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The Histidyl-tRNA Synthetase-Related Sequence in the eIF-2α Protein Kinase GCN2 Interacts with tRNA and Is Required for Activation in Response to Starvation for Different Amino Acids

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Protein kinase GCN2 is a multidomain protein that contains a region homologous to histidyl-tRNA synthetases juxtaposed to the kinase catalytic moiety. Previous studies have shown that in response to histidine starvation, GCN2 phosphorylates eukaryotic initiation factor 2 (eIF-2), to induce the translational expression of *GCN4*, a transcriptional activator of genes subject to the general amino acid control. It was proposed that the synthetase-related sequences of GCN2 stimulate the activity of the kinase by interacting directly with uncharged tRNA that accumulates during amino acid limitation. In addition to histidine starvation, expression of *GCN4* is also regulated by a number of other amino acid limitations. Questions that we posed in this report are whether uncharged tRNA is the most direct regulator of GCN2 and whether the function of this kinase is required to recognize each of the different amino acid starvation signals. We show that GCN2 phosphorylation of eIF-2, and the resulting general amino acid control pathway, is stimulated in response to starvation for each of several different amino acids, in addition to histidine limitation. Cells containing a defective aminoacyl-tRNA synthetase also stimulated GCN2 phosphorylation of eIF-2 in the absence of amino acid starvation, indicating that uncharged tRNA levels are the most direct regulator of GCN2 kinase. Using a Northwestern blot (RNA binding) assay, we show that uncharged tRNA can bind to the synthetase-related domain of GCN2. Mutations in the motif 2 sequence conserved among class II synthetases, including histidyl-tRNA synthetases, impair the ability of this synthetase-related domain to bind tRNA and abolish GCN2 phosphorylation of eIF-2 required to stimulate the general amino acid control response. These in vivo and in vitro experiments indicate that synthetase-related sequences regulate GCN2 kinase function by monitoring the levels of multiple uncharged tRNAs that accumulate during amino acid limitations.

Phosphorylation of eukaryotic initiation factor 2 (eIF-2) is an important mechanism regulating protein synthesis. The eIF-2 protein complexed with GTP and Met-tRNA$_i^{Met}$ participates in the selection of the start codon in the translation initiation process (19, 24, 29, 52). During the association of the ribosomal subunits to form the 80S complex, eIF-2–GTP is hydrolyzed to eIF-2–GDP and released from the ribosome. The GDP-bound form must be recycled to eIF-2–GTP to facilitate the next round of translation initiation. The eIF-2 protein is composed of three subunits, referred to as α, β, and γ. Phosphorylation of the α subunit at serine 51 reduces eIF-2 activity by impairing the rate of GDP-GTP exchange catalyzed by the initiation factor eIF-2B (19, 24, 29).

To date, genes encoding three protein kinases that phosphorylate the regulated site of eIF-2α have been isolated and characterized (8, 30, 38, 48). The kinase catalytic domains have similar sequence and structural features distinguishable from those other members of eukaryotic protein kinases (23, 37). The activities of two of these kinases inhibit general translation initiation in mammalian cells (24, 41, 47). RNA-dependent protein kinase participates in the interferon-mediated antiviral defense mechanism, reducing protein synthesis in response to viral infection. Heme-regulated inhibitor kinase is found principally in reticulocytes and bone marrow and couples the synthesis of globin, the primary translation product in these tissues, to hemin availability. The third eIF-2α kinase, GCN2, is found in the yeast *Saccharomyces cerevisiae*. In this instance, eIF-2α phosphorylation appears to regulate translation of a single species of mRNA, that encoding the GCN4 protein (10, 21, 47).

This report focuses on the regulation of the protein kinase GCN2. In *S. cerevisiae*, starvation for any one of several different amino acids, or a defect in an aminoacyl-tRNA synthetase, stimulates the expression of more than 30 genes encoding amino acid biosynthetic enzymes (20, 21). The GCN4 protein is a transcriptional activator that mediates this cross-pathway response, known as general amino acid control. The positive regulatory role of the protein kinase GCN2 in the general control resides in its ability to phosphorylate eIF-2α, thereby stimulating the translation of a preexisting pool of *GCN4* mRNA in response to an amino acid-limiting condition. Control of *GCN4* translation involves four short upstream open reading frames (uORFs) in the leader of *GCN4* mRNA (20, 21). During growth conditions not limiting for amino acids, the uORFs impede translation initiation of the *GCN4* protein-coding sequences. The inhibitory effect of these uORFs is overcome in amino acid-starved cells to allow increased *GCN4* translation (1).

Dever et al. (10) showed that phosphorylation of eIF-2α by the GCN2 kinase in vivo is stimulated when cells are starving for histidine. How does amino acid limitation control the activity of the GCN2 kinase? The carboxy-terminal portion of GCN2 was observed to contain significant similarity with the entire sequence of histidyl-tRNA synthetases (HisRSs) (Fig. 1) (48). Given that aminoacyl-tRNA synthetases bind uncharged tRNA as a substrate, and uncharged tRNA is thought to be the most immediate signal for the general control, it was proposed

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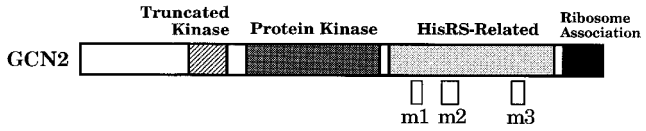

FIG. 1. Functional map of the GCN2 protein kinase. The box designated GCN2 indicates the 1,590-amino-acid-long sequence of the GCN2 protein kinase. In the middle portion of the protein is a domain homologous to protein kinases, and in the carboxy-terminal portion of GCN2 is a region with homology to HisRSs. This HisRS-related sequence is thought to activate GCN2 kinase through interaction with uncharged tRNA. In this synthetase-like sequence, there are three motifs, designated m1, m2, and m3, conserved among the class II aminoacyl-tRNA synthetases. The *gcn2-m2* mutation contains leucine residues substituted for the conserved Tyr and invariant Arg residues at positions 1050 and 1051, in the motif 2 sequence of *GCN2*. These positions correspond to Phe and Arg residues at positions 216 and 217, respectively, in the aspartyl-tRNA synthetase sequence from *E. coli* (9, 13). These aspartyl-tRNA synthetase residues are located at the interface between the motif 2 S4 β-strand and loop structure in the three-dimensional structure (39). Additionally, GCN2 associates with the ribosome, and this interaction requires sequences in the carboxy-terminal segment as indicated by the figure (36). The amino-terminal portion of GCN2 contains a sequence related to subdomains VIb to XI of eukaryotic protein kinases (16). This truncated kinase segment appears to be required for GCN2 function, since an in-frame deletion of this region abolishes GCN2 stimulation of the general control pathway (48).

that the HisRS-related domain in GCN2 functions to monitor the concentration of uncharged tRNA that accumulates during amino acid starvation (48). By this hypothesis, binding of uncharged tRNA to this synthetase-like domain would produce a conformational change in GCN2, resulting in activation of the adjacent protein kinase moiety and increasing GCN2 phosphorylation of the substrate eIF-2α.

Expression of *GCN4* is stimulated in response to starvation for at least 10 different amino acids (20). However, the role of GCN2 protein kinase in sensing starvation for amino acids other than histidine is uncertain. Although the HisRS-related domain of GCN2 does not contain any extensive homology to other aminoacyl-tRNA synthetases, this region does have three sequence motifs characteristic of the class II aminoacyl-tRNA synthetases, which include the HisRS family (3, 9, 37) (Fig. 1). On the basis of the three-dimensional structure of seryl-tRNA synthetase and aspartyl-tRNA synthetase complexed with their cognate tRNAs, the class II members are thought to share a core antiparallel β-sheet structure that binds to the enzyme substrates (4, 31, 39). The motif 2 sequence forms two β strands in this sheet structure, and residues in this motif interact directly with the acceptor stem of $tRNA^{Asp}$ (39).

There are two interesting possibilities concerning the role of GCN2 in sensing amino acid starvation conditions. In the first model, GCN2 phosphorylation of eIF-2α would be stimulated in response to starvation for each of the different amino acids that regulate the general control pathway. The HisRS-related domain of GCN2 might have diverged from the bonafide HisRSs such that this region can interact with many different uncharged tRNAs to elevate kinase activity. Alternatively, GCN2 might contain additional domains that function to monitor different amino acid limitations. In either case, starvation for multiple amino acids would regulate the activity of the GCN2 kinase to stimulate *GCN4* translation.

