Nitrogen Starvation and TorC1 Inhibition Differentially Affect Nuclear Localization of the Gln3 and Gat1 Transcription Factors Through the Rare Glutamine tRNA_{CUG} in *Saccharomyces cerevisiae*

Jennifer J. Tate, Rajendra Rai, and Terrance G. Cooper¹

Department of Microbiology, Immunology, and Biochemistry, University of Tennessee Health Science Center, Memphis, Tennessee 38163

ABSTRACT A leucine, leucyl-tRNA synthetase-dependent pathway activates TorC1 kinase and its downstream stimulation of protein synthesis, a major nitrogen consumer. We previously demonstrated, however, that control of Gln3, a transcription activator of catabolic genes whose products generate the nitrogenous precursors for protein synthesis, is not subject to leucine-dependent TorC1 activation. This led us to conclude that excess nitrogen-dependent down-regulation of Gln3 occurs via a second mechanism that is independent of leucine-dependent TorC1 activation. A major site of Gln3 and Gat1 (another GATA-binding transcription activator) control occurs at their access to the nucleus. In excess nitrogen, Gln3 and Gat1 are sequestered in the cytoplasm in a Ure2-dependent manner. They become nuclear and activate transcription when nitrogen becomes limiting. Long-term nitrogen starvation and treatment of cells with the glutamine synthetase inhibitor methionine sulfoximine (Msx) also elicit nuclear Gln3 localization. The sensitivity of Gln3 localization to glutamine and inhibition of glutamine synthesis prompted us to investigate the effects of a glutamine tRNA mutation (*sup70-65*) on nitrogen-responsive control of Gln3 and Gat1. We found that nuclear Gln3 localization elicited by short- and long-term nitrogen starvation; growth in a poor, derepressive medium; Msx or rapamycin treatment; or *ure2Δ* mutation is abolished in a *sup70-65* mutant. However, nuclear Gat1 localization, which also exhibits a glutamine tRNA_{CUG} requirement for its response to short-term nitrogen starvation or growth in proline medium or a *ure2Δ* mutation, does not require tRNA_{CUG} for its response to rapamycin. Also, in contrast with Gln3, Gat1 localization does not respond to long-term nitrogen starvation. These observations demonstrate the existence of a specific nitrogen-responsive component participating in the control of Gln3 and Gat1 localization and their downstream production of nitrogenous precursors. This component is highly sensitive to the function of the rare glutamine tRNA_{CUG}, which cannot be replaced by the predominant glutamine tRNA_{CAA}. Our observations also demonstrate distinct mechanistic differences between the responses of Gln3 and Gat1 to rapamycin inhibition of TorC1 and nitrogen starvation.

KEYWORDS Gat1; Gln3; glutamine tRNA; nitrogen starvation

MECHANISMS of nitrogen-responsive transcriptional regulation in *Saccharomyces cerevisiae* and other organisms have remained relatively obscure despite intensive investigation and identification of many required or involved components. The overall complexity of the problem and challenges in elucidating the mechanistic details of

overall nitrogen-responsive regulation derive from the fact that four or five distinguishable pathways operate in achieving it (Tate and Cooper 2013). Using Gln3 as the nitrogen-responsive reporter, each mode of regulation was shown to be associated with a distinct physiological condition: (1) short-term nitrogen limitation or growth with poor nitrogen sources, (2) long-term nitrogen starvation, (3) treatment with the glutamine synthetase inhibitor Msx, (4) rapamycin inhibition of TorC1, and (5) leucine starvation or inhibition of leucyl tRNA synthetase.

Gln3 and Gat1 are GATA-family transcription activators that have long been known to be responsible for catabolic nitrogen-responsive or nitrogen catabolite repression (NCR)-sensitive gene expression (Cooper 1982, 2004; Hofman-Bang 1999;

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¹Corresponding author: Department of Microbiology, Immunology and Biochemistry, University of Tennessee Health Science Center, Memphis, TN 38163.

E-mail: tcooper@uthsc.edu

Magasanik and Kaiser 2002; Broach 2012; Conrad *et al.* 2014). When cells are cultured with readily used nitrogen sources (also referred to as good, preferred, repressive, *e.g.*, glutamine), **Gln3** is restricted to the cytoplasm, and therefore, the NCR-sensitive transcription it activates is minimal (Cooper 1982). This cytoplasmic sequestration of **Gln3** requires the pre-prion protein **Ure2** (Blinder *et al.* 1996; Beck and Hall 1999; Cardenas *et al.* 1999; Hardwick *et al.* 1999; Bertram *et al.* 2000). In contrast, when poorly used nitrogen sources (poor, nonpreferred, derepressive, *e.g.*, proline) are provided, **Gln3** relocates to the nucleus, and GATA factor-mediated NCR-sensitive transcription increases dramatically.

The five physiological conditions that elicit nuclear entry of **Gln3** are distinguished by their protein phosphatase requirements (Tate *et al.* 2006, 2009, 2010; Georis *et al.* 2008, 2011; Rai *et al.* 2013, 2014; Tate and Cooper 2013). Nuclear **Gln3** localization in response to short-term nitrogen starvation or growth in a poor nitrogen source requires only **Sit4** phosphatase. Nuclear **Gln3** localization in response to long-term nitrogen starvation or Msx treatment exhibits no known phosphatase requirement, whereas a response to rapamycin treatment in glutamine-grown cells requires two phosphatases, **Sit4** and PP2A (Beck and Hall, 1999, Tate *et al.* 2006, 2009). Finally, **Gln3** localization does not demonstrably respond to leucine/leucyl tRNA synthetase activation of TorC1, which controls **Sch9** phosphorylation (Binda *et al.* 2009; Bonfils *et al.* 2012; Zhang *et al.* 2012; Panchaud *et al.* 2013; Tate and Cooper 2013). **Sch9** is a protein kinase that regulates protein synthesis, a major consumer of nitrogenous precursors.

Gat1, a homolog of **Gln3** and NCR-sensitive transcription activator in its own right, shares many regulatory characteristics with **Gln3**. These two GATA factors are not regulated identically, however (Georis *et al.* 2008, 2011). The most striking difference in the regulation of **Gln3** and **Gat1** is their responses to Msx and rapamycin. **Gln3** is exquisitely sensitive to Msx treatment, whereas **Gat1** localization is immune to it (Georis *et al.* 2011; Tate and Cooper 2013). Conversely, **Gat1** is exquisitely sensitive to rapamycin treatment, whereas **Gln3** is much less so.

GATA factor localization and function, however, are not the only nitrogen-responsive cellular processes. Others include sporulation, autophagy, and the formation of pseudohyphae in adverse nitrogen conditions (Gimeno *et al.* 1992). In nitrogen-rich conditions, diploid cells are ellipsoidal and bud in a bipolar manner. In contrast, when cultured under nitrogen conditions that verge on starvation, they bud in a unipolar manner that results in the formation of pseudohyphae (Gimeno *et al.* 1992). It has been suggested that pseudohyphal growth may facilitate scavenging for additional sources of environmental nitrogen. Positive correlations between the conditions that elicit NCR-sensitive transcription and dimorphic growth are striking.

Early on, Murray *et al.* (1998) noted these correlations and importantly reported that pseudohyphal growth occurred constitutively when a temperature-sensitive mutant containing an alteration in the glutamine tRNA_{CUG} molecule

itself (*sup70-65*) was grown in nitrogen-rich medium at 30° but not 22°. There are two glutamine tRNAs in *S. cerevisiae*. The more rare species possesses the anticodon 5'-CUG-3', which decodes the glutamine codon 5'-CAG-3', whereas the major species possesses the anticodon 5'-UUG-3', which decodes the codon 5'-CAA-3'.

Our discovery that nuclear **Gln3** localization in response to Msx inhibition of glutamine synthetase and long-term nitrogen starvation exhibits the same requirements piqued our interest in glutamine tRNA and hence the *sup70-65* mutant. Pseudohyphal growth and arginase (**CAR1**) gene expression occur constitutively in *sup70-65* mutant cells grown at a semi-nonpermissive temperature of 30° (Murray *et al.* 1998). Yet **DAL5** (encoding the catabolic allantoinase) expression rather than being constitutive, as expected, remained NCR sensitive and additionally was significantly lower in *sup70-65* mutant than wild-type cells (Beeser and Cooper 1999). This paradox and the prominent role played by glutamine availability in the regulation of **Gln3** prompted us to investigate the effects of the *sup70-65* mutation on all five modes of nitrogen-responsive control using **Gln3** localization, a more specific probe of nitrogen-responsiveness than NCR-sensitive transcription, as the reporter.

The results of those investigations showed, much to our surprise, that structurally unaltered glutamine tRNA_{CUG} is absolutely required for nuclear entry of **Gln3** and **Gat1**, even though cells are able to otherwise grow reasonably well in the presence of a specific tRNA_{CUG} mutation. Nuclear **Gln3** localization was completely abolished not only in the *sup70-65* mutant in response to the five physiological conditions known to elicit it but also in a *ure2Δ* mutant. Further, *sup70-65* and *ure2Δ* mutations exhibited a synthetic loss-of-growth phenotype. The *sup70-65*-dependent component was lost very slowly (in excess of four generations) following inactivation of glutamine tRNA_{CUG} but was reacquired in less than one generation when inactivation of tRNA_{CUG} ceased. We additionally identified new major differences in **Gln3** and **Gat1** regulation that significantly influence the interpretations of data measuring overall GATA factor-dependent NCR-sensitive transcription. The loss of rapamycin responsiveness in a *sup70-65* mutant was specific to **Gln3** localization. Rapamycin-elicited nuclear **Gat1** localization was not demonstrably affected in the mutant. Further, **Gln3** and **Gat1** responded oppositely to **Sit4**-independent long-term nitrogen starvation. Whereas long-term nitrogen starvation elicited strong tRNA_{CUG}-dependent nuclear **Gln3** localization, it had no demonstrable effect on **Gat1** localization.

