# Genome-wide Analysis of tRNA Charging and Activation of the eIF2 Kinase Gcn2p[*][S][◆]

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When cells are subjected to nutritional stress, uncharged tRNAs accumulate and activate Gcn2p phosphorylation of eukaryotic initiation factor-2 (eIF2) and the general amino acid control pathway. The Gcn2p regulatory domain homologous to histidyl-tRNA synthetases is proposed to bind to uncharged tRNA, directly contributing to activation of Gcn2p. Here we apply a microarray technology to analyze genome-wide changes in tRNA charging in yeast upon activation of Gcn2p in response to amino acid starvation and high salinity, a stress not directly linked to nutritional deficiency. This microarray technology is applicable for all eukaryotic cells. Strains were starved for histidine, leucine, or tryptophan and shown to rapidly induce Gcn2p phosphorylation of eIF2. The relative charging level of all tRNAs was measured before and after starvation, and Gcn2p activation and the intracellular levels of the starved amino acid correlate with the observed decrease in tRNA charging. Interestingly, in some cases, tRNAs not charged with the starved amino acid became deacylated more rapidly than tRNAs charged with the starved amino acid. This increase in uncharged tRNA levels occurred although the intracellular levels for these non-starved amino acids remained unchanged. Additionally, treatment of a wild-type strain with high salinity stress showed transient changes in the charging of several different tRNAs. These results suggest that Gcn2p can be activated by many different tRNA species in the cell. These results also depict a complex cellular relationship between tRNA charging, amino acid availability, and non-nutrient stress. These relationships are best revealed by simultaneous monitoring of the charging level of all tRNAs.

Reductions in nutrient availability trigger stress responses that lower protein synthesis coincident with changes in gene expression that provide for adaptive modifications in metabolism and nutrient uptake. A major contributor to this stress adaptation is the general amino acid control (GAAC)[3] pathway (1–3). In the GAAC, starvation for amino acids induces phosphorylation of eukaryotic initiation factor-2 (eIF2) by the protein kinase Gcn2p. The eIF2 binds GTP and Met-tRNA$_i^{Met}$ and participates in the ribosomal selection of the mRNA start site during translation. Phosphorylation of the $\alpha$ subunit of eIF2 at serine 51 lowers its activity. The resulting reduction in global protein synthesis conserves resources and allows cells time to reprogram the transcriptome to alleviate the underlying nutrient stress. In the yeast *Saccharomyces cerevisiae*, eIF2$\alpha$ phosphorylation also leads to preferential translation of GCN4 mRNA, encoding a transcriptional activator of a large number of genes involved in amino acid metabolism and the salvaging of nutrients (1–5). Although *S. cerevisiae* has only a single eIF2$\alpha$ kinase, Gcn2p, mammalian cells have expanded this stress response pathway to include additional eIF2$\alpha$ kinases, which each respond to different environmental stresses (6). Like yeast, phosphorylation of mammalian eIF2$\alpha$ leads to a block in global translation, accompanied by induced translational expression of ATF4, a basic zipper transcription factor related to Gcn4p (7–10).

A central question in the GAAC concerns the mechanism by which Gcn2p is activated during amino acid starvation. It is proposed that increased levels of uncharged tRNA that accumulate during severe amino acid depletion are the vital signal activating the GAAC (1, 3, 11–14). Consistent with this idea is the observation that a temperature-sensitive mutant in the histidyl tRNA synthetase gene (*HTS1*) elicits induced Gcn2p phosphorylation of eIF2$\alpha$ even in the presence of abundant histidine (12). Gcn2p is suggested to monitor uncharged tRNA levels by direct binding of deacylated tRNA to a regulatory region in Gcn2p with sequence homology to the entire length of histidyl-tRNA synthetase (HisRS) enzymes (1, 3, 11–15). Activation of Gcn2p by uncharged tRNA is proposed to involve a transition from an inhibited to catalytically active conformation that involves direct contacts between the protein kinase domain, HisRS-related region, and the extreme carboxyl terminus of Gcn2p (1, 14, 16–18). Gcn2p binding to uncharged tRNA is suggested to be obligate for induced eIF2$\alpha$ kinase activity in

[3] The abbreviations used are: GAAC, general amino acid control; eIF2, eukaryotic initiation factor-2; HisRS, histidyl-tRNA synthetase; SC, synthetic complete; 3-AT, 3-aminotriazole; HPLC, high pressure liquid chromatography; DMSO, dimethyl sulfoxide.

response to amino acid starvation because residue substitutions in the HisRS-related domain (Gcn2p-m2) that block binding to uncharged tRNA *in vitro* impair phosphorylation of eIF2$\alpha$ *in vivo* (1, 3, 12, 13).

Elevated concentrations of uncharged tRNA are proposed to be the central signal regulating Gcn2p activity in response to changes in amino acid availability. Although tRNAs are quite stable, charging can be dynamic, with rapid tRNA deacylation occurring upon depletion for the cognate amino acid. It is noteworthy that activation of Gcn2p is not restricted to histidine starvation. In fact, starvation for one of several different amino acids can induce the GAAC, suggesting that the HisRS-related domain of Gcn2p has diverged from the *bona fide* HisRS enzymes such that activation Gcn2p can be activated via binding to many different uncharged tRNAs (1, 3, 12). Therefore, Gcn2p is proposed to bind to many different uncharged tRNAs that accumulate in response to starvation for each corresponding amino acid. Where an amino acid has multiple isoacceptor tRNAs, there may even be some Gcn2p binding preference for isoacceptors, which differ in abundance or are prone to more rapid deacylation in response to amino acid depletion. An alternative version of this model is that Gcn2p binds and is activated by a limited number of uncharged tRNAs whose aminoacylation can be affected by starvation for both cognate and noncognate amino acids. According to this model, signaling tRNAs, such as tRNA$^{His}$, can be deacylated in response to starvation not only for histidine starvation but for deprivation for additional amino acids. Such changes in the charging of selected tRNAs may reflect the interconnections between the synthesis and catabolism of some amino acids, amino acid storage strategies, or the sensitivity of less abundant tRNAs to deacylation in response to general nutrient stress. In this case, the timing of Gcn2p phosphorylation of eIF2$\alpha$ in response to starvation for different amino acids may differ depending on how direct the nutrient limitation affects the charging of the key signaling tRNA.

In addition to amino acid starvation, Gcn2p is activated in yeast by diverse environmental stresses, including high salinity induced by elevated concentrations of sodium chloride, anesthetics isoflurane and halothane, oxidizing conditions such as methyl methanesulfonate and hydrogen peroxide, and the immunosuppressant rapamycin, an inhibitor of the target of rapamycin (TOR) pathway (1, 3, 19–25). Although these stress agents are not directly related to amino acid deprivation, it was proposed that many of these treatment regimens activate Gcn2p by deacylating tRNAs. In support of this idea, the tRNA-binding mutant, Gcn2p-m2, blocks the induction of the GAAC in response to several of these environmental stresses. However, it is currently not known whether tRNA charging is adversely affected upon exposure to these different environmental stresses. Activation of Gcn2p in response to rapamycin has also been shown to involve dephosphorylation of Gcn2p at residue Ser-577, so additional signaling pathways may contribute to activation of Gcn2p eIF2$\alpha$ kinase in concert, or independently, of Gcn2p binding of uncharged tRNA (23).

In this study, we addressed the changes in tRNA charging response to several different stresses, such as amino acid starvation and high salinity, in *S. cerevisiae*. Using tRNA microarrays, we measured the changes in the charging levels of every chromosomal-encoded tRNA expressed in yeast. During each amino acid-limiting condition, there was a rapid deacylation of the cognate tRNAs, coincident with induced eIF2$\alpha$ phosphorylation. Interestingly, in auxotrophic strains grown in the absence of essential amino acids, we also found deacylation of tRNAs not linked with the limiting amino acid. In response to treatment with high concentrations of sodium chloride, a stress not directly linked to nutritional deprivation, we found enhanced deacylation of tRNA$_i^{Met}$ and tRNA$^{Cys}$. This observation supports the idea that changes in tRNA charging levels contribute to activation of Gcn2p in response to stress conditions not directly involved with nutrient deprivation. Together these results support the model that upon diverse stress conditions, multiple tRNAs become deacylated, serving as key signaling molecules that trigger activation of Gcn2p and the GAAC.

## EXPERIMENTAL PROCEDURES

*Yeast Strains and Culture Conditions*—The *S. cerevisiae* strains used in this study were derived from EG328-1A (*MATα ura3-52 leu2 trp1*) (20, 26). Strain EG328-1A was transformed with DNA fragments containing the *LEU2, TRP1,* or *URA3* genes, generating a prototrophic strain, WY798 (*MATα URA3 LEU2 TRP1*), and strains WY795 (*MATα URA3 leu2 TRP1*) and WY900 (*MATα URA3 LEU2 trp1*), deficient for the synthesis of leucine and tryptophan, respectively. WY799 (*MATα gcn2::URA3 LEU2 TRP1*) and WY796 (*MATα gcn2::URA3 leu2 TRP1*) contain a deletion in the *GCN2* gene. WY909 (*MATα URA3 LEU2 TRP1 his3::kanMX4*) was constructed by PCR-mediated gene replacement, which replaced the entire *HIS3* coding region. The deletion cassette was amplified by PCR using genomic DNA from the *HIS3* deletion mutant in the BY4741 strain background, which was purchased from Open Biosystems (Huntsville, AL).

Yeast cells were grown overnight at 30 °C in synthetic complete (SC) media containing 2% dextrose, 0.5% ammonium sulfate, and all amino acids (27). Cells were then diluted in SC to $A_{600}$ ~0.1 and cultured to $A_{600}$ between 0.4 and 0.5, collected by centrifugation, and inoculated into SC medium or SC medium devoid of tryptophan, leucine, or histidine, as indicated. Cells were also inoculated into SC medium devoid of histidine supplemented with 10 mM 3-aminotriazole (3-AT). Cells were cultured in one of these amino acid-limiting conditions for 15, 60, or 120 min at 30 °C, as indicated. Finally, yeast cells were incubated in SC medium supplemented with 1 M NaCl at 30 °C for up to 120 min.