A second model for the role of GCN2 in general control is that phosphorylation of eIF-2α by GCN2 is elevated only in response to histidine limitation or a very restrictive subset of the amino acid starvation signals controlling *GCN4* expression. This model would require that there be GCN2-independent mechanisms that sense many of the amino acid starvations that stimulate the general control pathway. Such systems could involve additional eIF-2α protein kinases or proteins regulating other steps in *GCN4* translation initiation. There are two documented examples of GCN2-independent mechanisms regulating the translation of *GCN4*. In the first case, translation of *GCN4* is stimulated in response to a nutritional shiftdown (44). This GCN2-independent mechanism requires the activity of cyclic AMP protein kinase and may mediate translational induction of *GCN4* in response to UV irradiation (12).

The second system of GCN2-independent stimulation of *GCN4* translation, interestingly, appears to be activated by high levels of uncharged tRNA (25, 46). Vazquez de Aldana et al. (46) observed that elevated expression of an unchargeable mutant $tRNA^{Val}$ encoded by a high-copy-number plasmid stimulated *GCN4* translation by a mixture of GCN2-dependent and -independent responses. As no increase in GCN2 phosphorylation of eIF-2α was detected, it was proposed that the apparent GCN2 dependence reflected a requirement only for the basal level of phosphorylation seen in repressing conditions, which must be combined with the GCN2-independent response. The details of this GCN2-independent pathway are uncertain, although genetic evidence supports the model that it also involves a reduction in eIF-2 activity by a mechanism independent of the GCN2 phosphorylation site in eIF-2α (46). In higher eukaryotes, there is evidence that phosphorylation of eIF-2B subunits can alter the activity of this exchange factor, lowering the levels of eIF-2-GTP and thus mimicking the effect of eIF-2 phosphorylation (24, 50). Perhaps similar regulatory mechanisms are conserved in *S. cerevisiae*. This possible additive effect of GCN2-dependent and -independent pathways warrants caution when one is interpreting genetic linkage between GCN2 and *GCN4* translation; furthermore, it suggests that additional approaches, including measurements of GCN2 phosphorylation of eIF-2α, should be included when the stimulatory roles of different amino acid starvations on GCN2 activity are assessed.

In this report, we establish that GCN2 phosphorylation of eIF-2α is induced in response to starvation for one of several different amino acids in addition to histidine. Cells containing a defect in HisRS were also found to stimulate GCN2 phosphorylation of eIF-2α in the absence of a limiting amino acid, indicating that uncharged tRNA is the most direct regulator of GCN2 kinase function. Also consistent with the idea that GCN2 monitors uncharged tRNA levels, we show that the synthetase-related domain expressed in the form of a polyhistidine fusion protein in *Escherichia coli* can bind yeast tRNA in a Northwestern blot (RNA binding) assay. Mutations in conserved residues within the motif 2 sequence of the synthetase-related domain greatly reduce binding of tRNA to the GCN2 fusion protein and impair GCN2 phosphorylation of eIF-2α and stimulation of the general control system in response to different amino acid starvation conditions. Taken together, these in vivo and in vitro experiments indicate that the activity of the GCN2 kinase is regulated through the interaction of the HisRS-related domain with different uncharged tRNAs that accumulate when cells are starving for amino acids.

MATERIALS AND METHODS

Yeast strains. Genotypes of yeast strains used in this study are described in Table 1. Strain H1333 (*MAT*α *gcn2*::*URA3 ino1 ura3-52 leu2-3 leu2-112 hts1-1 HIS4-lacZ*) was crossed with A9914-1A (*MAT***a** *ura3-52 hts1-1*) (32), and a Ura^+ $LacZ^+$ temperature-sensitive spore was identified and designated WY184 (*MAT***a** *gcn2*::*URA3 ino1 ura3-52 leu2-3 leu2-112 HIS4*::*lacZ*). The *hts1-1* allele confers temperature sensitivity at 37°C. The *GCN2* and *gcn2-K559V* alleles were introduced into WY184 by using a previously described two-step gene replacement technique (17). Briefly, H1333 was transformed to Leu^+ by using the integrating plasmid p727 (*gcn2-K559V*) or p735 (*GCN2*) digested with *Sna*BI to direct integration to the *GCN2* locus. Ura^- Leu^- derivatives of these transformants were selected for by using 5-fluoro-orotic acid medium (5), resulting in

TABLE 1. Genotypes of yeast strains used in this study

Strain	Genotype	Source or reference
H1149	*MAT*α *gcn2*::*LEU2 ino1 ura3-52 leu2-3 leu2-112 hts1-1 HIS4-lacZ*	48
H1700	*MAT***a** *gcn2*::*LEU2 ino1 ura3-52 leu2-3 leu2-112 HIS4*::*lacZ*	A. Hinnebusch
H1816	*MAT***a** *ura3-52 leu2-3 leu2-112* Δ*gcn2* Δ*sui2 GCN4-lacZ* p1097 [*SUI2 LEU2*]	10
H1896	*MAT***a** *ura3-52 leu2-3 leu2-112* Δ*sui2 GCN4-lacZ* p1097 [*SUI2 LEU2*]	10
H1897	*MAT***a** *ura3-52 leu2-3 leu2-112* Δ*sui2 GCN4-lacZ* 1098 [*SUI2-S51A LEU2*]	10
WY205	*MAT*α *GCN2 ino1 ura3-52 leu2-3 leu2-112 trp1 hts1-1 HIS4-lacZ* pGN172 [*HTS1 URA3*], pSW94 [*SUI2 TRP1*]	This study
WY206	*MAT*α *GCN2 ino1 ura3-52 leu2-3 leu2-112 trp hts1-1 HIS4-lacZ* YCp50 [*URA3*] pSW94 [*SUI2 TRP1*]	This study
WY207	*MAT*α *gcn2-K559V ino1 ura3-52 leu2-3 leu2-112 trp1 hts1-1 HIS4-lacZ* pGN172 [*HTS1 URA3*], pSW94 [*SUI2 TRP1*]	This study
WY208	*MAT*α *gcn2-K559V ino1 ura3-52 leu2-3 leu2-112 hts1-1 trp1 HIS4-lacZ* YCp50 [*URA3*] pSW94 [*SUI2 TRP1*]	This study
WY229	*MAT*α *gcn2*::*LEU2 ser*$^{+/-}$ *ino1 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
WY230	*MAT***a** *gcn2*::*LEU2 arg*$^{+/-}$ *ura3-52 HIS4*::*lacZ*	This study
WY231	*MAT***a** *gcn2*::*LEU2 lys*$^{+/-}$ *ino1 ura3-52 HIS4*::*lacZ*	This study
WY232	*MAT***a** *gcn2*::*LEU2 his*$^{+/-}$ *ino1 ura3-52 HIS4*::*lacZ*	This study

strains WY187 (*MAT***a** *GCN2 ino1 ura3-52 leu2-3 leu2-112 HIS4*::*lacZ*) and WY188 (*MAT***a** *gcn2-K559V ino1 ura3-52 leu2-3 leu2-112 HIS4*::*lacZ*). To introduce the high-copy-number *TRP1*-based pSW94 into WY187 and WY188, the *TRP1* gene was disrupted as described by Alani et al. (2). Plasmid pSW94 was transformed into the *trp1* versions of WY187 and WY188 by selecting for the Trp$^+$ phenotype. Plasmid pGN172 encoding *HTS1* and vector YCp50 were transformed into WY197 containing pSW94 by selection for Ura$^+$, resulting in WY205 and WY206, respectively. Similar transformations of WY188 produced strains WY207 and WY208.

