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Identification of Positive-Acting Domains in GCN2 Protein Kinase Required for Translational Activation of *GCN4* Expression

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GCN4 is a transcriptional activator of amino acid-biosynthetic genes in the yeast *Saccharomyces cerevisiae*. GCN2, a translational activator of *GCN4* expression, contains a domain homologous to the catalytic subunit of eucaryotic protein kinases. Substitution of a highly conserved lysine residue in the kinase domain abolished GCN2 regulatory function in vivo and its ability to autophosphorylate in vitro, indicating that GCN2 acts as a protein kinase in stimulating *GCN4* expression. Elevated *GCN2* gene dosage led to derepression of *GCN4* under nonstarvation conditions; however, we found that *GCN2* mRNA and protein levels did not increase in wild-type cells in response to amino acid starvation. Therefore, it appears that GCN2 protein kinase function is stimulated posttranslationally in amino acid-starved cells. Three dominant-constitutive *GCN2* point mutations were isolated that led to derepressed *GCN4* expression under nonstarvation conditions. Two of the *GCN2*(Con) mutations mapped in the kinase domain itself. The third mapped just downstream from a carboxyl-terminal segment homologous to histidyl-tRNA synthetase (HisRS), which we suggested might function to detect uncharged tRNA in amino acid-starved cells and activate the adjacent protein kinase moiety. Deletions and substitutions in the HisRS-related sequences and in the carboxyl-terminal segment in which one of the *GCN2*(Con) mutation mapped abolished GCN2 positive regulatory function in vivo without lowering autophosphorylation activity in vitro. These results suggest that sequences flanking the GCN2 protein kinase moiety are positive-acting domains required to increase recognition of physiological substrates or lower the requirement for uncharged tRNA to activate kinase activity under conditions of amino acid starvation.

In the yeast *Saccharomyces cerevisiae*, starvation for any one of several amino acids or a defective aminoacyl-tRNA synthetase leads to increased transcription of over 30 genes encoding amino acid-biosynthetic enzymes in several different pathways (reviewed in reference 19). The coordinate derepression of these biosynthetic pathways is known as general amino acid control. GCN4 is a positive regulatory protein that acts directly to stimulate transcription by binding to 5′ noncoding sequences located upstream of each gene subject to the general control.

Expression of *GCN4* itself is regulated by amino acid availability at the level of translation initiation. This translational control involves four short upstream open reading frames (uORFs) present in the leader of *GCN4* mRNA that inhibit translation initiation at the *GCN4* start codon under nonstarvation conditions. The inhibitory effect of the four uORFs on *GCN4* expression requires *trans*-acting negative regulators encoded by multiple *GCD* genes. This group of negative effectors was recently shown to include *SUI2* and *SUI3*, the structural genes for the α and β subunits, respectively, of eucaryotic initiation factor 2 (42). Positive-acting factors encoded by the *GCN2* and *GCN3* genes are required to stimulate *GCN4* expression in response to amino acid starvation. GCN2 and GCN3 are thought to function indirectly by modifying the activity of one or more GCD-negative regulators, thereby eliminating the translational repression of *GCN4* expression mediated by the uORFs (19).

In a previous study, we characterized the positive regulator GCN2 by insertional mutagenesis and nucleotide sequencing of the *GCN2* complementation unit (41). The deduced amino acid sequence of GCN2 predicts a molecular weight of 182,000, a value consistent with that obtained by electrophoretic analysis of immunoprecipitated GCN2 protein synthesized in vivo. The GCN2 amino acid sequence contains two important homologies to known proteins. First, a segment of about 350 residues located roughly in the middle of GCN2 is homologous to the catalytic domain of eucaryotic protein kinases (35, 41) (Fig. 1). A recent compilation of 65 protein kinases identified 11 conserved subdomains containing 33 highly conserved or invariant residues (13). Each of these amino acids is present in the GCN2 protein kinase domain. One of the most conserved subdomains is the ATP-binding site, which includes a lysine residue thought to be directly involved in the phosphotransfer reaction. Substitutions of this lysine invariably abolish kinase activity (13). Mutations that change the corresponding lysine in GCN2 (position 559) to valine or arginine destroy the ability of GCN2 to derepress *HIS* genes subject to the general control in response to amino acid starvation (41) (*gcn2-K559R* in Fig. 1). In addition, a protein kinase activity associated with a protein of approximately 100,000 molecular weight was detected in vitro that is either a proteolytic fragment of GCN2 or a distinct kinase under *GCN2* control (35). These results strongly suggest that GCN2 functions as a protein kinase in stimulating expression of *GCN4* and the structural genes under its control in amino acid-starved cells.

