Integration of General Amino Acid Control and Target of Rapamycin (TOR) Regulatory Pathways in Nitrogen Assimilation in Yeast*

Kirk A. Staschke‡, Souvik Dey‡, John M. Zaborske‡, Lakshmi Reddy Palam‡, Jeanette N. McClintick‡, Tao Pan†, Howard J. Edenberg‡, and Ronald C. Wek‡†

From the ‡Department of Biochemistry and Molecular Biology, Indiana University School of Medicine, Indianapolis, Indiana 46202 and the †Department of Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois 60637

Two important nutrient-sensing and regulatory pathways, the general amino acid control (GAAC) and the target of rapamycin (TOR), participate in the control of yeast growth and metabolism during changes in nutrient availability. Amino acid starvation activates the GAAC through Gcn2p phosphorylation of translation factor eIF2 and preferential translation of GCN4, a transcription activator. TOR senses nitrogen availability and regulates transcription factors such as Gln3p. We used microarray analyses to address the integration of the GAAC and TOR pathways in directing the yeast transcriptome during amino acid starvation and rapamycin treatment. We found that GAAC is a major effector of the TOR pathway, with Gcn4p and Gln3p each inducing a similar number of genes during rapamycin treatment. Although Gcn4p activates a common core of 57 genes, the GAAC directs significant variations in the transcriptome during different stresses. In addition to inducing amino acid biosynthetic genes, Gcn4p in conjunction with Gln3p activates genes required for the assimilation of secondary nitrogen sources such as γ-aminobutyric acid (GABA). Gcn2p activation upon shifting to secondary nitrogen sources is suggested to occur by means of a dual mechanism. First, Gcn2p is induced by the release of TOR repression through a mechanism involving Sit4p protein phosphatase. Second, this eIF2 kinase is activated by select uncharged tRNAs, which were shown to accumulate during the shift to the GABA medium. This study highlights the mechanisms by which the GAAC and TOR pathways are integrated to reconfigure the transcriptome to alleviate nutrient stress. In the yeast Saccharomyces cerevisiae, eIF2α phosphorylation also leads to preferential translation of GCN4 mRNA. Gcn4p is a basic zipper transcriptional activator of genes subject to the GAAC, many of which are involved in amino acid biosynthesis (2, 5). GCN4 translational control occurs by a mechanism of delayed ribosomal reinitiation, allowing for the bypass of inhibitory upstream ORFs (uORFs) in the 5′-leader of the GCN4 mRNA (1, 3, 6). In mammalian cells, phosphorylation of eIF2α leads to preferential translation of a related basic zipper translation factor, ATF4 (7–9).

The TOR signaling pathway is also important for monitoring nutrient availability (10). Yeast contains two homologous TOR protein kinases, Tor1p and Tor2p, which form two distinct complexes, designated TOR complex 1 (TORC1) and TOR complex 2 (TORC2), both of which have parallels in mammalian cells (11). TORC1 regulates the GATA family of transcription activators such as Gln3p; these transcription activators direct the expression of genes encoding permeases and catabolic enzymes required for the utilization of secondary nitrogen sources (12). Repression of TORC1 in response to changes in nitrogen quality or treatment with the immunosuppressant drug rapamycin leads to Gln3p translocation to the nucleus and the activation of Gln3p target genes.

The drug rapamycin has been reported to enhance Gcn2p phosphorylation of eIF2α by a mechanism involving the release of inhibitory phosphorylation of Gcn2p at serine 577 (13). TORC1 is thought to facilitate indirectly the inhibitory Gcn2p phosphorylation by a process involving an unknown protein control (GAAC)2 pathway. In the GAAC, starvation of amino acids triggers phosphorylation of eukaryotic initiation factor-2 (eIF2) by protein kinase Gcn2p (1–3). Sensing of amino acid depletion by the Gcn2p involves direct binding of accumulating uncharged tRNAs that bind to a regulatory region in Gcn2p homologous to histidyl-tRNA synthetase (HisRS) enzymes (2–4). Gcn2p phosphorylation of the α-subunit of eIF2 at serine 51 reduces its activity, and the resulting lowered global translation allows cells to conserve resources and provides time to reconfigure the transcriptome to alleviate nutrient stress. In the yeast Saccharomyces cerevisiae, eIF2α phosphorylation also leads to preferential translation of GCN4 mRNA. Gcn4p is a basic zipper transcriptional activator of genes subject to the GAAC, many of which are involved in amino acid biosynthesis (2, 5). GCN4 translational control occurs by a mechanism of delayed ribosomal reinitiation, allowing for the bypass of inhibitory upstream ORFs (uORFs) in the 5′-leader of the GCN4 mRNA (1, 3, 6). In mammalian cells, phosphorylation of eIF2α leads to preferential translation of a related basic zipper translation factor, ATF4 (7–9).

Changes in nutrient availability direct programs of gene expression, which are important for adaptive modifications in metabolism and nutrient uptake. Multiple stress response pathways recognize nutritional deficiencies and contribute coordinately to the restructuring of the transcriptome. An important example of such a stress response is the general amino acid control (GAAC)2 pathway. In the GAAC, starvation of amino acids triggers phosphorylation of eukaryotic initiation factor-2 (eIF2) by protein kinase Gcn2p (1–3). Sensing of amino acid depletion by the Gcn2p involves direct binding of accumulating uncharged tRNAs that bind to a regulatory region in Gcn2p homologous to histidyl-tRNA synthetase (HisRS) enzymes (2–4). Gcn2p phosphorylation of the α-subunit of eIF2 at serine 51 reduces its activity, and the resulting lowered global translation allows cells to conserve resources and provides time to reconfigure the transcriptome to alleviate nutrient stress. In the yeast Saccharomyces cerevisiae, eIF2α phosphorylation also leads to preferential translation of GCN4 mRNA. Gcn4p is a basic zipper transcriptional activator of genes subject to the GAAC, many of which are involved in amino acid biosynthesis (2, 5). GCN4 translational control occurs by a mechanism of delayed ribosomal reinitiation, allowing for the bypass of inhibitory upstream ORFs (uORFs) in the 5′-leader of the GCN4 mRNA (1, 3, 6). In mammalian cells, phosphorylation of eIF2α leads to preferential translation of a related basic zipper translation factor, ATF4 (7–9).

The TOR signaling pathway is also important for monitoring nutrient availability (10). Yeast contains two homologous TOR protein kinases, Tor1p and Tor2p, which form two distinct complexes, designated TOR complex 1 (TORC1) and TOR complex 2 (TORC2), both of which have parallels in mammalian cells (11). TORC1 regulates the GATA family of transcription activators such as Gln3p; these transcription activators direct the expression of genes encoding permeases and catabolic enzymes required for the utilization of secondary nitrogen sources (12). Repression of TORC1 in response to changes in nitrogen quality or treatment with the immunosuppressant drug rapamycin leads to Gln3p translocation to the nucleus and the activation of Gln3p target genes.

The drug rapamycin has been reported to enhance Gcn2p phosphorylation of eIF2α by a mechanism involving the release of inhibitory phosphorylation of Gcn2p at serine 577 (13). TORC1 is thought to facilitate indirectly the inhibitory Gcn2p phosphorylation by a process involving an unknown protein.
kinase and the type 2A-related protein phosphatase, Sit4p (13). Rapamycin is suggested to release TORC1 inhibition of Sit4p, allowing for dephosphorylation of Gcn2p. However, the release of TORC1 phosphorylation of Gcn2p has not been found to involve nutrient stresses known to activate the GAAC, including starvation of amino acids such as histidine and deprivation of purine or glucose (13).

There are many unresolved questions concerning the regulatory linkages between the GAAC and TOR pathways. It is unclear which nutrient stress arrangements actually engage the TORC1/Gcn2p/Gcn4p pathway and whether TOR can function in conjunction with accumulating uncharged tRNAs to regulate Gcn2p. Prior transcriptome studies have suggested distinct patterns of gene expression during 3-aminotriazole (3-AT) and rapamycin treatments, and Gln3p is suggested to function only during rapamycin treatment or in response to changes in the quality of nitrogen in the medium (5, 14, 15). Do the GAAC and Gcn4p play a major role in the TOR-directed transcriptome, does Gln3p contribute significantly to gene expression during amino acid starvation, and is there significant overlap between the genes regulated by these two transcription factors? In this study we addressed these central questions, highlighting the mechanisms by which the GAAC is integrated with the TOR pathway in yeast to recognize changing nitrogen availability and to direct the transcriptome for optimal growth adaptation.

EXPERIMENTAL PROCEDURES

Yeast Strains and Culture Conditions—The S. cerevisiae strains used in this study are listed in Table 1, and all are derived from EG328-1A (16). Strains deleted for GCN2 or GCN4 have been described previously (17). All other knock-out strains were constructed by PCR-mediated gene replacement, which removes the entire coding region (18). For Kan-marked strains, deletion cassettes were amplified by PCR using genomic DNA from the corresponding deletion mutant in the BY4741 strain background (19), which was purchased from Research Genetics (Milwaukee, WI). Rapamycin, 1-methionine sulfoximine, and all amino acid supplements were obtained from Sigma and 3-AT was purchased from Fluka Chemical (Milwaukee, WI).

To minimize any unintended nutrient imbalances, we utilized isogenic strains that were prototrophic for amino acid biosynthesis (Table 1). Cells were cultured in synthetic medium (SD) containing 2% dextrose and 0.5% ammonium sulfate (20) and supplemented with all amino acids except histidine (SC medium). This culture arrangement ensured that cells were sated for amino acids, allowing for similar growth of strains defective for the GAAC and TOR pathways. Because 3-AT inhibits the synthesis of histidine, we deleted this amino acid from the non-stressed and rapamycin-treated cultures as well. Omission of this amino acid did not alter gene expression as judged by RT-PCR analysis of mRNAs such as HIS4 and GAP1 (data not shown). RT-PCR analysis was conducted using SuperScript One-step RT-PCR with Platinum Taq polymerase (Invitrogen). In experiments that analyzed the role of the GAAC and TOR pathways in nitrogen assimilation, amino acids were omitted, and alternative nitrogen sources (phenylalanine or γ-aminobutyric acid (GABA), each at concentrations of 10 mM) were substituted for ammonia in the SD medium as indicated. For some experiments, cells were grown in YPD medium (containing 1% yeast extract, 2% peptone, and 2% glucose) as indicated (20).