Materials and Methods

Yeast strains and culture conditions

S. cerevisiae strains used in this work are listed in Table 1. Cultures (50 ml) were grown to mid-log phase ($A_{600\text{nm}} = 0.5$) in Yeast Nitrogen Base (YNB, without amino acids or ammonia; Difco, Detroit, MI) minimal medium containing

Table 1 *S. cerevisiae* strains used in this work

Strain	Pertinent genotype	Complete genotype	Genetic background
LMDWLU	Wild type	<i>Mata/MATα SUP70/SUP70 ura3-52/ura3-52 leu2-3 112/leu2-3 112 ade1-1/ADE1</i>	LMDWLU
LMD65-1LU	<i>sup70-65</i>	<i>MATa/MATα sup70-65/sup70-65 leu2-3 112/leu2-3 112 ura3-52/ura3-52</i>	LMDWLU
RR232	<i>ure2Δ</i>	<i>Mata/MATα SUP70/SUP70 ura3-52/ura3-52 leu2-3 112/leu2-3 112 ade1-1/ADE1 ure2::kanMX/ure2::KanMX</i>	LMDWLU
RR234	<i>sup70-65, ure2Δ</i>	<i>MATa/MATα sup70-65/sup70-65 leu2-3 112/leu2-3 112 ura3-52/ura3-52 ure2::KanMX/ure2::KanMX</i>	LMDWLU
TB123	Wild-type <i>Gln3-Myc¹³</i>	<i>MATa leu2-3 112 ura3-52 rme1 trp1 his4 GAL⁺ HMLa GLN3-MYC¹³[KanMX]</i>	TB123
TB136-2a	<i>sit4Δ</i> <i>Gln3-Myc¹³</i>	<i>MATa leu2-3 112 ura3-52 rme1 trp1 his4 GAL⁺ HMLa GLN3-MYC¹³[KanMX], sit4::kanMX</i>	TB123
FV063	Wild-type <i>Gat1-Myc¹³</i>	<i>MATa leu2-3 112 ura3-52 trp1 his3 rme1 HMLa GAT1-MYC¹³[HIS3]</i>	TB50
FV066	<i>sit4Δ</i> <i>Gat1-Myc¹³</i>	<i>MATa leu2-3 112 ura3-52 trp1 his3 rme1 HMLa GAT1-MYC¹³[HIS3] sit4::kanMX</i>	TB50

the indicated nitrogen source at a final concentration of 0.1%. Leucine (120 μ g/ml), histidine (20 μ g/ml), tryptophan (20 μ g/ml), and uracil (20 μ g/ml) were added to the medium as needed to cover auxotrophic requirements. Where indicated, cells were treated with 200 ng/ml rapamycin or 2 mM methionine sulfoximine (Msx), as described earlier (Georis *et al.* 2011). All cells in the LMDWLU genetic background were cultured at the permissive temperature of 22° or the semi-nonpermissive temperature of 30°, as indicated in the text and figure legends. These are the temperatures used in previous investigations of the *sup70-65* mutant (Murray *et al.* 1998; Beeser and Cooper 1999). The latter temperature elicits pseudohyphal-like growth in the *sup70-65* mutant. This overall conclusion (not the data), however, remains controversial (Kemp *et al.* 2013). Strains TB123 and FV063 were cultured only at 30°. It is important to note that most of the experiments reported here were performed in diploid cells of the LMDWLU strain background, whereas haploid cells of the TB123/JK9-3da background were employed in many of our previously reported experiments (Georis *et al.* 2011; Tate and Cooper 2013). Although quantitative differences were noted when results from the two strain backgrounds were compared, qualitative conclusions remained the same.

Strain Construction: Constructions of strains RR232 and RR234 were performed as follows: diploid wild-type LMDWLU and mutant LMD65-1LU strains were first sporulated. *MATa* and *MAT α* spores containing the appropriate auxotrophic and *SUP70-65* or *sup70-65* alleles were chosen from the meiotic products of each sporulation. *URE2* then was deleted from these four strains using standard recombinant technologies and the following primers: 5'-GTTATTAGTCA TATTGTTTAAAGCTGCAAATTAAGTTGTACAC CAAATGCCT TGACAGTCTTGACGTGC-3' and 5'-CCTTCTTTTCTCCTTTT CTTCTTTCTTTCTTGTTTTAAAGCAGC CTTACGCACTTAA CTTTCGCA TCTG-3'. After DNA sequence verification of the *ure2* deletions, *MATa* and *MAT α* representatives of each strain pair were mated to yield homozygous diploid strains RR232 and RR234.

GFP- or Myc¹³-Tagged Gln3 and Gat1 visualization

GFP–GATA factor localization experiments were performed in real time with live cells, as described previously (Tate *et al.* 2010). Strains were transformed with *CEN*-based pRS416-*Gln3*-GFP and pRS416-*Gat1*-GFP, whose construction and detailed validation for normal regulation have been described previously (Liu *et al.* 2003; Giannattasio *et al.* 2005; Tate *et al.* 2010). All transformations were performed at 22°. Only freshly prepared transformants were assayed.

For *Gln3*-Myc¹³ and *Gat1*-Myc¹³ visualization, cell collection and immunofluorescent staining were performed as described previously (Cox *et al.* 2002, 2004; Tate *et al.* 2006, 2009; Georis *et al.* 2008). All cell images, whether derived from Myc¹³- or GFP-tagged proteins, were collected as described earlier (Tate and Cooper 2008; Tate *et al.* 2010). Nomarski images also were collected to permit assessment of the degree to which pseudohyphae were present.

Image Processing

Images were processed for presentation using Adobe Photoshop and Illustrator programs (Adobe Systems, San Jose, CA). Level settings (shadow and highlight only) were altered where necessary to avoid any change or loss in cellular detail relative to what was observed in the microscope; changes were applied uniformly to the image presented and were similar from one image to another. Midtone gamma settings were never altered. These processed images were used for illustrative presentation only, not for scoring GATA factor intracellular distributions.

Determination of intracellular Gln3-Myc¹³ and Gat1-Myc¹³ distributions

Given the subjective nature of image selection and potential errors of interpretation based on them, wherever possible, we quantified intracellular *Gln3* and *Gat1* distributions by manually scoring their localization in as many cells as our samples would permit. Irrespective of the tag used, scoring of *Gln3* and *Gat1* intracellular distribution was performed exclusively using unaltered primary .zvi image files viewed with Zeiss AxioVision 3.0 and 4.8.1 software (Carl Zeiss,

Thornwood, NY). For *Gln3*-Myc¹³ and *Gat1*-Myc¹³, 200 or more cells were scored for each data point. Cells containing *Gln3*-Myc¹³ or *Gat1*-Myc¹³ were classified into one of three categories where the GATA factors were cytoplasmic (cytoplasmic fluorescent material only; red histogram bars), nuclear-cytoplasmic (fluorescent material appearing in both the cytoplasm and co-localizing with DAPI-positive material, DNA; yellow histogram bars), or nuclear (fluorescent material co-localizing only with DAPI-positive material; green histogram bars). Representative “standard” images of these categories are shown in figure 2 of Tate *et al.* (2009) and figure 1 of Tate *et al.* (2010) along with descriptions of how the criteria were applied.

Determination of intracellular *Gln3*-GFP and *Gat1*-GFP distributions

Live, growing cultures were analyzed in real time using *Gln3*-GFP and *Gat1*-GFP. GFP-tagged proteins were required for the live-cell experiments because it was not possible to use the indirect immunofluorescence assay of *Gln3*-Myc¹³ or *Gat1*-Myc¹³ when *sup70-65* mutants were analyzed because the procedures required for sample preparation of Myc¹³ sheered and destroyed pseudohyphae-like cell chains formed in the *sup70-65* mutant.

As reported in earlier time-course experiments (Tate and Cooper 2008; Georis *et al.* 2011), the high background fluorescence that exists with GFP and the very low intracellular concentrations of *Gln3* protein do not permit nuclear-cytoplasmic *Gln3*-GFP localization to be unequivocally distinguished from exclusively nuclear localization in the unmodified .zvi images we used for scoring. Therefore, only two-category scoring was possible, *i.e.*, cells in which *Gln3*- or *Gat1*-GFP was completely cytoplasmic (red bars) *vs.* cells in which *Gln3*- or *Gat1*-GFP was nuclear-cytoplasmic and/or nuclear (yellow bars). As a result, the latter nuclear and nuclear-cytoplasmic categories normally employed in three-category scoring were scored cumulatively as nuclear-cytoplasmic. Hence, the effect of this limitation is that *Gln3*-GFP and *Gat1*-GFP appeared less nuclear than if exclusively nuclear localization could have been scored as a separate category.