The second homology detected between GCN2 and other known proteins is a 530-amino-acid segment that shows 22% identity to the complete sequence of histidyl-tRNA synthetase (HisRS) from *S. cerevisiae* (41). This region also shows similarity to HisRS sequences from humans and *Escherichia coli*. Several two-amino-acid insertions in the GCN2 HisRS-related domain abolish the derepression of *HIS* genes, suggesting that these residues are important for GCN2 positive regulatory function. Given that aminoacyl-

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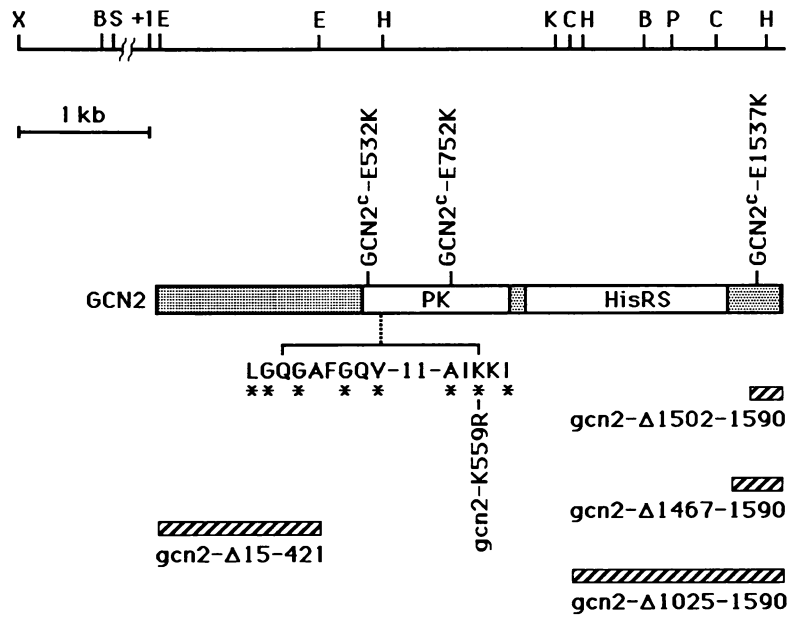

FIG. 1. Functional map of GCN2. The top line represents *GCN2* DNA marked with selected restriction endonuclease sites: B, *Bam*HI; C, *Bcl*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pvu*II; S, *Sna*BI; X, *Xba*I. The +1 marks the 5′ end of *GCN2* mRNA (35). An *Xba*I site at nucleotide position 2035 is not shown because its cleavage is inhibited by methylation. The box designated GCN2 indicates the *GCN2* protein-coding sequence, showing domains homologous to protein kinases (PK) and histidyl-tRNA synthetases (HisRS). Dominant constitutive *GCN2*(Con) alleles (GCN2^c) are indicated above the box at the positions of the mutations, with the wild-type residue, amino acid position, and mutant residue listed in that order. Below the box are *gcn2* mutations. The sequence of the putative ATP-binding site of the protein kinase domain is shown in the one-letter amino acid code, with asterisks indicating residues conserved among protein kinases. The position of mutation *gcn2-K559R* in the kinase domain is shown; this and a similar mutation, *gcn2-K559V*, were reported previously (41). The extent of deletions in *GCN2* is shown by hatched bars, with deleted residues listed beneath.

tRNA synthetases bind uncharged tRNA as a substrate and are able to distinguish between charged and uncharged tRNA (36), we proposed that the HisRS-related domain in GCN2 monitors the concentration of uncharged tRNA in the cell and activates the adjacent protein kinase moiety under starvation conditions when uncharged tRNA accumulates (41). This mechanism would provide a means of coupling *GCN4* expression to the availability of amino acids.

In this report, we use an in vitro assay to demonstrate directly that GCN2 is a protein kinase. In addition, we show that modulation of GCN2 regulatory function by amino acid availability does not require differential expression of *GCN2*, as there is little difference in *GCN2* mRNA and protein levels between repressing (nonstarvation) and derepressing (amino acid starvation) conditions. Consequently, we conclude that GCN2 protein kinase function is regulated posttranslationally. To gain further insight into the mechanism of GCN2 regulation, we isolated three dominant mutations in *GCN2* that lead to constitutive activation of its positive regulatory function. Two of these mutations alter residues in the protein kinase domain; the third substitutes an amino acid in the carboxyl-terminus just downstream from the HisRS-related sequences. Analysis of in-frame deletions of sequences outside the *GCN2* kinase domain indicates that these regions are dispensable for protein kinase activity in vitro but are absolutely required for GCN2 regulatory function in vivo. Together, our results suggest that multiple regions flanking the GCN2 protein kinase domain function in vivo to enhance recognition of the correct protein substrate(s) or to mediate a stimulatory effect of uncharged tRNA on GCN2 protein kinase activity.