Plasmids—Plasmids p180 and p227 have been described previously (21). The P_**GCN4**-_lacZ** reporter plasmid was derived from plasmid pME112 (22). Briefly, six copies of the GCRE were amplified by PCR and subcloned upstream of a minimal CYC1 promoter in the reporter plasmid p416-CYC1_TATA-_lacZ. The P_**GATA**-_lacZ** reporter plasmid contains two copies of a consensus GATA sequence (GATAAG) derived from the GLN1 gene (23). The P_**ARO9**-_lacZ, P_**UGA3**-_lacZ, and P_**UGA3**-_lacZ reporter plasmids contain the complete upstream noncoding sequences of the ARO9 (−608 to −1), UGA3 (−677 to −1), and UGA1 (−548 to −1) genes, respectively. These DNA segments were subcloned into the XbaI/EcoRI site of pRS416-_lacZ. Deletions of the UGA3 promoter (−371, −300, −200, and −103) were constructed by PCR. Site-directed mutagenesis of the minimal UGA3 promoter (−300 to −1) was carried out by PCR with oligonucleotides containing the specific nucleotide changes. The GATA element (CTTATG) at −206 and the GCRE (CGTGCAG) centered at −112 of the UGA3 promoter were changed to AGATCT and CGTGCAC, respectively. The sequences of all amplified regions were confirmed by nucleotide sequencing. Plasmid p722 encodes wild-type GCN2 and the selectable URA3 gene (24), and p299 includes the mutant version, gcen2-m2, which has Y1199L and R1120L substitutions in the HisRS-related domain of Gcn2p that block binding to uncharged tRNA (25–27). Plasmid pYB41 encoding a HisRS-related domain of Gcn2p that block binding to uncharged tRNA (25–27) was purchased from Research Genetics (Huntsville, AL). Rapamycin, L-methionine, and Open Biosystems (Huntsville, AL). Rapamycin,

Transcriptome and Sequence Analyses—Transcriptome analysis was carried out using RNA prepared from cells treated for 1 h with 10 mM 3-AT, 200 nM rapamycin, or no stress, in quadruplicate as indicated. The 1-h incubation time was shown to induce maximal expression of HIS4 and GAP1 mRNAs as judged by RT-PCR analysis (data not shown). Four independent cultures of strains WY857 (wild-type) WY799 (gcen4Δ), WY857 (gcen4Δ), WY858 (gln3Δ), WY859 (gcen2Δ gln3Δ), and WY860 (gcen4Δ gln3Δ) were cultured as described, and total RNA was purified using the hot phenol method (30). The RNA was labeled using the standard Affymetrix protocol for 3′-IVT arrays (Affymetrix, Santa Clara, CA). Labeled cRNA was hybridized for 17 h to yeast genome S98 GeneChips®. Signal values and detection calls were generated using Affymetrix Microarray Suite 5.0. Arrays were scaled to a target intensity of 1000, and detection calls were generated using the default parameters. Average signal intensities from four independent experiments for each of the 18 treatment groups were compared by performing a Welch’s unpaired t test (31), and false discovery rates (FDR) were calculated according to Benjamini.
and Hochberg (32). The fold change ratios and \( p \) values for each probe set and FDR values for each comparison are listed in supplemental Table S1. Microarray data have been deposited in GEO (gene expression omnibus) (www.ncbi.nlm.nih.gov/geo/) under accession number GSE15254. Pearson and Spearman correlations were calculated using GraphPad Prism version 4.03 (San Diego, CA).

The mRNA changes for select genes derived from the microarray analyses were confirmed independently by quantitative RT-PCR using SYBR Green. Briefly, cDNA was synthesized using 1 \( \mu \)g of total RNA and 2.5 \( \mu \)M random hexamers in the RT mixture according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). Quantitative PCR reactions consisted of 2.5 \( \mu \)l of RT product, 400 nM primers, and 1 \( \times \) SYBR Green PCR master mix (Applied Biosystems). Reactions were incubated for one initial cycle at 50 °C for 2 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primers were designed using PrimerExpress software (Applied Biosystems). The nucleotide sequences of the primers used to quantitate the ARO9, ARO10, and PDC6 genes, as well as those specific to the ACT1 gene used for internal normalization, have been described by Chen and Fink (33).

Gene list comparisons were performed using the compare class utility provided by the Regulatory Sequence Analysis Tools (34). Comparisons were made with previous 3-AT and rapamycin data sets (5, 14) and with several predefined gene lists such as genes induced by promoters bound in chromatin immunoprecipitation (ChIP-chip) experiments (35), genes in the MIPS functional catalogue (36), and gene ontology categories (37) as described by Godard et al. (38). The significance of overlap between gene lists was quantitatively determined by the hypergeometric distribution (39), using the number of probe sets on the S98 array as the population size, or by calculating the representation factor (40) using the web utility Microarray Analysis Tools. Upstream noncoding regulatory sequences were retrieved and analyzed using Regulatory Sequence Analysis Tools (34). The program DNA-Pattern was used to search for and catalogue occurrences of consensus GCRE (TGABTVW) and GATA (GATAAG, GATAAH, GATTA) motifs in yeast promoters. The program oligo-analysis (41) was used to search the promoter regions of co-regulated genes for overrepresented sequence motifs. Analysis of the 5’-noncoding regions of the GCN4-dependent activation core identified the consensus GCRE motif (TGABTVW).

**Immunoblot Analysis**—Yeast cells were cultured as described above, collected by centrifugation, washed with ice-cold water, and resuspended in a solution of 20 mM sodium phosphate (pH 7.2), 50 mM NaCl, 5 mM EDTA, 1 mM dithiothreitol, protease inhibitors (100 \( \mu \)M phenylmethylsulfonyl fluoride, 0.15 \( \mu \)M aprotinin, 1 \( \mu \)M leupeptin, and 1 \( \mu \)M pepstatin), and phosphatase inhibitors (50 mM NaF and 40 mM \( \beta \)-glycerophosphate). Cells were lysed by vortexing with glass beads followed by centrifugation to clarify the lysate. The protein content of the cell lysate was measured using the Bradford method (42). Equal amounts of each protein sample were separated by electrophoresis in an SDS-polyacrylamide gel and transferred to nitrocellulose filters. Immunoblot analyses were carried out using a polyclonal antibody that specifically recognizes phosphorylated eIF2α at Ser-51 (Research Genetics or StressGen) or M2-FLAG epitope tag (Sigma). Total eIF2α levels were measured using a rabbit polyclonal antibody against recombinant yeast eIF2α (17).

**lacZ Enzyme Assays**—Yeast cells expressing lacZ reporter genes were grown to early logarithmic phase in SD or SC medium and treated as indicated. Following incubation at 30 °C, the non-stressed cells were harvested after 4 h, and stressed cells were collected after 6 h, as described previously (17). Cells were collected by centrifugation, resuspended in 250 \( \mu \)l of breaking solution (100 mM Tris-HCl (pH 8.0), 20% glycerol, 1 mM \( \beta \)-mercaptoethanol, and 100 \( \mu \)M phenylmethylsulfonyl fluoride), and broken by vortexing with glass beads followed by centrifugation at 15,000 \( \times \) g to clarify the lysates. To measure the \( \beta \)-galactosidase activity, 20 \( \mu \)l of extract was added to 980 \( \mu \)l of Z-buffer (100 mM sodium phosphate (pH 7.5), 10 mM KCl, 2 mM MgSO\(_4\), and 4.5 mM \( \beta \)-mercaptoethanol), and the reaction was initiated by the addition of a 200-\( \mu \)l solution of o-nitrophenyl-\( \beta \)-D-galactopyranoside (4 mg/ml in Z-buffer). The reaction was terminated following a 10–20 min incubation at 30 °C by adding 0.5 ml of 1 M Na\(_2\)CO\(_3\), and the absorbance of the reaction mixture was measured at A\(_{420}\). Specific enzyme activity is represented as nanomoles of o-nitrophenol-\( \beta \)-D-galactopyranoside hydrolyzed/minute/milligram of total protein. Total protein concentration of the clarified lysate was determined using the Bradford method (42). The average \( \beta \)-galactosidase activity \( \pm \) S.E. from 2–3 independent cultures is presented for each experiment. Student’s \( t \) test was used to determine statistical significance.

**Polysome Analyses**—Ribosomal profiles were generated using sucrose gradient centrifugation as described previously (26). Briefly, wild-type or gcn2A cells were grown in SD medium containing ammonium as the sole nitrogen source and harvested in mid-logarithmic phase. Alternatively, these cells were shifted from SD to GABA medium, cultured for 1 h, and harvested. Just prior to harvesting, cycloheximide was added to a final concentration of 50 \( \mu \)g/ml to retain polysomes as described (43). Lysate preparation and sucrose gradient analyses were carried out as described previously (43, 44). In addition to cycloheximide, cells lysate preparations contained 10 mM MgCl\(_2\), required for association of ribosomal subunits. Aliquots of 20–25 A\(_{260}\) units were applied to 11.5 ml of 10–50% sucrose gradients and subjected to centrifugation at 40,000 rpm for 2 h in a Beckman SW41 rotor at 4 °C.

**Measurement of tRNA Charging**—Yeast cells were grown in SD medium and shifted to synthetic medium containing GABA as the sole nitrogen source for 15 or 60 min as indicated. Cells were collected by centrifugation, and tRNA preparations were prepared and analyzed for tRNA charging genome-wide as described (45). This method involves the preparation of RNA from collected cells under mildly acidic conditions, which allow retention of charged tRNAs. The RNA samples were then divided into two equal portions. One part was treated with periodate, which selectively oxidized uncharged tRNA and selectively blocked its subsequent ligation to a fluorophore-labeled oligonucleotide. The second portion was not treated with periodate, which allowed both charged and uncharged portions to be ligated subsequently to the labeled oligonucleotide.
Both tRNA preparations were then deacylated using alkaline pH. A fluorescently tagged oligonucleotide containing a stem-loop structure with a portion complementary to the 3′-CCA sequence that is conserved among all tRNAs was ligated onto only those tRNAs with intact 3′-ends. Each of the samples was labeled with Cy3 or Cy5 fluorophore, and after fluorescent labeling the tRNA preparations with opposite fluorophores, such as charged tRNA with Cy5 and total tRNA with Cy3, were combined and hybridized to microarrays. The labeled tRNA preparations were hybridized to custom-made microarrays, which contained multiple replicates for each probe as described (45). Two microarray analyses were carried out for each sample to minimize dye bias: the first microarray used Cy5-labeled charged tRNA and Cy3-labeled total tRNA, and the second microarray used Cy3-labeled charged tRNA and Cy5-labeled total tRNA.