Individual images in time-course experiments contained fewer cells because of the low cell densities that were required ($A_{600nm} = 0.02\text{--}0.5$). Any concentration of unfixed cells, irrespective of how gentle the technique, results in transient artifactual movement of *Gln3* (J. J. Tate, K. Cox, and T. G. Cooper, unpublished observations). Therefore, cultures were imaged without concentration. The average number of cells scored per histogram point was 59. Therefore, these time-course data cannot be presumed to possess as high precision as when using indirect immunofluorescence visualization of *Gln3*-Myc¹³ or *Gat1*-Myc¹³, where 200 or more cells were scored, *i.e.*, SD 7–10% (Tate *et al.* 2006, 2010; Tate and Cooper 2008; Rai *et al.* 2013, 2014). One can, however, obtain a reasonable estimate of time-course data's precision by assessing point-to-point variations

after *Gln3*-GFP or *Gat1*-GFP movement within the cell has slowed or ceased (usually the long time points in subsequent data). Experiments were performed two or more times with similar results.

Results

Glutamine tRNA_{CUG} is required for nitrogen starvation-elicited nuclear *Gln3*-GFP localization

To assess the effects of the *sup70-65* mutation on the five identifiable modes of nitrogen-responsive regulation, we chose *Gln3*-GFP localization as the reporter because (1) it is the most comprehensively studied reporter across the entire spectrum of catabolic nitrogen conditions and (2) *Gln3*-GFP localization is a more specific probe of nitrogen-responsive regulation than NCR-sensitive gene expression in that it avoids the complication that nitrogen-responsive mRNA levels derive from the cumulative actions of multiple transcription factors whose actions are not coordinately regulated (Messenguy *et al.* 1991, 2000; Kovari *et al.* 1993a, b; Smart *et al.* 1996; Dubois and Messenguy 1997; Park *et al.* 1999; van der Merwe *et al.* 2001; Rai *et al.* 2004).

Since GFP based GATA factor scoring has not been used previously in the LMDWLU genetic background containing the *sup70-65* mutation, it was necessary to assess whether the GFP fluorescence signals observed depended on GATA factor-containing *CEN* plasmids as opposed to background leak-through fluorescence emanating from the bandwidth of the barrier filter used in the fluorescence microscopy. To this end, wild-type LMDWLU was (Figure 1A, images B and D) or was not (Figure 1A, images A and C) transformed with *Gln3*-GFP. The transformed cultures then were grown in untreated (Figure 1A, images A and B) glutamine medium, where *Gln3* is cytoplasmic, or following rapamycin treatment (Figure 1A, images C and D), where *Gln3* is expected to be partially nuclear. The two cultures were sampled and images obtained at identical exposure times and thereafter processed identically. As a result, images of cells containing the *Gln3*-GFP plasmid were overexposed in order to sufficiently visualize untransformed cells. In the untransformed cultures, only a faint outline of the cells was present (Figure 1A, images A and C). Far stronger fluorescence was observed when cells were transformed with the *Gln3*-GFP plasmid (Figure 1A, images B and D). Further, the fluorescence became nuclear, co-localizing with DAPI-positive material, when the transformed cells were treated with rapamycin (Figure 1A, image D, and Figure 1B, images B and C).

We next assessed the effects of the *sup70-65* mutation on short- and long-term nitrogen starvation. Short-term starvation (~0–4 hr in the haploid TB123 background) exhibits the same *Sit4* phosphatase requirement as growth in a poor nitrogen source such as proline. Short-term starvation is more accurately a condition of nitrogen limitation during which intracellular nitrogen reserves are being consumed but cells still retain the ability to divide. In contrast, long-term

Figure 1 (A) Evaluation of background fluorescence from barrier filter bleed-through during the use of Gln3-GFP to follow the intracellular distribution of Gln3. To assess the amount of this “noise” signal compared with that emanating from authentic Gln3-GFP fluorescence, wild-type (LMDWLU) cells, devoid of or containing Cen-based pRS416-Gln3-GFP, were grown at 22° to mid-log phase in YNB-glutamine medium. Half the culture was left untreated (images A and B), while the other half was treated with rapamycin for ~20 min (images C and D). Photomicrographs of the four cultures then were taken using identical settings and exposure times. Primary .zvi images were prepared for publication using identical settings in Photoshop. These settings were chosen such that cells illuminated by the bleed-through light (images A and C) could be seen. (B) Lack of nuclear Gln3-GFP fluorescence in cell chains of *sup70-65* mutant cells cultured at 30° derives from failure of Gln3-GFP to accumulate in the nuclei rather than the absence of nuclei in cell chains themselves. *sup70-65* mutant (LMD65-1LU) cells containing pRS416-Gln3-GFP were cultured

starvation (occurs after about 4 hr of starvation in haploid TB123) is *Sit4* independent and occurs in parallel with cells G₁ arresting as internal nitrogen reserves are exhausted (Tate and Cooper 2013). Gln3-GFP localization in wild-type (LMDWLU) cells responded similarly to short- and long-term nitrogen starvation at both 22° and 30°. Gln3-GFP was largely cytoplasmic in unstarved ammonia-grown cells (Figure 2, A and B, 0 time point, red bars). Within 12 min (0.2 hr) of the cells being transferred to nitrogen-free medium, Gln3-GFP started relocating to the nucleus (Figure 2, A and B, yellow bars). Relocation of Gln3-GFP to the nucleus continued to increase, with nearly all the cells being scored as nuclear-cytoplasmic by 3–4 hr, the time at which long-term starvation sets in (Tate and Cooper 2013) (Figure 2, A and B, yellow bars).

The response of *sup70-65* mutant cells to short- and long-term nitrogen starvation at 22° was similar to that of the wild-type cells (Figure 2A vs. Figure 2C). In sharp contrast, Gln3-GFP totally failed to relocate to the nuclei of *sup70-65* mutant cell cultures at 30°. It remained staunchly cytoplasmic in all cells following the onset of nitrogen starvation irrespective of its duration (Figure 2D). This clearly indicated that relocation of Gln3-GFP from the cytoplasm to the nucleus in response to short- and long-term nitrogen starvation absolutely required the presence of unaltered glutamine tRNA_{CUG}.

Initial characterization of the *sup70-65* mutant showed it to exhibit constitutive pseudohyphal formation at 30° (Murray *et al.* 1998), although whether the cells were forming true pseudohyphae has been contested recently (Kemp *et al.* 2013). Since the formation of pseudohyphae and nuclear Gln3 localization are accepted to respond in parallel to nitrogen starvation, sequestration of Gln3 in the cytoplasm of 30°-grown *sup70-65* mutant cells forming pseudohyphae was paradoxical. Therefore, we monitored the formation of pseudohyphae-like chains of cells (cell chains; see *Discussion*) throughout the preceding experiment. Wild-type cells did not form cell chains at either temperature irrespective of whether or not they were nitrogen starved (Figure 2, A and B, images). In contrast, cell chain formation in the *sup70-65* mutant correlated with the culture temperature. At 22°, no cell chains were detected in either ammonia-grown or nitrogen-starved *sup70-65* mutant cells (Figure 2C, images). At 30°, cell chain formation was extensive in mutant cultures whether or not they were nitrogen starved (Figure 2D, images), thus confirming the mutant’s earlier characterization (Murray *et al.* 1998).

Together these data indicated that (1) neither short- nor long-term nitrogen starvation was sufficient to elicit cell

at 22° or 30° in YNB-glutamine medium to mid-log phase. At that time, rapamycin was added to cells cultured at each temperature, and images were collected ~20 min later. DAPI was added 10 min before imaging. Nomarski images also were collected to permit assessment of the degree to which cell chains were present.

Figure 2 Glutamine tRNA_{CUG} is required for nuclear Gln3-GFP localization in response to short- and long-term nitrogen starvation. Wild-type (LMDWL1U) and *sup70-65* (LMD65-1LU) Gln3-GFP transformants were cultured in YNB-ammonia medium at 22° (A and C) or 30° (B and D) to mid-log phase. These cultures were sampled six times over 20–26 min (only the first of these data points is presented in the figures, 0 hr). The cells then were transferred to nitrogen-free medium at the same temperature, after which 24 timed samples were collected and assayed for Gln3-GFP localization over the next 6 hr, a time previously demonstrated to achieve nuclear Gln3 localization in response to long-term nitrogen starvation. Data from 10 of the 24 timed samples in each experiment were not presented in the figures to reduce the apparent density/complexity of the data to be evaluated. It is important to emphasize, however, that the values of the omitted data points did not differ from those flanking them and that data from the same time points were omitted in the graphs of each experiment. This approach was used throughout the work whenever long-time-course experiments were performed. Samples were prepared for microscopic examination as described in *Materials and Methods*. The distribution of Gln3-GFP for each sample then was determined using two-category scoring as described in *Materials and Methods*. Red bars indicate exclusively cytoplasmic fluorescence, whereas yellow bars indicate fluorescence in the nucleus or in both the nucleus and cytoplasm. Inability to unambiguously distinguish exclusively nuclear from nuclear-cytoplasmic fluorescence is the reason these categories were combined. See *Materials and Methods* for a detailed explanation of the scoring procedures and criteria. Representative images of the cultures for each of the conditions appear above the histograms.

chain formation in wild-type cells irrespective of the temperature at which starvation was imposed, (2) the *sup70-65* mutation had not reverted, a common problem with suppressor mutations, and (3) cell chain formation negatively correlated with nuclear Gln3-GFP (scored as nuclear-cytoplasmic) localization in nitrogen-starved cells.

Glutamine tRNA_{CUG} is required for nuclear Gln3-GFP localization in cells provided with a poor nitrogen source or treated with rapamycin

Surprised by and skeptical of the preceding results, we further tested the conclusions by analyzing steady-state cultures

provided with a poor nitrogen source (proline), a derepressive condition that also elicits nuclear Gln3 localization (Cooper 1982). At 22°, Gln3-GFP was substantially nuclear-cytoplasmic in both wild-type and *sup70-65* mutant cells (Figure 3, A–C). At 30°, Gln3-GFP was again staunchly restricted to the cytoplasm of nearly all *sup70-65* mutant cells but not the wild-type cells (Figure 3, A–C). Therefore, these results supported the conclusion reached in the short-term nitrogen-starvation experiment.

