TorC1-dependent regulatory pathway. Therefore, by exclusion, this reasoning favors a Ure2 role in both pathways regulating Gln3 localization.

The Complexity of Ure2-HA\(^{3}\) Phosphorylation—Our results demonstrate Ure2 isoforms are significantly more complex than previously appreciated from data obtained using one-dimensional electrophoresis (14–17). This negative regulator exists as at least eight species with different isoelectric points. The Ure2-HA\(^{3}\) isoelectric point profile dramatically changed after in vivo rapamycin treatment or in vitro phosphatase treatment of cell extracts. By far the greatest changes occurred in wild type, YPD, or glutamine-grown cells. Moreover, no change in isoelectric profile was detected after phosphatase treatment of rapamycin-treated cell extracts. These observations indicate that at minimum some of the Ure2-HA\(^{3}\) species are rapamycin-sensitive phosphoforms. However, because the entire profile shifts after rapamycin treatment, it was not possible to identify particular species that are phosphoforms.

Ure2-HA\(^{3}\) and Gln3-Myc\(^{13}\) Phosphorylation and Its Responses to Nitrogen Availability—Given the dramatic rapamycin-elicited Ure2 dephosphorylation in glutamine- or YPD-grown cells, similarly strong effects would reasonably have been expected in response to nitrogen limitation. Surprisingly, however, rapamycin inhibition of TorC1 activity and limiting nitrogen do not generate parallel responses in Ure2-HA\(^{3}\) dephosphorylation. The differences between isoelectric point profiles observed with excess (glutamine) versus limiting nitrogen (proline) were minimal. Together, these results roughly parallel those reported earlier for nitrogen limitation-dependent Gln3-
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Myc\(^{13}\) phosphorylation, which also decreases after rapamycin treatment but remains the same or in some cases increases in YNB-proline medium (33, 52). On the other hand, Ure2-HA\(^{3}\) and Gln3-Myc\(^{13}\) phosphorylations markedly differed after Msx addition; the former remained unchanged, whereas the latter increased (52).

Ineffectiveness of TorC1 Pathway-associated Phosphatases on Ure2-HA\(^{3}\) Phosphorylation—Some but not all early views of Ure2 regulation suggested that specific Ure2 phosphorylation played a central role linking nitrogen availability via TorC1, Tap42, and Sit4 activities to the regulation of Gln3 and Gat1 localization and function (12, 13, 15, 16, 19, 17). That model generates the expectation that Ure2-HA\(^{3}\) isoelectric point profiles would be detectably altered by the loss of one or more of the TorC1 pathway-associated phosphatases, Sit4, PP2A, Ptc1, Siw14, or Ppz1. Indeed, Sit4 and PP2A have been shown to be strongly required for rapamycin-elicited Gln3-Myc\(^{13}\) dephosphorylation in nitrogen-replete medium. This expectation, however, was unfulfilled in our studies of Ure2, which is consistent with the observation of Shamiji et al. (17) that rapamycin-induced dephosphorylation of Ure2 occurred normally in a tap42–11 mutant strain. Although these observations substantially argue that Ure2 regulation occurs by a different mechanism independent of these phosphatases, it is important to keep three caveats in mind. (i) The lack of demonstrable phenotypes for the phosphatase deletion mutants could derive from one or more of them having overlapping functions thereby preventing observable phenotypes in single mutants. (ii) The particular determinative phosphorylation/dephosphorylation events were undetected in our experiments. (iii) The observed results could, alternatively, derive from strain-specific differences for which clear precedents exist. For example, Sit4 is required for nuclear Gln3-Myc\(^{13}\) localization in proline-grown, TB123-related strains but not in BY- or FY-derived strains used to construct the genome-wide deletion series (17). Similarly, Siw14 has been reported to be required for Gln3 regulation in strains derived by BY4742 (56). In contrast, we were unable to demonstrate loss of Gln3 or Gat1 control in siw14 mutants in TB123-related strains.\(^{3}\) What is clear from our experiments is that the requirements for individual phosphatases (Sit4 and PP2A) associated with nuclear Gln3 and Gat1 localization after rapamycin addition are not equivalently observed with Ure2 dephosphorylation under these conditions. Thus, it appears likely that although both Ure2 and Gln3 dephosphorylation are triggered by rapamycin addition, the detailed mechanisms involved may be different.

Acknowledgments—We thank Dr. Rajendra Rai for performing several control experiments and suggestions to improve the manuscript, Dr. Helian Boucherie who initiated the Ure2 phosphorylation analysis by two-dimensional gel experiments, and Fabienne Veriendeels for excellent technical assistance.

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