The array results were presented as histograms ± S.E., with the relative charging level between cells grown in SD medium compared with that cultured in GABA-containing medium. The tRNAs were grouped according to amino acid properties (hydrophobic, small, charged, or polar). Each tRNA measurement was derived from three independent culture preparations, and data analysis and statistics were provided by the scanner software (Axon Instruments, Foster City, CA). The median values of dye ratios for each array probe are presented. In parallel, Northern analyses were performed using 6.5% polyacrylamide acid-denaturing gels to separate total tRNA. The array results were presented as histograms of dye ratios for each array probe as described (45). The array results were then transferred to filters and hybridized to radiolabeled probe complementary to tRNA<sup>phe</sup> (45).

**RESULTS**

**Defects in the GAAC and TOR Pathways Alter Growth during Nutrient Stress**

To address the coordination of the GAAC and TOR pathways in the regulation of the transcriptome in response to nutrient stress, we constructed a set of isogenic strains that were deleted for the entire coding region of GCN2, GCN4, and GLN3, individually or in combination (gcn4<sup>Δ</sup> gln3<sup>Δ</sup> and gcn2<sup>Δ</sup> gln3<sup>Δ</sup>). This strain set was derived from strain EG328-1A, which has a robust nutrient stress response, and each strain was prototrophic for amino acid biosynthesis (Table 1). Deletion of GLN3 renders cells sensitive to L-methionine sulfoximine, an inhibitor of glutamine synthetase, which is consistent with the idea that GLN3 is central for gene expression directed by the TOR pathway in response to nutrient stress (Fig. 1A) (46). By contrast, deletion of GLN3 renders cells more resistant to rapamycin (Fig. 1A), implying that Gln3p alters genes contributing to control of the cell cycle and proliferation (47).

Consistent with their important roles in the GAAC, deletion of either GCN2 or GCN4 resulted in growth sensitivity to 3-AT, a potent inhibitor of histidine biosynthesis (Fig. 1A). Deletion of GLN3 led to a partial reduction in growth in the presence of 3-AT, suggesting that this transcription activator may function in conjunction with Gcn4p for the expression of certain biosynthetic genes. Loss of either GCN2 or GCN4 alone resulted in increased resistance to rapamycin (Fig. 1A). Combined deletion of GCN2/GLN3 or GCN4/GLN3 resulted in further resistance, comparable to the level seen in a dominant TOR1 mutant (TOR1-S1972I), which does not bind the Fpr1p-rapamycin complex (supplemental Fig. S1A) (48, 49). Therefore, the combined deletions suppress the inhibition of TOR function by rapamycin even in the presence of the functional TOR2 locus. These results support the idea that the two major nutritional stress pathways can work in synergy, with both GCN2 and GCN4 contributing to gene expression mediated by the TOR signaling pathway.

**Rapamycin Induces Gcn2p Phosphorylation of eIF2α and Gcn4p-mediated Transcription**

Treatment of the wild-type strain with either 3-AT or rapamycin increased Gcn2p-dependent phosphorylation of eIF2α and GCN4 translational control, as measured by a lacZ reporter fused to the 5′-leader of the GCN4 mRNA (Fig. 1, B and C) (13, 26, 50). Deletion of GCN2 blocked GCN4 expression in response to either stress, whereas gln3Δ cells showed a modest but significant (p ≤ 0.001) decrease in GCN4 expression in response to rapamycin exposure (Fig. 1C). Consistent with the idea that the GAAC regulates translation via uORFs in the 5′-leader of the GCN4 mRNA, high levels of GCN4 expression were measured in cells containing a similar lacZ reporter devoid of the GCN4 uORFs independent of the stress treatment (supplemental S1B).

Importantly, elevated eIF2α phosphorylation and GCN4 translation triggered increased Gcn4p-directed transcription as measured by a lacZ reporter containing a minimal CYC1 promoter containing consensus Gcn4p binding sites (GCRs) (Fig. 1D). Gcn4p transcriptional activity was blocked by deletion of either GCN4 or GCN2. Interestingly, cells devoid of GLN3 increased Gcn4p transcriptional activity by an additional fold in response to 3-AT, suggesting an underlying compensatory system in which loss of portions of the TOR pathway further enhances the GAAC.
**Gcn4p Is a Major Contributor to TOR-mediated Gene Expression**

To address the roles of Gcn4p and Gln3p in gene expression induced by either 3-AT or rapamycin treatment, we carried out whole genome transcriptional profiling experiments using Affymetrix GeneChips to measure mRNA levels in an isogenic set of strains. Cells deleted for GCN2, GCN4, or GLN3 or their combined mutations (gcnaΔ gln3Δ and gcn4Δ gln3Δ), were cultured for 1 h in the presence of either 3-AT or rapamycin and compared with a wild-type strain that was treated similarly. Of the genes that were defined by probe sets, a significant (p ≤ 0.05, FDR ≤ 0.2) change in expression (≥2-fold) was observed in 2532 probe sets representing 2103 unique genes (total of increases and decreases; Fig. 2A). This indicates that a substantial portion of the yeast transcriptome, ~30% of the encoded genes, is regulated by either amino acid starvation or rapamycin-induced inactivation of the TOR pathway.

**Genes Induced by 3-AT**—In cells exposed for 1 h to 10 mM 3-AT, there was a ~2-fold increased expression of 1029 genes (700 annotated; Table 2), which correlates well with those genes (both identity and magnitude of induction) reported to be induced by treatment with 100 mM 3-AT (5) (supplemental Fig. S3A). A histogram depicting the distribution of the fold changes for these genes is shown in supplemental Fig. S3B. Of the genes induced by 3-AT treatment, 209, 223, and 71 were dependent on GCN2, GCN4, or GLN3, respectively (Table 2 and supplemental Table S2). A large portion of the GAAC-depent genes activated by 3-AT are involved in amino acid metabolism, a major focus for Gcn4p (2, 5) (supplemental Table S2). Using this ≥2-fold induction criterion, Gcn4p was required for increased expression of 46 amino acid biosynthetic genes participating in the synthesis of each of the 20 amino acids, with the exception of proline. In this latter case, Gcn4p was required for a 1.6-fold increase in the expression of PRO2 (p < 0.001), encoding γ-glutamyl-phosphate reductase (catalyzes the second step in the biosynthesis of proline), which although significant was below the ≥2-fold threshold. Related to this biosynthetic process, Gcn4p also induced genes involved in vitamin metabolism (seven targeted genes involved in the biosynthesis of pyridoxal phosphate, NAD, folate, coenzyme A, or riboflavin), intermediary metabolism (12 genes), and transport processes (five genes) (highlighted in bold red in supplemental Table S2). It is noteworthy that Gcn4p also plays an important role in nitrogen utilization, targeting 12 genes in this functional category. The mechanistic role of Gcn4p in nitrogen utilization will be explored further below.

Among the genes requiring either GCN2 or GCN4 for full induction, a significant majority (77%; representation factor = 37.4) of these required both regulators of the GAAC, supporting the idea that Gcn2p is a central upstream activator of the Gcn4p. A subset of these (35 genes) was found to be signifi-
GCN4 Is Integral to Nitrogen Assimilation

A

≥ 2-fold increase

3-AT

Rap

Totals

616

141

157

60

413

10

66

11

511

103

≥ 2-fold decrease

3-AT

Rap

Totals

260

75

73

87

183

10

20

42

549

43

177

354

B

GCN4-dep

GLN3-dep

76

38

75

P ≤ 0.05, fdr ≤ 0.2

C

Amino acid biosynthesis

Nitrogen utilization

Intermediary metabolism

Signaling

Cellular transport

uncharacterized

D

Ribosomal proteins

Mitochondrial proteins

Intermediary metabolism

Protein processing

Transcription factor

Noncoding RNA

RPL9A
RPS9A
RPS22B
MRSPL17
MAS6
MAM33
IMD2
RNR1
PUS4
PMT2
MKC7
TOS4
SNR44
FYV4
SMC3
HLR1
4062_at
8092_s_at

GCRE copies

Avg

UGA3

Fold Change

wild-type

gcn4

gln3

gcn4, gln3

gcn4, Rap

gcn4, Rap, Raposome

GCRE copies
**TABLE 2**
Summary of gene expression profiling

| Treatment | Dependence | Number of probe sets<sup>a</sup> |
|-----------|------------|----------------------------------|
|           | Increase   | ≥2× Increase | Decrease | ≥2× Decrease |
| 3-AT      | Wild type  | 2186          | 1029     | 1521         | 443     |
| 3-AT      | GCN2-dep  | 541           | 209      | 319          | 85      |
| 3-AT      | GCN4-dep  | 579           | 223      | 333          | 93      |
| 3-AT      | GLN3-dep  | 383           | 71       | 289          | 129     |
| Rapamycin | Wild type  | 2236          | 924      | 1525         | 732     |
| Rapamycin | GCN2-dep  | 177           | 78       | 93           | 53      |
| Rapamycin | GCN4-dep  | 395           | 115      | 397          | 197     |
| Rapamycin | GLN3-dep  | 526           | 114      | 596          | 396     |

<sup>a</sup> Transcripts in which expression was dependent on GCN2, GCN4, or GLN3, identified as those showing a ≥2-fold (induced genes) or significant change (repressed genes) in the comparison between wild-type + treatment vs. gcnaΔ + treatment, gcnaΔ + treatment, or gln3Δ + treatment.