MATERIALS AND METHODS

Plasmids. Plasmid pC102-2 (9) contains *GCN2* on a 7.0-kilobase (kb) *Sau*3AI fragment inserted into the *Bam*HI site of the low-copy-number *URA3*-containing plasmid YCp50. The 3′ end of the insert in pC102-2 is a *Bgl*II site located 9 nucleotides downstream from the *GCN2* stop codon. Plasmid p585 was constructed from pC102-2 by inserting additional sequences present downstream from the chromosomal *GCN2* gene that were originally cloned on plasmid pAH15 (20). This was accomplished by exchanging the 6.0-kb *Sna*BI-*Sal*I fragment of pC102-2 with the corresponding 7.1-kb fragment of pAH15 (Fig. 1). (The *Sal*I site is present 3′ to the *GCN2* gene in the vector sequences.) pC102-2 and p585 are identical in their ability to complement a *gcn2* chromosomal deletion for the inability to derepress *GCN4*, *HIS4*, and *HIS3* expression under amino acid starvation conditions, assayed as described below.

Plasmid p722 is a low-copy-number *URA3*-containing plasmid constructed by first deleting the *Pvu*II-*Pvu*II fragment encompassing the multiple cloning sites in plasmid pRS316 (38) and replacing it with a double-stranded oligonucleotide containing *Xba*I and *Sal*I sites to form plasmid p713. The 8.2-kb *Xba*I-*Sal*I *GCN2* fragment was isolated from p585 and inserted into the corresponding sites in p713 to generate plasmid p722.

GCN2(Con) mutations were isolated by hydroxylamine mutagenesis of pC102-2, as described previously (32). Mutagenized plasmids were introduced into yeast strain H1053 (*MAT***a** *ura3-52 leu2-3 leu2-112 HIS4*::*lacZ*), and transformants were screened for increased resistance to 0.5 mM

5-fluorotryptophan (5-FT^r) (29, 42). Plasmids were recovered by transforming *E. coli* DH5α with total yeast DNA isolated from yeast clones of interest by the method of Hoffman and Winston (22), modified to include two ethanol precipitations following the phenol extraction step. Plasmids p628 and p708, containing mutations *GCN2*(Con)-*E532K* and *GCN2*(Con)-*E752K*, respectively, were constructed by replacing wild-type restriction fragments of p585 with the corresponding fragments from the mutant alleles containing only single-base-pair substitutions. Plasmid p693 is a similar substitution of a fragment containing the mutation *GCN2* (Con)-*E1537K* in plasmid pC102-2. *GCN2*(Con)-*E532K*+ *E1537K* was constructed by exchanging the 5.6-kb *Kpn*I fragment of p628 with the corresponding *Kpn*I fragment from p693. (The second *Kpn*I site is present 3′ to the *GCN2* gene in the vector sequences.)

The stop codon mutations in *gcn2-Δ1467-1590* and *gcn2-Δ1502-1590* were constructed by modifying linker-insertion alleles generated previously in pC102-2 (41) that contain the 6 bp corresponding to a *Sac*I restriction site inserted at *Taq*I sites in *GCN2* at positions 4422 and 4528, respectively. A 16-base-pair (bp) oligonucleotide was inserted at the *Sac*I sites, producing plasmids p779 and p780, respectively. Insertion of this oligonucleotide adds an in-frame AGC serine codon and two TAA stop codons immediately following *GCN2* codon 1466 or 1501, creating *gcn2-Δ1467-1590* and *gcn2-Δ1502-1590*, respectively.

The *gcn2-Δ15-421* allele, containing an in-frame deletion of *GCN2* coding sequences between nucleotides 69 and 1290, was constructed by eliminating the 1.2-kb *Eco*RI fragment containing these sequences from pC102-2, forming plasmid p498. *gcn2-Δ1025-1590* is a stop codon mutation constructed in pC102-2 by replacing the sequences between the *Kpn*I and *Bcl*I sites at positions 3076 and 3185 (Fig. 1), respectively, with a 33-bp oligonucleotide containing the seven codons normally present downstream from the *Kpn*I site, up to codon 1024, followed by an in-frame TGA termination codon (p358).

Plasmid p630 contains the 8.2-kb *Xba*I-*Sal*I fragment from p585 inserted between the *Nde*I and *Sal*I sites of the high-copy-number *URA3*-containing plasmid YEp24 (4). High-copy-number plasmids p776 and p775, containing mutant alleles *gcn2-Δ15-421* and *gcn2-Δ1025-1590*, respectively, are derivatives of p630.