Since nuclear Gln3 localization during short-term nitrogen starvation or growth with a derepressive nitrogen source (proline) similarly exhibits a Sit4 phosphatase requirement but no requirement for PP2A phosphatase (Beck and Hall

Figure 3 (A–C) Glutamine tRNA_{CUG} is required for nuclear Gln3-GFP localization in cells growing with a poor nitrogen source (proline). The strains [wild-type (LMDWLU) and *sup70-65* (LMD65-1LU) Gln3-GFP transformants] were cultured to mid-log phase in YNB-proline medium at 22° or 30°. Representative images of the cultures for each of the conditions appear to the left of the histograms. (D–F) Glutamine tRNA_{CUG} is required for nuclear Gln3-GFP localization in response to rapamycin treatment. Wild-type (LMDWLU) and *sup70-65* (LMD65-1LU) Gln3-GFP transformants were cultured in YNB-glutamine medium at 22° or 30° to mid-log phase and sampled for assay. Rapamycin then was added and the cultures sampled again. Representative images of the cultures for each of the conditions appear above the histograms.

Figure 4 Glutamine tRNA_{CUG} is partially required for nuclear Gln3-GFP localization in response to Msx treatment. Wild-type (LMDWL1U) and *sup70-65* (LMD65-1LU) Gln3-GFP transformants were cultured in YNB-ammonia medium at 22° (A and C) or 30° (B and D) to mid-log phase. These cultures were sampled six times over approximately 20 min. Msx then was added to each culture, and sampling continued as indicated for approximately 1 hr (14–16 samples per condition). The distribution of Gln3-GFP for each sample then was determined as described in Figure 2. Representative images of the cultures for each of the conditions appear above the histograms.

1999; Bertram *et al.* 2000; Tate and Cooper 2013), we assessed the glutamine tRNA_{CUG} requirement for a response to rapamycin addition. In this situation, nuclear Gln3-GFP localization requires both PP2A and *Sit4* (Tate *et al.* 2009; Tate and Cooper 2013). Rapamycin elicited strong nuclear-cytoplasmic Gln3 localization in glutamine-grown wild-type cells at either 22° or 30° (Figure 3, D and F). In contrast, nuclear-cytoplasmic Gln3-GFP localization in rapamycin-treated *sup70-65* mutant cells was highly temperature dependent. Its

localization was the same as in wild-type cells at 22°, highly nuclear-cytoplasmic (Figure 3, E and F). At 30°, Gln3-GFP was restricted to the cytoplasm of rapamycin-treated cells (Figure 3, E and F). Cell chain formation occurred only in *sup70-65* mutant cells cultured at 30°, the only condition where Gln3-GFP did not enter the nucleus (Figure 3E, images).

The rapid rapamycin response permitted the use of DAPI staining (*in vivo* nuclear visualization with DAPI is very short-lived) to answer an additional important question:

Figure 5 Four or more cell divisions at 30° are required for *sup70-65* mutant cells to lose their ability to relocate Gln3-GFP from the cytoplasm to the nucleus in response to rapamycin treatment. Wild-type (LMDWLU) and *sup70-65* (LMD65-1LU) Gln3-GFP transformants were cultured to mid-log phase in YNB-glutamine medium at 22° (A–H). These are designated as overnight cultures. At that time, two aliquots were removed from each culture. One aliquot of each strain was left untreated (A–D), while rapamycin was added to the other (E–H). The aliquots were sampled for ~30 min to determine the localization of Gln3-GFP. The untreated wild-type and mutant overnight cultures (22°) then were used to inoculate five cultures each of fresh 30° YNB-glutamine medium at low cell density ($A_{600\text{nm}} = 0.02$). Absorbances of the cultures were monitored, and at each doubling, wild-type and mutant cultures were sampled for Gln3-GFP localization (data not shown). Rapamycin then was added to the cultures, and sampling continued for 40–50 min. Multiple sampling ensured that we obtained representative observations of Gln3-GFP behavior despite the fact that the time Gln3-GFP remained in the nuclei of rapamycin-treated cells varied. Samples were prepared for mi-

was the absence of nuclear Gln3-GFP in cell chains caused by (1) a lack of nuclei in the chains of cells or (2) the lack of Gln3 accumulation in their nuclei? We simultaneously followed Gln3-GFP and DAPI fluorescence in rapamycin-treated *sup70-65* mutant cells at 22° and 30° (Figure 1B). DAPI-positive material was clearly present in the cells (22°) or cell chains (30°) of both samples, indicating that the absence of nuclear Gln3-GFP localization derived from a lack of nuclear Gln3-GFP accumulation, not an absence of nuclei.

Glutamine tRNA_{CUG} is partially required for nuclear Gln3-GFP localization following addition of Msx

A fourth method of eliciting nuclear Gln3 localization is by treating cells with the glutamine synthetase inhibitor Msx. Therefore, we treated 22°- and 30°-grown wild-type cells with Msx and observed that over the course of an hour, Gln3-GFP relocated to the nuclei of most cells, resulting in its localization being scored as predominantly nuclear-cytoplasmic (Figure 4, A and B). When *sup70-65* mutant cells were cultured at 22°, a similar if not stronger nuclear Gln3-GFP response was observed (Figure 4C). In contrast, Gln3-GFP relocated only weakly to the nuclei of *sup70-65* mutant cells when Msx was added to cultures grown at 30° (Figure 4D). Further, the time required for limited Gln3-GFP nuclear-cytoplasmic localization to occur increased substantially when compared with *sup70-65* mutant cells grown at 22° (Figure 4C vs. Figure 4D). Formation of cell chains in 30° cultures of *sup70-65* mutant cells was not affected by Msx addition despite the fact that Gln3-GFP relocated to the nuclei of ~40% of the cells. These experiments cumulatively demonstrated that glutamine tRNA_{CUG} function was central to Gln3 nuclear entry irrespective of the physiological condition employed to elicit it.

Alteration of glutamine tRNA alone is insufficient to elicit the *sup70-65* phenotypes

To determine whether glutamine tRNA_{CUG} also was required to retain as well as relocate Gln3-GFP to the nucleus, we cultured wild-type and *sup70-65* mutant cells to mid-log phase ($A_{600\text{nm}} = 0.5$) in ammonia medium at 22° and then transferred them to nitrogen-free medium for 4 hr, thus permitting Gln3-GFP to relocate to the nucleus (supporting information, Figure S1, A and B, left sides). We then increased the temperature of both cultures to 30° (Figure S1, A and B, right sides). We anticipated that the ability of Gln3-GFP to remain in the nucleus would be lost and accompanied by the appearance of cell chains in the *sup70-65* mutant within a short time after increasing the temperature of the culture. Instead, 4 hr after the temperature was increased to 30°, Gln3-GFP continued to be highly nuclear-cytoplasmic in the vast majority of wild-type and *sup70-65*

croscopic examination as described in *Materials and Methods*. Nomarski images also were collected to permit assessment of the degree to which cell chains were present.

Figure 6 Only 0.5–1.0 generation is required after shifting a *sup70-65* mutant culture from 30° to 22° to reacquire wild-type nuclear Gln3-GFP localization in response to rapamycin treatment. Wild-type (LMDWLU) and *sup70-65* (LMD65-1LU) Gln3-GFP transformants were cultured to mid-log phase in YNB-glutamine medium at 30° (A–H). These are designated as overnight cultures. At that time, two aliquots were removed from each overnight culture. One aliquot of each strain was left untreated (A–D), while rapamycin was added to the other. After incubating these aliquots for ~20–30 min, samples were taken to determine the localization of Gln3-GFP. The untreated wild-type and mutant overnight cultures (30°) then were used to inoculate four cultures each of fresh 22° YNB-glutamine medium at low cell density ($A_{600\text{ nm}}$ = 0.02). Absorbances of the cultures were monitored, and at each doubling, wild-type and mutant cultures were sampled for Gln3-GFP localization (data not shown). Rapamycin then was added to the cultures, and sampling continued for 40–50 min. Multiple sampling ensured that we obtained representative observations of Gln3-GFP behavior despite the fact that the time Gln3-GFP remained in the nuclei of rapamycin-treated cells varied. Samples were prepared for microscopic examination as described in *Materials and Methods*. Nomarski images also were collected to permit assessment of the degree to which cell chains were present. To more accurately represent variation that occurs in the cultures, two sets of images are presented for each indicated absorbance (0.031: I–L; 0.045: M–P; 0.058: Q–T; 0.080: U–X). Images I–X were obtained with rapamycin-treated cultures.

mutant cells (Figure S1). Paralleling the Gln3 response, none of the cells formed cell chains (data not shown). To assess whether we had merely misjudged the time required to abolish maintenance of nuclear/nuclear-cytoplasmic

Gln3-GFP localization and form cell chains, we left the cultures incubating at 30° overnight. The next morning, 19 hr (1154–1159 min) after the temperature had been increased, we assayed Gln3-GFP localization again and found that nothing had changed: it remained nuclear-cytoplasmic (Figure S1). This occurred despite the fact that these cells had been cultured at 30° for approximately the same length of time as *sup70-65* mutant cultures grown up at 30° from a small starting inoculum, the condition in which Gln3-GFP was absolutely sequestered in the cytoplasm.