To derive strain WY229, H1149 was crossed with F349 (*MAT***a** *ser*$^{+/-}$), and a 3-aminotriazole-sensitive (3-AT^s) Ser$^{+/-}$ Ura$^-$ LacZ$^+$ meiotic segregant was selected. H1149 was crossed with F351 (*MAT***a** *arg*$^{+/-}$), and a 3-AT^s Arg$^{+/-}$ Ura$^-$ LacZ$^+$ segregant was selected, generating WY230. H1700 was crossed with F348 (*MAT***a** *lys*$^{+/-}$), and a 3-AT^s Lys$^{+/-}$ Ura$^-$ LacZ$^+$ meiotic segregant was selected and designated WY231. To derive the *his*$^{+/-}$ strain WY232, H1700 was crossed with F352 (*his*$^{+/-}$). Because both *gcn2*::*LEU2* and *his*$^{+/-}$ mutations were 3-AT^s, we relied on the 5-methyltryptophan sensitivity (5-MT^s) of *gcn2* mutations and the *LEU2* marker for identification of the *gcn2*-disrupted allele. One segregant designated WY232 was 5-MT^s 3-AT^s His$^{+/-}$ Leu$^+$. To confirm that WY232 contained *gcn2*::*LEU2* and *his*$^{+/-}$ mutations, we transformed this strain with p722 encoding *GCN2* or parent vector pRS316. Expression of HIS4-LacZ enzyme activity in the *GCN2* transformant was greater when cells were grown in synthetic dextrose (SD) medium without histidine (930 U) or histidine-supplemented medium containing 5-MT (510 U) than when cells were grown under repressing conditions (100 U). In the vector-transformed strain, we did not detect any increase in HIS4-LacZ when cells were grown under these starvation conditions.

Plasmids. Plasmids were constructed by using standard recombinant techniques (40). Plasmid p722 (49) contains a 8.2-kb *Xba*I-to-*Sal*I *GCN2* fragment inserted into a modified version of the low-copy-number *URA3* plasmid pRS316 (43), which was engineered to contain these unique sites in the multiple cloning sequence. Plasmid p630 (49) contains the 8.2-kb *Xba*I-to-*Sal*I *GCN2* fragment inserted between the *Nde*I and *Sal*I sites of the high-copy-number *URA3* plasmid YEp24 (6). To generate the mutant allele *gcn2-m2*, the *GCN2* sequence TAC CGA encoding Tyr and Arg at positions 1050 and 1051, respectively, was altered by site-directed mutagenesis to TTGCTG encoding Leu-Leu. The *gcn2-m2* mutations were introduced into p722 and p630 to generate plasmids p299 and p332, respectively. Plasmid pC102-2 (11) is a second low-copy-number *URA3* plasmid containing a 7.0-kb *Sau*3AI *GCN2* fragment inserted into the *Bam*HI site of YCp50 (35). There is no detectable phenotypic difference between strains encoding *GCN2* on plasmids p722 and pC102-2 and those with chromosomally encoded *GCN2*. Plasmid pGN172 contains a 1,650-bp *Cla*I *HTS*1 fragment (32) inserted in the *Cla*I site of YCp50. Plasmid pSW94 contains the *SUI2* gene encoded on a 2.7-kb *Bam*HI restriction fragment inserted into the *Bam*HI site of the high-copy-number *TRP1* plasmid pRS424 (43). Plasmid p925 (10) contains the same *SUI2* gene fragment inserted into the *Bam*HI site of high-copy-number *URA3* vector YEp24.

Plasmid p274, used for expression of the GCN2 HisRS-related domain fused to an amino-terminal polyhistidine sequence in *E. coli*, was constructed in a multistep process. First, unique restriction endonuclease sites, *Sac*I, *Kpn*I, *Bgl*II, and *Sal*I, were introduced into the multiple cloning region between the *Nde*I and *Bam*HI sites of the T7 promoter expression vector pET15B (Novagen), generating plasmid p242. Plasmid p567 (48) contains a *Sac*I site introduced into the *GCN2* sequence at encoded amino acid position 930, and a 2,270-bp *Sac*I-to-*Sal*I fragment from p567 was inserted between the *Sac*I and *Sal*I sites of p242. The resulting plasmid, p245, encoded a polyhistidine amino-terminal sequence fused in frame to GCN2 positions 930 to 1590. To introduce a stop codon in the fusion protein after the encoded amino acid 1467, a 2,010-bp *Kpn*I-to-*Sal*I fragment was purified from plasmid p780, containing an in-frame stop codon inserted into a *Sac*I restriction site introduced at this encoded position 1467 (49), and this DNA fragment was inserted between the corresponding *Kpn*I and *Sal*I sites in p245, creating plasmid p274. A similar plasmid, p297, was constructed to express the *gcn2-m2* allele.

Assay of His4-LacZ and GCN4-LacZ fusion. Measurements of β-galactosidase activity were carried out as described previously (27). For steady-state repressing conditions, saturated cultures were diluted 1:50 in SD medium (42) containing only the required supplements and harvested in logarithmic growth after 5 h at 30°C or for a shorter duration of time as indicated. To elicit starvation conditions, strains were grown for 2 h under repressing conditions, and then 10 mM 3-AT, 0.5 mM 5-MT, or 0.5 μg of sulfometuron methyl (SM) per ml was added to the medium, and the culture was incubated for an additional 5 h at 30°C unless otherwise indicated. Experiments involving cultures starving for lysine, serine, or arginine used leaky auxotrophic strains described in Table 1. Cells were grown in SD medium supplemented with the limiting amino acid for 2 h at 30°C, pelleted by centrifugation, and resuspended in the identical volume of medium in the absence of the limiting amino acid at 30°C for 5 h unless otherwise indicated. Values reported are the averages from two to five independently derived transformants. β-Galactosidase activities are expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

Growth tests for amino acid sensitivity. Plasmid-borne *GCN2* alleles were tested for complementation in Δ*gcn2* strain H1816. The ability to stimulate *HIS3* expression was determined by measuring the growth rate of transformants replica printed onto 2% agar plates containing histidine dropout medium supplemented with 30 mM 3-AT and excess (40 mM) leucine at 30°C (22). Similar measurements of *ILV2* inducibility were carried out by using agar plates containing SD medium supplemented with 0.5 μg of SM per ml. Leaky auxotrophic mutations in amino acid biosynthetic pathways were screened for by replica plating cells onto agar plates containing either SD medium in the absence of the limiting amino acid or dropout medium (SD containing all amino acids except the limiting amino acid).

Isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE). With the exception of the focusing experiment shown in Fig. 3 and the analysis of strain H1897 shown in Fig. 4, all yeast strains were transformed with a high-copy-number plasmid containing *SUI2*, the gene encoding eIF-2α. In H1896 and H1816, this was achieved by transforming these strains with plasmid p925, a high-copy-number *URA3* plasmid encoding *SUI2*. In the leaky auxotrophic strains WY230, WY231, and WY232, the *trp1* gene was disrupted as previously described, and the *trp1*-derived strains were transformed with plasmid pSW94, a high-copy-number *TRP1* plasmid encoding the *SUI2* gene. Elevated expression of *SUI2* assisted separation and detection of eIF-2α in the focusing system. In two examples characterized, H1896 grown in the presence of 3-AT and in the presence of 5-MT, phosphorylation levels were similar between the strain transformed with *SUI2* on a high-copy-number plasmid and the strain expressing wild-type levels of eIF-2α. Levels of expression of HIS-LacZ and GCN4-LacZ were also similar, although we did note that high-copy-number *SUI2* strains showed a modest reduction in the levels of stimulation of the general control pathway compared with the levels in strains containing only chromosomally encoded *SUI2*.

Yeast strains were grown under repressing and starvation conditions as described above. Cells were chilled, collected by centrifugation, and washed with water. Protein extracts were prepared as described previously (10), and 40 μg of total protein from strains containing only chromosomally encoded *SUI2* or 5 to 20 μg of protein from strains containing *SUI2* on a high-copy-number plasmid was analyzed by using a vertical IEF-PAGE system. This focusing system was used essentially as described by Dever et al. (10) except that the polarity of the

electrophoresis was reversed. The anode was at the top, and the upper chamber was filled with 50 mM histidine; the cathode was at the bottom of the gel, with 20 mM aspartic acid in the bottom chamber. As noted by Feng et al. (15), this modification can provide improved resolution to the focusing of eIF-2α. However, we note that this modified procedure can vary between different lots of Pharmalytes (Pharmacia/LKB). After electrophoresis, proteins separated in the acrylamide gel were transferred to nitrocellulose paper. The filter was incubated with a 1:200 dilution of a rabbit polyclonal antiserum prepared against TrpE-SUI2 fusion protein (10). Complexed antibody was detected by using [^{125}I]protein A. Filters were washed, dried, and exposed to Kodak XAR-5 X-ray film.

