Methionine Sulfoximine Treatment and Carbon Starvation Elicit Snf1-independent Phosphorylation of the Transcription Activator Gln3 in Saccharomyces cerevisiae*

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Tor proteins are global regulators situated at the top of a signal transduction pathway conserved from yeast to humans. Specific inhibition of the two Saccharomyces cerevisiae Tor proteins by rapamycin alters many cellular processes and the expression of hundreds of genes. Among the regulated genes are those whose expression is activated by the GATA family transcription activator, Gln3. The extent of Gln3 phosphorylation has been thought to determine its intracellular localization, with phosphorylated and dephosphorylated forms accumulating in the cytoplasm and nucleus, respectively. Data presented here demonstrate that rapamycin and the glutamine synthetase inhibitor, methionine sulfoximine (MSX), although eliciting the same outcomes with respect to Gln3-Myc13 nuclear accumulation and nitrogen catabolite repression-sensitive transcription, generate diametrically opposite effects on Gln3-Myc13 phosphorylation. MSX increases Gln3-Myc13 phosphorylation and rapamycin decreases it. Gln3-Myc13 phosphorylation levels are regulated by at least three mechanisms as follows: (i) depends on Snf1 kinase as observed during carbon starvation, (ii) is Snf1-independent as observed during both carbon starvation and MSX treatment, and (iii) is rapamycin-induced dephosphorylation. MSX and rapamycin act additively on Gln3-Myc13 phosphorylation, but MSX clearly predominates. These results suggest that MSX- and rapamycin-inhibited proteins are more likely to function in separate regulatory pathways than they are to function tandemly in a single pathway as thought previously. Furthermore, as we and others have detected thus far, Gln3 phosphorylation/dephosphorylation is not a demonstrably required step in achieving Gln3 nuclear localization and nitrogen catabolite repression-sensitive transcription in response to MSX or rapamycin treatment.

The potential utility of rapamycin and its derivatives as drugs that diminish tissue rejection in transplant patients and cell division in some forms of cancer, particularly those associated with defects in the pTEN tumor suppressor gene, have stimulated interest and research into the mechanisms through which the target of the drug, the Tor protein, regulates cellular processes (1–4). Moreover, studies in Saccharomyces cerevisiae are contributing significantly to our understanding of the Tor signal transduction pathway in eukaryotic cells. These studies have identified many of the downstream targets of Tor1/2 control as well as participants in the yeast signal transduction pathway itself, including peptidylprolyl isomerase/rotomase, Fpr1 (5, 6), protein kinase Tap42 (7–14, 46), Tip41 (11, 15), type 2A protein phosphatases, Pph21/22 (8, 14), Sit4 (7, 8, 14, 16–18), Pph3 (16), Sit4-associated proteins or SAPS (11, 17, 19, 20), Lst8, which participates in protein trafficking (21–23), and proteins found in complexes with Tor1/2, Kog1, Avo1–3, Tec89, and Bit6 (21, 22).

The transcription factor often used as a downstream reporter of the Tor1/2 signal transduction pathway in S. cerevisiae is Gln3, the transcription activator responsible for nitrogen catabolite repression (NCR)-sensitive expression of the nitrogen catabolic genes (24–27). It is through NCR-sensitive Gln3 regulation that S. cerevisiae selectively utilizes good nitrogen sources in preference to poor ones. When cells are provided with good nitrogen sources, such as glutamine or (in some strains) ammonia, Gln3 is localized in the cytoplasm, and NCR-sensitive transcription is minimal. In contrast, when poor nitrogen sources, such as proline, are available, Gln3 is nuclear and NCR-sensitive transcription is high (for reviews of the physiology and regulation see Refs. 24–27). The correlations that Gln3 is nuclear with a poor nitrogen source (28) or in rich medium containing rapamycin (9, 16) were consistent with the suggestion that Tor1/2 transmit the signal of nitrogen excess to Gln3. The observations that rapamycin induces Gln3 dephosphorylation and that this dephosphorylation depends on Sit4, a type-2A serine/threonine phosphatase regulated by Tor1/2, Tap42, and Tip41, further connected Gln3 intracellular localization to the upper portion of the Tor1/2 pathway (see Refs. 9, 11, 16, 29, and 30, and see Ref. 31 for a large group of reviews on TOR and rapamycin). Recently, treating cells with the glutamine synthetase inhibitor L-methionine sulfoximine (MSX) has been reported to increase Gln3 dephosphorylation, nuclear accumulation, and Gln3-activated transcription (32). MSX data also led to the conclusion that the nitrogen nutrient sensed by the Tor pathway is glutamine or a glutamine metabolite (32). Overall, rapamycin treatment, nitrogen limitation, glutamine starvation, and MSX treatment all generate the same outcomes, nuclear localization of Gln3 and increased Gln3-mediated gene expression. In bare outline, the Tor pathway has been envisioned as glutamine levels positively regulating Tor1/2, which in turn positively regulates Tip41 and/or Tap42, the latter negatively regulating Sit4 phosphatase activity, preventing it from dephosphorylating Gln3, which thereby restricts Gln3 to the cytoplasm. Addition of either MSX, which inhibits production of the positive input signal sensed by Tor1/2...

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1 The abbreviations used are: NCR, nitrogen catabolite repression; MSX, methionine sulfoximine; CIP, calf intestine alkaline phosphatase.
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(16, 33). Although much of the available data support the existing view of Tor regulation, several observations suggest that we do not yet fully understand how Tor1/2 regulate Gln3 intracellular localization. (i) The results of genetic studies with tap42 mutants are more consistent with the suggestion of Tap42 functioning as a positive rather than a negative regulator of Sit4 activity (7, 8, 13, 14). (ii) Gln3 phosphorylation levels do not correlate with intracellular Gln3 localization in cells provided with a range of nitrogen sources and during nitrogen and carbon starvation in the same way as they do with rapamycin treatment (34). (iii) Nuclear accumulation of phosphorylated Gln3 during carbon starvation derives not from starvation for glucose but for nitrogen. Nitrogen starvation occurs because glucose-starved cells cannot produce the α-ketoglutarate needed to assimilate the nitrogen source (ammonia) used in the experiment. Therefore, both phosphorylated Gln3, generated by α-ketoglutarate-mediated nitrogen starvation, and dephosphorylated Gln3, in response to rapamycin addition, accumulate in the nucleus (35). This occurs despite the fact that nitrogen starvation and rapamycin are both thought to regulate Gln3 through their inhibition of Tor1/2 (9, 16, 29, 30).