Concerned that the protocol we used had perhaps caused Gln3-GFP to become irreversibly stuck in the nucleus, we added glutamine (0.1% final concentration) to the preceding nitrogen-starved cultures and assayed them again. Within 3 min, Gln3-GFP completely relocated to the cytoplasm of both wild-type and *sup70-65* mutant cells (Figure S1, +Gln). Gln3 had not lost its ability to exit the nucleus in either wild-type or *sup70-65* mutant cells provided with a good nitrogen source. Control experiments demonstrated that the outcomes were the same whether the temperature was shifted to 30° before or after nuclear-cytoplasmic Gln3 localization was elicited experimentally (data not shown). Additional control experiments, including medium swaps, indicated that the failure of Gln3-GFP to leave the nuclei of *sup70-65* mutant cells shifted to 30° did not derive from changes in the medium (data not shown).

Four or more cell divisions required to acquire the *sup70-65* phenotypes at 30° but only 1.5 generations to reacquire the wild-type phenotype at 22°

The preceding experiments clearly indicated that increasing the temperature and, by inference, altering the glutamine tRNA_{CUG} molecule were insufficient to elicit the *sup70-65* phenotypes at 30°. However, growth of *sup70-65* mutant cells at 30° from a small starting inoculum was sufficient. This suggested that the concentration of a functional component, either a complex of glutamine tRNA_{CUG} with another molecule or another molecule whose production required native glutamine tRNA_{CUG}, was being decreased as a result of cell division. This reasoning prompted the following question: how many divisions at 30° are actually required to achieve the mutant phenotype?

To answer this question, we grew wild-type and *sup70-65* mutant cells overnight from a small inoculum ($A_{600\text{ nm}}$ = 0.02) in glutamine medium at 22° to mid-log phase ($A_{600\text{ nm}}$ = 0.5). Under these conditions, *sup70-65* mutant cells exhibited a wild-type phenotype; i.e., there were no cell chains, and Gln3-GFP was nuclear-cytoplasmic in most rapamycin-treated cells (Figure 5, A–H). These wild-type and mutant cultures were used to inoculate five identical fresh aliquots of 30° medium for each strain. The 10 resulting aliquots then were cultured at 30° for 1–4.5 generations. At the end of each successive generation, we added rapamycin to one of the wild-type and mutant aliquots and assayed the ability of Gln3-GFP to relocate to the nuclei of these rapamycin-treated cells. Assays were performed at multiple times for 40–50 min

Figure 7 The constitutive presence of nuclear Gln3 does not prevent cell chain formation. *sup70-65, ure2Δ* mutant (RR234) Gln3-GFP transformants were cultured to mid-log phase in YNB-glutamine medium at 22° and sampled to determine the intracellular distribution of Gln3-GFP, as described in Figure 6, A and B, and Figure 7, A and B. This culture then was used to inoculate fresh 30° YNB-glutamine medium at low cell density ($A_{600\text{ nm}} = 0.02$). The absorbance of the culture was monitored, and at each indicated absorbance, the culture was sampled to determine the

to avoid being misled by potential changes in the kinetics of the rapamycin responses. The initial cell density of the 30° aliquots was $A_{600\text{ nm}} = 0.02$.

For the first generation ($A_{600\text{ nm}} = 0.04$), *sup70-65* mutant cells behaved the same as wild-type cells; i.e., there were no detectable cell chains, and nuclear-cytoplasmic Gln3-GFP localization was observed in nearly all (~80%) the rapamycin-treated cells (Figure 5, I–L). Over the next two generations ($A_{600\text{ nm}} = 0.08$ and 0.16), cell chains remained undetectable in the *sup70-65* mutant cells, but the fraction of rapamycin-treated cells in which Gln3-GFP was nuclear-cytoplasmic decreased markedly (Figure 5, O and P, S and T). *sup70-65* cells moving into the fourth generation ($A_{600\text{ nm}} = 0.32$) began clumping together, and cell chains became apparent (Figure 5, W and X). A half-generation later ($A_{600\text{ nm}} = 0.48$), nuclear Gln3-GFP was no longer evident, whereas cell chain formation was pervasive (Figure 5, AA and BB). Unlike in the *sup70-65* mutant cells, rapamycin treatment elicited nuclear-cytoplasmic Gln3-GFP localization in wild-type cells at each cell division (Figure 5, left two columns). Collectively, these observations suggested that three to four generations were required for the gradual loss of the ability of *sup70-65* mutants to relocate Gln3-GFP into the nuclei of rapamycin-treated cells. Equally important, these losses began to occur prior to detection of cell chains, which occurred most convincingly in the fourth to fifth generation at 30°.

If simple cell division-driven dilution of some cellular component or complex accounted for the delay in onset of the mutant phenotypes, the functional determinant required for rapamycin-elicited nuclear Gln3-GFP localization had to decrease to about 6–12% of its original concentration. A further twofold decrease in this component or, additionally, effective exclusion of Gln3 from the nucleus was required for cell chains to form. If this reasoning was valid, a wild-type phenotype should be much more rapidly reacquired when a small inoculum of *sup70-65* mutant cells precultured at 30° was used to inoculate 22° medium. This was the expectation, because only a small amount of the hypothesized functional glutamine tRNA_{CUG}-dependent determinant (complex or molecule) appeared to be required to support rapamycin-elicited nuclear Gln3-GFP localization.

To test this explanation, we cultured wild-type and *sup70-65* mutant cells overnight from small inocula ($A_{600\text{ nm}} = 0.015$) to mid-log phase ($A_{600\text{ nm}} = 0.55$) at 30°. In contrast with wild-type cells cultured under these conditions, most *sup70-65* mutant cells were clumped, cell chains predominated, and Gln3-GFP largely failed to relocate to the nuclei when the cells were treated with rapamycin (Figure 6, A–H). Samples of the preceding untreated *sup70-65* mutant culture were then inoculated into four aliquots of fresh 22° medium.

localization of Gln3-GFP. Samples were prepared for microscopic examination as described in *Materials and Methods*. Nomarski images also were collected to permit assessment of the degree to which cell chains were present. *0.151 (M and N) was taken the following morning after 11 additional hours of incubation at 30°.

Figure 8 Intracellular Gln3-Myc¹³ localization responds to Sit4-dependent short-term and Sit4-independent long-term nitrogen starvation, whereas intracellular Gat1-Myc¹³ localization moderately responds to only Sit4-dependent short-term starvation and not at all to long-term starvation. Wild-type (TB123, Gln3-Myc¹³ and FV063, Gat1-Myc¹³) and *sit4*Δ (TB136-2a, Gln3-Myc¹³ and FV066, Gat1-Myc¹³) strains were grown to mid-log phase ($A_{600nm} = 0.5$) in YNB-glutamine medium. After sampling (0 time point), the cultures were transferred to nitrogen-free medium, and sampling continued for 10 hr. Samples were prepared for microscopic examination as described in *Materials and Methods*. (A and B) Representative images from which the corresponding histograms were generated. Red bars indicate Gln3-Myc¹³ or Gat1-Myc¹³ indirect immunofluorescence in the cytoplasm only, yellow bars indicate both cytoplasmic and nuclear fluorescence, and green bars indicate fluorescence in the nucleus only. The particular images presented in the figures are those that were the most evaluative for the arguments presented. Histograms depict all the data collected. For example, in A, it is important that Gln3-Myc¹³ was almost completely nuclear in wild-type cells after 4 hr of starvation, whereas in the *sit4*Δ cultures it remained highly cytoplasmic at 4 hr. The use of the TB123 genetic background for this experiment permits more direct comparison with previously published extensive characterizations of Gln3 and Gat1 regulation.

Over the next two generations, at each of the cell densities indicated, rapamycin was added to one of these aliquots, and Gln3-GFP localization was assayed at multiple times for 40–50 min (Figure 6). Two images are presented for each cell density to capture the degree of variation observed.

The initial cell density of the 22° aliquots was $A_{600nm} = 0.02$. Within a half-generation ($A_{600nm} = 0.031$), Gln3-GFP was already nuclear-cytoplasmic in a small percentage of the rapamycin-treated *sup70-65* mutant cells (~20%) (Figure 6, I and K). Though clumped, cells with nuclear-cytoplasmic Gln3-GFP were consistently those at the ends of cell chains or not demonstrably part of cell chains. By the end of one generation ($A_{600nm} = 0.045$), Gln3-GFP was nuclear in most rapamycin-treated cells (Figure 6, M and O). Between the ends of the first and second generations ($A_{600nm} = 0.058$

and 0.080), the culture also was increasingly composed of single budding cells (Figure 6, Q–X). Cells in which rapamycin still failed to elicit nuclear-cytoplasmic Gln3-GFP localization either were associated with cell chains or were enlarged cells with evidence of having been associated with cell chains (Figure 6, Q–T, arrows). As we predicted, rapamycin-elicited nuclear-cytoplasmic Gln3-GFP localization was reacquired in cells transferred from 30° to 22° medium much more rapidly than it was lost in mutant cells transferred from 22° to 30° medium.