<sup>b</sup> Number of probe sets representing transcripts that increased or decreased significantly (p ≤ 0.05, FDR ≤ 0.2) following treatment with 3-AT or rapamycin and number of these significant probe sets in which the change was at least 2-fold.

**Genes Repressed by 3-AT**—Fewer genes were repressed by 3-AT treatment (443 were repressed ≥2-fold), with a total of 93 genes displaying significant dependence on Gcn4p (Table 2 and Fig. 2A). Included among the genes requiring Gcn4p for repression are those involved in protein synthesis, ribosomal proteins, and ribosomal biogenesis, as well as genes involved in protein folding, targeting, and sorting (supplemental Table S2). It is also noteworthy that there was significant overlap among the Gcn4p-repressed genes and those reduced during the so-called environmental stress response (51) (105 of 261 annotated genes; p < 1.4 × 10^-5). This suggests that Gcn4p-dependent repression can occur in response to diverse environmental stresses.

Gln3p contributes to repression of an even larger set of genes compared with Gcn4p (129 versus 93 genes; Table 2), and these repressed genes are involved in the same processes as described above for Gcn4p. Of these genes, 44 require both Gcn4p and Gln3p, suggesting significant regulatory overlap between the genes repressed by the TOR and GAAC pathways (supplemental Fig. S2B and Table S2). We concluded that Gln3p could be a significant contributor to regulation of the transcriptome in response to amino acid starvation.

**Genes Induced by Rapamycin**—Treatment of cells with rapamycin resulted in 924 transcripts (695 annotated genes) being induced ≥2-fold, which correlates well with those reported by Marion et al. (14) (supplemental Fig. S3C). The distribution of the fold changes for these genes is shown in supplemental Fig. S3B. There is a moderate correlation (Pearson r = 0.73, Spearman r = 0.78) between those genes regulated by rapamycin and those regulated by 3-AT (supplemental Fig. S3D). Of these induced transcripts, 115 and 78 genes showed a dependence on GCN4 and GCN2, respectively (Fig. 2A and Table 2).

Almost one-third of the Gcn4p target genes did not show any significant requirements for GCN2, supporting the idea that basal amounts of Gcn4p are significant contributors to the transcriptome in response to rapamycin, an idea noted above for 3-AT stress. The genes induced by Gcn4p in response to rapamycin can be divided into two groups. The first group includes 57 genes that were also activated during 3-AT stress. We will refer to those genes requiring Gcn4p for induction in response to either 3-AT or rapamycin stress as the Gcn4p activation core (Fig. 2C and supplemental Table S3). As discussed further below, the core genes are involved in amino acid biosynthesis, nitrogen utilization, intermediary metabolism, and cellular transport. The second group includes those Gcn4p-

**FIGURE 2. Role of the GAAC and TOR in the changes in the yeast transcriptome following treatment with rapamycin or 3-AT.** A. Venn diagrams illustrating the number of genes in which encoded mRNAs require GCN2, GCN4, or GLN3 for a 2-fold change in expression following 3-AT or rapamycin treatment. Red, indicates those gene transcripts changed only by 3-AT; green, those changed only in response to rapamycin treatment; yellow, those changed by both stress treatments. The total numbers of transcripts changed for each mutant and the percentage of the total number of different mRNAs changed in the wild-type strain are indicated at the right. B, top, Venn diagram illustrating the number of genes in which encoded mRNAs require GCN4 or GLN3 for a ≥2-fold increase following rapamycin exposure. Red, indicates those gene transcripts requiring only GCN4; green, those requiring only GLN3; yellow, those requiring both transcriptional regulators. Bottom, rapamycin-induced fold changes for gene transcripts in wild-type, gcnaΔ, gln3Δ, and gcnaΔ gln3Δ strains are plotted individually in black. The average fold change for all gene transcripts is shown in red, and the fold change values for UGA3 mRNA are highlighted in green. C and D, heat maps illustrate the levels of gene transcripts that require GCN4 for increased (C) or decreased (D) expression following treatment with 3-AT or rapamycin. Genes in the Gcn4p activation or repression core are listed along with their general biological functions. The legend at the bottom of the heat maps illustrates the changes in transcript levels between the paired samples listed at the top of each map. The number of GCREs present in each gene promoter region is represented to the right of the heat maps. In C, the asterisks indicate promoters reported to bind Gcn4p in chromatin immunoprecipitation (ChIP-chip) experiments (35); genes highlighted in blue were reported to have increased transcription in medium supplemented with secondary group B nitrogen compounds (38).
targeted genes specifically induced in response to rapamycin, which were centered on the generation and utilization of energy-related compounds (11 genes) (supplemental Fig. S2A and Table S2). The significant differences between the genes induced by Gcn4p by 3-AT and rapamycin indicate that GAAC transcriptional regulation can be tailored to meet specific stress arrangements.

The number of genes dependent on Gcn4p for induced expression during rapamycin treatment was similar to those requiring GLN3 (115 versus 114 in Table 2). This result demonstrates that Gcn4p has a major role in the induction of TOR-regulated genes, comparable with that of its known effector, Gln3p. An analysis of genes requiring both Gcn4p and Gln3p for enhanced expression in response to rapamycin indicated that 38 were reduced by the loss of either transcriptional activator (Fig. 2B). When GCN4 and GLN3 were deleted individually or in combination, 24 genes showed loss of induction in response to rapamycin stress as illustrated in Fig. 2B. The average fold change for this collection of genes is illustrated in red (Fig. 2B). Many of these genes are suggested to carry out catalytic processes (Table 3 and supplemental Table S4), and we will explore further below the coordinate regulation of genes involved in GABA utilization. Paradoxically, among the 38 genes, 14 showed full or enhanced induction when both GCN4 and GLN3 were deleted, suggesting that there are compensatory mechanisms when both are ablated.

**Genes Repressed by Rapamycin**—Rapamycin treatment led to a 2-fold repression of 732 genes, with 197 of these dependent on Gcn4 (Fig. 2A and Table 2). The largest functional classes among the Gcn4p repressed genes are involved in ribosome biogenesis, including rRNA processing and modification, protein synthesis, transcription, and nucleotide metabolism (supplemental Table S2). Although these genes overlap in their functional classes with those repressed during 3-AT, their identities are largely unique. There were 20 probe sets (Fig. 2A), representing 16 identifiable genes, that were repressed in response to either stress arrangement (Fig. 2D and supplemental Table S5, Gcn4p repression core). These genes encode ribosomal proteins as well as those involved in the mitochondria, intermediary metabolism, and protein processing. It is interesting to note that only 53 of the genes repressed by rapamycin required GCN2 (Table 2). Thus, as noted above for 3-AT, a large number of genes requiring Gcn4p for regulation did not show significant requirements for GCN2, which would suggest an important role for basal amounts of Gcn4p for coordination of the transcriptome.

Gln3p contributes to the repression of 396 probe sets (Table 2), which represent genes involved in the same processes as described above for Gcn4p. In fact, 149 of the genes repressed by rapamycin require both Gcn4p and Gln3p (supplemental Fig. S2B). This finding reinforces the idea that Gcn4p is a major effector of the TOR pathway, with this GAAC regulator contributing to gene repression as well as activation in response to rapamycin. Relief of repression of these genes may be at least one reason for the growth resistance of gcn2Δ, gcn4Δ, and gln3Δ cells to rapamycin treatment (Fig. 1A).

**The Gcn4p Activation Core Is Induced by Either 3-AT or Rapamycin Treatment**

Analysis of the overlap between genes induced in response to either 3-AT or rapamycin identified 68 and 66 genes that required GCN2 and GCN4, respectively, for full induction (Fig. 2A). By contrast, far fewer (11 genes) required GLN3 for enhanced expression during both stress arrangements (Fig. 2A). As discussed further below, the two additional genes that were dependent on GCN2, but not on GCN4, were ARO9 and ARO10. We refer to the 66 probe sets (57 identifiable genes) induced by either 3-AT or rapamycin treatment by a mechanism requiring Gcn4p as the Gcn4 activation core (Fig. 2C and supplemental Table S3). A total of 53 genes in the Gcn4p activation core contain at least one copy of a GCRE, with promoters of 46 members previously shown to bind to Gcn4p in ChIP experiments (Fig. 2C) (35).

The Gcn4p activation core can be viewed as those genes in which expression is the foundation for GAAC-directed adaptation to different stress arrangements. A large portion of these core genes participate in amino acid metabolism (23 of 57 genes; \(p \leq 2 \times 10^{-19}\)). The most represented among these biosynthetic genes are those involved in the aromatic amino acids (ARO3, ARO8, TRP2, TRP3, and TRP4) and arginine (ARG2, ECM40 [ARG7], CPA1, CPA2, and ARG1) (Fig. 2C and supplemental Tables S2 and S3). Other major functional categories are genes involved in nitrogen utilization (11 genes), a point that will be discussed further below, and those contributing to cellular transport (seven genes). One of these metabolism-related genes is BSC5 (YNR069C), which encodes the amino-terminal
portion of a coding region juxtaposed out-of-frame with a downstream ORF YNR068C. Together these combined ORFs encode a predicted polypeptide with homology to Bul1p, an ubiquitin-binding protein that is important for the sorting of amino acid permeases and growth during stress conditions (52).

The Gcn4p activation core also includes three transcription factors: Gln3p, suggesting a regulatory intersection between the GAAC and TOR pathways; Stb4p, involved in histone deacetylation (53); and Uga3p, a zinc finger transcriptional activator of genes required for nitrogen assimilation from GABA (54). The coordination of Gcn4p and Gln3p in GABA catabolism will be addressed further below. Also part of the Gcn4p activation core is Pcl5p, a cyclin partner for the Pho85p protein kinase that is required for phosphorylation and subsequent feedback degradation of Gcn4p (55, 56). Gcn4p induces the expression of two protein kinase genes, PKP2 and YDL025C (RTK1). Pkp2p inhibits mitochondrial pyruvate complex, which catalyzes the oxidative decarboxylation of pyruvate to acetyl-CoA (57). This suggests that the GAAC restricts carbon flux between glycolysis and the tricarboxylic acid cycle. Rtk1p has been tied to glucose homeostasis and is closely related to the Hrk1p protein kinase that regulates plasma membrane transporters (58, 59). Finally, two TY elements and six probe sets that map to unannotated regions of the yeast genome are among the Gcn4p activation genes, suggesting that noncoding RNAs also contribute to Gcn4p-mediated stress responses.