*Immunoblot Analysis*—Yeast cells were cultured in SC medium or subjected to amino acid starvation, as described above. Cells were then collected by centrifugation, washed with ice-cold water, and resuspended in a breaking solution containing 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 with glass beads and vortexing for 2 min at 15-s intervals, maintaining the samples on ice between each mixing period. Lysed cells were clarified by centrifugation to remove glass beads and cell debris, and the

protein content of the lysate preparations was measured using the Bradford method and bovine serum albumin as a standard (28). Equal amounts of each protein sample were separated by electrophoresis in a SDS-12% polyacrylamide gel and transferred to nitrocellulose filters. Immunoblot analyses were carried out using a polyclonal antibody that specifically recognizes eIF2α phosphorylated at Ser-51 (Cell Signaling). Total eIF2α levels were measured using a rabbit polyclonal antibody against recombinant yeast eIF2α (12). The antibody-bound eIF2α was visualized by using horseradish peroxidase-conjugated anti-rabbit IgG and chemiluminescent substrate.

*Isolation and Characterization of Charged tRNA*—Yeast cells were cultured as described above and collected by centrifugation. Cell pellets were resuspended in lysis buffer solution (0.3 M NaOAc/HOAc (pH 4.5) and 10 mM EDTA) and then vortexed with acetate-saturated phenol/CHCl$_3$ (pH 4.5) three times each for 30 s. Lysates were placed in an ice bath following each vortex mixing to ensure that the preparations remain chilled. Following cold centrifugation at 18,600 relative centrifugal force, the aqueous layers were removed and subjected to another extraction with acetate-saturated phenol/CHCl$_3$ (pH 4.5) solution. The aqueous layers were removed, and the RNA was precipitated by adding 2.7× volumes of ethanol and centrifuging at 18,600 relative centrifugal force. RNA pellets were resuspended in lysis buffer solution and then precipitated with ethanol a second time. Finally, precipitated RNA was resuspended in a solution containing 10 mM NaOAc/HOAc (pH 4.5) and 1 mM EDTA for subsequent microarray analyses.

The tRNA microarray experiments were adapted from a previous study that measured the charging level of all tRNAs in *Escherichia coli* (29). RNA samples were divided into two equal parts. One portion was treated with periodate, which selectively oxidizes uncharged tRNA and blocks the subsequent ligation to the fluorophore-labeled oligonucleotide. The second portion was not treated with periodate, and both charged and uncharged tRNAs were subsequently ligated to the fluorophore-labeled oligonucleotide. For yeast studies, we used three *E. coli* tRNAs (tRNA$^{Phe}$, tRNA$^{Tyr}$, and tRNA$^{Lys}$ from Sigma-Aldrich) as standards to control for variations in the sample treatment and array hybridization. tRNA standards were charged in a solution containing 10 μM tRNA, 60 mM Tris-HCl (pH 7.5), 12.5 mM MgCl$_2$, 10 mM KCl, 3 mM dithiothreitol, 1.5 mM ATP, 1 mM spermine, 1 mM of the respective amino acid, and 4.2 units/μl *E. coli* aminoacyl-tRNA synthetases (from Sigma-Aldrich). The reaction was incubated at 37 °C for 15 min and then mixed with equal volumes of 0.5 M NaOAc/HOAc (pH 4.8), extracted with acetate-saturated phenol/CHCl$_3$, and precipitated with ethanol. Charged tRNA standards were stored in 50 mM NaOAc/HOAc (pH 4.8) and 1 mM EDTA at −80 °C for up to one month. 0.66 pmol each of the charged tRNA standards were added to 1 μg of total RNA prior to periodate oxidation.

Periodate oxidation was performed by incubating total RNA premixed with charged tRNA standards (0.1 μg/μl) with 50 mM NaIO$_4$ in 100 mM NaOAc/HOAc (pH 4.8). The samples were incubated at room temperature for 30 min and then quenched by adding glucose to 100 mM followed by incubation at room temperature for 5 more minutes. The RNA preparations were
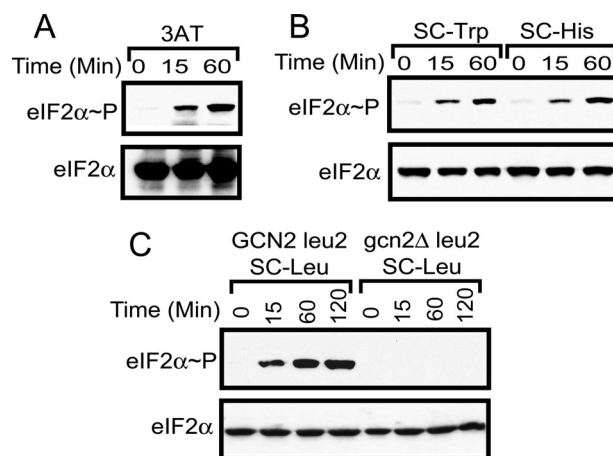


**FIGURE 1. Phosphorylation of eIF2α is induced in response to starvation for different amino acids.** Isogenic yeast strains were subjected to amino acid starvation by treating a prototrophic strain WY798 with 3-AT (*A*) or introducing auxotrophic strains WY900 (*trp1*) and WY909 (*his3*) into SC medium depleted of tryptophan (*SC-Trp*) or histidine (*SC-His*), respectively, for up to 60 min (*B*). WY795 (*GCN2 leu2*) and WY796 (*gcn2 leu2*) were also cultured in SC medium devoid of leucine (*SC-Leu*) for up to 120 min (*C*). Cells cultured in SC medium containing a full complement of amino acids are represented as zero time in each of the panels. Equal amounts of protein lysates were separated by SDS-PAGE, and the levels of eIF2α phosphorylated specifically at serine 51 (*eIF2α~P*) or total eIF2α were measured by immunoblot analyses using antibodies specific to each.

subjected to a G25 spin column (GE Healthcare) and then precipitated with ethanol. To deacylate tRNAs, samples were resuspended in 50 mM Tris-HCl (pH 9) and incubated at 37 °C for 30 min. The RNA samples were ethanol-precipitated and subsequently resuspended in water. RNA preparations not subjected to periodate oxidation were prepared in the same manner except that NaIO$_4$ was replaced with NaCl. Ligations of the tRNA samples to the fluorescent tag were performed in 1× ligase buffer (USB Corp.), 15% DMSO, 0.1 μg/μl deacylated RNA, 4 μM Cy3- or Cy5-containing oligonucleotides (Integrated DNA Technologies), 0.5 units/μl T4 DNA ligase (USB Corp.), and yeast exo-phosphatase (5,000 units/μl). The reactions were incubated at 16 °C for over 16 h, mixed with 3 volumes of 50 mM KOAc (pH 7), 200 mM KCl, and then extracted with an equal volume of phenol/CHCl$_3$. Following centrifugation, RNA preparations were precipitated with ethanol and resuspended in water.

Microarray analysis was performed as described (29–31) with PerfectHyb Plus (Sigma) as the hybridization buffer, and GenePix Pro 6.0 was used to analyze the arrays. Microarrays were custom printed by Integrated Genomics (Chicago, IL) or Microarray Inc. (Nashville, TN). Each array contains up to 54 replicates of complementary DNA oligonucleotide probes covering the entire length of tRNA minus the 3′-CCA sequence. The same array has probes for all nuclear-encoded yeast tRNAs plus tRNAs from *E. coli* and *Yersinia pestis* as hybridization controls. As demonstrated previously, very little overlapping signals were observed between total bacterial and yeast tRNAs (30). Data analysis and statistics were provided by the scanner software (Axon Instruments, Foster City, CA), and the median values of dye ratios for each array probe were presented.