GCN2 immunoblot. Yeast strains were grown under repressing or starvation conditions as described above. Cell breakage and the immunoblot analysis were carried out essentially as described previously (18). Briefly, cells were collected by centrifugation and broken by using glass beads, and 200 μg of protein was analyzed by electrophoresis in a sodium dodecyl sulfate (SDS)–7.5% polyacrylamide gel. After transfer of proteins to nitrocellulose paper, the filter was incubated with a rabbit polyclonal antiserum prepared against a TrpE-GCN2 fusion protein (48). The GCN2-antibody complex was detected by using [^{125}I]protein A.

Northwestern gel blot assay. To express the polyhistidine–GCN2 HisRS-related domain fusion protein in *E. coli*, plasmids p274 and p297 were transformed into strain BL21(DE3) (F^- *ompT* r_B^- m_B^- containing lysogen DE3). Transformants were grown to mid-logarithmic phase in Luria-Bertani medium (40) supplemented with 100 μg of ampicillin per ml at 30°C, and 0.5 mM isopropylthiogalactopyranoside (IPTG) was added to the culture to induce expression of the fusion gene. Bacterial cultures were grown for an additional 3 h, and cells were collected by centrifugation, resuspended in 20 mM Tris-HCl (pH 7.9)–0.5 M NaCl–10% glycerol–1 mM phenylmethylsulfonyl fluoride solution containing 5 mM imidazole, and lysed in a French press. Polyhistidine fusion proteins were purified by using His-Bind metal chelation resin (Novagen, Madison, Wis.). Protein extract was loaded onto a column containing affinity resin charged with Ni(II)SO_4, the resin was washed with a 20 mM Tris-HCl (pH 7.9)–0.5 M NaCl–10% glycerol–1 mM phenylmethylsulfonyl fluoride solution containing 28 mM imidazole, and the fusion protein was eluted from the column by using a 60 mM imidazole solution. The molecular weight of the polyhistidine–HisRS-related domain fusion protein was 64,000, in agreement with that predicted from the DNA sequence, and this protein was absent from an identically prepared extract from a strain transformed with vector pET15B. Additionally, a thrombin cleavage site was engineered between the polyhistidine tag and the HisRS-related domain. Cleavage of the fusion protein with thrombin lowered the size of the protein by about 2 kDa as predicted.

Northwestern assays were carried out essentially as described previously (7, 28). The polyhistidine–GCN2 HisRS-related domain fusion protein (GCN2 residues 930 to 1467) was analyzed by SDS-PAGE, and the proteins were electrotransferred to a nitrocellulose filter membrane. The filter membrane was incubated in a solution of standard binding buffer (50 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid [HEPES; pH 7.5], 50 mM KCl, 0.05% Triton X-100, 0.04% Ficoll, 0.04% polyvinylpyrrolidone, 0.08% bovine serum albumin, 2.5 mM EDTA) containing 20 μg of sheared herring sperm DNA per ml at 25°C for 5 h. Total uncharged yeast tRNA (Boehringer Mannheim) was radiolabeled at the 5′ terminus by dephosphorylation with calf alkaline phosphatase followed by phosphorylation with [γ-^{32}P]ATP and polynucleotide kinase. Membranes were then incubated with a binding buffer solution containing 0.5 μg of ^{32}P-labeled tRNA and 20 μg of DNA per ml for 1 h at 25°C. Filters were washed four times in standard buffer, and the membrane filter was dried and exposed to autoradiography.

RESULTS

The HisRS-related domain of GCN2 kinase is required for stimulation of the general control pathway in response to different amino acid starvation conditions. To address whether GCN2 kinase stimulates the general control pathway in response to different amino acid limitations, we used two approaches to starve cells for defined amino acids. First, chemical inhibitors of biosynthetic enzymes were used to block the synthesis of different amino acids. A second method was to grow leaky auxotrophic strains containing mutations in genes encoding amino acid biosynthetic enzymes in minimal medium in the absence of the limiting amino acid. We grew strains containing different *GCN2* alleles under these starvation conditions and measured the effects on *GCN4* expression by assaying a GCN4-LacZ fusion containing the translational control elements (uORFs) in the mRNA leader region. As seen in Table 2, the level of GCN4-LacZ enzyme activity was about fivefold higher in the *GCN2* strain grown in the presence of 3-AT, an inhibitor of the histidine biosynthetic enzyme, encoded by the *HIS3* gene, than in cells grown under repressed or nonstarved conditions. The induction of GCN4-LacZ activity was greatly diminished during this starvation condition in the Δ*gcn2* strain. The requirement for *GCN2* function in the stimulation of *GCN4* translation is also illustrated by the observation that the Δ*gcn2* mutant was unable to grow in agar medium supplemented with 3-AT (Table 2). This growth inhibition is consistent with the idea that the reduced levels of GCN4 protein in the Δ*gcn2* strain are not sufficient for transcriptional activation of *HIS3* to levels required to overcome the inhibitory effects of 3-AT (20).

TABLE 2. GCN2 kinase stimulates *GCN4-lacZ* expression in response to starvation for different amino acids

Plasmid-borne allele	GCN4-LacZ enzyme activity (nmol/min/mg of protein)[a]				Growth[b]	
	R	D				
		3-AT	5-MT	SM	3-AT	SM
Low-copy-number *GCN2*	25	130	71	260	+	+
Δ*gcn2*	10	20	24	26	−	−
Low-copy-number *gcn2-m2*	10	33	19	37	−	−
High-copy-number *gcn2-m2*	12	33	23	65	−	−

[a] β-Galactosidase enzyme activity was assayed in extracts prepared from transformants of H1816 *gcn2*::*LEU2* containing the indicated *GCN2* allele on a plasmid as described in Materials and Methods. R, nonstarvation growth conditions; D, derepressing growth conditions imposed by the addition of 3-AT, 5-MT, or SM for 5 h. Each *GCN2* allele was encoded on a *URA3*-based plasmid as follows: *GCN2* on pC102-2; Δ*gcn2* on vector YEp24; low-copy-number *gcn2-m2* on p299; and high-copy-number *gcn2-m2* on p332. The results shown are averages of assays of from two to five independent transformants, and the individual measurements deviated from the average values shown by 25% or less.

[b] Growth of H1816 transformants as a measure of *HIS3* (3-AT) and *ILV2* (SM) enzyme derepression. Symbols: +, confluent growth of replica-plated patches of cells after 2 days at 30°C; −, no discernible growth under the same conditions.

The isogenic *GCN2* and Δ*gcn2* strains were also grown in the presence of two additional inhibitors: 5-MT, an inhibitor of tryptophan biosynthesis (34), and SM, an inhibitor of the *ILV2* product, acetohydroxy acid synthase, which catalyzes the first enzymatic step in the synthesis of the branched-chain amino acids (14) (Table 2). As previously reported (51), *GCN4* expression was about threefold higher in cells grown in the presence of 5-MT than in cells grown under repressing conditions. This more limited stimulation does not appear to be due to less stringent starvation conditions, as the growth rate of the Δ*gcn2* strain in liquid medium supplemented with 5-MT was very similar to that of cells grown in 3-AT or SM (data not shown). Rates measured in the presence of these chemical inhibitors were also similar to one another in the *GCN2* strain. Addition of SM to the medium stimulated GCN4-LacZ expression to levels about 10-fold higher than that measured under repressing conditions. As observed for histidine starvation, inactivation of *GCN2* greatly diminished the expression of GCN4-LacZ in response to either 5-MT or SM.