Plasmid p500 was constructed from plasmid p484, which contains the 4.5-kb *GCN2 Bam*HI fragment (Fig. 1) inserted at the *Bam*HI site of a modified version of pUC18 (45), in which the unique *Eco*RI and *Hin*dIII sites were removed by end-filling (28). The *GCN2* sequences between the *Eco*RI and *Hin*dIII sites at positions 63 and 3284, respectively (codons 15 to 1086), were deleted from p484 and replaced by a 7-bp oligonucleotide containing a *Bgl*II site, forming p495. The 2.8-kb *LEU2 Bgl*II fragment from YEp13 (7) was inserted at the *Bgl*II site of p495 to form plasmid p500.

Yeast strains. Plasmid-borne *GCN2* alleles were introduced into yeast strain H1149 (*MATα ura3-52 ino1 leu2-3 leu2-112 gcn2-Δ63-3284::LEU2 HIS4::lacZ*) or H1354 (*MATα ura3-52 leu2-3 leu2-112 gcn2-1 GCN4::lacZ*) by the lithium acetate transformation technique (24). The *gcn2* deletion allele in H1149 was constructed by transforming strain H113 (*MATα gcn2-1 ura3-52 HIS4::lacZ leu2-3 leu2-112*) to Leu$^+$ (33) by using the 7.3-kb *Bam*HI fragment of plasmid p500, described above. The structure of the resulting deletion-insertion allele was verified by DNA blot hybridization analysis (28), using as a probe the 4.5-kb *GCN2 Bam*HI fragment. H1149 was obtained as a Ura$^-$ Leu$^+$ 3-aminotriazole-sensitive (3-AT^s) segregant from a cross between this transformant and strain H46 (*MATa ino1 HIS4::lacZ*). (All *gcn* mutations produce sensitivity to 3-AT, an inhibitor of histidine biosynthesis [44].) The *HIS4::lacZ* (27) and *GCN4::lacZ* (17) fusions are integrated on chromosome V between two copies of *ura3-52*; thus, both H1149 and H1354 are Ura3$^-$.

Strains H4 (*MATα ura3-52 leu2-3 leu2-112*), H1080 (*MATα ura3-52 leu2-3 leu2-112 gcn4::LEU2*), and H1081 (*matα ura3-52 leu2-3 leu2-112 gcn2-Δ2030-2403::LEU2*) used for RNA analysis are isogenic strains constructed previously (42). The *gcn2-Δ2030-2403::LEU2* deletion-insertion in H1081 contains the same *LEU2* fragment described above inserted in place of *GCN2* sequences between the *Bgl*II sites at positions 2030 and 2403. The *gcn4* deletion in H1080 contains *LEU2* sequences inserted in place of nucleotides 88 and 1092, numbered relative to the start site of *GCN4* transcription.

Analysis of *GCN2* expression and regulatory function. With the exception of the mRNA analysis done on strains H4, H1080, and H1081, all experiments were done with transformants of *gcn2* deletion strain H1149 or *gcn2-1* strain H1354, which contains *GCN2* alleles on low-copy-number plasmids.

(i) Amino acid analog sensitivity. Plasmid-borne *GCN2* alleles were tested for complementation of *gcn2-Δ63-3284::LEU2* for the inability to derepress *HIS3* expression by measuring the growth rate of H1149 transformants replica-plated to medium containing 30 mM 3-AT and excess (40 mM) leucine (20). Constitutive derepression of tryptophan-biosynthetic enzymes was assayed by replica-plating transformants to medium containing 0.5 mM 5-FT.

(ii) Assay of *HIS4::lacZ* and *GCN4::lacZ* fusions. Assays were conducted as described previously (27) after transformants were grown in SD medium (37) containing only the required supplements: 2 mM leucine, 0.5 mM isoleucine, 0.5 mM valine, and 0.2 mM inositol. For steady-state repressing conditions, saturated cultures were diluted 1:50 and harvested in mid-logarithmic phase after 6 h of growth. For derepressing conditions, cultures were grown for 2 h under repressing conditions and then for 6 h after 3-AT was added to 10 mM. β-Galactosidase activities are expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

(iii) Analysis of *GCN2* and *HIS4* mRNA abundance. Strains were grown under the same repressing and derepressing conditions described above except that H4, H1080, and H1081 required a supplement of 0.2 mM uracil. RNA was extracted, and formaldehyde-agarose gel electrophoresis and blot hybridization analysis were performed as described previously (21). *GCN2*, *HIS4*, *GCN3*, and *GCN4* mRNAs were probed, respectively, with the 5.6-kb *GCN2 Sna*BI-*Bst*EII fragment isolated from p585, the 2.8-kb *HIS4 Eco*RI fragment from pR5 (8), the 0.6-kb *GCN3 Dra*I-*Cla*I fragment isolated from plasmid Ep157 (14), and the 550-bp *GCN4 Kpn*I-*Mlu*I fragment from p136 (17). Densitometry was used to scan autoradiographs, and peak areas were determined.