*Analysis of tRNA Aminoacylation by Northern Hybridization*— Northern analyses were performed using 6.5% polyacrylamide
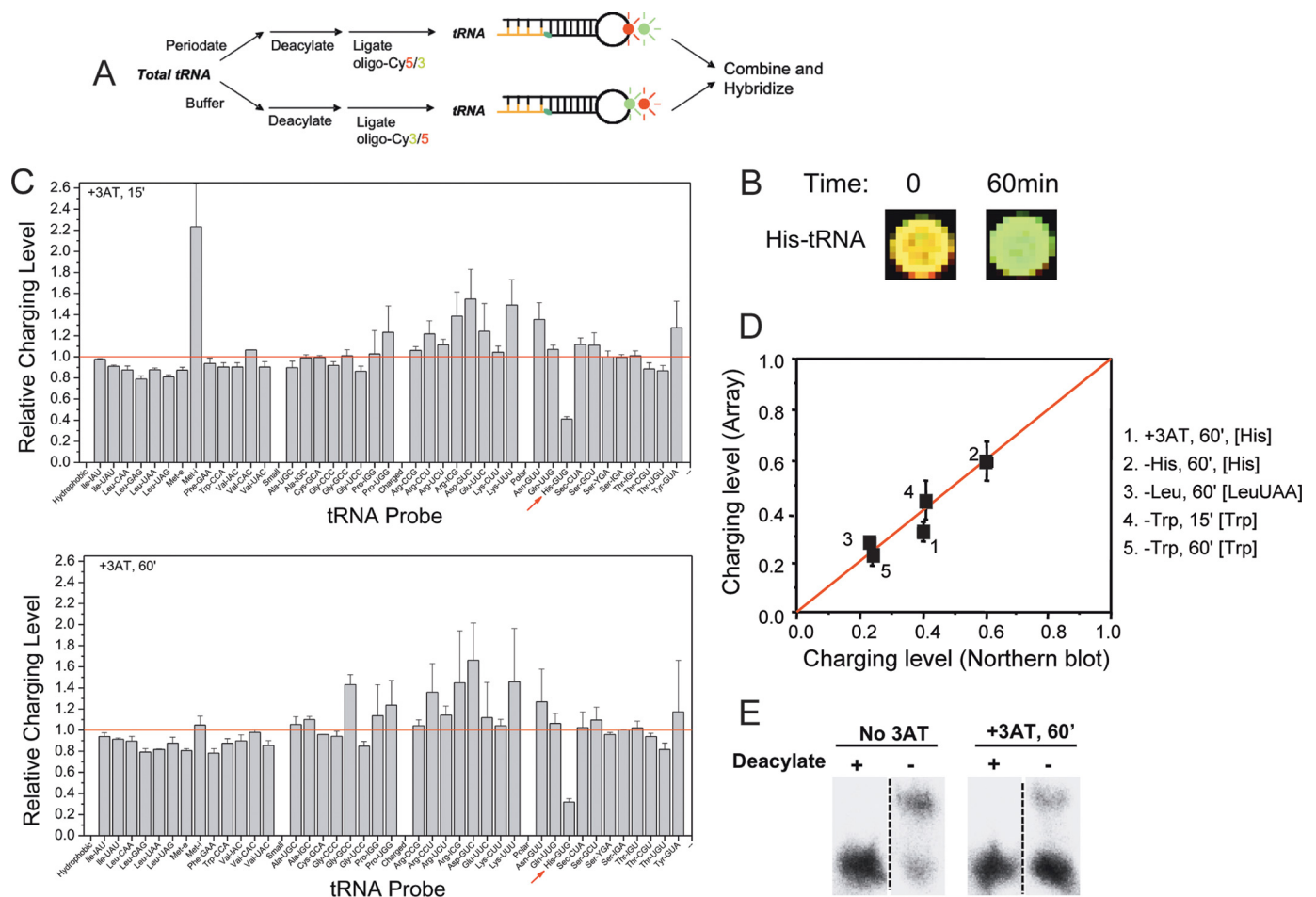
FIGURE 2. **Genome-wide analysis of tRNA charging.** *A*, the charging of each chromosomal-encoded tRNA in *S. cerevisiae* was measured by a method that involved selective labeling of charged tRNA with Cy3 or Cy5 fluorophore and hybridization to tRNA microarrays containing complementary probes to each tRNA. Yeast cells were starved for amino acids, and total tRNA was extracted under conditions that retain aminoacylated tRNAs. The total RNA samples were divided into two portions. One part was treated with periodate, which oxidizes the 3′-acceptor stem of uncharged tRNA, whereas the other part was exposed only to buffer solution. The tRNAs in both parts were deacylated and then ligated to a fluorescent-tagged oligonucleotide, which contains a stable stem-loop structure and a portion complementary to the 3′-CCA sequence that is conserved among all tRNAs. Both of the samples were labeled with Cy3 or Cy5 fluorophore and were combined and hybridized to microarrays that contained probes for each chromosomal-encoded tRNA. The first microarray used Cy5-labeled charged tRNA and Cy3-labeled total tRNA followed by a second microarray, which utilized Cy3-labeled charged tRNA and Cy5-labeled total tRNA. *B*, representative spots from scanned fluorescent images of tRNA[His] (*His-tRNA*) hybridized to a complementary probe in the microarrays. The tRNA preparations were from cells treated with 3-AT for 60 min (*60min*) or no starvation (*0*). *Green* intensity measurements indicate decreased tRNA[His] charging, and *yellow* indicates no difference in tRNA aminoacylation. *C*, relative tRNA charging levels are presented as the ratio of charging of each tRNA listed prepared from cells cultured in the presence of 3-AT for 15 min (*+3AT, 15′*) or 60 min (*+3AT, 60′*) when compared with non-starved cells. The *x* axis indicates each of the different tRNAs, separated into hydrophobic, small, charged, and polar groups. In the *y* axis, the value of 1.0 represents equal tRNA charging in the presence of 3-AT when compared with absence of 3-AT (non-starved control). Values less than 1.0 indicate reduced tRNA charging in response to 3-AT treatment, whereas values greater than 1.0 represent enhanced charging of the indicated tRNA. Heat map representations of the genome-wide tRNA charging in response to 3-AT and other subsequent stress conditions are represented in supplemental Fig. S1. *Error bars* indicate S.E. *D*, comparison of the tRNA charging levels measurements by the microarray method when compared with that measured by Northern blot analysis of acid-denaturing gels. The *numbers* indicate the charging of specific tRNAs charging in the prototroph strain treated with 3-AT or the auxotroph strains starved for histidine, leucine, or tryptophan, as indicated by the legend. *+3AT, 60′*, cells cultured in the presence of 3-AT for 60 min ; *−His, 60′*, cells cultured in SC medium devoid of His for 60 min; *−Leu, 60′*, cells cultured in SC medium devoid of Leu for 60 min; *−Trp, 15′*, cells cultured in SC medium devoid of Trp for 15 min; *−Trp, 60′*, cells cultured in SC medium devoid of Trp for 60 min. *E*, Northern blot analysis of acid-denaturing gels measuring the charging of tRNA[His] in strain WY798 treated with 3-AT for 1 h (*+3AT, 60′*) or with no amino acid starvation (*No 3AT*). The panels illustrate an autoradiogram representing hybridization of a radiolabeled probe complementary to charged (slower migrating band) and uncharged (faster migrating band) tRNA[His]. As a control, the tRNA[His] was deacylated prior to the Northern analysis (+) and when compared with the tRNA preparations, which were not subjected to deacylation *in vitro* prior to the Northern analysis (−).

acid denaturing gels as described (32). Briefly, gels were prerun for 30 min at 500 V with 0.1 M NaOAc/HOAc (pH 4.8). 2.5 $\mu$g of RNA were loaded in each lane with acid denaturing sample buffer solution (0.1 M NaOAc/HOAc (pH 4.8), 8 M urea, 5% glycerol, 0.05% bromphenol blue, and 0.05% xylene cyanol). Charged and uncharged tRNAs were separated by gel electrophoresis for 16 h at 500 V. The RNA was transferred by layering 3MW blotting paper (MIDSCI), Hybond-XL membrane (GE Healthcare), the gel, and plastic wrap on a gel dryer with the

blotting paper facing the dryer. Gels were dried for 2 h at 80 °C, which both transfers and fixes the RNA to the membrane. The gels were removed from the membranes by soaking the membrane in distilled water. Membranes were prehybridized for 30 min at room temperature in hybridization solution (300 mM NaCl, 1% SDS, and 20 mM phosphate buffer (pH 7)). tRNAs were visualized by hybridization with 5′-$^{32}$P-labeled DNA oligonucleotide probes for 16 h at 60 °C. The membranes were washed three times for 20 min each in a solution containing 0.3

His-starvation



**A** Time: 0 60min

His-tRNA

Asp-tRNA

**B**
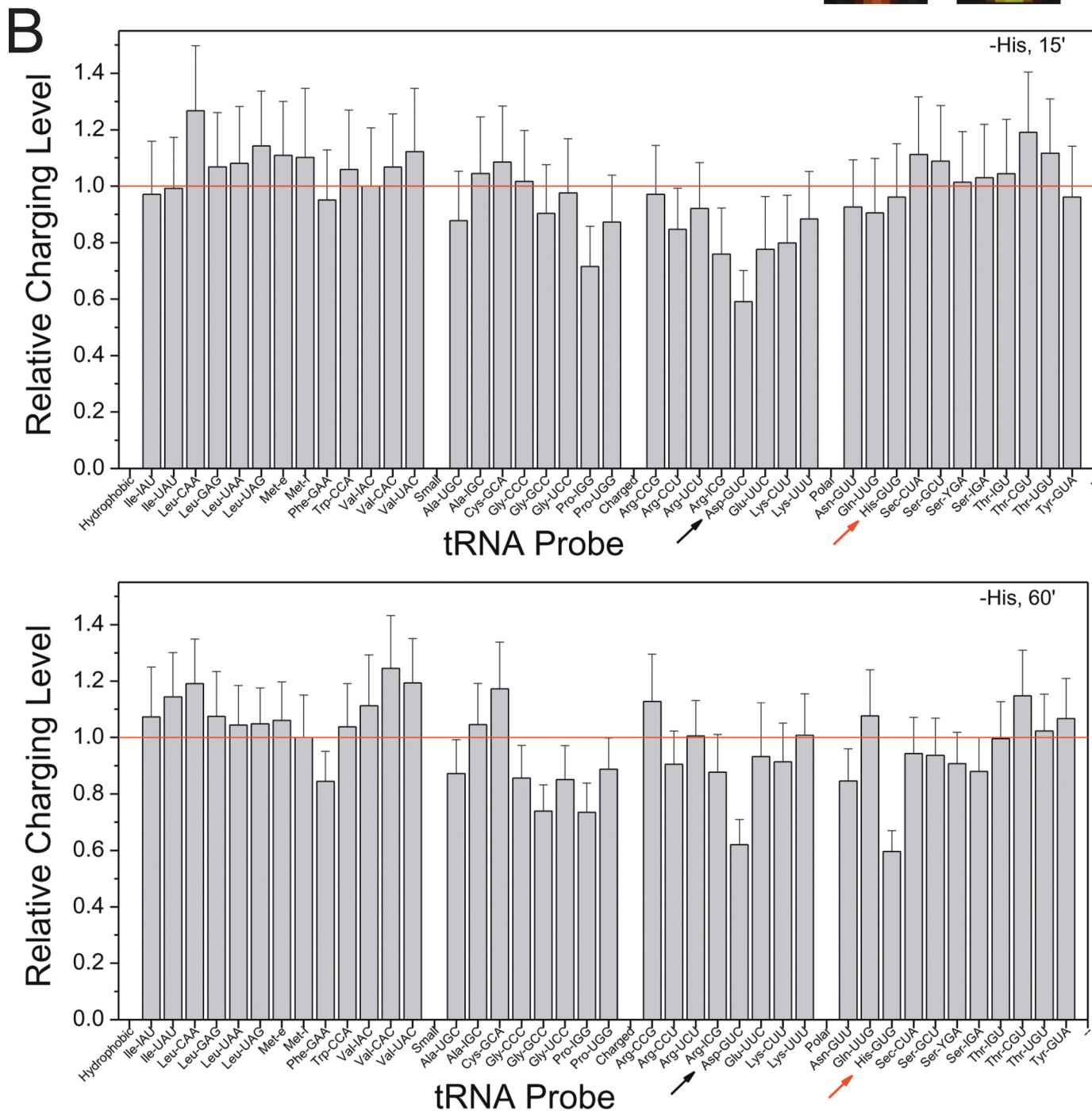
M NaCl, 20 mM phosphate buffer (pH 7.2), 2 mM EDTA, and 0.1% SDS and exposed to imaging plates and quantified using a PhosphorImager (GE Healthcare).

*Measurements of Amino Acids*—Yeast cells were cultured in amino acid starvation conditions, as described above. Cell lysates were prepared and amino acid compositions were analyzed as described (33). Briefly, cell cultures were collected by filtration using a Millipore 0.45-micron filter followed by extensive washing with chilled water. The filtrate was then resuspended and incubated in 500 $\mu$l of 5% trichloroacetic acid solution on ice for 30 min accompanied by gentle agitation. After centrifugation to remove cellular debris, the supernatant was filtered a second time using an Amicon Ultra centrifugal filter (molecular weight 10,000). Amino acid levels were measured using ninhydrin to determine the total levels in the cell preparation, and equal amounts were analyzed for each amino acid profile. Amino acid profiles were analyzed by the Quantitative Amino Acid Core Facility at Indiana University School of Medicine (IUSM) by using the Waters Pico-Tag method that involves derivatization with phenylisothiocyanate, forming phenylthiocarbamyl derivatives. Prepared samples were analyzed by reversed phase HPLC separation and UV detection by using a Waters Alliance HPLC system. Results are presented as means $\pm$ S.E. that were derived from three independent experiments. The Student's *t* test was used to determine the statistical significance of differences between starved and non-starved preparations, with an *asterisk* indicating a *p* value less than 0.05 in Figs. 6 and 7.