**GAAC Directs Transcription of Genes Involved in Assimilation of Aromatic Amino Acids**

Our transcriptome analysis suggested that the GAAC is important for directing gene transcription involved in nitrogen assimilation, as reported previously for the TOR pathway. Included among these genes are ARO9 and ARO10, which are required for reclamation of nitrogen from amino acids via the Ehrlich pathway (60). As noted above, the microarray analysis showed increased expression of a small number of genes requiring Gcn2p in response to either 3-AT or rapamycin treatment but largely independent of Gcn4p (Fig. 3, A and B). The fold induction of the ARO9 and ARO10 mRNAs is also represented in supplemental Fig. S4, with an independent confirmation by quantitative RT-PCR of these transcript levels and additional select mRNAs shown in supplemental Fig. S5.

The transcription factor Aro80p is suggested to facilitate ARO9 and ARO10 transcription (61). We found that the levels of ARO80 mRNA were not significantly changed during either a stress condition or in response to deletion of GCN2, GCN4, or

**FIGURE 3. The GAAC and ARO80 are required for expression of aromatic catabolism genes.** ARO9 (A) and ARO10 (B) mRNA levels were measured by microarray in wild-type cells or in cells deleted for GCN2, GCN4, or GLN3, individually or in combination, following treatment with 3-AT or rapamycin or no treatment (Control) as indicated. Transcript levels are plotted as mean fluorescence intensity (MFI). Changes in ARO9 and ARO10 mRNA levels were confirmed independently by quantitative RT-PCR (supplemental Fig. S5), C, wild-type (WY837), gcnaΔ (WY838), and aro80Δ (WY962) strains containing a P<sub>ARO9-laZ</sub> reporter plasmid were cultured in synthetic complete medium with (+AA) or without amino acids (−AA), as indicated, and treated with 3-AT or rapamycin or not treated (Control). β-Galactosidase activity was measured from two independent cultures and is presented as the mean ±S.E. D, prototrophic strains were grown on synthetic agar plates containing ammonia (NH₄⁺), all 20 amino acids (20 AA), or Phe as the nitrogen source as indicated.
**GCN4 Is Integral to Nitrogen Assimilation**

**FIGURE 4.** Gcn4p and Gln3p co-regulate gene expression in response to rapamycin treatment. 

A. A wild-type (WT) strain and those containing the indicated gene deletions were grown on synthetic agar plates containing either ammonia (NH$_4^+$) or GABA as the nitrogen source for 3 days at 30 °C. 

B. Yeast cells deleted for GCN4, GLN3, and UGA3, as indicated, were transformed with a plasmid encoding a lacZ reporter gene fused to the UGA1 promoter. Cells were cultured in synthetic medium containing ammonia (NH$_4^+$) as the nitrogen source and then switched to synthetic medium containing phenylalanine as the sole nitrogen source for 6 h. 

C. Gcn4p and Gln3p co-regulate gene expression in response to either 3-AT or rapamycin, with the most robust increase during histidine starvation (Fig. 3C). Loss of GCN2 resulted in a dramatic reduction in P$_{ARO9}$-lacZ activity in both control and stress conditions. This reporter assay supports the idea that the changes in ARO9 mRNA levels measured in our microarray analysis were the result of a GCN2p-dependent transcription from P$_{ARO9}$. As expected, there was minimal P$_{ARO9}$-lacZ expression in cells deleted for ARO80 (Fig. 3C). The induction of P$_{ARO9}$-lacZ by 3-AT or rapamycin was observed only when amino acids were supplemented to the minimal medium, suggesting that cells recognize starvation signals and induce the expression of key catabolic enzymes only when their respective substrates are available in the media (Fig. 3C).

These results suggest that the Gcn2p functions upstream or in a pathway parallel to Aro80p activation of genes that are important for catabolism of aromatic amino acids. Illustrating the key role of ARO80 in aromatic catabolism, deletion of ARO80 partially reduced growth in synthetic medium containing phenylalanine as the sole nitrogen source (Fig. 3D). No reduction in growth was found when the aro80 strain cells were grown in synthetic medium containing the nitrogen source ammonia or in medium containing all 20 amino acids.

Cells deleted for either GCN2 or GCN4, individually or in combination with ARO80, were also cultured in these media. All mutant strain combinations grew in medium containing all 20 amino acids. Although deletion of GCN2 alone did not elicit a growth defect in synthetic medium containing ammonia or phenylalanine as the nitrogen source, the combined gcn2Δaro80Δ strain selectively displayed a severe growth defect in the phenylalanine medium (Fig. 3D). Cells deleted for GCN4 often have growth defects in minimal medium supplemented with ammonia but no amino acids, presumably because of their reduced capacity to synthesize amino acids. Interestingly, gcn4Δ cells grew to wild-type levels in synthetic medium supplemented with the nitrogen source phenylalanine (Fig. 3D). 

As observed for gcn2Δ mutants, introduction of gcn4Δ into the aro80Δ strain exacerbated the growth defect in the phenylalanine medium. These results indicate that the Gcn2p contributes to catabolism of aromatic amino acids via a pathway linked to Aro80p-directed transcription of ARO9 and ARO10. As Gcn4p did not appear to be required for induced transcription of either ARO9 or ARO10, Gcn4p may contribute to growth in media containing phenylalanine as a nitrogen source by coordinating amino acid biosynthesis and related metabolic pathways.

**Gcn4p and Gln3p Stimulate GABA Catabolism**

One of the target genes in the Gcn4p activation core is LIGA3, which also is regulated jointly by Gln3p (Fig. 2C and Table 3). Gcn4p is suggested to have an important role in nitrogen utili-
zation, and UGA3 encodes a transcription factor that directs expression of UGA1, UGA2, and UGA4, all of which facilitate the catabolism of GABA. Deletion of either UGA3 or UGA1 significantly blocked growth in minimal medium containing GABA as the nitrogen source but had no effect on growth in media containing ammonia (Fig. 4A). By comparison, deletion of GLN3 showed a partial reduction in growth in GABA medium, whereas gcn2Δ cells showed wild-type levels of growth. However, when gcn2Δ and gln3Δ were combined, growth in GABA medium was reduced to levels comparable with that in cells deleted for UGA1 or UGA3 (Fig. 4A). These results further support the idea that the GAAC and TOR pathways function together to regulate GABA catabolism. Consistent with these findings, deletion of either GCN4 or GLN3 reduced UGA1 expression as measured by a lacZ reporter assay in response to the addition of GABA in the medium as the sole nitrogen source (Fig. 4B). Combined GCN2 and GLN3 deletions led to a further lowering of PUGAAT-lacZ expression, although this reduction did not equal that found in the uga3Δ cells (Fig. 4B). These results suggest that the GAAC and TOR pathways function upstream of UGA3 in the regulation of UGA1 expression in response to GABA.

To address more directly the role of the GAAC in GABA catabolism, we assayed Gcn4p transcriptional activation as measured by the PGCRE-lacZ reporter, in GABA medium. There was a marked increase in PGCRE-lacZ activity in the GABA medium compared with that containing ammonia (Fig. 4C). GABA induction of Gcn4p transcriptional activity in fact exceeded that measured for rapamycin. Loss of Gcn2p elf2α kinase function by gcn2Δ (Fig. 4C) or substitution of serine 51 in elf2α to alanine (SUI2-S51A) (data not shown) significantly reduced PGCRE-lacZ activity in the GABA medium as well as during rapamycin treatment.

Similar assays using a lacZ reporter expressed from a promoter containing GATA elements showed that GABA was also a potent inducer of this TOR-targeted transcriptional activator, which exceeded that measured following treatment with rapamycin (Fig. 4D). Deletion of GLN3 partially reduced the PGATA-lacZ activity in response to GABA medium or in response to the addition of rapamycin to synthetic medium containing ammonia. Gat1p is another transcriptional activator in the GATA family that is induced by TOR, and gat1Δ cells showed a similar reduction in PGATA-lacZ activity, which was further exacerbated with the combined deletion of GLN3 and GAT1 (Fig. 4D). It is noted that although rapamycin and GABA media significantly increased PGATA-lacZ activity, treatment with 3-AT in fact led to a 50% reduction in PGATA-lacZ expression (data not shown). In our transcriptome analysis, loss of GLN3 significantly reduced the expression of 71 genes during 3-AT stress (Table 2). This suggests that the TOR pathway per se is not repressed by 3-AT, but rather the basal activity of Gln3p is a contributor to the expression of these genes under amino acid-limiting conditions. Finally, deletion of GCN4 did not significantly alter PGATA-lacZ expression in response to rapamycin treatment but did lower induced expression by 60% in GABA medium (data not shown). The fact that GLN3 is a member of the Gcn4p activation core (Fig. 2C) suggests that in select nutrient arrangements, Gcn4p can enhance Gln3p expression and its activity.

**Gcn2p Phosphorylation of elf2α Is Increased in Cells Shifted to GABA Medium**

We next addressed the underlying mechanisms facilitating activation of Gcn4p transcription in GABA medium. Gcn2p phosphorylation of elf2α was induced within 15 min of transfer from synthetic medium supplemented with ammonia into that containing GABA (Fig. 5A). Elevated elf2α phosphorylation was accompanied by lowered translation initiation, as judged by a sharp decrease in polysomes, coincident with elevated monosomes, as judged by sucrose gradient centrifugation (Fig. 5B). The gcn2Δ cells shifted to the GABA medium showed high levels of polysomes, supporting the idea that Gcn2 phosphorylation of elf2α was required for this reduction in global protein synthesis. Translational expression of GCN4, as assayed by the GCN4-lacZ reporter, was also significantly increased in response to GABA (Fig. 5C). Consistent with the idea that the GAAC regulates translation via uORFs in the 5′-leader of the GCN4 mRNA, high levels of GCN4 expression were measured in cells containing a similar lacZ reporter devoid of the GCN4 uORFs independent of the stress treatment (supplemental Fig. S6). We also directly measured Gcn4p levels by immunoblot analysis and found accumulation of Gcn4p beginning within 1 h of shifting to GABA medium with maximum levels after 3 h (Fig. 5D). These studies support the idea that Gcn2p phosphorylation of elf2α triggers both global and gene-specific translational control in the GABA medium.