This study was initiated in an effort to rectify instances in which the available data cited above are not congruent with expectations derived from the current model describing how the S. cerevisiae Tor proteins regulate Gln3. The report of MSX triggering dephosphorylation and nuclear localization of Gln3 provided a new way of perturbing Gln3 regulation (32) and prompted us to employ MSX in our investigations. Here we confirm the earlier report that MSX treatment elicits nuclear localization of Gln3-Myc13 and high level NCR-sensitive gene expression. But to our surprise, MSX markedly increased rather than decreased Gln3-Myc13 phosphorylation. Our results demonstrate Gln3-Myc13 phosphorylation occurs by at least two mechanisms: one is Snf1-dependent and the other Snf1-independent. MSX-induced Gln3 phosphorylation is Snf1-independent, whereas both mechanisms function to increase Gln3 phosphorylation during carbon starvation. Furthermore, the effects of MSX and rapamycin treatment on Gln3 phosphorylation are additive, but those of MSX clearly predominate. Although MSX and rapamycin treatment generate the same final outcome, nuclear localization of Gln3, the phosphorylation data are more consistent with MSX and rapamycin acting in parallel pathways to achieve that outcome than they are with their inhibiting tandem steps in a single regulatory pathway as proposed previously. These observations also suggest that something more or other than the Gln3 phosphorylation/dephosphorylation levels, detected thus far, determine the intracellular localization of Gln3.
H3 and HHT-1 (H3), and samples being collected. Radiolabeled PCR products for source. Inhibitors, where indicated (H11001), were used as hybridization probes with ACT1 as a positive control. We were surprised that MSX treatment decreased Phosphorylation—Analogously, MSX appears to be an even more useful perturbant for investigating control of Gln3. This inhibitor is useful both in the study of Tor function and Gln3 regulation of Crespo et al. (34, 35).

RESULTS

MSX Induces Decreased Gln3-Myc13 Mobility Suggesting Increased Phosphorylation—Rapamycin inhibition of Tor1/2 has been useful both in the study of Tor function and Gln3 regulation. Analogously, MSX appears to be an even more useful perturbant for investigating control of Gln3. This inhibitor is more specific than rapamycin in that it altered nuclear localization of Gln3 and Rtg1 but not Gat1, Msn2, Msn4, and an unknown factor involved in the regulation of ribosomal protein gene expression (32).

Our studies were initiated with control experiments testing whether MSX elicited the same response in our hands as reported previously (32). In support of that earlier work, we observed that treating cells with MSX markedly increased both Gln3- and Rtg3-mediated expression of DAL5 and CIT2, respectively (Fig. 1). Moreover, increased expression occurred with each nitrogen source tested except glutamine. The data, however, exhibited a somewhat unexpected characteristic. DAL5 expression increased more in glutamate-grown cells treated with MSX than when induced by rapamycin (Fig. 1A, lanes C–E). This was exceptional because rapamycin inhibits proteins (Tor1,2) considered to be downstream of the nutrient-sensing signal altered by MSX inhibition of glutamine synthetase in the current model of Tor regulation (32). Given these positive results, we assessed the effect of MSX on Gln3 phosphorylation, and we were surprised that MSX treatment decreased Gln3-Myc13 mobility rather than increasing it (Fig. 2A, lanes B and C). This change in mobility suggested Gln3-Myc13 phosphorylation was increased rather than decreased by MSX treatment, a finding unaccountably opposite to the observations of Crespo et al. (32).

**Fig. 1.** Northern blot analysis of the effects of 2 mM 1-methionine sulfoximine (MSX) or 200 ng/ml rapamycin (RAP) treatment on NCR-sensitive (DAL5) (A) and retrograde (CIT2) (B) gene expression in wild type (TB123). S. cerevisiae cells, growing in YNB medium, were provided with 0.1% proline (PRO), glutamate (GLU), urea, ammonia (NH4), or glutamine (GLN) as sole nitrogen source. Inhibitors, where indicated (+), were present for 30 min prior to samples being collected. Radiolabeled PCR products for DAL5, CIT2, DAL80, HHT-1 (H3), and ACT1 were used as hybridization probes with H3 and ACT1 serving as controls to assess RNA loading and transfer.

Effects of Culture Medium and Method of Cell Harvest on Gln3-Myc13 Mobility—The striking difference in MSX-induced Gln3-Myc13 mobility we observed compared with that reported earlier prompted us to look more carefully at the experimental conditions used in present and past experiments. The first difference was the concentration of ammonia provided in the medium; we used 0.1% compared with 0.5% used in the earlier analysis (32). This, however, did not account for the differing results. MSX treatment decreased Gln3-Myc13 mobility irrespective of the ammonia concentration (Fig. 2A, lanes B–E). Minor differences did, however, occur in the migration rates of minor Gln3-Myc13 species derived from untreated cells grown at the two ammonia concentrations. Gln3-Myc13 migrated slightly faster with glutamine than with low (0.1%) ammonia as nitrogen source; this difference was greater at the higher (0.5%) concentration of ammonia (compare Fig. 2A, lanes A and B with lanes E and F).