## RESULTS

*Starvation for Different Amino Acids Induces eIF2$\alpha$ Phosphorylation*—Starvation for one of several different amino acids was reported to activate Gcn2p and the GAAC (1, 3, 12). We addressed this central idea by measuring the induction of eIF2$\alpha$ phosphorylation in yeast cells subjected to starvation for histidine, tryptophan, or leucine (Fig. 1). First, a wild-type version of yeast strain EG328-1A that can synthesize all amino acids was treated with 3-AT, a potent inhibitor of histidine biosynthesis. Within 15 min of 3-AT treatment of this prototrophic strain, there was a significant induction of eIF2$\alpha$ phosphorylation as measured by immunoblot analysis using phospho-specific antibody (Fig. 1*A*). We independently confirmed that starvation for histidine rapidly induced eIF2$\alpha$ phosphorylation by shifting an isogenic *HIS3*-deficient yeast strain from SC medium to medium devoid of histidine. Phosphorylation of eIF2$\alpha$ was enhanced within 15 min of histidine starvation of this auxotrophic strain (Fig. 1*B*). Phosphorylation of eIF2$\alpha$ phosphorylation was also rapidly induced in *trp1* and *leu2* mutant strains upon culturing in medium devoid of tryptophan and leucine, respectively (Fig. 1, *B* and *C*). As expected,

loss of *GCN2* blocked eIF2$\alpha$ phosphorylation during leucine starvation. These results support the idea that starvation for several different amino acids rapidly induces high levels of Gcn2p phosphorylation of eIF2$\alpha$.

*Amino Acid Starvation Leads to Deacylation of tRNAs*—To measure changes in tRNA charging of all tRNA species in *S. cerevisiae*, we adapted a method involving tRNA microarrays that includes complementary probes to each chromosomal-encoded tRNA (Fig. 2*A*). Cells were starved for histidine, tryptophan, or leucine as above, and RNA was extracted under mild acidic conditions, which retain aminoacylated tRNAs. The RNA sample was split into two portions; one-half was subjected to periodate oxidation, which destroys the 3'-ends of all uncharged tRNAs, whereas the other half served as a control and was not treated with periodate (Fig. 2*A*). Both samples were then deacylated using alkaline pH, and a fluorescent-tagged oligonucleotide was ligated onto only those tRNAs with intact 3'-ends. The fluorescent oligonucleotide contains a stable stem-loop structure, with a portion complementary to the 3'-CCA sequence that is conserved among all tRNAs. Both samples were labeled with Cy3 or Cy5 fluorophore. After fluorescent labeling, tRNA preparations with opposite fluorophores (*e.g.* charged tRNA with Cy5 and total tRNA with Cy3) were combined and hybridized to microarrays. The custom-made microarrays contained multiple replicates for each probe, and at least 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, whereas the second microarray used Cy3-labeled charged tRNA and Cy5-labeled total tRNA. The array results show the relative charging level between starved *versus* unstarved samples and are presented as histograms where the tRNAs are grouped according to amino acid properties (hydrophobic, small, charged, polar) (Figs. 2–5). Corresponding heat maps for each of the tRNA analyses are presented in supplemental Fig. S1. As described later, we also validated key array results by standard Northern blot analyses (Fig. 2, *D* and *E*).

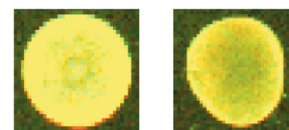*Histidine Starvation*—In response to histidine starvation induced by 3-AT treatment, there was a sharp decrease in the charging of tRNA^His (Fig. 2). Deacylation of tRNA^His occurred within 15 min of 3-AT exposure and was sustained after 60 min of treatment, with ~30% of aminoacylation of tRNA^His when compared with untreated cells (Fig. 2*C*). Northern analysis of tRNA^His in response to 3-AT exposure confirmed a reduction in charging, with 40% of aminoacylation of tRNA^His when compared with non-treated cells (Fig. 2, *D* and *E*). Importantly, there were no other tRNAs whose charging was significantly reduced in response to 3-AT, although some showed elevated charging levels during this treatment regime. For example, ini-

FIGURE 3. **Changes in tRNA charging in response to starvation for histidine.** The auxotrophic strain WY909 (*his3*) was cultured in SC medium depleted of histidine for up to 60 min, and the levels of charged tRNAs were measured using the microarray method. *A*, scanned fluorescent images of tRNA^His (*His-tRNA*) and tRNA^Asp (*Asp-tRNA*) hybridized to complementary probe spots in the microarray. The tRNA preparations were from cells starved for histidine for 60 min (*60min*) or no starvation (*0*). *Green* specifies decreased tRNA charging, and *yellow* indicates no change in the aminoacylation levels of the tRNA. *B*, relative tRNA charging levels are presented as the charging ratio of each tRNA prepared from cells cultured in SC medium devoid of histidine for 15 min (−*His, 15'*) or 60 min (−*His, 60'*) when compared with cells grown in SC medium containing all amino acids. The *x* axis indicates each of the different tRNAs, and the value of 1.0 in the *y* axis represents that tRNA charging in the histidine starvation condition equals that in the non-starved control. Reduced tRNA charging during histidine starvation is less than 1.0, whereas values greater than 1.0 represent increased tRNA charging. *Error bars* indicate S.E.
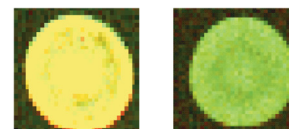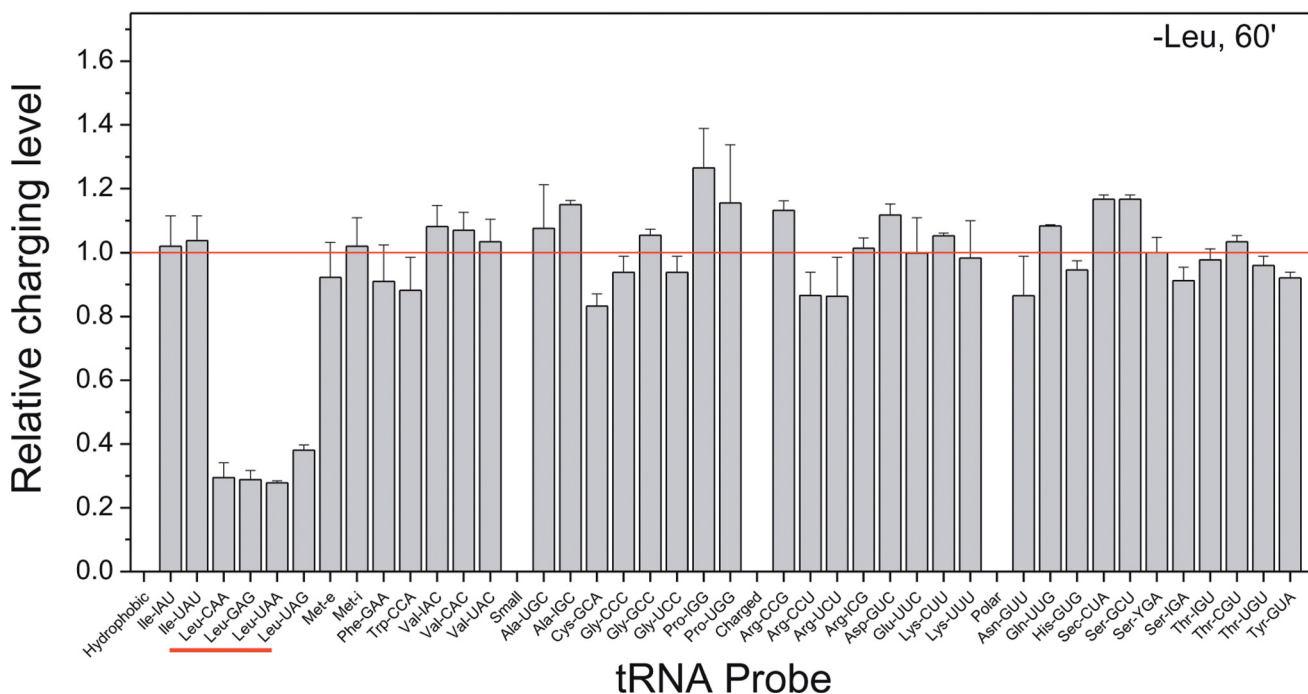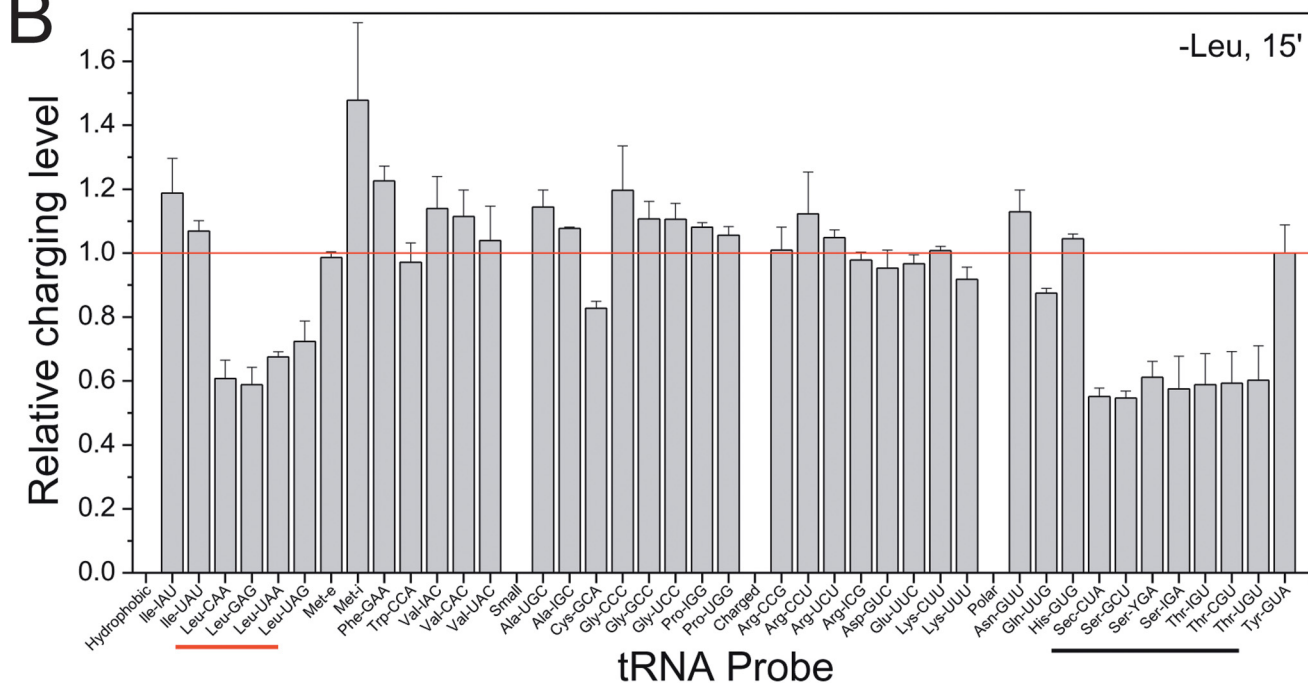
tiator tRNA$_i$$^{Met}$ showed a transient increase in aminoacylation within 15 min of 3-AT treatment.