**Sit4p Facilitates GCN4 Translation in GABA Medium**

Gcn2p phosphorylation of elf2α is essential for enhanced GCN4 translation control in GABA medium as viewed by the observation that gcn2Δ (Fig. 6A) and SUI2-S51A (supplemental Fig. S6B) cells displayed significantly lowered GCN4-lacZ reporter activity. Sit4p protein phosphatase also contributes to the activation of Gcn2p when TORC1 is repressed by rapamycin treatment. Consistent with an earlier report (13), the sit4Δ mutant suppressed GCN4 translational control in response to rapamycin treatment (Fig. 6B). Importantly, deletion of SIT4 also reduced GCN4-lacZ expression in response to GABA medium (Fig. 6B). However, the sit4Δ mutant did not affect GCN4 expression during 3-AT treatment (data not shown), suggesting that TORC1 is not required for the regulation of the GAAC in response to amino acid depletion (13). Together these results are consistent with the idea that shifting to a secondary nitrogen source such as GABA relieves TOR-mediated repression of Gcn2p through a mechanism involving Sit4p protein phosphatase.

**Increased Decacylation of tRNAAsp and tRNAPhe in Cells Shifted to GABA Medium**

Our studies suggest that repression of TORC1 contributes to the activation of the GAAC in response to a shift from synthetic medium supplemented with ammonia into that containing GABA. Elevated uncharged tRNA levels are also central to activation of Gcn2p. To address directly whether the levels of tRNA...
charging are reduced upon a shift to GABA medium, we measured changes in charging of all tRNA species by a method involving tRNA microarrays that includes complementary probes to each chromosome-encoded tRNA (45). A prototrophic strain and its gcn2Δ counterpart were shifted from SD medium to synthetic medium containing GABA for up to 120 min, and RNA was extracted under mild acidic conditions, during which aminoacylated tRNAs were retained. The RNA sample was split into two parts. One half was subjected to periodate oxidation, destroying the 3′-ends of all uncharged tRNAs, and the other half, which served as a control, was not treated with periodate. Both samples were then deacylated using alkaline pH and a fluorescently tagged oligonucleotide was ligated onto only those tRNAs with intact 3′-ends. These samples were labeled with Cy3 or Cy5 fluorophore. After fluorescent labeling, tRNA preparations with opposite fluorophores, for example charged tRNA with Cy5 and total tRNA with Cy3, were combined and hybridized to microarrays.

The array results, presented as histograms and heat maps, show the relative tRNA charging levels between cells shifted to GABA medium compared with the SD control (Fig. 7). Within 60 min of the shift of the wild-type cells to GABA medium, there was a significant reduction in the charging of tRNA\(^{\text{Phe}}\) and tRNA\(^{\text{Cys}}\), along with a more modest lowering in tRNA\(^{\text{Trp}}\) aminoacylation (Fig. 7, A–C). These tRNA charging levels were further diminished with culturing in this medium for 120 min (Fig. 7C). The gcn2Δ strain showed a different pattern of tRNA charging genome-wide, with increased deacylation of tRNA\(^{\text{Cys}}\), tRNA\(^{\text{Amp}}\), and tRNA\(^{\text{Pro}}\) in both the SD and synthetic GABA media (Fig. 7C). Upon shifting to the GABA medium, there

**FIGURE 5.** Gcn2p phosphorylation of eIF2α reduces global translation and enhances GCN4 expression upon shifting to GABA medium. **A,** wild-type (GCN2) and gcn2Δ cells were grown in synthetic medium lacking amino acids and containing ammonia (NH\(_4^+\)) as the nitrogen source and then switched to minimal medium containing GABA as the nitrogen source and grown for up to 120 min as indicated. As a control, GCN2 cells were grown in ammonia containing medium supplemented with 3-AT for 60 min (GCN2 3-AT). **A,** levels of phosphorylated and total eIF2α were measured by immunoblot analyses. **B,** GCN2 and gcn2Δ cells were grown in medium containing ammonia (NH\(_4^+\)) or shifted to GABA medium for 1 h, and lysates were analyzed by sucrose gradient centrifugation. The panels show the A\(_{260}\) profile of the gradients, with free 40 S and 60 S subunits, 80 S ribosomes, and polysomes indicated. The profile for the gcn2Δ cells grown in synthetic medium with ammonia was not shown, as it was unchanged from the wild-type cells cultured in this medium. The ratio of polysomes (dimers or greater) compared with monosomes (P/M) is illustrated above each panel. **C,** β-galactosidase activity was measured from wild-type cells containing a GCN4-lacZ reporter plasmid with uORFs. Cells were grown in synthetic medium containing ammonia as the nitrogen source in the presence (+AA) or absence (−A) of all 20 amino acids, or they were treated with 10 mM 3-AT or 200 nM rapamycin (Rap) or grown in medium containing GABA as the nitrogen source for 6 h as indicated. **D,** a low-copy plasmid p1025 encoding the GCN4 gene with an encoded carboxyl-terminal FLAG epitope for detection by immunoblot was introduced into the wild-type strain WY837. This plasmid contains the wild-type GCN4 promoter and encoded GCN4 uORFs. Cells were grown in synthetic medium with ammonia as the nitrogen source (0) or shifted to GABA medium for 1, 3, or 6 h. Alternatively, these cells were cultured in SD medium containing 3-AT for 6 h or in SC medium containing ammonia and all amino acids (NH\(_4^+\) + AA). For controls, the WY837 strain containing vector alone or p1025, which expresses the GCN4 gene devoid of uORFs expressed from a constitutive ADH promoter (GCN4\(^{\text{AC}}\)), was cultured in SD medium. Equal amounts of protein lysates were analyzed by immunoblot using FLAG-specific antibody to visualize the tagged Gcn4p. In the lower panel, eIF2α protein was measured by immunoblot to show that equal amounts of total protein were analyzed in each of the lanes.
uncharged tRNA would thwart activation of the GAAC. Indeed, we found that the gcn2-m2 mutation blocked enhanced GCN4-lacZ expressed in response to GABA medium (Fig. 7E). This GCN2 mutation also diminished GCN4 expression in response to rapamycin exposure, suggesting that tRNA binding to Gcn2p is also required for activation of the GAAC during this drug treatment (see “Discussion”). These findings suggest that enhanced levels of uncharged tRNA also contribute to the activation of Gcn2p and the GAAC upon shifting from SD to synthetic GABA medium.

Gcn4p and Gln3p Activate UGA3 Transcription

Our transcriptome studies suggested that Gcn4p and Gln3p activate UGA3 transcription in response to stress and therefore, that the role of the GAAC and TOR pathways is to coordinate the transcription of UGA1 and other GABA catabolic genes. This idea was further supported by our observation that expression of a lacZ reporter fused to the UGA3 promoter (PUGA3) was increased in response to rapamycin or 3-AT treatment (Fig. 8A). Deletion of either GCN2 or GCN4 significantly lowered UGA3 transcription in response to either stress. By comparison, loss of GLN3 lowered PUGA3-lacZ expression only during rapamycin exposure, with no change in the 3-AT medium (Fig. 8A). There are four predicted Gln3p binding sites in the PUGA3 region and two Gcn4p binding elements (Fig. 8B). To determine the minimum elements in the PUGA3 required for enhanced transcription in response to 3-AT or rapamycin stress, a progressive 5′-deletion analysis was carried out in the PUGA3-lacZ reporter. Removal of the most 5′ elements, including two Gln3p binding sites and a single Gcn4p binding element, in the PUGA3-lacZ reporter construct did not significantly reduce transcription in response to either 3-AT or rapamycin treatment (Fig. 8B). Further deletion of the Gln3p binding site in the PUGA3 lowered β-galactosidase activity by 2-fold during rapamycin treatment but had no effect in response to 3-AT. Importantly, the removal of the lone GCRE blocked UGA3 transcription in response to either stress condition, suggesting that Gcn4p binding to the PUGA3 region is central for increased UGA3 transcription (Fig. 8B).

To further address the roles of the Gcn4p and Gln3p binding elements in UGA3 transcription, these elements were mutated individually or in combination in the proximal −300 version of the of the PUGA3-lacZ reporter, which retained maximal transcription in response to either 3-AT or rapamycin (Fig. 8C). Consistent with our deletion analysis, mutations in the Gcn4p binding element blocked PUGA3−300−lacZ expression in response to either stress condition. By comparison, alteration of the Gln3p binding element partially lowered PUGA3−300−lacZ activity in response to rapamycin.

We also addressed whether these two binding elements are important for UGA3 transcription when GABA is the sole nitrogen source in the medium. The PUGA3−300−lacZ reporter was assayed in cells cultured in medium containing ammonia and a complete complement of amino acids or in medium devoid of amino acids and with only GABA as the nitrogen source. GABA medium led to an 8-fold increase in PUGA3−300−
GCN4 Is Integral to Nitrogen Assimilation

lacZ activity (Fig. 8D). Mutations in either the Gcn4p or the Gln3p binding element significantly reduced P_{UGA3-300-lacZ} expression in GABA medium. Therefore, Gcn4p and Gln3p are essential for increasing UGA3 transcription in response to a variety of nutrient stresses. These findings support the idea that Gcn4p is a major transcriptional effector of the TORC1 pathway and that this regulatory pathway has an important role in the assimilation of secondary nitrogen sources such as GABA (Fig. 8E).