Present and past experiments technically differed in two further respects. YNB medium was used in current experiments, whereas those reported earlier used SD medium. Although the two media are similar in composition, they are not identical, and differences in results obtained with them have been noted in the past.2 In the present instance, however, media differences were not important; MSX treatment elicited decreased Gln3-Myc13 mobility in both media (Fig. 2B, lanes B and C and D and E). The second difference was the method of cell harvest. In previous experiments, centrifugation was used to harvest the cells (34), whereas filtration and flash-freezing was used here. We changed our protocol because Wilson et al. (41) reported Snf1 kinase is activated by centrifugation, and Snf1 is required for Gln3 phosphorylation during glucose starvation (33). As shown in Fig. 2C (lanes A and B and D and E), centrifugation decreased Gln3-Myc13 mobility. In Fig. 2C, the uppermost band (upper black dot) in lanes A and D (prepared by centrifugation) is absent in lanes B and E (filtration). Furthermore, although not as clear in the photograph as the original films, Gln3-Myc13 is about equally distributed between the

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2 T. G. Cooper and J. Bossinger, unpublished results.
cells immediately broken, and the extracts subjected to electrophoresis. Gln3-Myc\(^{13}\) from the extract of rapamycin-treated cells exhibited significantly greater mobility than the one from MSX-treated cells (Fig. 3A, lanes B and C). The third sample, containing a mixture of cells separately treated with the two inhibitors, yielded a profile that was very nearly the sum of the profiles observed in lanes B and C (Fig. 3A, lanes A and D). This indicated it was unlikely that significant artifacts occurring after the cells were broken had generated the profile seen in extracts from MSX-treated cells. If there was a difference of the profiles in Fig. 3A, lanes B and C versus A and D, it was a small amount of artifactual, \textit{in vitro} dephosphorylation of the slowly migrating Gln3-Myc\(^{13}\) species generated by MSX treatment in lanes A and D.

To determine whether decreased Gln3-Myc\(^{13}\) mobility observed with extracts from MSX-treated cells derived from increased phosphorylation, we tested its sensitivity to CIP. Incubating extracts from urea-grown, MSX-treated cells with CIP prior to electrophoresis increased the mobility of Gln3-Myc\(^{13}\) such that it nearly co-migrated with that from rapamycin-treated cells (Fig. 3B, lanes D–F). The CIP-dependent increase in Gln3-Myc\(^{13}\) mobility was prevented by including 40 mM sodium pyrophosphate, a CIP inhibitor, in the reaction mixture (Fig. 3B, lanes C–E). Extracts derived from similarly treated ammonia-grown cells yielded the same results (Fig. 3C). It is not clear why Gln3-Myc\(^{13}\) was dephosphorylated by CIP to a level one band above that observed with rapamycin treatment rather than to the same level, but we did encounter some inhibition of CIP by our extraction buffer.

Three conclusions were derived from these experiments. (i) Gln3 phosphorylation levels are sensitive to even minor differences in culture conditions and method of cell harvest. (ii) Centrifugation, as reported for other substrates of Snf1 (41), increases Gln3 phosphorylation. (iii) Treating cells with MSX markedly increases rather than decreases Gln3 phosphorylation irrespective of technical differences evaluated above.

Increased Nuclear Localization of Gln3-Myc\(^{13}\) Induced by MSX Occurs with All Nitrogen Sources Tested but Glutamine—Increased Gln3-Myc\(^{13}\) phosphorylation in MSX-treated cells prompted two questions. (i) Did MSX and rapamycin treatment differentially affect Gln3 nuclear localization? (ii) Were the effects of MSX treatment influenced by the nitrogen source? The pertinence of these questions derived from the fact that MSX and rapamycin were reported to affect Gln3 phosphorylation, nuclear localization, and Gln3-mediated gene expression in the same way, \textit{i.e.} through their influence on Tor1/2 activity (32).

As reported previously, treating ammonia-grown cells with MSX resulted in strong nuclear accumulation of Gln3-Myc\(^{13}\) similar to that seen in rapamycin-treated cells (Fig. 4A, microscopio images). This accumulation correlated well with high level Gln3-mediated \textit{DAL5} expression under the same conditions (Fig. 4A, images, and Fig. 1A, lanes H and I). On the other hand, little Gln3-Myc\(^{13}\) nuclear localization or \textit{DAL5} expression occurred in MSX-treated, glutamine-grown cells (Fig. 4F, images, and Fig. 1A, lanes J and K). This contrasts with the response of glutamine-grown cells to rapamycin (Fig. 4F, images, and Fig. 1A, lanes J and L). Glutamine, however, was unique in its ability to prevent nuclear accumulation of Gln3-Myc\(^{13}\). With all of the other nitrogen sources we assayed (urea, proline, and glutamate), Gln3-Myc\(^{13}\) nuclear accumulation and \textit{DAL5} expression was elicited by MSX treatment, albeit with some quantitative differences (Fig. 4, B, D, and E, and Fig. 1A).

The close correlation described above was not, however, observed in Gln3-Myc\(^{13}\) phosphorylation. Gln3-Myc\(^{13}\) phosphorylation, in preparations derived from ammonia- or urea-grown

![Image](http://www.jbc.org/)

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**Fig. 3.** A, effect of \textit{in vitro} mixing of frozen cells treated with MSX or rapamycin on the electrophoretic mobility of Gln3-Myc. Wild type cells were cultured in YNB/urea (0.1%) in the presence (+) or absence (−) of MSX or rapamycin. In lanes B and C, cells were harvested by filtration and processed normally for Western blot analysis. The combined cell samples in A, lanes A and D (one from rapamycin-treated cells and the other from MSX-treated cells), were harvested and frozen separately, but with freezing taking place in the same microcentrifuge tube as described in the text. Thereafter, all of the cell samples were processed normally for Western blot analysis. The combined cell samples in B, lanes A–D, were harvested and frozen as well (Fig. 3A, lane A and repeated in D). In cells treated with rapamycin, the method of harvest seemed to make less difference in Gln3-Myc mobility (Fig. 2C, lanes C and F).