Changes in the charging of tRNA genome-wide were also measured in response to histidine starvation involving culturing of the *HIS3*-deficient strain WY909 in SC medium devoid of histidine (Fig. 3). In this case, reduced charging of tRNA$^{His}$ occurred only following 1 h of histidine starvation, with ~60% of aminoacylation of tRNA$^{His}$ when compared with non-starved cells (Fig. 3*B*). This result was confirmed by Northern analysis (Fig. 2*D*). There was a more rapid and sustained reduction in tRNA$^{Asp}$, with an ~60% level of aminoacylation when compared with unstarved cells following 15 min of histidine depletion (Fig. 3*B*). This result, however, could not be compared by Northern analysis because the negative charge of Asp eliminates the gel shift of the charged tRNA. There were no tRNAs that showed significant elevation in charging in response to this histidine starvation protocol. These results indicate that histidine starvation can elicit deacylation of both cognate and non-cognate tRNAs. Furthermore, histidine starvation conditions induced by 3-AT treatment of a prototrophic strain and by shifting a *his3::kanMX4* auxotrophic strain to histidine-depleted medium can result in different patterns of charging of tRNA$^{His}$ and non-cognate tRNAs (tRNA$^{Asp}$). Given that eIF2$\alpha$ phosphorylation was induced within 15 min of the shift to histidine-depleted medium (Fig. 1*B*), this suggests that tRNA$^{Asp}$ may serve as an initial activating signal of Gcn2p. At 60 min of histidine depletion, there were significant levels of uncharged tRNA$^{His}$ and tRNA$^{Asp}$ present, and both may contribute to the induction of the GAAC.

*Leucine Starvation*—Phosphorylation of eIF2$\alpha$ was significantly induced at 15 and 60 min of leucine starvation (Fig. 1*C*). Following 60 min of leucine starvation, all four tRNA$^{Leu}$ isoacceptors showed a marked decrease in charging, ~4-fold lower than non-starved cells (Fig. 4). For the isoacceptor tRNA$^{Leu(UAA)}$, this amount of deacylation was confirmed by Northern analysis (Fig. 2*D*). Because the charging level of tRNA$^{His}$ did not change during the leucine starvation condition, this result suggests that one or more of the tRNA$^{Leu}$ isoacceptors contributes to activation of Gcn2p. However, with 15 min of leucine starvation, the pattern of tRNA charging showed some unexpected results. At this time point, there was only ~1.4-fold reduction in the aminoacylation of each of the four tRNA$^{Leu}$ isoacceptors. Interestingly, there was lowered charging of each of the tRNA$^{Ser}$ and tRNA$^{Thr}$ isoacceptors, which in fact exceeded that measured for the leucyl-tRNAs. Again, charging of tRNA$^{His}$ remained unchanged at 15 min of leucine deficiency. Only tRNA$_i$$^{Met}$ showed a modest increase in charging only after 15 min of starvation for leucine. These results suggest a dynamic pattern of tRNA deacylation in response to leucine starvation. Within 15 min of leucine depletion, there

was reduced charging of multiple tRNAs, representing serine, threonine, as well as leucine. By 60 min of leucine starvation, there was deacylation of only the four tRNA$^{Leu}$ isoacceptors, which were uniformly affected. Given that eIF2$\alpha$ phosphorylation was significantly elevated at 15 and 60 min of leucine starvation, this suggests that there are multiple uncharged tRNAs activating Gcn2p and the GAAC. For example, Gcn2p may be activated by multiple different cognate and non-cognate tRNAs early in leucine starvation and then switch to the uncharged leucyl-tRNAs for Gcn2p activation when starvation persists over time.

*Tryptophan Starvation*—The charging of tRNA$^{Trp}$ decreased by ~2-fold at 15 min and ~4-fold at 60 min of starvation for tryptophan (Fig. 5). The charging levels of tRNA$^{Asp}$ and tRNA$^{Glu}$ were also decreased at both the 15-min and the 60-min time points. Levels of deacylation of tRNA$^{Trp}$ were confirmed by Northern blot analysis (Fig. 2*D*). There were also decreases in the aminoacylation of tRNA$^{Arg}$ isoacceptors, with tRNA$^{Arg(ICG)}$ showing some charging reductions following 15 and 60 min of tryptophan starvation. The levels of tRNA$^{His}$ charging did not change in response to tryptophan deficiency, nor were there any increases in the levels of tRNA aminoacylation when compared with non-starved cells. These results suggest a complex pattern of tRNA charging during tryptophan starvation, with increases in the levels of multiple uncharged tRNAs, including tRNA$^{Trp}$, tRNA$^{Asp}$, and tRNA$^{Glu}$, each being possible contributors to induced Gcn2p phosphorylation of eIF2$\alpha$.

*Reduced Levels of Selected Amino Acid in Cells in Response to Starvation*—We next addressed whether there were changes in the intracellular levels of amino acids in response to the different starvation regimens that coincided with reductions in charged tRNAs. Amino acid profiles were first measured in the prototrophic cells treated with 3-AT for 15 or 60 min. Significant changes in amino acid levels are illustrated in Fig. 6*A*, with the remaining measured amino acid levels presented in supplemental Fig. S2. As expected, there were reduced amounts of intracellular histidine, with significantly lowered histidine levels within 1 h of 3-AT treatment. There were no other amino acids showing significant declines. This result is consistent with the idea that 3-AT reduces histidine synthesis, leading to lowered intracellular histidine levels and deacylation of tRNA$^{His}$. A reduction in intracellular histidine levels was also measured in the *HIS3*-deficient strain shifted to histidine-depleted medium (Fig. 6*B*). It is noted that although there were increased levels of uncharged tRNA$^{Asp}$ during this starvation regimen (Fig. 3), there was no significant change in aspartate levels (supplemental Fig. S2). This observation argues against the simple idea that lowered intracellular amino acid levels are obligate for decreased charging of the corresponding tRNAs. Rather, there