(iv) Analysis of rates of GCN2 and HIS4-LacZ protein synthesis. Transformants were grown in 5-ml cultures for 6 h under the repressing and derepressing conditions described above. Then, 300 μCi of [^{35}S]methionine (1,000 Ci/mmol) was added, and after 20 min of incubation, unlabeled methionine was added to 1 mM for another 10 min. Preparation of protein extracts and immunoprecipitation were performed by the method of Klionsky et al. (26) with the following modifications. All solutions were supplemented with 1 μM pepstatin, 1 μM leupeptin, 0.15 μM aprotinin, and 100 μM

phenylmethylsulfonyl fluoride (PMSF). Only a single immunoprecipitation step was performed, and three additional washing steps were added (twice with 100 mM Tris hydrochloride [Tris-HCl, pH 7.5], 200 mM NaCl, 2 M urea, 0.5% Tween-20; once with 0.1% sodium dodecyl sulfate [SDS]). Immunoprecipitations of GCN2 were conducted with antiserum raised against a *trpE::GCN2* fusion (41); a monoclonal antibody against β-galactosidase (Promega) was used to immunoprecipitate HIS4-LacZ fusion proteins. Samples for immunoprecipitations were adjusted to contain equal counts of [^{35}S]methionine incorporated into protein (15).

(v) Analysis of steady-state GCN2 protein levels. Transformants were grown under the repressing and derepressing conditions described above, and total protein extracts were prepared as described below for immune complex kinase assays. Samples containing 200 μg of total cellular protein, or immunoprecipitates prepared from the equivalent amount of extract with GCN2-specific antiserum, were suspended in SDS sample buffer (15) and separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose (15) and blocked with a solution of 7% nonfat milk and 0.2% Triton X-100 in phosphate-buffered saline (PBS) for 2 h at room temperature. Blots were incubated overnight in blocking solution containing GCN2-specific antiserum or β-galactosidase monoclonal antibody. After being washed four times in PBS, blots were incubated in a blocking solution containing alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G (IgG) or goat anti-mouse IgG. Blots were washed three times in PBS and five times in water and then developed by the 5-bromo-4-chloro-3-indoyl phosphate/Nitro Blue Tetrazolium alkaline phosphatase assay system (Bio-Rad Laboratories).

Phosphorylation of GCN2 protein in vivo. Transformants were grown in 4-ml cultures in low-phosphate minimal medium supplemented with 2% dextrose. This medium is identical to SD (Difco manual "Dehydrated Culture and Media Reagents for Microbiology," 10th ed; Difco Laboratories, Detroit, Mich.) except for the following modifications. Potassium phosphate was present at 0.2 mM, and 1 g of KCl, 1.25 g of citric acid, and 5.6 g of sodium citrate were added per liter. After 2 h of growth in this medium, 1 mCi of $^{32}P_i$ was added. For repressing conditions, growth continued for another 4 h. For derepressing conditions, cultures were incubated for 3 h and then in the presence of 10 mM 3-AT for 1 h. Immunoprecipitations of GCN2 were carried out as described above except that the phosphatase inhibitors NaF (10 mM), sodium orthovanadate (100 μM), and β-glycerolphosphate (50 mM) were added to all solutions. Samples used for immunoprecipitations contained equal amounts of total protein as determined by the Bradford assay (5); ^{32}P counts per milligram of protein varied between different samples by twofold or less. Immunoprecipitated samples were treated with 2 μg of RNase A for 10 min at 4°C prior to SDS-PAGE. After electrophoresis, the polyacrylamide gel was boiled for 30 min in 5% trichloroacetic acid to reduce the background of labeled nonphosphoprotein material (31).

Immune complex assay of GCN2 protein kinase activity. Transformants were grown under the repressing and derepressing growth conditions described above. Cultures were chilled on ice prior to harvesting by centrifugation. The cell pellets were washed once with 50 mM Tris-HCl (pH 7.5)–150 mM NaCl (TN buffer). Unless otherwise indicated, this and all subsequent steps were performed at 4°C. The cells were suspended in TN buffer supplemented with 1 μM pepstatin, 1 μM leupeptin, 0.15 μM aprotinin, and 100 μM PMSF. (These protease inhibitors were present in all solutions used subsequently.) Glass beads were added, and cells were broken with a Vortex mixer. An equal volume of 2% Triton X-100, 1% sodium deoxycholate, and 0.2% SDS in TN buffer was added to the cell extract, followed by gentle mixing. Cell debris was removed by centrifugation at 3,000 rpm in a Beckman model J-6B centrifuge, and the supernatant was divided into equal portions and stored at −70°C. Each sample was assayed for total protein by the Bradford assay (5).