**Decreased MSX-induced Gln3-Myc Mobility Derives from Increased Phosphorylation**—Since differences we could identify between current and past experimental techniques did not account for differences in our results with MSX compared with those reported earlier, we determined whether the electrophoretic behavior of Gln3-Myc\(^{13}\) from MSX-treated cells (i) was an artifact created during extract preparation or (ii) was derived from verifiable hyperphosphorylation. We tested the first possibility by using an \textit{in vitro} mixing experiment. Four wild type cultures were prepared. The first two were treated with rapamycin for 30 min, while the third and fourth were similarly incubated with MSX. The first and third cultures were harvested by filtration and processed normally (Fig. 3A, lanes B and C, respectively). The second culture (rapamycin-treated) was harvested, flash-frozen, and held in liquid nitrogen until the fourth cell sample (MSX-treated) was harvested, added to it, and frozen as well (Fig. 3A, lane A and repeated in D). In other words, the third experimental sample contained two sets of cells that were harvested and frozen separately but were contained in the same tube. This protocol prevented the cell samples from being exposed to a second drug before or during harvest. The three samples were subsequently thawed, the two predominant bands in Fig. 2C, lanes B and E. In contrast, in Fig. 2C, lanes A and D, where centrifugation was used, more of the protein is situated in the uppermost of these two bands (lower black dot). In cells treated with rapamycin, the method of harvest seemed to make less difference in Gln3-Myc\(^{13}\) mobility (Fig. 2C, lanes C and F).
cells treated with MSX versus rapamycin, behaved oppositely. Gln3-Myc\textsuperscript{13} phosphorylation increased upon MSX treatment and, overall, decreased in the presence of rapamycin (Fig. 4, A and B, lanes B and C and F and G). To ensure that we were not misinterpreting the behavior of Gln3-Myc\textsuperscript{13} phosphorylation relative to wild type (Fig. 4C, lanes A–C), whereas rapamycin treatment decreased it (lanes D–F), the difference was even more dramatic when Gln3-Myc\textsuperscript{13} from MSX-treated cells was electrophoresed between samples from rapamycin-treated cells (Fig. 4C, lanes G–I).

Gln3-Myc\textsuperscript{13} phosphorylation in untreated versus MSX-treated cells provided with proline or glutamate as nitrogen source overall behaved similarly to ammonia- and urea-grown cells, with the exception that the increase in phosphorylation, relative to untreated cells, was not as great (compare Fig. 4, A and B with D and E). This derives from the fact that Gln3-Myc\textsuperscript{13} in untreated cells already exhibits greater phosphorylation with both proline and glutamate than observed with ammonia or urea (compare with Gln3-Myc\textsuperscript{13} derived from control Gln-grown cells, Fig. 4, A, B, D, and E, lanes A, D, E, and H) as reported earlier (34). MSX had no demonstrable effect on phosphorylation levels in glutamine-grown cells (Fig. 4F).

Beyond major changes of Gln3-Myc\textsuperscript{13} phosphorylation in MSX-treated cells, additional, more subtle differences were also noted in Fig. 4. When using the filtration method of cell harvest, the most rapidly migrating Gln3-Myc\textsuperscript{13} species seen in rapamycin-treated cells frequently migrated similarly to the most rapidly migrating species of Gln3-Myc\textsuperscript{13} from untreated samples. In other words, we did not always see the marked increase in electrophoretic mobility in Gln3-Myc\textsuperscript{13} species that was often, but not always, observed when we used the centrifugation

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**FIG. 4.** A and B and D–F, the effects of MSX and rapamycin treatment on the electrophoretic mobility and intracellular localization of Gln3-Myc\textsuperscript{13} derived from cells cultured with various nitrogen sources. Cells were grown in YNB medium with 0.1% ammonia (\(\text{NH}_4\)), glutamine (GLN), urea, proline (PRO), or glutamate (GLU) as sole nitrogen source. Cells were treated with MSX or rapamycin as described in Fig. 2. Samples were harvested by filtration and processed as described under “Materials and Methods” for Western blot analysis (top portions of each panel). Gln3-Myc\textsuperscript{13} intracellular localization was assessed by indirect immunofluorescence imaging (bottom microscopic images of each panel). Gln3-Myc\textsuperscript{13} (red) is shown in columns A, C, and E. Gln3-Myc\textsuperscript{13} merged with 4,6-diamidino-2-phenylindole-positive material (magenta) is shown in columns B, D, and F. Solid lines connect Western blots and microscopic images derived from cells cultured under the same conditions. C, effect of MSX or rapamycin on Gln3-Myc\textsuperscript{13} mobility in wild type cells grown in YNB/urea medium. Inhibitor conditions were as in Fig. 2 and the cultures harvested by filtration.
method of harvest (34) and in earlier reports of phosphorylation experiments (9, 16). This is clearly observed in cells provided with ammonia, urea, and glutamine as nitrogen source (Fig. 4, A and B, lanes F–H, and F, lanes C and D). The effect is not seen with glutamate or proline, because rapamycin treatment of cells provided with these nitrogen sources does not increase mobility of Gln3-Myc13; this is in agreement with our earlier observations (34).

Because MSX treatment of urea-grown cells produced the largest change in Gln3-Myc13 mobility, we chose this nitrogen source for subsequent experiments. However, experiments using ammonia-grown cells were also performed in most cases to ensure the results we observed did not derive from using urea as the nitrogen source.

**Snf1-dependent and -independent Gln3-Myc13 Phosphorylation during Carbon starvation**—Increased Gln3-Myc13 phosphorylation following MSX treatment was similar to that observed earlier during carbon starvation of ammonia-grown cells (33, 34). Moreover, as occurs with MSX treatment above, Gln3-Myc13 localizes to the nuclei of ammonia-grown cells (33–35). These correlations raised the possibility that carbon starvation-activated Snf1 kinase might affect NCR-sensitive Gln3-mediated gene expression, and whether all of the increased Gln3-Myc13 phosphorylation observed during carbon starvation was Snf1-dependent. Wild type and snf1Δ strains are equally NCR-sensitive (Fig. 5A, lanes A–D). However, strains in this genetic background are overall less NCR-sensitive than M970 in the Σ1278b background; this is more clearly evident in the case of DAL80 expression (compare data in this work with analogous experiments in Refs. 39 and 40). Similarly, the CIT2 expression profile with various conditions is not identical to that in M970 (compare Fig. 1B to data in Refs. 39 and 40). SNF1 expression was not demonstrably affected by NCR, and the message was totally absent, as expected, in the snf1Δ (Fig. 5A, lanes E–H). Furthermore, Gln3-Myc13 accumulated in the nuclei of snf1Δ cells treated with either rapamycin or MSX in nearly the same ways as wild type (Fig. 5B). There did appear, however, to be greater Gln3-Myc13 nuclear localization in the glutamine-grown, MSX-treated snf1Δ.