The constitutive presence of nuclear Gln3 does not prevent cell chain formation

The preceding experiments demonstrated that the loss of ability for Gln3 to enter the nuclei of rapamycin-treated cells

Figure 9 Glutamine tRNA_{CUG} is required for nuclear Gat1-GFP localization in response to rapamycin treatment. Wild-type (LMDWLU) and *sup70-65* (LMD65-1LU) Gat1-GFP transformants were cultured in YNB-glutamine medium at 22° (A, C, and D) or 30° (B, C, and E) to mid-log phase. The cultures were sampled for assay, and then rapamycin was added to each culture, and sampling continued for 40–50 min. The intracellular distribution of Gat1-GFP for each sample was determined as described in Figure 2.

occurred prior to significant cell chain formation. Further, the reacquisition of the ability of Gln3-GFP to relocate to the nucleus preceded or occurred concomitantly with loss of cell chain formation. This suggested that cell chain formation might be due to the loss of the ability of Gln3 to enter the nucleus in *sup70-65* mutant cells at 30°. Therefore, would cell chain formation still occur in 30°-grown cells if Gln3 was constitutively nuclear?

To answer this question, we deleted *URE2* from both wild-type and *sup70-65* mutant cells, as described in *Materials and Methods*. As expected, Gln3-GFP was constitutively nuclear-cytoplasmic in glutamine-grown *sup70-65, ure2Δ* cells at 22° (Figure 7, A and B; data not shown for wild-type cells). These *sup70-65, ure2Δ* cells then were used to inoculate fresh glutamine medium at a low cell density ($A_{600nm} = 0.02$) and cultured at 30° using the same protocol described in Figure 5.

There was no detectable change in cell morphology for the first generation (Figure 7, C–F). Halfway through the second generation ($A_{600nm} = 0.061$), cell chains were prevalent, and Gln3-GFP was present in the nuclei of most cells situated in chains (Figure 7, G and H). By the end of the second full generation ($A_{600nm} = 0.081$) and halfway into the third generation ($A_{600nm} = 0.122$), there was extensive cell chain formation in nearly all fields viewed (Figure 7, I–L). Now, however, the number of cells in which Gln3-GFP was nuclear began decreasing. We incubated the cultures for an additional 11 hr. By this time, the culture had nearly completed only its third generation ($A_{600nm} = 0.151$) but grew no further. Cell chain formation was extensive, but most cells were now devoid of nuclear-cytoplasmic Gln3-GFP (Figure 7, M and N). Moreover, these cells were quite fragile; often just focusing the microscope oil objective generated sufficient pressure on the cells to rupture them.

These data generated several conclusions. The presence of Gln3-GFP in the nuclei of *sup70-65, ure2Δ* cells did not prevent formation of cell chains. In fact, constitutive nuclear Gln3-GFP substantially shortened the time of cell chain formation from four to five generations in the *sup70-65* mutant strain to about 1.5 generations in the *sup70-65, ure2Δ* double mutant (Figure 5, U–BB, vs. Figure 7, G and H). Finally and importantly, growth of the *sup70-65, ure2Δ* mutant cells at 30° could only be sustained for about three generations. The simultaneous presence of the two mutations exhibited a synthetic loss of ability for continued cell division. This strong synthetic relationship also was observed using plate assays (data not shown).

Rapamycin-elicited nuclear Gat1-GFP localization does not require glutamine tRNA_{CUG}

The preceding experiments focused on the glutamine tRNA_{CUG} requirement for nitrogen-responsive Gln3 localization, which raised the question of whether nuclear Gat1 localization possessed a similar requirement? Although Gln3 and Gat1 are both GATA family transcription activators, they are in some instances regulated quite differently (Georis *et al.*

Figure 10 The *sup70-65* mutation is epistatic to a *ure2Δ* mutation in untreated cells irrespective of whether the reporter is Gat1-GFP or Gln3-GFP. (A and B) Wild-type (LMDWLU), *ure2Δ* (RR232), *sup70-65* (LMD65-1LU), and *sup70-65, ure2Δ* (RR234) Gat1-GFP transformants were grown in YNB-glutamine medium at 22° (A) or 30° (B). These cultures were left untreated (left two columns) or treated with rapamycin (right two columns). The cultures then were sampled during the next 20–35 min of incubation. Multiple sampling ensured that we obtained representative observations of Gat1-GFP behavior despite the fact that the time Gat1-GFP spent in the nuclei of rapamycin-treated cells varied from strain to strain. (C) Wild-type (LMDWLU), *ure2Δ* (RR232), *sup70-65*

2008, 2011; Tate *et al.* 2010). *Gat1* localization is remarkably more responsive to rapamycin treatment than is *Gln3*. Conversely, *Gln3* localization is highly responsive to Msx treatment and NCR, whereas *Gat1* localization is immune to Msx treatment and only modestly relocates to the nuclei of cells grown with a derepressive nitrogen source (e.g., proline). Finally, the *Gln3* responses to Msx treatment and long-term nitrogen starvation exhibit the same lack of *Sit4* and PP2A requirements, but whether or not *Gat1* localization responds to long-term nitrogen starvation is not known.

Together these observations and correlations generated two important testable predictions. The lack of a *Gat1* response to Msx addition predicted that *Gat1* localization either might not respond to *Sit4*-independent long-term nitrogen starvation or might require intact glutamine tRNA_{CUG} to move from the cytoplasm to the nuclei of rapamycin-treated *sup70-65* mutant cells cultured at 30°.

We tested the first of these predictions by comparing intracellular *Gln3*-Myc¹³ and *Gat1*-Myc¹³ localization following the transfer of glutamine-grown wild-type and *sit4Δ* mutant cells to nitrogen-free medium. *Gln3*-Myc¹³ relocated to the nucleus during both *Sit4*-dependent short-term (0–4 hr) and *Sit4*-independent long-term (>4 hr) nitrogen starvation, as reported earlier (Figure 8A) (Tate and Cooper 2013). In contrast, nuclear *Gat1*-Myc¹³ localization responded only modestly to *Sit4*-dependent short-term nitrogen starvation in a manner similar to that observed earlier in proline-grown cells (Tate *et al.* 2010). *Gat1* failed to respond further to long-term nitrogen starvation, remaining substantially cytoplasmic and nuclear-cytoplasmic rather than becoming more highly nuclear in a *Sit4*-independent manner as the time in the nitrogen-free medium progressed and starvation became more severe (Figure 8B). Even after extended starvation, *Gat1*-Myc¹³ remained cytoplasmic in the *sit4Δ* mutants, further indicating that it had responded to short-term starvation/limitation but not to long-term starvation. Parenthetically, the modest cyclic movement of *Gat1*-Myc¹³ in and out of wild-type nuclei over the time course of short-term nitrogen starvation was reproducible, but we do not understand the source or significance of this cyclic movement.

We next queried whether rapamycin-elicited nuclear *Gat1*-GFP localization required glutamine tRNA_{CUG}, as did *Gln3*-GFP. We cultured wild-type and *sup70-65* mutant cells at 22° and 30°, assayed *Gat1*-GFP localization, added rapamycin to the cultures, and re-assayed them thereafter. Rapamycin elicited similar nuclear *Gat1*-GFP localization in both *sup70-65* mutant and wild-type cells irrespective of the temperature at which they were cultured (Figure 9). These data indicated that *Gln3* and *Gat1* localizations responded very differently to the conditions we tested. The requirement of glutamine tRNA_{CUG} for rapamycin-elicited nuclear localization was ex-

quisitely *Gln3*-specific and correlated with the responses of *Gln3* to long-term nitrogen starvation and Msx treatment. Rapamycin-elicited nuclear *Gat1*-GFP localization, in contrast, did not require unaltered glutamine tRNA_{CUG}.

Epistasis relationships of *ure2Δ* and *sup70-65* mutations using *Gat1*-GFP and *Gln3*-GFP as reporters

The differing requirements for nuclear localization of *Gat1*-GFP and *Gln3*-GFP, especially with respect to rapamycin addition, prompted us to query whether glutamine tRNA_{CUG} was participating in nuclear GATA factor localization upstream or downstream of *Ure2*, the protein responsible for maintaining *Gln3* and *Gat1* in the cytoplasm of cells cultured in excess nitrogen. Therefore, we compared the epistasis relationships of the *sup70-65* and *ure2Δ* mutations using *Gat1*-GFP and *Gln3*-GFP as reporters. At 22°, *Gat1*-GFP was mostly cytoplasmic in both glutamine-grown wild-type and *sup70-65* mutant strains (~70%) (Figure 10A, images A and I) and strongly nuclear-cytoplasmic following rapamycin addition (~90–100%) (Figure 10A, images C and K). *Gat1*-GFP was mostly nuclear-cytoplasmic in untreated glutamine-grown *ure2Δ* single and *sup70-65,ure2Δ* double mutants (~80%) (Figure 10A, images E and M) and even more so when these strains were treated with rapamycin (Figure 10A, images G and O).

At 30°, *Gat1*-GFP localization exhibited similar phenotypes in wild-type and *sup70-65* mutant cells; *Gat1*-GFP was cytoplasmic in untreated, glutamine-grown cells (~90–100%) (Figure 10B, images A and I). Here, as at 22°, *Gat1*-GFP localization responded well to rapamycin (~95–100% and ~80% nuclear-cytoplasmic, respectively, for wild-type and *sup70-65* mutant cells) (Figure 10B, images C and K). When *URE2* was deleted, *Gat1*-GFP was again nuclear-cytoplasmic in many of the untreated cells (Figure 10B, image E). However, the response was not nearly as robust as that observed following rapamycin addition (Figure 10B, image G).