It has been proposed that the HisRS-related domain stimulates GCN2 kinase phosphorylation of eIF-2α by interacting with uncharged tRNA that accumulates during amino acid starvation (48). As discussed earlier, the HisRS-related domain contains three sequence motifs representative of the class II aminoacyl-tRNA synthetases. To address whether the HisRS-related domain of GCN2 participates in the recognition of each of the chemically induced starvation conditions, we

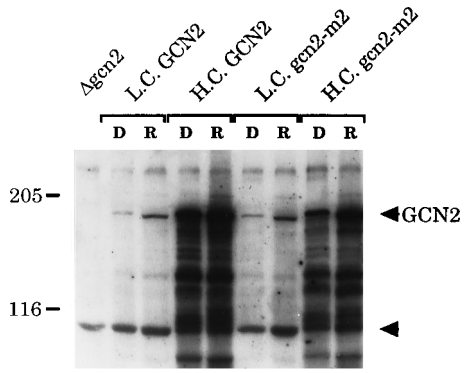

FIG. 2. Immunoblot analysis of GCN2 protein. Protein extracts were prepared from Δ*gcn2* strain H1816 transformed with different plasmid-borne *GCN2* alleles. Strains were grown under repressing conditions (R) or in the presence of 3-AT (derepressing conditions [D]) as described in Materials and Methods. Equal amounts of total protein were separated by SDS-PAGE (7.5% polyacrylamide gel), transferred to nitrocellulose paper, and probed with a rabbit antiserum prepared against a TrpE-GCN2 fusion protein. Each lane is designated by the *GCN2* allele and the copy number of the encoding plasmid. Lane Δgcn2 corresponds to Δ*gcn2* strain H1816 transformed with vector YEp24. L.C. GCN2 and H.C. GCN2 are wild-type *GCN2* encoded on low- and high-copy-number plasmids, respectively, transformed into H1816. L.C. gcn2-m2 and H.C. gcn2-m2 correspond to the *gcn2-m2* allele, encoding a motif 2 mutation in the HisRS-related domain, encoded on low- and high-copy-number plasmids, respectively, transformed into H1816. Note that the lanes for high-copy-number strains are overexposed to allow detection of GCN2 and gcn2-m2 proteins expressed from low-copy-number plasmids. Shorter autoradiographic exposures indicate that GCN2 and gcn2-m2 proteins are expressed at similar levels when encoded on the high-copy-number plasmids. Autoradiographic bands below the high-copy-number lanes are thought to be GCN2 degradation products that are visibly enhanced in the overexposed autoradiogram. In addition to normalization of the lanes for total protein, a protein with a molecular weight of 105,000 that cross-reacts with the TrpE-GCN2 antiserum, indicated by the lower arrowhead on the right, was used as an internal standard between lanes. This protein is detected at similar levels in all lanes and is independent of GCN2, as it is detected even in the Δ*gcn2* strain. Sizes are indicated in kilodaltons on the left.

altered conserved residues in motif 2 and assayed the effects of these mutations on the stimulation of *GCN4* expression. The *gcn2-m2* allele was introduced into the GCN4-LacZ strain H1816 on either a low- or high-copy-number plasmid to examine the effects of increased expression of the mutant protein (Table 2). The mutant *gcn2-m2* allele contains Leu residues substituted for the chemically conserved Tyr and the invariant Arg residues at positions 1050 and 1051, respectively, in GCN2 (48). Transformants expressing the *gcn2-m2* product encoded on a low-copy-number vector failed to grow on agar medium supplemented with either 3-AT or SM. Consistent with this growth inhibition, the levels of GCN4-LacZ enzyme activity in the presence of the chemical inhibitors were similar to those measured in the isogenic Δ*gcn2* strain. In the high-copy-number *gcn2-m2* strain, GCN4-LacZ expression in the presence of SM was twofold higher than in the Δ*gcn2* strain. This level of stimulation, however, was not sufficient for growth on agar medium in the presence of SM.

To ensure that the *gcn2-m2*-encoded protein was stably expressed, the steady-state levels of the gcn2-m2 protein were compared with those of wild-type GCN2 protein in an immunoblot assay. In both the repressed and starved (also referred to as derepressed) growth conditions, the levels of this kinase expressed from low-copy-number plasmids were similar in the *GCN2* and *gcn2-m2* strains (Fig. 2). It is noted that the level of GCN2 protein during starvation conditions is slightly lower than that under repressing conditions. The basis of the reduction is not known, although a similar reduction was noted previously in an in vitro autophosphorylation assay, which is a useful measure of steady-state levels of GCN2 protein (36, 49). These results demonstrate that the failure of gcn2-m2 protein to stimulate GCN4-LacZ expression was not due to reduced levels of the mutant protein. Expression of *GCN2* encoded on a high-copy-number plasmid leads to a large increase in the level of GCN2 kinase compared with the low-copy-number expression level (49). While this level of wild-type GCN2 protein stimulates the general control pathway even in the absence of amino acid starvation (45, 49), similarly elevated levels of gcn2-m2 protein failed to significantly affect GCN4-LacZ enzyme activity (Table 2). Taken together, these results are consistent with the idea that the HisRS-related domain of GCN2 is essential for the recognition of these three different starvation conditions.

TABLE 3. Effects of plasmid-borne *GCN2* alleles on the derepression of *HIS4-lacZ* expression in response to starvation for different amino acids

Plasmid-borne allele	HIS4-LacZ enzyme activity (nmol/min/mg of protein)[a]				
	R	D			
		−Lys	3-AT	5-MT	SM
Low-copy-number *GCN2*	210	1,250	1,140	760	2,280
Δ*gcn2*	140	190	78	73	240
Low-copy-number *gcn2-m2*	120	190	180	170	400
High-copy-number *gcn2-m2*	250	340	210	180	390

[a] β-Galactosidase enzyme activity was assayed in extracts prepared from transformants of WY231 *lys*$^{+/-}$ *gcn2*::*LEU2* containing the indicated *GCN2* allele on a plasmid as described in Materials and Methods. R, nonstarvation growth conditions; D, derepressing growth conditions imposed by omitting lysine from the media or by adding 3-AT, 5-MT, or SM to lysine-supplemented media for 5 h at 30°C. Each *GCN2* allele was encoded on a *URA3*-based plasmid as follows: *GCN2* on p722; Δ*gcn2* on vector pRS316; low-copy-number *gcn2-m2* on p299; and high-copy-number *gcn2-m2* on p332. The results shown are averages of assays of from two to five independent transformants, and the individual measurements deviated from the average values shown by 30% or less.

Another assay for stimulation of the general control pathway is measurement of expression of a HIS4-LacZ fusion. Stimulation of *HIS4* expression is completely dependent on the levels of the GCN4 transcriptional activator (20). Strain WY231 containing *HIS4-lacZ* Δ*gcn2* and a leaky lysine auxotrophic mutation (*lys*$^{+/-}$) was transformed with different plasmid-borne *GCN2* alleles and grown in media supplemented with lysine in the presence of one of the chemical inhibitors. In the *GCN2* transformant grown in the presence of each of the chemical inhibitors, HIS4-LacZ enzyme activity showed a degree of stimulation similar to that detected for the GCN4-LacZ fusion in strain H1816 (Tables 2 and 3). For example, there was about a 10-fold increase in GCN4-LacZ and HIS4-LacZ expression in the *GCN2* strains grown in medium supplemented with SM. Also, consistent with the previously described results, both the Δ*gcn2* and *gcn2-m2* strains were greatly impaired in the stimulation of HIS4-LacZ expression in response to each of the amino acid starvation conditions (Table 3). The *lys*$^{+/-}$ WY231 transformants were also subjected to starvation for lysine by removing this supplement from the media. In the *GCN2* strain grown in the absence of lysine, HIS4-LacZ enzyme activity was about sixfold higher than when this strain was grown in the presence lysine. The isogenic mutant Δ*gcn2* and *gcn2-m2* strains showed only a modest increase in *HIS4-lacZ* expression in the absence of lysine compared with level found under repressed growth conditions.

Two additional yeast strains containing leaky auxotrophic mutations defective for the biosynthesis of serine or arginine

TABLE 4. GCN2 kinase is required for stimulation of *HIS4-lacZ* expression in response to starvation for serine or arginine

Plasmid-borne allele	HIS4-LacZ enzyme activity (nmol/min/mg of protein)[a]					
	WY229 ($ser^{+/-}$)			WY230 ($arg^{+/-}$)		
	R	D		R	D	
		−Ser	3-AT		−Arg	3-AT
Low-copy-number *GCN2*	200	1,170	1,090	150	420	1,230
Δ*gcn2*	120	80	70	120	140	92
Low-copy-number *gcn2-m2*	120	120	110	100	150	140
High-copy-number *gcn2-m2*	220	230	210	180	270	210

[a] β-Galactosidase enzyme activity was assayed in extracts prepared from transformants of WY229 ($ser^{+/-}$ *gcn2*::*LEU2*) or WY230 ($arg^{+/-}$ *gcn2*::*LEU2*) containing the indicated *GCN2* allele on a plasmid as described in Materials and Methods. R, nonstarvation growth conditions; D, derepressing growth conditions imposed in WY229 by omitting serine from the media or adding 3-AT to serine-supplemented media for 5 h at 30°C. In WY230, derepressing growth conditions were achieved by omitting arginine from the media or adding 3-AT to arginine-supplemented media for 5 h at 30°C. Each *GCN2* allele was encoded on a *URA3*-based plasmid as follows: *GCN2* on p722; Δ*gcn2* on vector pRS316; low-copy-number *gcn2-m2* on p299; and high-copy-number *gcn2-m2* on p332. The results shown are averages of assays of from two to five independent transformants, and the individual measurements deviated from the average values shown by 25% or less.