DISCUSSION

This study addresses the regulatory linkages between the GAAC and TOR pathways in directing the transcriptome in
response to amino acid starvation and rapamycin treatment. Three central questions were addressed. The first is whether the transcriptome directed by Gcn4p in response to amino acid depletion is similar to that in rapamycin treatment, or can different stress conditions allow for significant variations in Gcn4p-directed transcription? We found that there is a core of 57 genes that are activated by Gcn4p in response to either stress condition; these include genes involved in amino acid biosynthesis, nitrogen utilization, signaling, and gene expression (Fig. 2C). Among these core genes, we focused on the relationship between the GAAC and nitrogen utilization. Specifically, we delineated the underlying mechanisms by which Gcn4p facilitates GABA utilization and the processes contributing to the activation of the GAAC upon shifting to this secondary nitrogen source. These results indicate that the GAAC has a focal set of genes that are central to cellular adaptation to different stresses. Gcn4p and the GAAC also retain the versatility to direct stress-specific gene expression, as illustrated by the large collection of genes induced specifically by 3-AT or rapamycin treatment (Fig. 2A). The Gcn4p-targeted genes activated specifically by histidine depletion emphasized amino acid and intermediary metabolism, whereas those induced by rapamycin included generation and utilization of energy-related compounds (supplemental Tables S2 and S3).

The second central question concerned the role of Gcn4p in TOR-directed gene expression and the mechanisms underlying the regulatory linkages between the GAAC and TOR pathways. Importantly, we found that Gcn4p is a major transcriptional effector of the TOR pathway, with the number of genes requiring Gcn4p for activation in response to rapamycin treatment being similar to the number dependent on the canonical Gln3p transcription factor (Table 2). In response to rapamycin, Gcn4p induced genes involved in amino acid biosynthesis, intermediary metabolism, and transport processes. Furthermore, analogous to the TOR pathway, Gcn4p targets many genes that are central for nitrogen utilization.

The GAAC and TOR pathways intersect at multiple points to regulate the transcriptome. Rapamycin inhibition of TORC1 is suggested to reduce inhibitory phosphorylation of Gcn2p by activating type 2A-related protein phosphatases such as Stp4p and by inhibiting an unknown protein kinase (13). Our study suggests that the shift to a secondary nitrogen source such as GABA is a physiological condition that releases TORC1 inhibition of Gcn2p and the GAAC. We propose that there is a dual mechanism regulating the GAAC upon shifting to GABA medium (Fig. 9). Gcn2p activation can occur by both the release of TOR repression of Gcn2p and the activation of this elf2α kinase by uncharged tRNAs that accumulate in response to a shift to a secondary nitrogen source. We found that a shift to GABA medium led to significant deacylation of tRNAs (tRNA^Phe and tRNA^Cys) (Fig. 7), which is suggested to also contribute to the activation of Gcn2p by direct binding to this elf2α kinase. It is noted that deletion of GCN2 alters the pattern of accumulated uncharged tRNA (Fig. 7C). In the gcn2Δ cells grown in synthetic medium containing ammonia there were significant levels of many different uncharged tRNAs, including tRNA^Asp, tRNA^Cys, tRNA^Lys, tRNA^Phe and tRNA^Tyr. This finding suggests that the loss of Gcn2p creates a deficiency in the amino acid biosynthetic pathways that can increase the levels of many different deacylated tRNAs. Interestingly, upon shifting the GCN2-deficient cells to GABA, there continued to be significant levels of uncharged tRNA, albeit the pattern was modified compared with the wild-type strain (Fig. 7C).

Additional points of intersection occur downstream in the TOR and GAAC pathways. The Gcn4p activation core includes GLN3, suggesting that activation of the GAAC can further Gln3p activity at least under some nutrient conditions (Fig. 2C). Therefore, the requirement for Gcn4p for stress-induced expression of some genes may be indirect. The transcriptional activators Gcn4p and Gln3p also can coordinately induce transcription of target genes such as UGA3 by binding at different elements within their respective promoters (Fig. 8E). This regulatory circuit also illustrates that Gcn4p serves as an indirect transcriptional activator of the UGA1, UGA2, and UGA4 catabolic genes.

The third central question addressed in this study is whether Gcn2p can regulate the transcriptome independently of Gcn4p or vice versa. The predominant picture is that genes requiring Gcn2p for induction in response to either rapamycin or 3-AT are also dependent on Gcn4p. These results support the model in which the primary role of Gcn2p in yeast is to enhance Gcn4p expression in response to stress. However, Gcn2p is suggested to be required for a small number of genes for activation in response to both 3-AT and rapamycin independently of

**FIGURE 7. Increased uncharged tRNA levels in cells shifted to GABA medium.** The protrophic strain WY798 and its isogenic gcn2Δ counterpart (WY799) were cultured in SD medium and then shifted to synthetic medium containing GABA as the sole nitrogen source for 15, 30, and 120 min. The genome-wide charging of tRNA was measured using the microarray method. A, scanned fluorescent images of tRNA^TM hybridized to the complementary probe in the microarrays. The tRNA preparations were cultured in SD medium (0 min) or GABA medium for 120 min. **Yellow** represents no change in the charging of tRNA^TM, and **green** indicates low tRNA charging. **B**, the relative levels of tRNA charging are presented as the ratio of each charged tRNA prepared from the wild-type strain cultured in GABA medium for 60 min compared with those cultured in SD medium. The x axis lists each of the different tRNAs collated into hydrophobic, small, charged, and polar groups. The value of 1.0 in the y axis indicates that the tRNA charging in cells cultured in GABA is equal to that in the SD control. Values less than 1.0 indicate reduced tRNA charging, and values greater than 1.0 denote tRNA charging that is greater upon shift of the cells to GABA medium. **Error bars** represent ±S.E. **C**, heat map representation of genome-wide tRNA charging in response a shift to GABA medium. The levels of tRNA charging were measured in the GCN2 and gcn2Δ strains upon shifting to GABA medium for 15, 60, and 120 min. Those cells that were shifted to SD medium as a control are represented as 0 min. Green indicates decreased tRNA charging in the GABA medium compared with the SD control, and red represents enhanced tRNA charging, as listed on the scale to the right of the figures. **D**, Northern blot analysis of acid-denaturing gels measuring the charging of tRNA^TM in the GCN2 and gcn2Δ strains upon shifting the cultures from SD medium to GABA medium for up to 120 min as indicated. The **panels** comprise an autoradiogram representing hybridization of a radiolabeled probe complementary to charged (slower migrating band) and uncharged (faster migrating band) tRNA^TM. As a control, the tRNA^TM was deacylated prior to Northern analysis (−) and compared with samples that were not subjected to deacylation in vitro prior to Northern analysis (+). **E**, strain FY139 (gcn2Δ) containing plasmid pYB41 encoding a GCN4-lacZ reporter with u0RS intact was transformed with a low-copy plasmid encoding GCN2, mutant gcn2-m2, or vector alone. Cells grown in synthetic medium containing ammonia as the nitrogen source (NH4^+) were treated with 10 mM 3-AT (NH4^+ + 3-AT) or 200 mm rapamycin (NH4^+ + Rap), or grown in medium containing GABA as the nitrogen source for 6 h. The average β-galactosidase activity measured from three independent cultures ± S.E. is shown.
GCN4p and Gln3p co-regulate the UGA3 promoter. A, a P\textsubscript{UGA3}-lacZ reporter plasmid was introduced into wild-type cells or into cells deleted for GCN2, GCN4, or GLN3, individually or in combination, as indicated. Cells were cultured in synthetic complete medium supplemented with all amino acids except histidine, treated with 3-AT or rapamycin (Rap), or not treated (Control) for 6 h, and β-galactosidase activity was measured. The average β-galactosidase activity from two independent cultures ± S.E. is shown. B, wild-type cells containing a lacZ reporter gene under the control of the UGA3 promoter (−677) or a deletion of the UGA3 promoter (−371, −300, −200, or −103), as indicated, were cultured and treated as described in A. The predicted Gln3p and Gcn4p binding sites in the UGA3 promoter are indicated. C, the consensus Gln3p (GATA) and Gcn4p (GCRE) binding sites present in the minimal UGA3 promoter at −206 and −112, respectively, were mutated by site-directed mutagenesis, as indicated. Wild-type cells containing a lacZ reporter gene under the control of the wild-type minimal UGA3 promoter (−300) were cultured and analyzed as described in A. Alternatively, a minimal promoter containing mutant Gln3p (GATA) and Gcn4p (GCRE) binding sites, individually or in combination, was analyzed. D, wild-type cells containing P\textsubscript{UGA3}-lacZ reporter genes as described in C were cultured in synthetic medium containing ammonia as the nitrogen source in the presence of all 20 amino acids (NH\textsubscript{4}\textsuperscript{+} + AA) or the absence of all 20 amino acids (NH\textsubscript{4}\textsuperscript{+}) or were grown in medium containing GABA as the nitrogen source for 6 h as indicated. E, model depicting the role of TOR-mediated regulation of Gln3p and Gcn4p in the control of UGA3 mRNA expression, which impacts the subsequent utilization of GABA as a nitrogen source in yeast.

FIGURE 8. GCN4 Is Integral to Nitrogen Assimilation

GCN4p, ARO9 and ARO10 (Fig. 3, A and B) are central to the catabolism of aromatic amino acids, and interestingly their induced mRNA expression was independent of GCN4. However, deletion of either GCN2 or GCN4 led to a significant growth defect in medium containing phenylalanine as the sole nitrogen source when combined with aro80/H9004 (Fig. 3D). These results suggest that although Gcn4p is not involved directly in ARO9 and ARO10 transcription, Gcn4p contributes to the metabolic conditions required for assimilation of aromatic amino acids.

It is also noteworthy that almost a third of the genes targeted by Gcn4p displayed significantly reduced requirements for Gcn2p, suggesting that basal levels of Gcn4p retain important biological functions (Table 2). This idea is consistent with the findings that gcn4Δ cells have a growth defect in the absence of supplemented amino acids, whereas the loss of GCN2 in an otherwise prototrophic strain does not have a phenotype in SD medium devoid of amino acids (Fig. 3D). Analogous to Gcn4p, the basal activity of Gln3p is also suggested to have activating functions in the transcriptome. This was illustrated in cells treated with 3-AT, where 71 genes required Gln3p for full induction (Table 2). However, 3-AT did not lead to any increase in the expression of the P\textsubscript{GATA}-lacZ reporter (data not shown). This suggests that the basal activity of Gln3p is a significant contributor to the transcriptome during amino acid starvation, although the precise mechanisms are currently unclear. Supporting this idea is the finding that deletion of this transcription factor reduces growth in 3-AT containing medium (Fig. 1A).
were transcriptionally induced during either amino acid starvation or rapamycin treatment. Gcn4p can also increase the expression of additional genes that are unique to the specific stress (Table 2). Currently, we understand only the general mechanistic schemes for how Gcn4p can delineate between core and stress-specific gene transcription. Gcn4p activation of transcription is largely thought to be a consequence of its increased concentration in the cell, which involves translational control induced by eIF2α phosphorylation and its turnover, triggered by Pho85p/Pcl5p (55, 62, 63). Elevated levels of Gcn4p lead to its enhanced binding at the GCREs in the promoters of its target genes, which then serves to recruit different co-activators that facilitate association with RNA polymerase II (64).