To prepare GCN2 immunoprecipitates, samples containing 200 μg of protein were added to a final volume of 1 ml of TN buffer supplemented with 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS. The sample was precleared with protein A-Sepharose CL-4B beads (15), and 5 to 10 μl of GCN2-specific antiserum was added and incubated overnight at 4°C. Immune complexes were collected with protein A-Sepharose beads and washed three times with 800 μl of TN buffer supplemented with 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS, once in TN buffer alone, and twice in kinase assay (KIN) buffer (20 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl, 1 mM dithiothreitol [DTT]), leaving a 40-μl suspension of Sepharose beads after the final centrifugation. (The kinase assay requires $MgCl_2$, and reactions carried out in the absence of $MgCl_2$ or in the presence of EDTA show no autophosphorylation of GCN2.) After a 5-min incubation at 30°C, the kinase reaction was initiated by the addition of 10 μCi of [γ-^{32}P]ATP to a final concentration of 1 μM ATP. The reaction mix was incubated at 30°C for between 2.5 and 30 min, and the reaction was terminated by the addition of 40 μl of 2× SDS sample buffer and boiled for 5 min. Samples were analyzed by SDS-PAGE. The gel was stained with Coomassie blue and dried, and autoradiography was performed with Kodak XAR film.

Kinase assays were also performed with immunoprecipitates prepared in the absence of detergents by the method just described. In these experiments, KIN buffer supplemented with protease inhibitors was used exclusively for washing and breaking cells, immunoprecipitations, and washes of immune complexes. Unlike kinase assays done on immune complexes isolated in the presence of detergents, in which GCN2 was the only phosphoprotein detected (Fig. 2), many labeled phosphoproteins were observed in addition to GCN2 in assays done on immune complexes isolated without detergents.

RESULTS

GCN2 is a protein kinase. We showed previously that a highly conserved lysine residue (position 559) in the presumptive ATP-binding site of the GCN2 kinase domain (Fig. 1) is required for derepression of biosynthetic genes subject to the general control in response to amino acid starvation (41). Substitution of this residue with arginine impaired derepression of both the *HIS4* gene, leading to reduced expression of a *HIS4::lacZ* fusion under starvation conditions, and the *HIS3* gene, leading to increased sensitivity to 3-AT, a competitive inhibitor of the *HIS3* gene product (Table 1). Derepression of a *GCN4::lacZ* fusion was also impaired in the *gcn2-K559R* mutant, indicating that the inability to derepress *HIS* gene expression in this strain results from reduced synthesis of the transcriptional activator GCN4. The *gcn2-K559R* mutation was comparable to a deletion of *GCN2* in the degree to which derepression was impaired for both *GCN4* and *HIS* genes under its control (Table 1). These results are consistent with the idea that GCN2 protein kinase activity is required for derepression of genes subject to general amino acid control.

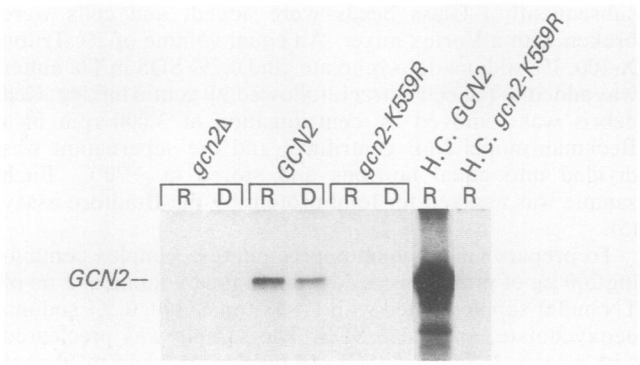

FIG. 2. GCN2 immune complex kinase assay. Protein extracts were prepared from Δ*gcn2* strain H1149 transformed with different plasmid-borne *GCN2* alleles, grown in repressing (R) or derepressing (D) conditions. GCN2 was immunoprecipitated from samples containing equal amounts of total protein extracted in the presence of detergents, and immune complexes were incubated in KIN buffer in the presence of [γ-^{32}P]ATP for 30 min. Radiolabeled samples were analyzed by 7.5% SDS-PAGE, followed by autoradiography. Each lane is designated by the *GCN2* allele present in the strain from which the extract was prepared; lane gcn2Δ corresponds to strain H1149 transformed with the vector YCp50; H.C., high copy number. GCN2 phosphoprotein is indicated.