We next measured Gln3-Myc13 phosphorylation in carbon-starved wild type and snf1Δ cells. As quickly as 30 min following onset of carbon starvation, Gln3-Myc13 phosphorylation in the wild type strain increased (Fig. 6A, compare lane B to lanes C–F) as reported by Bertram et al. and subsequently ourselves (33, 34). In addition, the heterogeneity of Gln3-Myc13 species increased compared with the unstarved sample. When the above experiment was repeated in a snf1Δ, carbon starvation increased Gln3-Myc13 phosphorylation but to a lesser degree than wild type (compare Fig. 6B, lane D, black dots). Characteristic differences in Gln3-Myc13 phosphorylation between carbon-starved wild type and snf1Δ cells are more clearly seen in Fig. 6C, lanes C–E. These results suggest that carbon starvation elicits both Snf1-dependent and -independent Gln3-Myc13 phosphorylation.

The expected phenotype of deleting a protein kinase gene is decreased phosphorylation of its target substrates, as observed with Gln3-Myc13 phosphorylation in carbon-starved snf1Δ versus wild type (Fig. 6C, lanes C–E). However, in addition, we observed a slight increase in Gln3-Myc13 phosphorylation (by one band, black dot) in the snf1Δ grown in high glucose (Fig. 6C, lanes A and B). The effect is most clearly seen by comparing
Gln3-Myc\textsuperscript{13} phosphorylation in glutamine- versus urea-grown wild type cells (Fig. 6A, lanes A and B) with those in the snf1\textDelta (Fig. 6C, lanes A and B and F and G). This suggests Snf1, in high glucose medium, indirectly influences Gln3-Myc\textsuperscript{13} phosphorylation in a way opposite that seen in carbon-starved cells. MSX-induced Gln3-Myc\textsuperscript{13} Phosphorylation Is Snf1-independent—Given that both Snf1-dependent and -independent Gln3-Myc\textsuperscript{13} phosphorylation occurred, we wanted to ascertain which mechanism was associated with MSX-elicted Gln3-Myc\textsuperscript{13} phosphorylation. Therefore, an extract from MSX-treated cells was placed between two extracts from carbon-starved, wild type (Fig. 7A, lanes C–E) or snf1\textDelta (Fig. 7B, lanes C–E) cells. The Gln3-Myc\textsuperscript{13} phosphorylation profile in MSX-treated wild type cells resembled that observed in a carbon-starved snf1\textDelta more than it did in carbon starved wild type cells, suggesting phosphorylation was Snf1-independent. This was confirmed by demonstrating Gln3-Myc\textsuperscript{13} phosphorylation increased in unstarved snf1\textDelta cells treated with MSX compared with untreated snf1\textDelta cells (Fig. 7D, lanes A–C; also observed in Fig. 7B, lanes B and D). Consistent with these results, the Snf1-dependent heterogeneity of hyperphosphorylated Gln3-Myc\textsuperscript{13} species observed in carbon-starved wild type cells was lacking in MSX-treated cell extracts (Fig. 7, A, lanes C–E and B, lanes C–E). The question remained, however, whether or not Gln3-Myc\textsuperscript{13} phosphorylation levels observed in MSX-treated wild type and snf1\textDelta cells were the same. Data in Fig. 7C (lanes D and E), argue that Gln3-Myc\textsuperscript{13} species from the two cell samples co-migrate. The slight apparent lag in mobility of the upper-most band in Fig. 7C, lane E (black dot), relative to that in lane D derives from “blooming” of the more intense signal in lane E. On the other hand, the small increase in Gln3-Myc\textsuperscript{13} phosphorylation previously seen in snf1\textDelta relative to wild type is also present in Fig. 7C, lanes B and C. Note that the bottom band in lane B of Fig. 7C is missing in lane C. Together these data suggest that (i) Gln3 is phosphorylated during carbon starvation by both Snf1-dependent and -independent mechanisms, and (ii) MSX elicits Snf1-independent Gln3 phosphorylation. During these and other experiments, we also noticed that Gln3-Myc\textsuperscript{13} phosphorylation did not respond in quite the same way to rapamycin treatment of wild type versus snf1\textDelta cells (Fig. 7D, lanes D–F; data not shown). The extent of Gln3-Myc\textsuperscript{13} dephosphorylation in response to rapamycin was greater (by one band) in the wild type than in a snf1\textDelta, i.e. there was less dephosphorylation in the snf1\textDelta. Recall the same observation was made above in untreated snf1\textDelta extracts (Fig. 6C, lanes A and B, and F and G versus A, lanes A and B). This again argued in favor of Snf1 kinase indirectly influencing Gln3-Myc\textsuperscript{13} dephosphorylation. Data in Fig. 4, A and B, indicated that increased Gln3-Myc\textsuperscript{13} phosphorylation was similarly observed in both ammonia- and urea-grown cells. To ensure this similarity extended to the responses elicited by carbon starvation and snf1\textDelta, we carbon-starved ammonia-grown cells. MSX-induced Gln3-Myc\textsuperscript{13} phosphorylation occurred in ammonia-grown cells just as it had with urea (Fig. 8A). In both cases, the bottom band in extracts from MSX-treated cells was lost, and the signal in the top band increased (carefully compare black dots in Fig. 8A, lanes A and C, with Fig. 7A, lanes B and D). Similarly, the three phosphorylated species observed in MSX-treated, urea-grown snf1\textDelta cells were also observed in snf1\textDelta ammonia-grown samples (Fig. 8B, lanes B and E). Increased heterogeneity of Gln3-Myc\textsuperscript{13} phosphorylation observed when ammonia-grown wild type cells were starved for carbon was similar to that seen with urea, as was disappearance of the heterogeneity in the snf1\textDelta (Fig. 8B, lanes C and D). Although results with ammonia as nitrogen source were very similar to those observed with urea, one observation in the experiment with ammonia-grown cells was unsettling. In our
earlier studies, Gln3-Myc\textsuperscript{13} phosphorylation increased much more dramatically in ammonia-grown, carbon-starved cells (180 min) than we observed here (compare the right panel of Fig. 7B, lanes C and D, in Ref. 34 with Fig. 8A, lanes A and D of the present work). However, upon comparison, we found the two experiments differed in one important respect, i.e. filtration was used to harvest cells in the present case, whereas we previously used centrifugation (34). This technical difference clearly accounts for the markedly different observations. Gln3-Myc\textsuperscript{13} was phosphorylated to a much greater extent in the centrifuge-harvested, carbon-starved cells than those collected by filtration (Fig. 8C, compare lanes A, C, D, and F).