For stress-specific gene transcription, one anticipates additional mechanisms for controlling Gcn4p activity or supplementary transcription factors that interact with the promoters of a subset of Gcn4p-targeted genes and are regulated by alternative stress pathways. Supporting the idea that additional mechanisms contribute to Gcn4p activity, we found that GABA induction of Gcn4p transcriptional activity in fact exceeded that measured for other stresses, 3-AT and rapamycin, despite GABA being a more moderate inducer of GCN4 translational control (Figs. 4C and 5C). Regarding supplemental transcription factors, we showed that Gcn4p and Gln3p were required for full induction of 24 different genes during rapamycin stress, including regulation of the UGA3 promoter in response to rapamycin treatment, as well as during a shift to the GABA medium (Table 3 and Fig. 8, C and D). Cooperation between Gcn4p and a second transcription factor, which can be regulated independently of Gcn4p and the GAAC during certain stresses, would facilitate variations in the transcriptome. Many of these central concepts concerning Gcn4p and stress-specific gene expression are germane to the transcriptome induced by eIF2α phosphorylation in mammalian cells subjected to different stress arrangements, such as those influenced by nutrient deficiencies or unfolded proteins (65–69).

Gcn4p was also shown to be required for the repression of a number of genes in response to 3-AT or rapamycin, with 16 genes constituting the so-called Gcn4p repression core (Fig. 2D and supplemental Table S5). The mechanism by which Gcn4p contributes to repressed transcription is not understood. It has been proposed that elevated levels of Gcn4p may squelch the transcription of certain target genes by binding to and impeding the function of key co-activator proteins, therefore compromising the induction of these target genes (70, 71). Interestingly, many of these Gcn4p repression core genes have GCREs in proximity to their promoters, which suggests that Gcn4p repression may result through direct DNA binding. Gln3p contributed to a ≈2-fold repression of an even greater number of genes compared with Gcn4p (396 versus 197 genes; Table 2 and supplemental Table S2). The underlying mechanisms for Gln3p repression are also unclear. It has been suggested that Gln3p repression of transcription of amino acid biosynthetic genes may occur indirectly through imbalanced changes in the expression of permease genes that facilitate nutrient uptake (72). We note that many of the genes requiring GLN3 for repression have identifiable GATA elements in their promoters (supplemental Table S5), suggesting that Gln3p may also repress select promoters by direct binding.

**TOR Regulates the GAAC to Facilitate Utilization of Secondary Nitrogen Sources**—Our study suggests that TOR regulation of the GAAC is central for adaptation to secondary nitrogen sources in the medium. The introduction of GABA as a nitrogen source enhances Gcn2p phosphorylation of eIF2α, leading to increased Gcn4p translation and transcriptional activity (Figs. 4C and 5, A, C, and D). Supporting the idea that GABA activation of Gcn2p is facilitated through inhibition of TORC1, we found that GABA induces Gln3p activity (Fig. 4D) and that GABA induction of GCN4 mRNA translation is diminished by loss of Sit4p (Fig. 6B), which is similar to findings reported for rapamycin treatment (13). By contrast, amino acid starvation elicited by 3-AT does not lead to repression of TORC1, as judged by only diminished Gln3p-directed transcription during this stress condition, and activation of Gcn2p in response to 3-AT does not require Sit4p (data not shown and Ref. 13).

Together, these results support a model in which dual mechanisms contribute to the activation of Gcn2p and the GAAC in response to changes in nutrient availability. In the first of these mechanisms, amino acid deprivation, such as that elicited by 3-AT, rapidly increases the levels of uncharged tRNA (45). Elevated levels of uncharged tRNA would then directly bind to the HisRS-related domain of Gcn2p, resulting in enhanced Gcn2p phosphorylation of eIF2α (Fig. 9). The second mechanism would occur when yeast cells are shifted to medium containing certain secondary nitrogen sources such as GABA. Repression of TORC1 upon shifting to GABA medium can induce eIF2α kinase activity by a mechanism consistent with the release of inhibitory phosphorylation of Gcn2p (Fig. 9). This release of Gcn2p inhibitory phosphorylation would involve the signaling pathway including TAP42 and the type 2A-related protein phosphatase encoded by SIT4 and the SIT4-associated protein (SAP) genes.
Uncharged tRNA is also suggested to be an activating ligand for Gcn2p in GABA medium; therefore TORC1 signaling and Gcn2p binding to uncharged tRNA appear to work in combination to enhance Gcn2p phosphorylation of eIF2α in response to a shift to secondary nitrogen sources. It is noteworthy that phosphorylation of eIF2α occurs within 15 min of a shift from SD to synthetic GABA medium and is retained after 120 min (Fig. 5A). However, significant decaylation of tRNA\(^{\text{Phe}}\) and tRNA\(^{\text{Cys}}\) occurs after culturing for 1 h in the GABA medium (Fig. 6C). The timing of these events suggests that the two mechanisms may contribute at different time periods during the course of activation of Gcn2p. Release of TOR repression through dephosphorylation of Gcn2p would occur rapidly followed by measurable levels of decylated tRNAs, which would enhance Gcn2p by direct binding to its HisRS-related regulatory domain. A central feature in this mechanism of activation is that the TORC1 and Gcn2p protein kinases are reciprocally regulated in response to changes in nitrogen source, which would contribute to their opposing control of protein synthesis and Gcn4p activation (Fig. 9).

It is noteworthy that the gcn2-m2 mutant, defective for binding to uncharged tRNA, blocks enhanced GCN4-lacZ expressed in response to shifting to GABA medium or during rapamycin treatment (Fig. 7E). Previously, Cherkasova and Hinnebusch (13) argued that activation of Gcn2p by rapamycin may be dependent on binding to uncharged tRNA. As rapamycin does not appear to reduce tRNA aminoacylation levels, it was proposed that Gcn2p could be activated by rapamycin in cells in the presence of amino acids because dephosphorylation of serine 577 allows Gcn2 to have a greater affinity for uncharged tRNA. In this way, the dephosphorylated Gcn2p can bind even basal concentrations of uncharged tRNA. Thus, although the underlying mechanisms are still not fully understood, these results suggest that eIF2α kinases may also be integrated with the mTOR pathway to control gene expression.

Integration of eIF2α Kinases and TOR in Mammals—Although a clear connection between TOR and Gcn2 protein kinase in yeast has been established, less is known about the regulation of Gcn2 by mammalian TOR. Recent studies from this laboratory have shown that a reduction in 4E-BP and S6K1 phosphorylation (two key downstream targets of mTOR (74)) following leucine starvation is blocked in liver from Gcn2\(^{-/-}\) mice (75). This suggests that there is a direct or indirect linkage between activation of the Gcn2 and mTOR pathways in mammalian cells starved for essential amino acids. Mammalian TOR (mTOR) is regulated by diverse stress conditions by a mechanism involving inhibitory TSC1 and TSC2 proteins (76).

Increased translation in Tsc2\(^{-/-}\) mouse embryo fibroblasts was shown to induce endoplasmic reticulum stress, activating the eIF2α kinase PERK (PKR-like endoplasmic reticulum kinase)/PEK (77). In this case, it is suggested that elevated protein synthesis overcomes the endoplasmic reticulum processing capacity, leading to elevated levels of malfolded protein and activation of PERK and the associated unfolded protein response. Interestingly, recent work in Drosophila has shown that RNA interference knockdown of dGCN2 suppresses the increased cell size associated with knockdown of Tsc2\(^{-/-}\) (78). Thus, although the underlying mechanisms are still not fully understood, these results suggest that eIF2α kinases may also be integrated with the mTOR pathway to control gene expression.

Acknowledgments—We thank Li Jiang for technical assistance, Alan Hinnebusch, Gerhard Braus, and Stephen Zheng for providing reagents, and the Biochemistry Biotechnology Facility at Indiana University for DNA sequencing. We also thank Ron Jerome at the Center for Medical Genomics for processing of microarray chips. Microarray experiments were carried out at the Center for Medical Genomics at Indiana University School of Medicine, which is supported in part by the Indiana Genomics Initiative and the Lilly Endowment, Inc.

REFERENCES

1. Hinnebusch, A. G. (1997) J. Biol. Chem. 272, 21661–21664
2. Hinnebusch, A. G. (2005) Annu. Rev. Microbiol. 59, 407–450
3. Wek, R. C., Staschke, K. A., and Narasimhan, J. (2004) Topics in Current Genetics: Nutrient-induced Responses in Eukaryotic Cells (Winderickx, J. G., and Taylor, P. M., eds) pp. 177–199, Springer, Berlin/Heidelberg
4. Wek, R. C., Jackson, B. M., and Hinnebusch, A. G. (1989) Proc. Natl. Acad. Sci. U. S. A. 86, 4579–4583
5. Natarajan, K., Meyer, M. R., Jackson, B. M., Slade, D., Roberts, C., Hinnebusch, A. G., and Marton, M. J. (2001) Mol. Cell. Biol. 21, 4347–4368
6. Abastado, J. P., Miller, P. F., Jackson, B. M., and Hinnebusch, A. G. (1991) Mol. Cell. Biol. 11, 486–496
7. Harding, H. P., Novoa, I., Zhang, Y., Zeng, H., Wek, R., Schapira, M., and Ron, D. (2000) Mol. Cell 6, 1099–1108
8. Vattem, K. M., and Wek, R. C. (2004) Proc. Natl. Acad. Sci. U. S. A. 101,