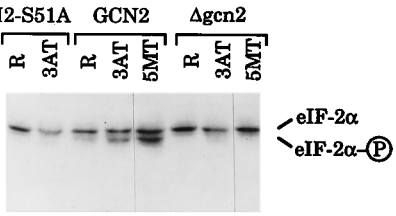

FIG. 3. Analysis of eIF-2α phosphorylation by IEF-PAGE. Cell extracts were prepared from isogenic strains grown under repressing conditions (R) or in the presence of 3-AT (3 AT) or 5-MT (5 MT). Proteins were separated by IEF on a vertical slab gel and detected by immunoblot analysis using a polyclonal antiserum prepared against a TrpE-SUI2 fusion protein. Phosphorylated and nonphosphorylated forms of eIF-2α are indicated at the right. The relevant genotypes of the strains from which the extracts were prepared are shown above the lanes. SUI2-S51A designates extracts prepared from strain H1897, containing wild-type *GCN2* and substitution of alanine for the phosphorylated serine 51 in *SUI2*. GCN2 lanes are from H1896. Lanes designated Δ*gcn2* were prepared from strain H1816.

were characterized for stimulation of *HIS4-lacZ* expression. In the *GCN2*-transformed strain WY229 ($ser^{+/-}$) grown in the absence of serine, HIS4-LacZ enzyme activity was almost sixfold higher than in cells grown in the medium supplemented with serine (Table 4). No increase in *HIS4-lacZ* expression was detected when the isogenic Δ*gcn2* strain was grown in the absence of serine. Arginine starvation in WY230 ($arg^{+/-}$) also showed a *GCN2*-dependent stimulation of HIS4-LacZ expression, although a somewhat modest threefold increase in HIS4-LacZ activity was detected, compared with about an eightfold induction when the *GCN2* strain was grown in the presence of 3-AT. In both the $ser^{+/-}$ and $arg^{+/-}$ strains, wild-type *GCN2* function was required for full stimulation of the general control pathway. It is noted, however, that strains expressing gcn2-m2 from a high-copy-number plasmid showed modest increases in the levels of HIS4-LacZ enzyme activity compared with the Δ*gcn2* strain (Table 4). The level of HIS4-LacZ in the *gcn2-m2* strain was unchanged during derepressed and repressed growth conditions, suggesting that the *gcn2-m2* product has a low-level activity that can constitutively stimulate to a modest degree the general control pathway in strains overexpressing the mutant protein.

In summary, six different amino acid starvation conditions were examined for stimulation of the general control pathway. In each of the six conditions, *GCN2* function was required for stimulation of *GCN4-lacZ* or *HIS4-lacZ* expression. Additionally, mutations in motif 2, a sequence conserved between the class II aminoacyl-tRNA synthetases, greatly reduced GCN2 positive regulation of the general control system in response to each of the starvation conditions. These results strongly support the idea that the HisRS-related domain of the GCN2 kinase participates in the recognition of a diverse range of amino acid starvations that signal the stimulation of *GCN4* expression.

Different amino acid starvation conditions elevate in vivo phosphorylation of eIF-2α by GCN2 kinase. To investigate more directly the stimulation of GCN2 kinase function in response to different amino acid starvation conditions, we measured in vivo phosphorylation of eIF-2α by IEF-PAGE. Consistent with previous observations, GCN2 phosphorylation of eIF-2α increases when yeast cells are grown in the presence of 3-AT (10) (Fig. 3). Phosphorylation of eIF-2α is dependent on serine 51 because a mutant strain containing an alanine substitution for serine 51 abolished both phosphorylation of eIF-2α and stimulation of *GCN4-lacZ* expression in response to the histidine starvation (10) (Fig. 3). It was also previously noted that only a portion of eIF-2α was phosphorylated in response to limiting histidine. This level of phosphorylation is proposed to alter the rate of translational reinitiation at the leader of the *GCN4* mRNA, leading to elevated expression of the *GCN4* coding sequences (10, 21, 47). Higher levels of eIF-2α phosphorylation detected in certain constitutively activated $GCN2^c$ alleles appear to reduce eIF-2 activity sufficiently to inhibit general translation initiation, resulting in severe slow-growth phenotypes (37).

We used IEF-PAGE to determine whether eIF-2α is also phosphorylated when strains are grown in the presence of 5-MT for 5 h. As shown in Fig. 3, phosphorylation of eIF-2α was higher in strains grown in medium supplemented with either of the chemical inhibitors, 3-AT or 5-MT, than in strains grown under the repressed condition. It is noted that there was a small amount of detectable phosphorylation in the *GCN2* strain in the absence of starvation. Presumably, this represents a basal activity of GCN2 kinase, as no phosphorylation of eIF-2α was detected in the Δ*gcn2* cells grown under identical conditions. Consistent with this idea, the GCN4-LacZ enzyme activity was almost threefold higher in the *GCN2* strain grown under repressing growth conditions than in the Δ*gcn2* cells (Table 2).

During initial experiments to ascertain whether GCN2 kinase activity is altered in response to SM, we detected no appreciable difference in the levels of phosphorylation of eIF-2α compared with levels in cells grown under the repressing growth conditions. We rationalized that phosphorylation of eIF-2α might be transitory, and therefore we assayed phosphorylation at different times during growth under SM starvation conditions. As illustrated in Fig. 4, eIF-2α phosphorylation was elevated after 1 h of growth in the presence of SM, but phosphorylation after 3 h had decreased to levels near those found under repressing growth conditions. In comparison, the strain grown in the presence of 3-AT showed high levels of eIF-2α phosphorylation after 1 h of growth; although the extent of phosphorylation was slightly diminished after 3 and 5 h of growth in the presence of 3-AT, it was still higher than in the repressed cells (Fig. 4).

Similar focusing experiments were carried out to measure

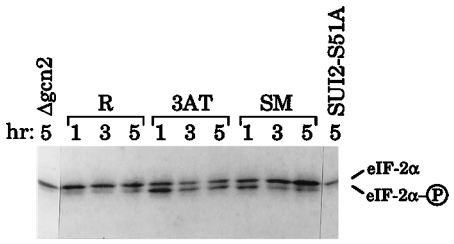

FIG. 4. GCN2 phosphorylation of eIF-2α is increased in response to 3-AT and SM. Protein extracts were prepared from isogenic strains grown under conditions not limiting for amino acids (repressing [R]) or in the presence of 3-AT (3 AT) or SM for 1, 3, or 5 h as indicated. After separation by IEF on a vertical slab gel, eIF-2α was detected by immunoblotting. Lane Δgcn2 is H1816, containing *SUI2* on high-copy-number *URA3* plasmid p925, grown for 5 h in the presence of 3-AT. Lane SUI2-S51A is strain H1897, containing wild-type *GCN2* and an alanine substitution at the phosphorylation site serine 51 in *SUI2*, grown for 5 h in the presence of 3-AT. All other lanes are *GCN2* strain H1896 containing *SUI2* on a high-copy-number *URA3* plasmid. As discussed in Materials and Methods, elevated expression of eIF-2α assisted detection of the protein in the IEF system. Phosphorylated and nonphosphorylated forms of eIF-2α are indicated at the right.

eIF-2α phosphorylation in response to starvation for lysine. An increase in eIF-2α phosphorylation was detected at the 3-h time point that was further elevated after 5 h of growth in the absence of lysine (Fig. 5A). It is noted that the fainter secondary bands visible below both the phosphorylated and nonphosphorylated eIF-2α bands in these immunoblots are often visible under our experimental conditions when extracts from certain strain backgrounds are used. For as yet unknown reasons, there appears to be a reduction in the quality of resolution in the IEF system in these strain backgrounds. This phosphorylation time course is consistent with intracellular levels of lysine being slowly depleted in this leaky auxotrophic strain. Only minimal phosphorylation is detected in the *GCN2* strain