MSX and Nitrogen Starvation-induced Gln3-Myc\textsuperscript{13} Phosphorylation—MSX is suggested to elicit increased NCR-sensitive transcription by starving cells of the signal for nitrogen excess (32), predicting the Gln3-Myc\textsuperscript{13} phosphorylation profiles of MSX-treated and nitrogen-starved cells should be the same. Testing this prediction, we observed that nitrogen starvation of ammonia-grown cells only modestly increased Gln3-Myc\textsuperscript{13} phosphorylation levels (Fig. 9, lanes A, F, C, and D). Similar results were observed when cells were pre-grown in YNB-urea medium (Fig. 9A, lanes A, F, C, and D). In contrast, Gln3-Myc\textsuperscript{13} phosphorylation increased substantially in MSX-treated cells (Fig. 9, A and B, lanes B and E). There was a modest increase in Gln3-Myc\textsuperscript{13} phosphorylation after 60 min of nitrogen starvation, but it was still significantly less than observed with MSX (Fig. 9, A and B, lanes B–E). Similarly, modest nitrogen source-dependent differences were observed earlier (34). Finally, deletion of SNF1 had no detectable effect on the Gln3-Myc\textsuperscript{13} phosphorylation profile generated by nitrogen starvation (data not shown).

Effect of Sequential MSX and Rapamycin Treatment on Gln3-Myc\textsuperscript{13} Phosphorylation—Extracts of MSX- and rapamycin-treated cells exhibit clearly distinguishable Gln3-Myc\textsuperscript{13} phosphorylation profiles, permitting us to test whether MSX and rapamycin inhibit tandem steps in a single regulatory pathway. In the best circumstance, treating cells with these inhibitors might generate the same effects as null mutations of the proteins they inhibit. MSX treatment is thought to generate the signal that is received by and inhibits Tor1/2 function of minor species in Fig. 10, B and C, lanes B–D; note greater mobility of minor species in Fig. 10C, lanes C and E). In any case, we felt these control experiments were sufficiently positive to proceed. Two control extracts were electrophoresed on either side of samples from the ordered addition protocol: (i) cells treated with MSX alone (Fig. 10A, lanes B–E), arguing that (i) the MSX-inhibited step was downstream of the one inhibited by rapamycin, (ii) the inhibited steps did not occur in tandem with the MSX-inhibited step being either the later or predominant of the two, or (iii) MSX somehow inhibited rapamycin action by preventing uptake or interfering with its inhibition of Tor1/2.

The second experiment consisted of treating cells sequentially with rapamycin and MSX. In the first phase, MSX was added to a log phase culture, and following a 30-min incubation, rapamycin was added. 30 min later, the cells were harvested, and Gln3-Myc\textsuperscript{13} phosphorylation was assayed. In the second phase, the order of inhibitor addition was reversed. We could not perform the ordered addition experiment by transferring cells from a medium containing one inhibitor to medium containing the other, because merely transferring cells to a fresh sample of the same medium, with no other change at all, generates marked differences in Gln3-Myc\textsuperscript{13} localization (see Fig. 1B of Ref. 45).

Since, in these experiments, the first inhibitor added to the cells would be present for 60 min, it was necessary to ensure that treating cells with MSX or rapamycin for 30 min versus 60 min generated the same Gln3-Myc\textsuperscript{13} phosphorylation profiles. This was found to be the case, except that Gln3-Myc\textsuperscript{13} phosphorylation was a little greater at 60 min of MSX treatment than 30 min (Fig. 10, B and C, lanes B–D; note greater mobility of minor species in Fig. 10C, lanes C and E). In any case, we felt these control experiments were sufficiently positive to proceed. Two control extracts were electrophoresed on either side of samples from the ordered addition protocol: (i) cells treated with rapamycin alone, and (ii) a 1:1 mixture of cells that had been treated separately with rapamycin or MSX and frozen separately in the same tube as occurred for the experiment in Fig. 3A, lanes A and D. These control samples appear in Fig. 10D, lanes A, B, E, and F.
Results from the first phase of the ordered inhibitor addition experiment, *i.e.* MSX added before rapamycin, are shown in Fig. 10D, lane C. Gln3-Myc\textsuperscript{13} was highly phosphorylated, and the two faster migrating, dephosphorylated species of Gln3-Myc\textsuperscript{13} characteristic of rapamycin-treated cells (*two black dots* in Fig. 10D, lanes A, E, and F) were absent. In the second phase of the experiment, *i.e.* rapamycin was added before MSX, hyperphosphorylated Gln3-Myc\textsuperscript{13} was still observed as was the continued absence of dephosphorylated Gln3-Myc\textsuperscript{13} species characteristic of rapamycin-treated cells (Fig. 10D, lane D). These results occurred in spite of the fact that Gln3-Myc\textsuperscript{13} had been dephosphorylated by rapamycin treatment (Fig. 10, B, lane C and D, and Fig. 10D, lanes A and F) prior to MSX being added to the cells. In other words, Gln3-Myc\textsuperscript{13} was hyperphosphorylated even though Tor1/2 kinase activities were inhibited by rapamycin. This result argued that Tor1/2 was not responsible for the MSX-induced increase in Gln3-Myc\textsuperscript{13} phosphorylation (Fig. 10D, lane D *versus* A and F).