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FIGURE 4. **Multiple leucyl-tRNA isoacceptors are deacylated in response to leucine starvation.** The auxotrophic strain WY795 (*leu2*) was cultured in SC medium depleted of leucine for up to 60 min, and tRNA charging was measured using the microarray method. *A*, scanned fluorescent images of tRNA$^{His}$ (*His-tRNA*) and tRNA$^{Leu(UAA)}$ (*Leu(UAA)*) hybridized to the microarrays. The tRNA preparations are from cells starved for leucine for 60 min (*60min*) or no starvation (*0*). *Green* and *yellow* specify decreased tRNA charging and no change, respectively. *B*, relative levels of tRNA charging are presented as the charging ratio of each tRNA prepared from the strain cultured in SC medium devoid of leucine for 15 min (−*Leu, 15′*) or 60 min (−*Leu, 60′*) when compared with cells grown in SC medium containing all amino acids. The *x* axis includes each of the chromosomal-encoded tRNAs, and the value of 1.0 in the *y* axis indicates that the tRNA charging in cells starved for leucine is equal to the non-starved control. Values lower than 1.0 indicate reduced tRNA charging, whereas values greater than 1.0 represent tRNA charging that is greater in response to leucine starvation. *Error bars* indicate S.E.
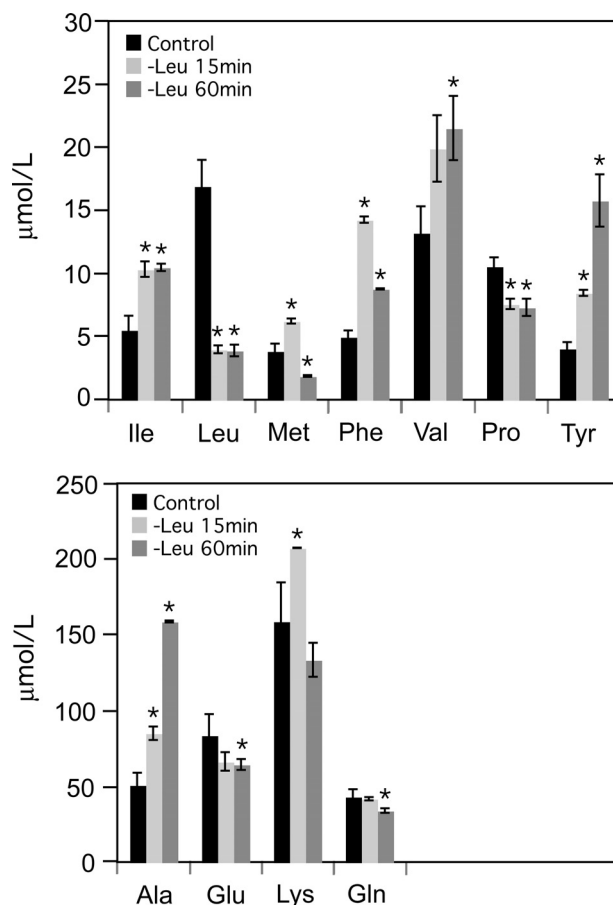
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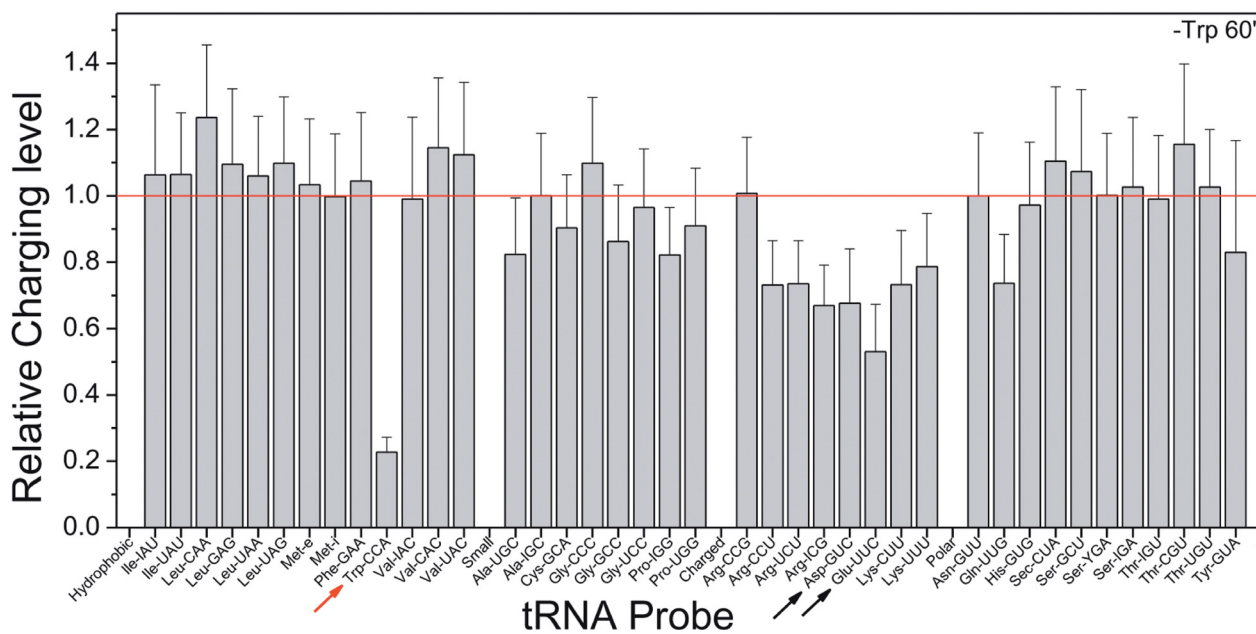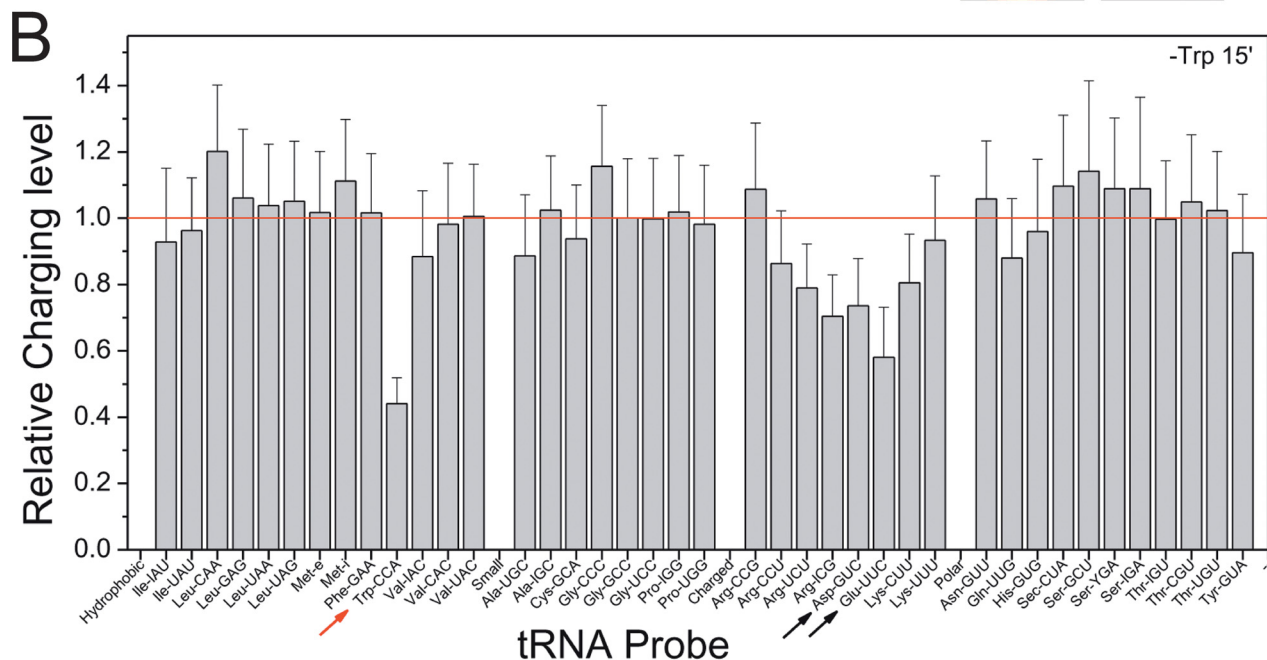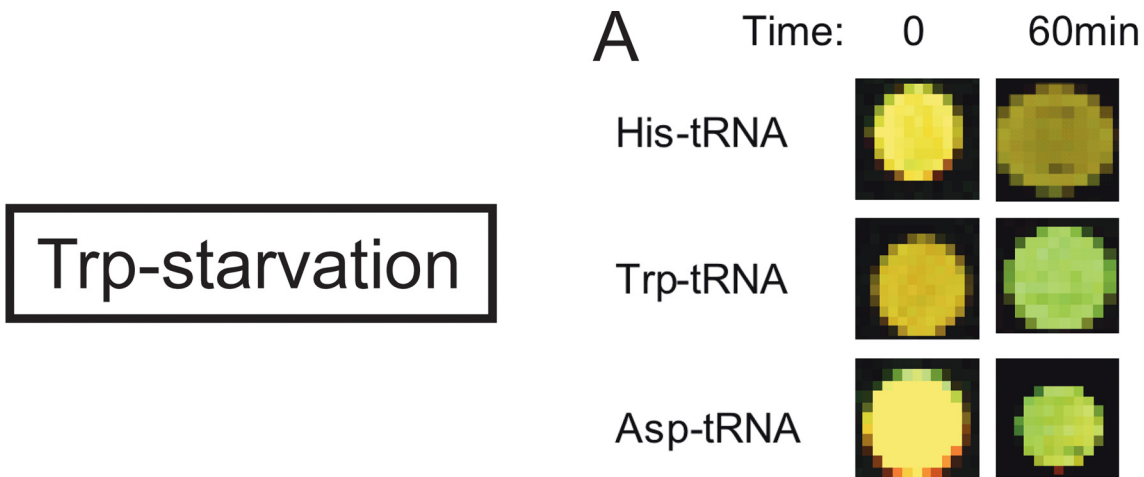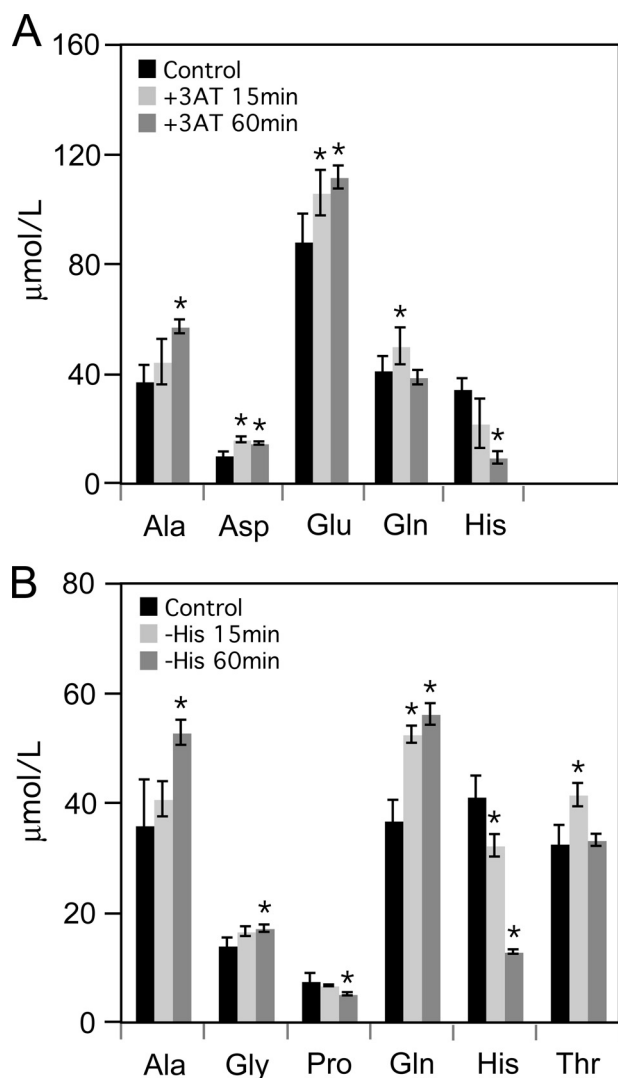
FIGURE 6. **Amino acid composition in yeast cells subjected to histidine starvation by treatment with 3-AT or histidine depletion of an auxotrophic strain.** *A,* yeast strain WY798 was cultured in SC medium devoid of histidine containing 3-AT for 15 min (*+3AT 15min*) or 60 min (*+3AT 60min*). Alternatively, non-starved cells (*Control*) were grown in the SC medium without 3-AT. Amino acid profiles in whole cell lysates were measured by the Waters Pico-Tag method. *B,* strain WY909 (*his3*) was cultured in SC medium depleted of histidine for 15 min (−*His 15min*) or 60 min (−*His 60min*). Additionally, cells were cultured in SC medium containing all amino acids (*Control*). Amino acid compositions were measured in whole cell lysates. Amino acid measurements with significant changes during these starvation conditions when compared with control are illustrated with an *asterisk* where the *asterisk* indicates *p* < 0.05. The levels of amino acids that did not significantly change in response to 3-AT treatment are presented in supplemental Fig. S2. *Error bars* indicate S.E.



FIGURE 7. **Composition of amino acids in an auxotrophic strain starved for leucine.** Strain WY795 (*leu2*) was cultured in SC medium devoid of leucine for 15 min (−*Leu 15min*) or 60 min (−*Leu 60min*) or in SC medium containing all amino acids (*Control*). The amino acid levels were determined in the whole cell lysates using the Waters Pico-Tag method. Amino acid measurements with significant changes in response to leucine deprivation when compared with control are illustrated with an *asterisk* where the *asterisk* indicates *p* < 0.05. The levels of amino acids that did not significantly change in response to leucine starvation are presented in supplemental Fig. S2. *Error bars* indicate S.E.

appear to be additional signals, some possibly indirect, that can lower charging of certain tRNAs.