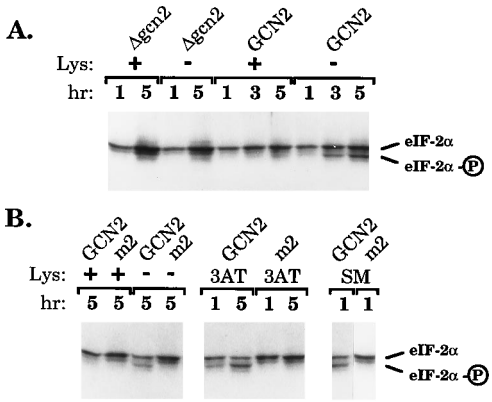

FIG. 5. Lysine starvation stimulates GCN2 phosphorylation of eIF-2α by a mechanism requiring the HisRS-related sequences. Leaky auxotrophic strain WY231 (*lys*$^{+/-}$) containing wild-type *GCN2*, Δ*gcn2*, or *gcn2-m2* was grown in the presence or absence of lysine as described in Materials and Methods. Protein extracts were prepared from these cell cultures and separated by IEF-PAGE, and eIF-2α was detected by immunoblot analysis. Phosphorylated and nonphosphorylated eIF-2α are indicated at the right. (A) Strain WY231 (Δ*gcn2 lys*$^{+/-}$) containing *GCN2* encoded on p722 (GCN2) or vector plasmid pRS316 (Δgcn2) was grown in the presence (+) or absence (−) of lysine for 1, 3, or 5 h as indicated. (B) Strain WY231 containing plasmid p722 (GCN2) or p299 (m2) was grown in medium supplemented with lysine or in the absence of lysine for 5 h. Alternatively, transformants were grown in the presence of lysine medium supplemented with 3-AT or SM for the indicated time. To assist in the detection of eIF-2α, each of the strains used in these experiments contained *SUI2* on a high-copy-number plasmid (see Materials and Methods). All lanes in panel B are from a single IEF-PAGE experiment.

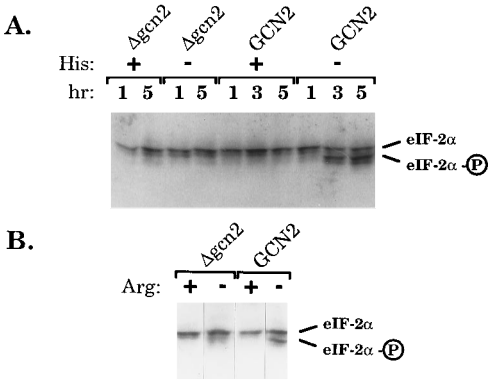

FIG. 6. Amino acid starvation in different leaky auxotrophic strains stimulates GCN2 phosphorylation of eIF-2α. Leaky auxotrophic strains containing wild-type *GCN2* or Δ*gcn2* were grown in the presence or absence of the limiting amino acid, and protein extracts were prepared and separated by IEF-PAGE. eIF-2α was visualized by immunoblot analysis. Phosphorylated and nonphosphorylated eIF-2α are indicated at the right. (A) The Δ*gcn2 his*$^{+/-}$ strain WY232 was transformed with p722 (GCN2) or pRS316 (Δgcn2) and grown in the presence (+) or absence (−) of histidine for 1, 3, or 5 h as indicated. (B) Strain WY230 (Δ*gcn2 arg*$^{+/-}$) containing plasmid p722 (GCN2) or pRS316 (Δgcn2) was grown in medium supplemented with arginine (+) or in the absence of arginine (−) for 5 h. To assist in the detection of eIF-2α, each of the strains used in these experiments contained *SUI2* on a high-copy-number plasmid (see Materials and Methods).

grown in the lysine-supplemented media. We did not observe any phosphorylation of eIF-2α in Δ*gcn2* or *gcn2-m2* strains grown in the absence of lysine or in *gcn2-m2* cells grown in the presence of the chemical inhibitor 3-AT or SM in lysine-supplemented medium (Fig. 5).

Phosphorylation of eIF-2α was also examined in the leaky auxotrophic strains WY232 (*his*$^{+/-}$) and WY230 (*arg*$^{+/-}$) (Fig. 6). When histidine was absent from the growth medium, phosphorylation of eIF-2α in the *his*$^{+/-}$ cells was detectable at 3 h, with a further increase visible after 5 h. This time course was comparable to that determined for the *lys*$^{+/-}$ strain grown in the absence of lysine (Fig. 5A). In contrast, eIF-2α phosphorylation was detected within 1 h in a *GCN2* strain starving for histidine in the presence of the chemical inhibitor 3-AT (Fig. 4). These different time courses are therefore independent of the limiting amino acid and would be more reflective of the method used to starve the cells. The chemical inhibitor 3-AT appears to rapidly deplete histidine within the cell, leading to activation of GCN2 kinase. Alternatively, the leaky auxotrophic strains would reduce the pools of a limiting amino acid more slowly. This gradual increase in eIF-2α phosphorylation was also detected in the *arg*$^{+/-}$ strain. Phosphorylation was visible only after the *arg*$^{+/-}$ strain was grown in the absence of arginine for 5 h (Fig. 6B). No phosphorylation was detected in *GCN2* cells grown in the presence of arginine or in the isogenic Δ*gcn2* strain grown under arginine-limiting conditions.

Elevated levels of uncharged tRNA^{His} stimulate GCN2 phosphorylation of eIF-2α in the absence of amino acid starvation. Uncharged tRNA that accumulates during amino acid starvation is proposed to be the direct signal regulating the activity of GCN2 kinase. This idea is derived in part from the observation that temperature-sensitive mutations in isoleucyl-tRNA synthetase and lysyl-tRNA synthetase can stimulate the general control pathway even in the absence of amino acid starvation (26, 33). We examined whether a temperature-sensitive mutation in the HisRS gene, *hts1-1*, could stimulate the general control pathway. The *hts1-1* mutation was previously shown to reduce the level of charged tRNA^{His} (32). After

TABLE 5. GCN2 kinase stimulates *HIS4-lacZ* expression in response to elevated uncharged $tRNA^{His}$ in aminoacyl-tRNA synthetase mutants

Strain	Relevant genotype	HIS4-LacZ enzyme activity (nmol/min/mg of protein)[a]
WY205	*GCN2 HTS1*	250
WY206	*GCN2 hts1-1*	760
WY207	*gcn2-K559V HTS1*	71
WY208	*gcn2-K559V hts1-1*	110

[a] β-Galactosidase enzyme activity was assayed in extracts prepared from the indicated strains as described in Materials and Methods. The results shown are averages from two independent experiments, and the individual measurements deviated from the average values shown by 20% or less.

growth of the *GCN2 hts1-1* strain for 5 h at the semipermissive temperature (34°C), the HIS4-LacZ enzyme activity was about three times that measured in the *GCN2 HTS1* strain (Table 5). This increase in HIS4-LacZ enzyme activity clearly requires GCN2 kinase function, as substitution of valine for the conserved lysine 559 in the ATP-binding region of the GCN2 kinase domain abolished derepression of the general control pathway in the *hts1-1* strain. Previous studies have shown that mutations altering lysine 559 in GCN2 abolish in vitro autophosphorylation and eIF-2α phosphorylation (10, 49).

Stimulation of the GCN2 kinase function in response to elevated levels of uncharged $tRNA^{His}$ was examined by using the IEF system. In the presence of *GCN2* function, phosphorylation of eIF-2α was higher in the *hts1-1* strain than in the wild-type *HTS1* cells (Fig. 7). This increase was visible at 1, 3, and 5 h after growth at 34°C. No phosphorylation of eIF-2α was detected in the *gcn2-K559V* mutant containing the *hts1-1* allele. These data indicate that elevated levels of uncharged $tRNA^{His}$ can stimulate GCN2 kinase function and the attendant induction of the general control pathway even in the absence of histidine starvation.