To demonstrate by an in vitro approach that GCN2 is a protein kinase, we immunoprecipitated GCN2 from cell extracts by using antiserum raised against a *trpE*::*GCN2* fusion protein (41), incubated the isolated immune complexes with [γ-^{32}P]ATP, and analyzed the radiolabeled products by SDS-PAGE. For this and all subsequent in vitro kinase assays, each sample contained the same amount of total cellular protein. A radiolabeled species with the molecular weight of GCN2 was produced by immune complexes prepared from a strain containing *GCN2* in single copy, but not by complexes prepared from a Δ*gcn2* strain (Fig. 2). In addition, the yield of labeled phosphoprotein was greater in complexes prepared from a strain containing *GCN2* on a high-copy-number plasmid. Immune complexes prepared from mutant strains containing *gcn2-K559R* on single or high-copy-number plasmids produced no detectable labeled phosphoprotein. Experiments discussed later show that the steady-state level of the *gcn2-K559R* product is similar to that of wild-type GCN2. Finally, the kinase assay performed with [α-^{32}P]ATP gave no radiolabeled GCN2 product (data not shown). Together, these results indicate that GCN2 is phosphorylated in the immune complexes and that this reaction occurs by autophosphorylation. There was a small decrease in the amount of GCN2 autophosphorylation with complexes prepared from strains grown under starvation versus nonstarvation conditions (Fig. 2). This decrease may be attributable to a small reduction in the steady-state level of GCN2 protein under starvation conditions that we observed in some experiments.

GCN2 is a phosphoprotein in vivo. Protein phosphorylation is involved in the regulation of many protein kinases (reviewed in references 10, 13, 23, and 46). As seen in the immune complex assay, GCN2 was autophosphorylated in vitro. To determine whether GCN2 is a phosphoprotein in vivo, we grew strains in low-phosphate minimal medium containing $^{32}P_i$, immunoprecipitated GCN2 from the ^{32}P-labeled extracts, and analyzed the radiolabeled species by SDS-PAGE (Fig. 3). A phosphoprotein with the molecular weight of GCN2 was present in wild-type cells and absent in a Δ*gcn2* strain and occurred at elevated levels in a strain containing *GCN2* on a high-copy-number plasmid. These results demonstrate that GCN2 is a phosphoprotein in vivo. Because phosphorylation of GCN2 was also evident in the *gcn2-K559R* mutant, which is defective for autophosphorylation, it appears that GCN2 is phosphorylated in vivo by another protein kinase. The level of GCN2 phosphorylation was similar under repressing and derepressing growth conditions, suggesting that differential phosphorylation may not be an important factor in regulating GCN2 function. However, if there are multiple phosphorylation sites on GCN2, differential phosphorylation of a particular site might not be easily discernible by one-dimensional gel electrophoresis. The location of the phosphorylation site(s) in GCN2 and the nature of the additional protein kinase remain to be defined.

TABLE 1. Effect of plasmid-borne *GCN2* mutations on the derepression of *GCN4* and amino acid-biosynthetic genes subject to general control in response to histidine starvation[a]

Plasmid-borne allele	Enzyme activity (nmol/min/mg of protein)				Growth	
	HIS4-LacZ		GCN4-LacZ			
	R	D	R	D	5-FT	3-AT
GCN2	200	800	14	120	−	+
High-copy *GCN2*	780	830	ND	ND	+	+
Δ*gcn2*	130	200	6	5	−	−
gcn2-K559R	130	310	9	7	−	−
High-copy *gcn2-K559R*	130	220	ND	ND	−	−
gcn2-K559V	140	260	ND	ND	−	−
Truncations of *GCN2* coding sequences						
High-copy *gcn2-Δ15-421*	170	250	ND	ND	−	−
High-copy *gcn2-Δ1025-1590*	150	250	ND	ND	−	−
gcn2-Δ1467-1590	130	260	8	7	−	−
gcn2-Δ1502-1590	140	270	9	7	−	−
Constitutively derepressed alleles						
GCN2(Con)-*E532K*	1,000	810	58	110	+	+
GCN2(Con)-*E752K*	580	870	31	130	+	+
GCN2(Con)-*E1537K*	1,200	1,100	110	120	+	+
GCN2(Con)-*E532K*+*E1537K*	2,500	2,100	270	220	+	+
GCN4(Con)-(uORFs deleted)	1,200	950	ND	ND	+	+