After both 15 and 60 min of leucine starvation, there was a dramatic reduction in the leucine levels (Fig. 7). This finding

is consistent with the observed deacylation of tRNA[Leu] isoacceptors in response to leucine depletion (Fig. 4) and supports the idea that one or more of the tRNA[Leu] isoacceptors contributes directly to activation of Gcn2p. The only other amino acids that showed a mild reduction in the intracellular levels were proline, glutamine, and glutamate. It is noted that serine and threonine, whose tRNAs were transiently deacylated upon leucine starvation, were unaffected (supplemental Fig. S2). Interestingly, the intracellular levels of many amino acids were increased, including alanine, isoleucine, phenylalanine, tyrosine, and valine. These results suggest that cells starving for leucine adjust the metabolism

FIGURE 5. **Multiple tRNAs are deacylated in response to tryptophan starvation.** The auxotrophic strain WY900 (*trp1*) was cultured in SC medium depleted of tryptophan for up to 60 min, and tRNA charging was measured by the microarray method. *A,* scanned fluorescent images of tRNA[His] (*His-tRNA*), tRNA[Trp] (*Trp-tRNA*), and tRNA[Asp] (*Asp-tRNA*) hybridized to the complementary probes in the microarrays. The tRNA preparations were from cells starved for tryptophan for 60 min (*60min*) or no starvation (*0*). *Green* indicates decreased tRNA charging, and *yellow* represents no change. *B,* the relative levels of tRNA charging are presented as the charging ratio of each tRNA prepared from the strain cultured in SC medium devoid of tryptophan for 15 min (−*Trp, 15′*) or 60 min (−*Trp, 60′*) when compared with cells grown in SC medium containing all amino acids. The *x* axis lists each tRNA. The value of 1.0 in the *y* axis indicates that the tRNA charging in the tryptophan starvation condition is equal to cells grown in non-starved conditions. Values less than 1.0 indicate reduced tRNA charging, whereas values greater than 1.0 represent increased tRNA charging in response to tryptophan starvation. *Error bars* indicate S.E.
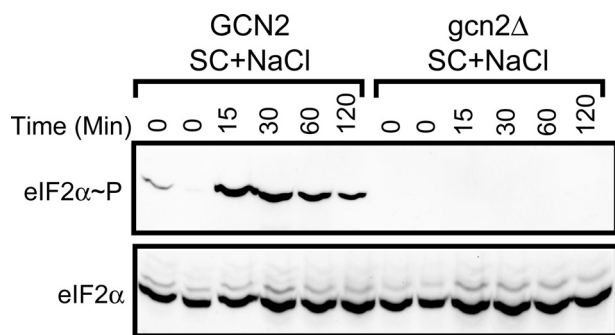
FIGURE 8. **Gcn2p phosphorylation of eIF2$\alpha$ is enhanced during treatment with 1 M NaCl.** Prototrophic cells WY798 and an isogenic *gcn2* strain (WY799) were cultured in SC medium containing 1 M NaCl for up to 120 min. Cells grown in SC and not subject to the high salt stress are indicated by the zero times. Equal amounts of protein lysates were separated by SDS-PAGE. Levels of eIF2$\alpha$ phosphorylated at serine 51 (*eIF2$\alpha$~P*) or total eIF2$\alpha$ were measured by immunoblot analyses.

of many key amino acids, coincident with reducing global protein synthesis by eIF2$\alpha$ phosphorylation.

*Environmental Stress Not Directly Related to Nutrient Deprivation Also Triggers Deacylation of tRNA*—We also investigated whether treating yeast cells with an environmental stress not related to nutrient deprivation can decrease tRNA charging (Figs. 8 and 9). High concentration of NaCl is a commonly applied stress condition that generates osmotic pressure and can interfere with transport of a variety of molecules (19, 20). Treatment of the prototrophic yeast strain with 1 M NaCl stress led to a rapid increase in phosphorylation of eIF2$\alpha$ (Fig. 8). Gcn2p phosphorylation of eIF2$\alpha$ by the high salinity stress persisted for up to 60 min, with a modest reduction following 120 min of the treatment with 1 M NaCl. We also observed a rapid reduction in the charging of several different tRNAs, with tRNA$_i^{Met}$, tRNA$^{Cys}$, and tRNA$^{Arg(ICG)}$ showing the greatest changes after 15 min of 1 M NaCl treatment (Fig. 9, *A* and *B*). We confirmed that there was an increase in uncharged tRNA$_i^{Met}$ by Northern analysis (Fig. 9*C*). These reductions of charged tRNA species, however, were transient, and by 60 min, charging of these tRNAs returned to the levels of non-stressed cells (Fig. 9*B*). After 60 min, tRNA$^{His}$ had the lowest charging level, although this change was modest. We conclude that there are increases in the levels of uncharged tRNAs in response to environmental stresses not directly related to nutrient starvation. These changes in tRNA charging were not uniform and were transient, with deacylation of selected tRNAs occurring rapidly following treatment of yeast cells with 1 M NaCl. These results support the model that the eIF2 kinase Gcn2p can recognize and be activated by diverse environmental stresses by increases in the levels of uncharged tRNAs.

## DISCUSSION

Gcn2p phosphorylation of eIF2$\alpha$ is rapid, occurring within 15 min of starvation for one of several different amino acids (Fig. 1). It is suggested that uncharged tRNAs, which accrue in response to the starvation, are the key signal that bind to Gcn2p and activate this eIF2$\alpha$ kinase. Two models were presented for activation of Gcn2p by uncharged tRNA. In the first model, Gcn2p is proposed to bind to many different uncharged tRNAs, each rapidly accumulating in response to starvation for the cor-

responding amino acid. An alternative version of this model is that activation of Gcn2p occurs by direct interaction with one or a limited number of select tRNAs, and the charging of these select tRNAs is reduced in response to starvation for both cognate and non-cognate amino acids. To delineate between these models, we developed a microarray-based method to measure changes in all chromosomally encoded tRNAs in yeast. Our results suggest a dynamic pattern of changes in tRNA charging involving deacylation of both cognate and non-cognate tRNAs in response to starvation for amino acids. Deacylation of these different tRNAs was coincident with induced eIF2$\alpha$ phosphorylation, suggesting that multiple uncharged tRNAs contribute to activation of Gcn2p. This suggests that multiple uncharged tRNAs can contribute to activation of Gcn2p during the course of an amino acid starvation. We also observed transient increases in uncharged tRNAs levels, coincident with eIF2$\alpha$ phosphorylation, in response to treatment with high salinity conditions. These results support the idea that uncharged tRNA can also serve as key signals for activation of Gcn2p during environmental stresses not directly linked to nutritional deficiencies.

*Multiple Different Uncharged tRNAs Can Accumulate during Amino Acid Starvation*—Within 15 min of 3-AT treatment, we measured a sharp increase in the levels of uncharged tRNA$^{His}$, which continued after 60 min of exposure to this inhibitor of histidine biosynthesis (Fig. 2). No other tRNAs were significantly deacylated during 3-AT treatment, although charging of initiator tRNA$_i^{Met}$ was transiently enhanced at 15 min of the 3-AT stress when compared with non-starved cells. Reduced charging of tRNA in response to 3-AT treatment coincided with lowered intracellular histidine levels (Fig. 6*A*), supporting the idea that the addition of 3-AT rapidly blocked histidine biosynthesis, triggering high levels of uncharged tRNA$^{His}$. This scenario for Gcn2p regulation by 3-AT treatment supports facets of the first model for Gcn2p activation. In this model, uncharged tRNAs, each accumulating during starvation for the cognate amino acid, would serve as the direct signal inducing Gcn2p phosphorylation of eIF2$\alpha$.