Although results of the ordered addition experiment were clear, they were not absolute, *i.e.* Gln3-Myc\textsuperscript{13} was not as extensively phosphorylated in the second phase of the experiment as when MSX was added before or in combination with rapamycin (Fig. 10D, lane D). Note that Gln3-Myc\textsuperscript{13} was more uniformly distributed between the bands in Fig. 10D, lane D than in lane C. The species in Fig. 10D, lane D, that slightly predominated (*black dot*) was two species lower than the predominant pair of species in lane C (*black dot*). Also note as a point of reference that the uppermost species observed during rapamycin treatment alone co-migrates with the lowermost species observed after MSX treatment (Fig. 3A, lanes A–D). In the first experiment (Fig. 10A), the effects of MSX clearly predominated over those of rapamycin, whereas in the second experiment (Fig. 10D) there was detectable additivity of the effects even though the MSX-generated Gln3-Myc\textsuperscript{13} species again predominated. In neither experiment were the data as expected if the proteins inhibited by rapamycin were downstream of the nitrogen excess signal whose generation was posited to be inhibited by MSX.

**DISCUSSION**

The above data demonstrate that MSX and rapamycin, although eliciting the same outcomes with respect to Gln3-Myc\textsuperscript{13} nuclear accumulation and NCR-sensitive transcription, generate opposite effects on Gln3-Myc\textsuperscript{13} phosphorylation, *i.e.* MSX increases it and rapamycin decreases it. The additive effects of the two inhibitors on Gln3-Myc\textsuperscript{13} phosphorylation favor them functioning nonaddemely in different pathways or in different branches of one pathway more than they support the current model positing them to function tandemly in a single pathway (32). Furthermore, inhibitors oppositely affecting Gln3-Myc\textsuperscript{13} phosphorylation, but eliciting the same outcomes on its intracellular localization, suggest that Gln3-Myc\textsuperscript{13} phosphorylation/dephosphorylation, detected thus far in this and the work of others, is likely associated with something other than determining Gln3 intracellular localization. This suggestion is also consistent with the lack of correlation observed between Gln3-Myc\textsuperscript{13} phosphorylation and intracellular localization in cells provided with various nitrogen sources (34).

We also showed that Gln3-Myc\textsuperscript{13} phosphorylation levels are regulated by at least three separate mechanisms. The first depends on the carbon regulatory kinase, Snf1. The second is independent of it; MSX-induced phosphorylation occurs via the Snf1-independent mechanism. Third, Gln3-Myc\textsuperscript{13} is dephosphorylated in response to rapamycin inhibition of Tor1/2. The existence of Snf1-dependent Gln3-Myc\textsuperscript{13} phosphorylation is not surprising as it had been observed earlier in response to carbon starvation of ammonia-grown cells (33, 34). The novel observation of the present work is that carbon starvation also triggers Gln3-Myc\textsuperscript{13} phosphorylation in a *snf1Δ*. This phosphorylation level increases to even higher levels in the presence of wild type Snf1. However, the profile of Snf1-dependent and -independent phosphorylation responses differ. Snf1-independent phosphorylation appears to generate three characteristic Gln3-Myc\textsuperscript{13} species, whereas the Snf1-dependent Gln3-Myc\textsuperscript{13} species are more heterogeneous; appearing largely as a fainter, slower mobility smear. This would be an expected result if the Snf1-independent mechanism possessed greater substrate specificity. However, given the number of phosphorylated Gln3-Myc\textsuperscript{13} species we can detect, in addition to those in the smear that we can not individually resolve, such an interpretation is probably speculative.

Although our experiments demonstrate increased Gln3-Myc\textsuperscript{13} phosphorylation derives from at least two distinguishable regulatory pathways, they cannot identify the kinases directly responsible for it. The literature supports Snf1 and Tor1/2 as the most likely candidates: (i) both kinases interact with Gln3 in two-hybrid assays (16, 33) and (ii) both Tor1/2 and Snf1 phosphorylate Gln3 in *vitro* (16, 33). Consistent with Snf1 phosphorylating Gln3-Myc\textsuperscript{13} during carbon starvation is the correlation that Snf1 activity is activated by centrifugation (41), a characteristic also observed in our Snf1-dependent data (Fig. 8C). To conclude that Tor1/2 phosphorylate Gln3-Myc\textsuperscript{13} is more difficult. First, the data in Fig. 10D demonstrate that MSX induces Gln3-Myc\textsuperscript{13} phosphorylation in the presence of rapamycin, which inactivates Tor1/2. Second, if Tor1/2 are responsible for Gln3-Myc\textsuperscript{13} phosphorylation, a dilemma arises in trying to reconcile current data with the earlier model of Tor regulation (9, 32). The dilemma arises from the fact that both MSX treatment and carbon starvation of ammonia-grown cells (urea is also degraded solely to ammonia) are thought to generate nitrogen starvation, but yet unlike rapamycin treatment, they increase Gln3-Myc\textsuperscript{13} phosphorylation. According to the existing model, nitrogen starvation and MSX and rapamycin treatment are all envisioned to inhibit Tor1/2 kinase activities, which in turn should all result in dephosphorylation of Gln3-Myc\textsuperscript{13} (32). By this reasoning, we can account for present data only by suggesting (i) that Tor1/2 mediate Gln3 phosphorylation in their inactive forms or (ii) that Tor1/2 are not directly responsible for MSX-induced and/or nitrogen starvation-induced Gln3 phosphorylation. Although we do not favor it, one possibility that the data in Fig. 10D do not eliminate is that Tor1/2 were responsible for the most highly phosphorylated species of Gln3-Myc\textsuperscript{13} in lane C (where Tor1/2 were not inhibited by rapamycin for the first 30 min) that were missing in lane D.

The final dilemma generated by the above experiments is that MSX and rapamycin treatment, in our hands, elicit effects on Gln3-Myc\textsuperscript{13} phosphorylation opposite to those reported previously (32). Since the strains used here were the same as those used earlier (9, 32), we searched for differences in culture conditions. Although such differences were found, they do not account for the results obtained. Our failed attempts to rectify the differing observations notwithstanding, there is clearly one or more differences between present and earlier experiments that remain to be identified.