In sharp contrast, *Gat1*-GFP was nuclear in only an occasional untreated *sup70-65,ure2Δ* double-mutant cell at 30° (~10–15% nuclear-cytoplasmic) (Figure 10B, image M, arrow). Moreover, when *Gat1*-GFP was observed to be nuclear-cytoplasmic, it was most often in cells that had not formed extensive cell chains. In other words, the *sup70-65* mutation was epistatic to the *ure2Δ* mutation in the vast majority of untreated cells, especially when they had formed cell chains. However, in rapamycin-treated cells at 30°, *Gat1*-GFP was nuclear-cytoplasmic in most of the *sup70-65,ure2Δ* mutant cells (~70–80%) whether or not they exhibited chain cell formation (Figure 10B, image O). Although the rapamycin response was somewhat less robust than that observed with rapamycin-treated wild-type or *ure2Δ* cells, a positive response was clearly present (compare Figure 10B, images C, G, K, and O).

(LMD65-1LU), and *sup70-65,ure2Δ* (RR234) *Gln3*-GFP transformants were grown in YNB-glutamine medium at 30°. The transformant cultures were grown overnight. During that time, the wild-type and single mutant cultures grew to mid-log phase ($A_{600nm} = 0.5$), whereas the double-mutant (RR234) culture divided only ~2.5 times. The latter *sup70-65,ure2Δ* (RR234) culture was sampled again after 19 additional hours of incubation at 30°.

These data argued that nuclear Gat1-GFP localization in response to the loss of Ure2 differed significantly from that elicited by rapamycin treatment. The former response required unaltered glutamine tRNA_{CUG}, whereas the latter did not. The fact that nuclear Gat1 localization in rapamycin-treated sup70-65 mutant cells at 30° did not require glutamine tRNA_{CUG} precluded identification of an epistasis relationship for this response.

When Gln3 was employed as the reporter, Gln3-GFP was cytoplasmic in wild-type and sup70-65 mutant cells and highly nuclear-cytoplasmic in ure2Δ mutant cells (>80%) (Figure 10C, images A–F). In the sup70-65,ure2Δ double mutant, the phenotype was more complex. Gln3-GFP was nuclear-cytoplasmic in some cells (~50–60%) (Figure 10C, image G) but cytoplasmic in others (~40–50%) (Figure 10C, image G). Localization correlated with the degree of cell chain formation. When cell chains were clearly present, Gln3-GFP was cytoplasmic. However, when the cells appeared to be single, budded, and clumped together, as occurred in the upper-right portion of Figure 10C, image G, Gln3-GFP was present in the nuclei of most of the cells.

When evaluating these observations, it is important to keep in mind that the sup70-65,ure2Δ double-mutant cells were only capable of growing for slightly more than three generations. Therefore, there was likely insufficient time for all the cells to form cell chains or to deplete the glutamine-tRNA_{CUG}-dependent component required for nuclear Gln3-GFP localization. If the sup70-65,ure2Δ double-mutant culture was permitted to incubate overnight, i.e., about 16 hr longer, cell chains predominated throughout the culture, and Gln3-GFP was exclusively cytoplasmic (~100%) (Figure 10C, images I and J). These data indicated to us that when sup70-65 mutant cells were grown to the point of extensive cell chain formation, i.e., more than three generations, the sup70-65 mutation was epistatic to the ure2Δ mutation, irrespective of whether Gat1-GFP or Gln3-GFP was employed as the reporter.

Glutamine tRNA_{CUG} is required for nitrogen-responsive nuclear Gat1-GFP localization

The sup70-65,ure2Δ double-mutant epistasis data generated an important and very surprising conclusion. Glutamine tRNA_{CUG} was required for nuclear Gat1-GFP localization that resulted from deletion of URE2, but it was not required if rapamycin was used as the trigger. This indicated that Gat1-GFP localization was subject to both glutamine tRNA_{CUG}-dependent and independent regulation. Since Ure2 has long been associated with NCR-sensitive GATA factor control, these observations generated two testable predictions: nuclear Gat1-GFP localization in response to growth with a poor nitrogen source (proline), i.e., NCR-sensitive regulation, and short-term nitrogen starvation should depend on glutamine tRNA_{CUG}.

We tested the first prediction by following Gat1-GFP localization in cells provided with glutamine or proline as a nitrogen source. In sup70-65 mutant cells cultured at 22°, Gat1-GFP was largely cytoplasmic in glutamine-grown cells and became

substantially nuclear-cytoplasmic when proline was the nitrogen source (Figure 11, A and C). In sharp contrast, Gat1-GFP remained staunchly cytoplasmic in proline-grown sup70-65 mutant cells cultured at 30° (Figure 11, B and C).

Moving to the second prediction, we subjected sup70-65 mutant cells cultured at 22° and 30° to short-term nitrogen starvation over a 4-hr period. Gat1-GFP largely relocated from the cytoplasm to the nuclei of the sup70-65 mutant cells (became nuclear-cytoplasmic) following transfer to nitrogen-free medium at 22° (Figure 11, D and E). At 30°, however, Gat1-GFP remained securely sequestered in the cytoplasm of sup70-65 mutant cells transferred to nitrogen-free medium (Figure 11, F and G), thus positively fulfilling the second prediction. Together these experiments demonstrated that nuclear Gat1-GFP localization in response to deleting URE2, growth in derepressive medium, and short-term nitrogen starvation all required glutamine tRNA_{CUG} even though a similar Gat1-GFP outcome in response to rapamycin treatment did not.

Discussion

The most important conclusions of the data presented in this article are (1) unaltered glutamine tRNA_{CUG} is required for normal catabolic nitrogen-responsive GATA factor regulation, (2) rapamycin-elicited nuclear Gln3 but not Gat1 localization requires tRNA_{CUG}, and (3) Gat1 localization does not respond to long-term Sit4-independent nitrogen starvation, whereas that of Gln3 does. Since Gln3 is not demonstrably controlled by the leucyl tRNA synthetase-Gtr-Ego-TorC1 activation pathway, these data raise the possibility that more than one tRNA-dependent mechanism is required to achieve overall nitrogen-responsive regulation in *S. cerevisiae*. Consistent with this proposal, we have shown previously that substitution of three serine residues in a short (~17 amino acids) putative α -helix in Gln3 abolishes its ability to interact with Tor1 but only partially eliminates Gln3 cytoplasmic sequestration in nitrogen-rich conditions (Rai *et al.* 2013). This indicates that another regulatory system is responsible for the remainder of the Gln3 cytoplasmic localization.

We have demonstrated that unaltered glutamine tRNA_{CUG} is absolutely required for nuclear Gln3-GFP localization irrespective of the different physiological conditions eliciting it: short- and long-term nitrogen starvation, growth in derepressive conditions (i.e., with proline as nitrogen source), and treatment of cells with rapamycin or Msx and even in a ure2Δ mutant (Figure 12A). These correlations prompt a basic question: does the rare glutamine tRNA_{CUG} participate as a primary component in sensing the metabolic signal of nitrogen excess/limitation or in the more downstream response to that metabolic sensing. In addressing this question, it is important to recall that five distinct physiological situations with equally distinct phosphatase requirements elicit nuclear Gln3 localization, which argues in favor of multiple distinct mechanisms through which the cell senses its nitrogen physiology (Tate and Cooper 2013). The fact that Gln3 responses to all five physiological conditions were summarily abolished when

Figure 11 Glutamine tRNA_{CUG} is required for nuclear Gat1-GFP localization in cells growing with a poor nitrogen source (proline) and in response to short-term nitrogen starvation. (A–C) *sup70-65* mutant cells were grown at 22° and 30° in YNB-glutamine or YNB-proline medium. (D–G) *sup70-65* mutant cells were grown at 22° and 30° in YNB-glutamine

glutamine tRNA_{CUG} was altered suggests that the glutamine tRNA-dependent component, be it a complex involving tRNA_{CUG} or a protein whose production is particularly sensitive to the availability of functional tRNA_{CUG}, more likely participates in or regulates a step in the downstream response to nitrogen availability than the mechanisms sensing it. This interpretation also correlates with (1) epistasis data indicating that the glutamine tRNA_{CUG}-dependent component most likely functions downstream of *Ure2* and (2) the generally accepted view that dissociation of *Gln3* from *Ure2* is immediately proximal to *Gln3* binding to α /*Srp1* and subsequently entering the nucleus (Carvalho *et al.* 2001; Carvalho and Zheng 2003).

Another of our observations further supports this interpretation and potentially narrows down the site at which *Ure2* functions. The pertinent observation is that *ure2Δ* and *sup70-65* mutations exhibit a synthetic no-growth phenotype. The single mutants grow reasonably well at 30°, whereas the double mutants grow for only three generations after being shifted to 30° before growth ceases. This is roughly the same amount of time required for nuclear *Gln3* entry to be lost when a *sup70-65* single mutant is shifted to 30°. Importantly, cells with deletions of *URE2* also exhibit synthetic loss of growth with mutations in VPS Class C and D proteins (*vps3*, *vps34*, *vps45*, *pep3*) that participate in endomembrane vesicular trafficking (Fayyadkazan *et al.* 2014). These synthetic interactions, along with the observations that (1) *Gln3*-Myc¹³ associates with a tubular membranous structures as it enters and exits the nucleus (Cox *et al.* 2002, 2004) and (2) *Gln3*-Myc¹³ partially co-localizes with *Vps10*, a late-Golgi/endosomal marker (Puria *et al.* 2008; Nickerson *et al.* 2009; Kingsbury *et al.* 2014), suggest that the component or complex that is specifically sensitive to glutamine tRNA_{CUG} alteration and required for nuclear *Gln3* entry may well be associated with the membranous system *Gln3* uses when entering and exiting the nucleus.