The GCN2 synthetase-related domain binds tRNA. Uncharged tRNA that accumulates during amino acid starvation appears to be the direct regulator of GCN2 kinase function. To determine whether the synthetase-related sequence of GCN2 can bind tRNA, we expressed this portion of GCN2 in the form of a polyhistidine fusion protein. Using the amino-terminal polyhistidine tag, we greatly enriched for this protein by affinity chromatography using metal chelation resin (see Materials and Methods). The enriched protein was separated by SDS-PAGE, and proteins were transferred to a nitrocellulose filter and

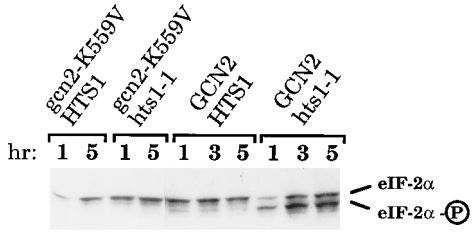

FIG. 7. Uncharged $tRNA^{His}$ stimulates GCN2 phosphorylation of eIF-2α. Cell extracts were prepared from isogenic strains grown at the semipermissive temperature (34°C) for 1, 3, or 5 h as indicated. Proteins were separated by IEF-PAGE, and eIF-2α was detected by immunoblot analysis. Phosphorylated and nonphosphorylated forms of eIF-2α are indicated at the right. Lanes gcn2-K559V HTS1 were prepared from strain WY207 containing a kinase-deficient *gcn2-K559V* mutant and wild-type HisRS gene, *HTS1*. Lanes gcn2-K559V hts1-1 were derived from strain WY208 containing the temperature-sensitive HisRS mutation, *hts1-1*. The lanes GCN2 HTS1 and GCN2 hts1-1 were extracts prepared from strains WY205 and WY206, respectively, containing wild-type *GCN2*.

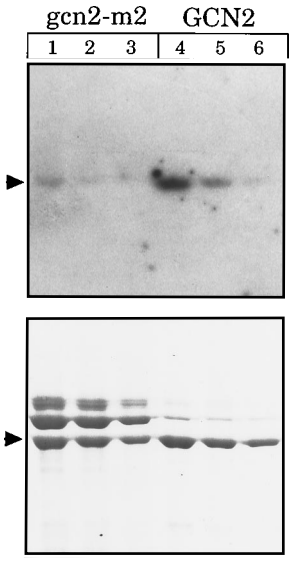

FIG. 8. Uncharged yeast tRNA binds to the synthetase-related domain of GCN2. The polyhistidine–GCN2 HisRS-related domain fusion protein was expressed in *E. coli* and greatly enriched for by affinity chromatography by using metal chelation resin. (Top) The fusion protein was analyzed by SDS-PAGE, transferred to a nitrocellulose filter membrane, and incubated with ^{32}P-labeled tRNA in a Northwestern blot assay. (Bottom) Coomassie blue stain of fusion protein following SDS-PAGE. Lanes 4 to 6 are the wild-type GCN2 HisRS-related domain fused to the polyhistidine, and lanes 1 to 3 are the gcn2-m2 mutant fusion protein. Arrowheads designate the GCN2 fusion proteins; higher-molecular-weight proteins visible primarily in the gcn2-m2 preparation are endogenous bacterial proteins. Amounts of fusion protein: lanes 1 and 4, 15 pmol; lanes 2 and 5, 10 pmol; lanes 3 and 6, 5 pmol.

incubated with radiolabeled uncharged yeast tRNA in a Northwestern blot assay. As shown in Fig. 8, the wild-type synthetase-related domain of GCN2 clearly bound tRNA, while minimal binding was detected when a similarly prepared mutant gcn2-m2 fusion protein was used. No tRNA associated with similar amounts of bovine serum albumin or polyhistidine–eIF-2α fusion protein characterized in the Northwestern assay (data not shown). These results illustrate that GCN2 can interact with tRNA by a mechanism requiring motif 2 sequences and support the idea that the failure of the gcn2-m2 protein to stimulate *GCN4* expression was due to the inability of the mutant protein to recognize uncharged tRNAs that accumulated during the different amino acid starvation conditions.

DISCUSSION

A family of protein kinases phosphorylate the α subunit of eIF-2 to regulate translation initiation in response to different stress signals. In this report, we focused on one of these eIF-2α kinases, GCN2, a positive regulator of the general amino acid control system in *S. cerevisiae*. The GCN2 kinase participates in sensing amino acid starvation in the cell and transducing this signal, via phosphorylation of eIF-2α, to increase translation of *GCN4*, a transcriptional activator of amino acid biosynthetic genes. In the carboxy-terminal portion of GCN2, there are sequences homologous to HisRSs, and this region is proposed to activate the adjacent protein kinase moiety during starvation conditions when uncharged tRNA accumulates.

The questions that we posed in this report were whether the GCN2 kinase functions in the recognition of each of the amino acid starvation signals known to induce the general control system and whether uncharged tRNA is a more direct regula-

tor of GCN2 than amino acid limitation per se. We used chemical inhibitors or leaky auxotrophic mutations to starve cells for a variety of different amino acids, including histidine, tryptophan, the branched-chain amino acids, lysine, arginine, and serine. In each of these starvation conditions, loss of GCN2 kinase function greatly impaired the general control response, as measured by GCN4-LacZ or HIS4-LacZ enzyme expression (Tables 2 to 4). To assay for the activity of GCN2 kinase more directly, we characterized the in vivo phosphorylation of eIF-2α by using the IEF method. In each of the conditions examined, phosphorylation of eIF-2α was increased in response to amino acid starvation (Fig. 3 to 6). No increase in phosphorylation was detected when *GCN2* function was absent or when the cells were not limited for amino acids. These findings indicate that the activity of GCN2 kinase can be stimulated in response to starvation for a range of different amino acids. Furthermore, each of these starvation signals appears to be recognized by a common mechanism involving the GCN2 HisRS-related domain, as point mutations in the motif 2 sequences of this region abolished phosphorylation of eIF-2α and greatly diminished the stimulation of the general control response (Fig. 6B and Tables 2 to 4).

To address whether the stimulatory effect of amino acid starvation on GCN2 kinase is mediated more directly by uncharged tRNA levels, we characterized the general control response in strains containing a temperature-sensitive mutation in the HisRS gene (*hts1-1*). In strain WY206 (*GCN2 hts1-1*) grown at the semipermissive temperature, HIS4-LacZ enzyme activity and eIF-2α phosphorylation were higher at each of the time points examined than in the wild-type *HTS1* strain (Table 5 and Fig. 7). This response was completely dependent on *GCN2* kinase activity, as an isogenic *hts1-1* strain containing the defective *gcn2-K559V* gene showed reduced levels of HIS4-LacZ and no detectable phosphorylation of eIF-2α. We conclude that the levels of uncharged tRNA regulate GCN2 phosphorylation of eIF-2α more directly than does a limitation for an amino acid. Indeed, when the synthetase-related sequences were examined for the ability to bind uncharged tRNA in a Northwestern blot assay, there was a clear interaction that was dependent on the motif 2 sequences (Fig. 8).

Previous reports also support the model that the general control pathway is stimulated by different species of uncharged tRNA that accumulate during amino acid starvation. In cells containing an *ils1-1* mutation affecting isoleucyl-tRNA synthetase, there is an induction of certain biosynthetic enzymes under GCN4 control in amino acid-complete medium (33); however, it was not determined whether this response was dependent on *GCN2* function. During the course of our study, we observed that an *ils1-1* mutant strain shows increased HIS4-LacZ enzyme activity when cells were grown under nonstarvation conditions, whereas no increase in the expression of *HIS4-lacZ* was detected in an isogenic strain containing a kinase-deficient *gcn2-K559V* allele. Lanker et al. (26) also reported that a mutation in the lysyl-tRNA synthetase gene stimulated the translational expression of *GCN4* independent of amino acid starvation. Genetic evidence suggested a role for GCN2 kinase in recognizing this charging defect. In this study, we used IEF-PAGE to directly show that starvation for lysine increases phosphorylation of eIF-2α by GCN2 kinase by a mechanism requiring the function of the HisRS-related sequences (Fig. 5).

Our observation that GCN2 kinase is activated in cells defective for charging $tRNA^{His}$, in conjunction with these earlier reports of stimulation of the general control pathway in strains impaired for isoleucyl- or lysyl-tRNA synthetase activity, supports the idea that GCN2 monitors the aminoacylation level of different cognate tRNAs that accumulate during starvation for amino acids. This interaction with uncharged tRNA requires the synthetase-like domain of GCN2, as mutations in the motif 2 region block tRNA binding to the synthetase-related domain in the Northwestern blot assay and impair eIF-2α phosphorylation and the resulting stimulation of the general control pathway in response to each of the amino acid starvation conditions that we examined. Such an interaction supports the model that the HisRS-related domain of GCN2 has diverged from bona fide HisRSs such that it can associate with many different tRNA species. Thus, characterization of GCN2-tRNA interactions could provide useful insight into the mechanisms by which synthetases discriminate between their tRNA substrates.

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