Another question that arose during this work was whether MSX treatment elicited Gln3-Myc\textsuperscript{13} phosphorylation profiles similar to those that occur in response to nitrogen starvation as expected if MSX treatment inhibits glutamine formation, the signal of excess nitrogen proposed earlier to be relayed to Tor1/2 (32). Our MSX-induced Gln3-Myc\textsuperscript{13} phosphorylation profiles share characteristics with profiles generated both by carbon and nitrogen starvation. To conclude the Gln3-Myc\textsuperscript{13} phosphorylation profile resembles that of nitrogen starvation,
however, would require that MSX treatment generates a more extreme case of nitrogen starvation than incubating cells in nitrogen-free medium for 60 min. Although certainly not difficult to imagine, if this were the case, then one would also expect Gα1 to be accumulated in the nucleus following MSX treatment, because like Gln3, Gα1-mediated transcription is NCR-sensitive. Furthermore, Gα1 has been proposed to be subject to the same phosphorylation/dephosphorylation regulatory mechanism as Gln3 (9). This expectation contrasts with the report that MSX does not elicit nuclear localization of Gα1 (32). On the other hand, MSX-elicited Gln3-Myc phosphorylation does possess a profile similar to the one seen in carbon-starved, wild type cells and identical to the one caused by carbon starvation of a snf1Δ mutant. This suggests MSX treatment directly or indirectly triggers responses shared by those that occur with carbon starvation.

Most importantly, the MSX data reported above argue that both phosphorylated and dephosphorylated forms of Gln3-Myc can be accumulated in the nucleus, a conclusion also reported in studies of the effects of carbon starvation on Gln3 regulation (33). These observations make it difficult to understand how data concerning Gln3 phosphorylation levels, which we and others have detected thus far, can be rectified with the current regulatory model. At the same time, we must hasten to emphasize that these difficulties would quickly disappear if the phosphorylated species detected so far are not the ones associated with nitrogen-dependent Gln3 regulation. Current data also prompt a new question, if phosphorylation levels we detect are their functions? The importance of this question to future studies is enormous, especially those involving carbon starvation or limitation. Centrifugation-mediated activation of Snf1 was originally reported with respect to carbon regulation (41), but the above data suggest cell harvest methods could influence the data obtained with other proteins as well. Such artifacts appear to be avoided, or at least decreased, when cells are harvested by filtration rather than centrifugation.

Acknowledgments—We thank Dr. Michael Hall for strain TB123, Tim Higgins for preparing the artwork, and the University of Tennessee Yeast Group for suggestions to improve the manuscript.
STRUCTURAL BASIS FOR THE COORDINATED REGULATION OF TRANSGlutaminase 3 BY GUANINE NUCLEOTIDES AND CALCIUM/MAGNESIUM.

Bijan Ahvazi, Karen M. Boeshans, William Idler, Ulrich Baxa, Peter M. Steinert, and Fraydoon Rastinejad

This paper has been withdrawn.

METHIONINE SULFOXIMINE TREATMENT AND CARBON STARVATION ELICIT Snf1-INDEPENDENT PHOSPHORYLATION OF THE TRANSCRIPTION ACTIVATOR Gln3 IN SACCHAROMYCES CEREVISIAE.

Jennifer J. Tate, Rajendra Rai, and Terrance G. Cooper

PAGES 27196–27197:
Under “Materials and Methods,” subheading “Phosphatase Treatment,” the first sentence should read: “Crude protein extracts were prepared as described above with the following extraction buffer: 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM EDTA, 2 mM phenylmethylsulfonyl fluoride, and a commercial mixture of protease inhibitors (Roche Applied Science).”

FORMATION, STRUCTURE, AND DISSOCIATION OF THE RIBONUCLEASE S THREE-DIMENSIONAL DOMAIN-SWAPPED DIMER.

Jorge P. López-Alonso, Marta Bruix, Josep Font, Marc Ribó, Maria Vilanova, Manuel Rico, Giovanni Gotte, Massimo Libonati, Carlos González, and Douglas V. Laurents

Add the following to the grant support footnote: This work was also supported by Spanish “Ministerio de Educacion y Ciencia” Grant GEN2003-20642-C09-03.

ANTIANGIOGENIC ANTITHROMBIN BLOCKS THE HEPARAN SULFATE-DEPENDENT BINDING OF PROANGIOGENIC GROWTH FACTORS TO THEIR ENDOTHELIAL CELL RECEPTORS. EVIDENCE FOR DIFFERENTIAL BINDING OF ANTIANGIOGENIC AND ANTICOAGULANT FORMS OF ANTITHROMBIN TO PROANGIOGENIC HEPARAN SULFATE DOMAINS.

Weiqing Zhang, Richard Swanson, Yan Xiong, Benjamin Richard, and Steven T. Olson

PAGE 37302:
Benjamin Richard was supported by a fellowship from the Fondation pour la Recherche Médicale.
Chemokine CXCL12 induces binding of ferritin heavy chain to the chemokine receptor CXCR4, alters CXCR4 signaling, and induces phosphorylation and nuclear translocation of ferritin heavy chain.

Runsheng Li, Cherry Luo, Marjelo Mines, Jingwu Zhang, and Guo-Huang Fan

PAGE 37616:

The first line of the support footnote should read as follows (with mention of Veterans Affairs Merit Award and SNRP grant deleted): “This work was supported by Science and Technology Commission of Shanghai Municipality Project Grant 04DZ14902 and Research Center for Minority Institute Grant RR03032-19.”

ZHX proteins regulate podocyte gene expression during the development of nephrotic syndrome.

Gang Liu, Lionel C. Clement, Yashpal S. Kanwar, Carmen Avila-Casado, and Sumant S. Chugh

PAGE 39689:

The first two lines of the legend for Fig. 7 should read as follows: “FIGURE 7. Effect of ZHX mRNA knockdown and overexpression on protein distribution. Knockdown studies (panels A–D) were performed in cultured mouse GECs, whereas overexpression studies (panels E–L) to specifically identify tagged ZHX proteins were performed in HEK 293 cells.”