The participation of glutamine tRNA_{CUG} in protein synthesis makes it equally plausible to argue that it is the general slowing of protein synthesis *per se* that is responsible for the loss of nuclear *Gln3* entry and cell chain formation in the *sup70-65* mutants. Four observations argue against this interpretation. (1) The impact of inhibiting protein synthesis on *Gln3* localization has been studied in the past, and it does not respond as observed in the *sup70-65* mutants (Tate and Cooper 2013). (2) The second, more prevalent

medium. After sampling the glutamine-grown cultures (0 time points), the cells were transferred to nitrogen-free YNB medium, and sampling was continued for 4 hr. The experimental and data-presentation formats are as described in Figure 2. It is important to note that the LMDWLU diploid genetic background used in this figure is somewhat more sensitive to nitrogen limitation than the haploid TB123 background used in Figure 9. Beyond potential differences in the genetic backgrounds themselves, this probably derives from the fact that the demands for biosynthetic precursors are greater in diploid cells, and hence their response is greater when nitrogen flux decreases.

Figure 12 Catabolic nitrogen-responsive regulation coalesces at the requirement of glutamine tRNA_{CUG}. This figure presents a schematic summary of data demonstrating the conditions under which glutamine tRNA_{CUG} is required for nuclear localization of Gln3 and Gat1. Rapamycin-elicited nuclear Gat1-GFP localization, however, does not require tRNA_{CUG}.

glutamine tRNA_{CAG} is able to decode CUG codons; otherwise, protein synthesis and cell growth would terminate when *sup70-65* mutant cells are shifted to 30°. Although there is disagreement over the degree of efficiency with which CAG- and CAA-rich mRNAs can be translated in the *sup70-65* mutants because of the fact that different heterologous reporters were used in the experiments addressing this question (synthesis of *Escherichia coli* β-galactosidase vs. firefly luciferase to which 5–40 CAG or CAA codons were added to the 5' termini of the mRNAs), it was the degree of translation, not its absence, that was at issue (Murray *et al.* 1998; Kemp *et al.* 2013). (3) The *sup70-65* mutants retain their ability to serve as a suppressor (Weiss and Friedberg 1986; Murray *et al.* 1998). (4) The kinetics with which nuclear Gln3 entry is abolished when *sup70-65* mutant cells are shifted from 22° to 30° (over four generations) and reacquired when 30°-grown cells are returned to 22° (about one-half to one generation) indicate that only a small amount of the component required for nuclear Gln3 entry is necessary and can be relatively quickly produced at 22°. However, one must concede that this is negative circumstantial evidence. The most likely way in which tRNA_{CUG} alteration would decrease the levels of gross protein synthesis to a point of producing insufficient amounts of the component or complex needed to generate the observed Gln3 response kinetics would be if that production possessed an exquisitely specific and concentration-sensitive codon bias for glutamine tRNA_{CUG}.

Glutamine tRNA_{CUG} is required for Ure2-related and nitrogen-responsive Gat1 localization but not that associated with rapamycin treatment

A second major outcome of this work is the demonstration of newly discovered ways in which Gln3 and Gat1 are each uniquely subjected to forms of regulation not shared by the other. Gat1 possesses a strong glutamine tRNA_{CUG}-independent response to rapamycin, as well as insensitivity to Msx treatment and long-term nitrogen starvation. In contrast, Gln3 is highly sensitive to long-term nitrogen starvation or Msx treatment, and all of its responses, including that to rapamycin treatment, are highly dependent on tRNA_{CUG}.

The requirement of tRNA_{CUG} observed for nuclear Gat1 localization centers on whether or not the condition eliciting that nuclear localization is related to nitrogen catabolism. Conditions directly associated with controlling the catabolic production of nitrogenous precursors are regulated by Ure2 and are now shown to require unaltered glutamine tRNA_{CUG} for both Gln3 and Gat1 (Figure 12). In contrast, the physiological condition most directly associated with responding to the use of nitrogenous precursors (rapamycin inhibition of TorC1) is, for Gat1 at least, distinguishable from its regulation by Ure2 (Figure 10B) in that the rapamycin response is independent of glutamine tRNA_{CUG} (Figure 12B). This observation suggests that the degree to which Ure2 is involved in the chemical events whereby rapamycin elicits nuclear Gat1 localization may be much smaller than previously accepted and may be quite different from those associated with nuclear Gln3 entry. Finally, the differences we have documented in the requirements for nuclear Gln3 and Gat1 localization will require some reevaluation of GATA factor-dependent transcription data because it has not been shown previously that Gat1 localization does not respond to long-term nitrogen starvation.

Relationship of nuclear Gln3 and Gat1 localization to cell chain formation and NCR-sensitive transcription

Because the formation of pseudohyphae and nuclear localization of Gln3 normally occur in adverse nitrogen environments, one would *a priori* expect them to respond in parallel to alteration of glutamine tRNA_{CUG}. However, as far as we could determine, it did not matter whether or not Gln3 was nuclear for *sup70-65* mutant cell chains to form at 30°. Nuclear Gln3-GFP localization began disappearing about a generation or so before cell chains began appearing in the culture, and yet they continued to be formed even when Gln3 was constitutively nuclear, *i.e.*, in a *ure2Δ* mutant. In the end, however, conditions that bring about the formation of cell chains also precluded nuclear Gln3-GFP localization, and when those conditions were reversed, Gln3-GFP reappeared in the nuclei and cell chains disappeared. The most likely explanation to rectify the paradoxical behavior of Gln3 localization and cell chain formation is to speculate that while both effects are triggered by a common process,

beginning with the alteration of glutamine tRNA_{CUG}, their occurrence is probably independent of each other. This reasoning and the observation that cell chains did not form in nitrogen-starved *sup70-65* mutant cells at 22° support the conclusions of Kemp *et al.* (2013) that the *sup70-65* cell chains are not true pseudohyphae.

Finally, it is useful to rectify present and previous results (Beeser and Cooper 1999). The purpose of the 1999 paper was to determine whether or not catabolic *CAR1* (arginase) expression, reported to be constitutive in parallel with pseudohyphal formation in *sup70-65* mutants (Murray *et al.* 1998), remained NCR sensitive at 30°. We found that steady-state *CAR1* and *DAL5* (allantoate permease) mRNA levels were both NCR sensitive, with *CAR1* expression being greater than wild type, whereas *DAL5* was far less. Here, in contrast, we have shown that *Gln3* and *Gat1*, the activators of NCR-sensitive transcription, are cytoplasmic at 30° in *sup70-65* mutants irrespective of the nitrogen source, leading to the expectation that very little NCR-sensitive transcription should exist. In rectifying the two sets of data, it is first important to recognize that radioactive assays of concentrated RNA isolated from a total population of cells are far more sensitive than and somewhat different from assays that evaluate the behavior of single cells at a specific instant in time (the time of quenching). This likely explains our ability to detect NCR-sensitive transcription in *sup70-65* mutants. From this perspective, the very low levels of *DAL5* mRNA relative to wild type can be explained by the present demonstration that the *DAL5* transcription activators *Gln3* and *Gat1* were predominantly cytoplasmic. The high levels of *CAR1* mRNA likely derived from two sources. First, the *CAR1* promoter contains multiple demonstrably functional *cis*-acting elements (12 sequences), including three *CAR1* UAS_i elements that mediate strong arginine-induced *CAR1* transcription and one that is homologous to a *Gln3/Gat1* binding site (Kovari *et al.* 1990, 1993a, b; Viljoen *et al.* 1992). The vacuole contains millimolar levels of arginine that are mobilized during starvation; this induces arginase (*CAR1*) production, accounting for the high levels of *CAR1* expression observed (Wiemken *et al.* 1970; Wiemken and Durr 1974; Zacharski and Cooper 1978; Sumrada and Cooper 1978). This leaves the necessity of explaining how the *sup70-65* mutant cells were starving if they were growing in minimal proline medium at 30°. *Gln3* and *Gat1*, being predominantly cytoplasmic at 30°, were largely unavailable to support transcription of the NCR-sensitive *PUT* genes required to transport and catabolize proline, thus compromising its use even as a poor nitrogen source (Daugherty *et al.* 1993).

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Supporting Information

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Nitrogen Starvation and TorC1 Inhibition Differentially Affect Nuclear Localization of the Gln3 and Gat1 Transcription Factors Through the Rare Glutamine tRNA_{CUG} in *Saccharomyces cerevisiae*

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

Figure S-1

Figure S1. Increasing the temperature at which *sup70-65* cells are cultured (30°) is alone insufficient to result in loss of nuclear Gln3-GFP localization in response to nitrogen starvation. Wild type (LMDWLU) and *sup70-65* (LMD65-1LU) Gln3-GFP transformants were cultured in YNB-ammonia medium at 22° to mid-log phase (*Panels A and B*). These cultures were sampled six times over 23-28 mins and then transferred to nitrogen-free medium at the same temperature (22°) after which sampling was continued as indicated for four hrs; 13 samples assayed. This was sufficient time for Gln3-GFP to move from the cytoplasm to the nucleus in response to nitrogen starvation. At this time, the temperature of the cultures was quickly increased to 30° and sampling continued for another four hrs; 12 samples assayed (*Panels A and B*, right half). The cultures were permitted to continue growing at 30° overnight (~1154-1159 mins total) and again sampled twice. Glutamine was then added to each of the cultures at a final concentration of 0.1% and sampling continued for another ~10 mins. Samples were prepared for microscopic examination as described in Materials and Methods. The distribution of Gln3-GFP for each sample was then determined using two category scoring as described in Figure 2.