More complex patterns of tRNA charging were observed in response to starvation elicited by introducing auxotrophic strains into medium depleted for the essential amino acid (Figs. 3–5). After 15 min of histidine depletion in *his3* mutant cells, there was a marked increase in uncharged tRNA$^{Asp}$ (Fig. 3). Only at 60 min of histidine starvation were there measurable levels of uncharged tRNA$^{His}$, which were accompanied by continued high levels of uncharged tRNA$^{Asp}$. Although intracellular histidine levels were reduced during this starvation period, there were no other reductions in the measured amino acids. This suggests that uncharged tRNA$^{Asp}$ results from mechanisms other than lowered intracellular aspartate (Fig. 6*B*). It is noteworthy that eIF2$\alpha$ phosphorylation was induced at both 15 and 60 min of histidine starvation (Fig. 1*A*). This suggests that at least during the early histidine starvation period, uncharged tRNA$^{Asp}$, a non-cognate tRNA, is a signal for activation of Gcn2p. These observations support the idea that multiple uncharged tRNAs can activate Gcn2p during a given starvation period and that each of these tRNA are not necessarily charged by the amino acid subject to starvation.
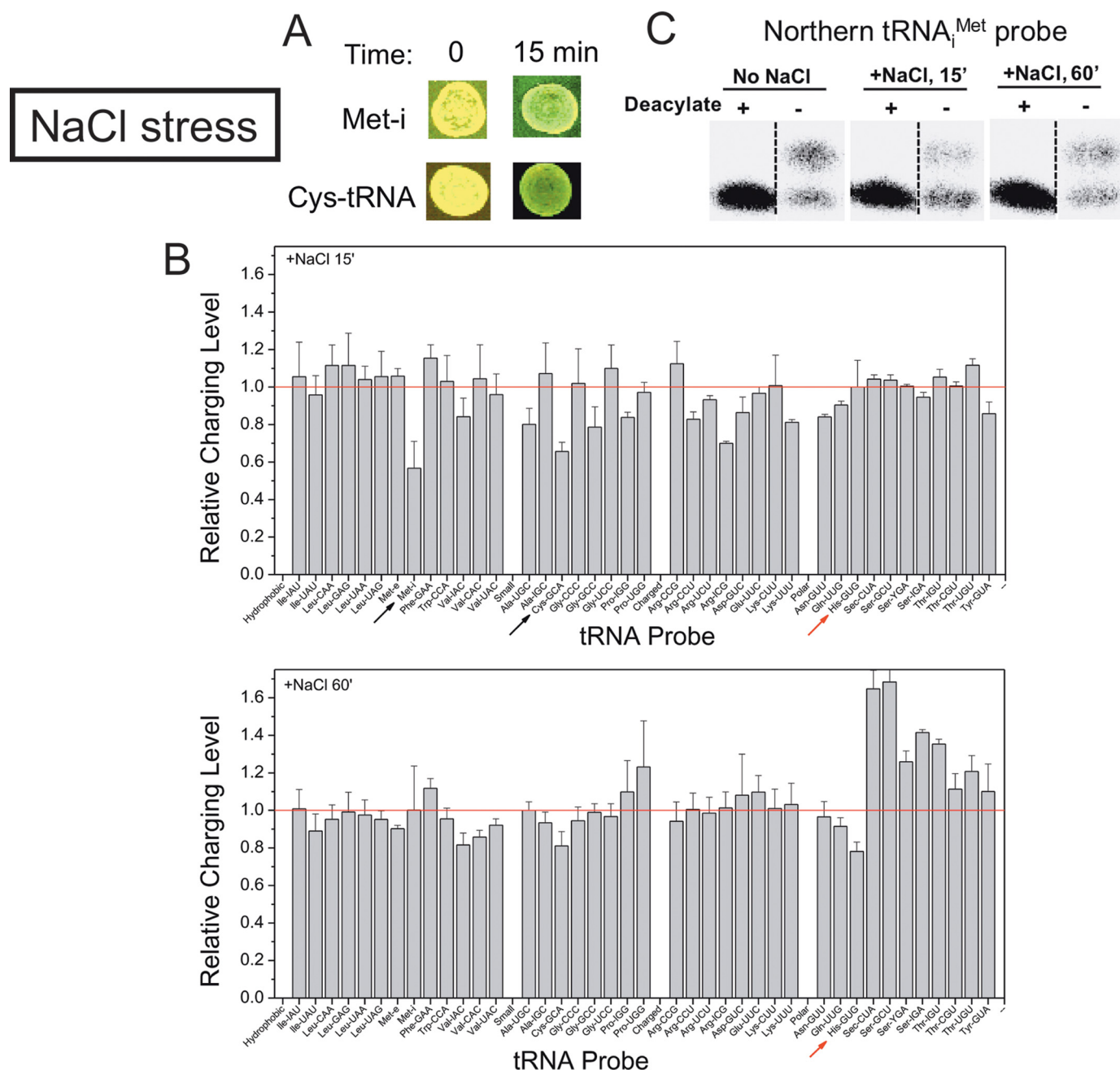
FIGURE 9. **Increased uncharged tRNA levels in response to treatment with high salt treatment.** The prototrophic strain WY798 was cultured in SC medium containing 1 M NaCl for up to 60 min, and the charging of tRNA genome-wide was measured by the microarray method. *A*, scanned fluorescent images of tRNA$_i^{Met}$ (*Met-i*) and tRNA$^{Cys}$ (*Cys-tRNA*) hybridized to the complementary probes in the microarrays. The tRNA preparations were from cells treated with 1 M NaCl for 15 min (*15 min*) or no stress (*0*). *Green* indicates low tRNA charging, and *yellow* represents no change in the charging of tRNA. *B*, the relative levels of tRNA charging are presented as the ratio of each charged tRNA prepared from the strain cultured in high salt conditions for 15 min (*+NaCl, 15'*) or 60 min (*+NaCl, 60'*) when compared with cells grown in the absence of stress. The *x* axis lists each tRNA, and the *y* axis represents changes in tRNA charging between stressed and non-stressed cultures. Values less than 1.0 indicate reduced tRNA charging, whereas values above 1.0 denote increased tRNA charging in response to the 1 M NaCl treatment. *Error bars* indicate S.E. *C*, Northern blot analysis of acid-denaturing gels measuring the charging of tRNA$_i^{Met}$ in strain WY798 treated with 1 M NaCl for 15 min (*+NaCl, 15'*), 60 min (*+NaCl, 60'*), or no stress (*No NaCl*). Autoradiograms represent the charged (slower migrating band) and uncharged (faster migrating band) tRNA$_i^{Met}$. As a control, the tRNA$_i^{Met}$ was deacylated prior to the Northern analysis (*+*) and when compared with those not subjected to deacylation *in vitro* prior to the Northern analysis (*−*).

In the cases of tryptophan and leucine starvation, there was increased deacylation of cognate tRNAs, along with elevated levels of uncharged tRNAs not linked with the limiting amino acid (Figs. 4 and 5). Within 15 min of leucine starvation, there was significant deacylation of each of the four tRNA$^{Leu}$ isoacceptors, which was further lowered after 60 min of the starvation condition (Fig. 4). Interestingly, each of the tRNA$^{Ser}$ and

tRNA$^{Thr}$ isoacceptors were subject to deacylation only during leucine starvation for 15 min. Although intracellular leucine levels were sharply reduced in response to the leucine limitation, serine and threonine did not significantly change (Fig. 7). As noted above, these findings suggest that lowered intracellular amino acid level is not obligate for deacylation of the corresponding cognate tRNAs. In response to both 15 and 60 min of

tryptophan starvation, there was significant uncharged tRNA$^{Trp}$, as well as tRNA$^{Asp}$, tRNA$^{Glu}$, and to a lesser extent, tRNA$^{Arg(ICG)}$ (Fig. 5). For both leucine and tryptophan starvation, there was increased eIF2$\alpha$ phosphorylation after 15 and 60 min of the starvation period (Fig. 1, *B* and *C*).

These results further support the idea that multiple uncharged tRNAs can activate Gcn2p. Clearly, starvation for a given amino acid can trigger significant levels of uncharged cognate tRNAs, albeit the timing can differ depending on the limiting amino acid. However, starvation for amino acids can also elicit deacylation of non-cognate tRNAs. The timing of their deacylation, in conjunction with induced eIF2$\alpha$ phosphorylation, suggests that the different uncharged tRNAs can jointly contribute to enhanced Gcn2p phosphorylation of eIF2$\alpha$. Deacylation of the non-cognate tRNAs may reflect lowered levels of amino acids other than that limiting in the medium. In this case, limitation for an amino acid, such as leucine, appears to lead to changes in the metabolism of non-limiting amino acids. However, lowered deacylation of certain tRNAs occurred despite there being no significant reduction in the cellular levels of the corresponding amino acids. The underlying mechanism(s) for this tRNA deacylation is not fully understood. This could reflect differences in the compartmentation of amino acids, with an influx of amino acid pools from the cytosol to the vacuole (26, 34). The lowered cytosolic levels of the amino acids may then lead to lowered charging for their cognate tRNAs. Alternatively, many aminoacyl-tRNA synthetases are organized into a complex that functions in the channeling of certain tRNAs during translation (35–38). This multiaminoacyl-tRNA synthetase complex, in conjunction with changes in amino acid levels, may contribute to adjustments in the charging of different tRNAs. Finally, trans-editing proteins have been identified that can deacylate specific tRNAs, and regulation of such proteins during certain amino acid-limiting conditions may contribute to the rapid accumulation of selected uncharged tRNAs (39–43).

*Accumulation of Uncharged tRNAs Occurs in Response to Environmental Stress Not Directly Related to Nutrient Deprivation*—Several different environmental stresses induce Gcn2p phosphorylation of eIF2$\alpha$, and it has been proposed that many of these stresses activate Gcn2p by deacylating tRNAs (1, 3). Indeed we found elevated levels of several uncharged tRNAs, including tRNA$_i$$^{Met}$, tRNA$^{Cys}$, and to a lesser extent, tRNA$^{Arg(ICG)}$, after 15 min of exposure to 1 M NaCl (Fig. 9). Elevated levels of these uncharged tRNAs was rapid, coincident with induced Gcn2p phosphorylation of eIF2$\alpha$ (Fig. 8). These results suggest that accumulation of multiple uncharged tRNAs can serve as the central activator of Gcn2p during environmental stresses not directly linked to nutrition deprivation. Our finding that charging of initiator tRNA$_i$$^{Met}$ is reduced by high salinity indicates that this stress may reduce the levels of the eIF2 ternary complex and block translation initiation by mechanisms not fully dependent on eIF2$\alpha$ phosphorylation. This idea is supported by a report that deletion of *GCN2* in yeast does not fully restore protein synthesis in cells treated with 1 M NaCl (19).

It is not precisely known how treatment with 1 M NaCl leads to accumulation of uncharged tRNAs and induced eIF2$\alpha$ phosphorylation. The high salinity stress may impair certain bio-

chemical processes or nutrient uptake (19, 20, 44). It is noted that we and others found that simple osmotic stress alone is not the primary reason for Gcn2p activation by 1 M NaCl (19, 20). For example, treatment of yeast with elevated levels of KCl does not induce eIF2$\alpha$ phosphorylation. Rather, it has been proposed that the Na$^+$/K$^+$ ion balance plays a pivotal role, suggesting that the electric potential across the plasma membrane is altered by treatment of yeast cells with 1 M NaCl (20). This could deleteriously affect many cellular processes, including nutrient transport and compartmentation within the cell. Concerning the nutrient transport explanation, we carried out our 1 M NaCl treatment using a prototrophic strain, which is capable of synthesizing all amino acids. This would argue against 1 M NaCl treatment inducing eIF2$\alpha$ phosphorylation by depleting amino acids via a simple defect in amino acid transport.

Like yeast, eIF2$\alpha$ phosphorylation is significantly enhanced in mammalian cells treated with high levels of NaCl (45). It is not known which of the different mammalian eIF2 kinases are activated by salt stress. We have found over a 5-fold increase in eIF2$\alpha$ phosphorylation within 1 h of treatment with 0.2 M NaCl.[4] Although deletion of Gcn2 significantly reduces eIF2$\alpha$ phosphorylation in response to amino acid starvation, such as histidine or leucine depletion (8, 46, 47), loss of Gcn2 does not lower eIF2$\alpha$ phosphorylation in mouse embryo fibroblasts elicited by treatment with high levels of NaCl.[4] These results suggest that Gcn2 is the primary eIF2 kinase activated by nutrient limitation in mammalian cells, but another eIF2 kinase is activated by salt stress independently of, or in parallel with, Gcn2.

*Measurements of Charging of Eukaryotic tRNA Genome-wide*—This study emphasizes the utility of measuring the charging levels of all eukaryotic tRNAs simultaneously in the context of regulation of Gcn2p and accompanying changes in protein synthesis. Although this study focused on the tRNA charging in yeast, the experimental approaches are applicable to measurements in mammalian cells to address the role of uncharged tRNA in key regulatory processes. For example, mammalian Gcn2 is not only involved in resistance to nutritional deprivations (48–50) but is also central to learning and memory, certain behavior responses, and immunoregulatory actions (51–56). Many of these mammalian Gcn2 functions do appear to involve environmental stress, and it is important to determine whether uncharged tRNA is a central regulator of Gcn2 during these physiological arrangements.

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