[a] β-Galactosidase enzyme activity was assayed in transformants of *gcn2-Δ63-3284*::*LEU2 HIS4*::*lacZ* strain H1149 or *gcn2-1 GCN4*::*lacZ* strain H1354 containing the designated *GCN2* or *GCN4* allele on a plasmid. The *GCN4*(Con) allele contains a deletion of the four uORFs at *GCN4*. R, Repressing conditions (nonstarvation); D, derepressing conditions (histidine starvation with 3-AT). Values are the averages of results obtained from assays on two to four independently derived transformants; for each construct, the individual measurements deviated from the average value by 30% or less. Growth in the presence of 3-AT and 5-FT was assayed for transformants of H1149 as a measure of *HIS3* and tryptophan pathway enzyme derepression, respectively. Symbols: +, confluent growth of replica-plated patches of cells after 2 days at 30°C; −, little or no discernable growth under the same conditions. ND, Not determined.

***GCN2* expression is constitutive.** Regulation of *GCN2* expression by amino acid availability could play an important role in modulating the level of GCN2 protein kinase activity. It was noted previously that *GCN2* on a high-copy-number plasmid results in partial derepression of *GCN4* expression in the absence of amino acid starvation (35). In accord with this observation, data in Table 1 show that multicopy *GCN2* led to constitutive derepression of *HIS4* expression. Measurements of steady-state protein levels indicate that GCN2 was overproduced more than 20-fold

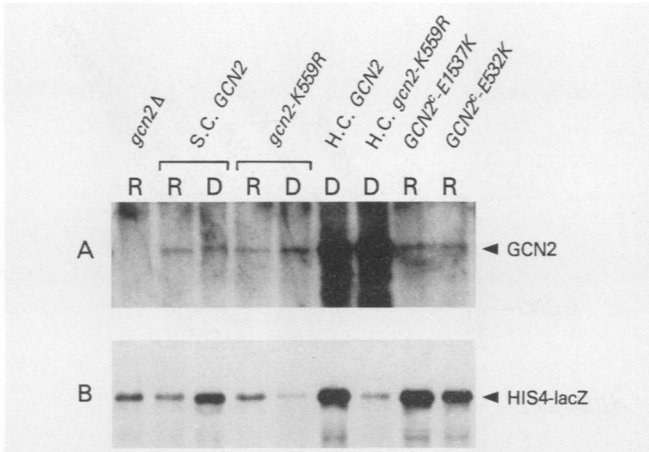

FIG. 3. In vivo phosphorylation of GCN2. (A) GCN2 was immunoprecipitated from Δ*gcn2* strain H1149 containing different plasmid-borne *GCN2* alleles, grown in repressing (R) or derepressing (D) conditions in the presence of $^{32}P_i$. Samples containing equal amounts of protein were immunoprecipitated and analyzed by 7.5% SDS-PAGE, followed by autoradiography. Each lane is designated by the *GCN2* allele present in the strain from which the extract was prepared; lane gcn2Δ corresponds to strain H1149 transformed with the vector YCp50; S.C. and H.C., single copy and high copy number, respectively. GCN2 phosphoprotein is indicated. (B) HIS4-LacZ fusion protein was immunoprecipitated from extracts identical to those analyzed in panel A except that cells were grown without labeled P_i and were pulse-labeled with [^{35}S]methionine just prior to harvesting. Immunoprecipitated samples were analyzed by 7.5% SDS-PAGE, followed by flourography. HIS4-LacZ protein is indicated.

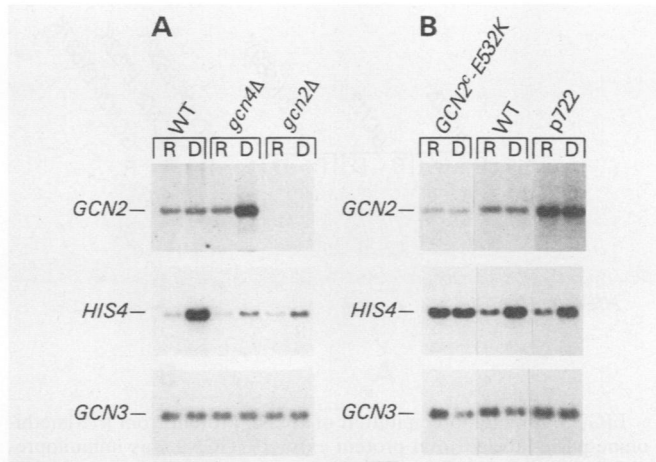

FIG. 4. Blot hybridization analysis of *GCN2* mRNA. (A) *GCN2*, *HIS4*, and *GCN3* mRNAs were examined in equal amounts of total RNA isolated from wild-type, Δ*gcn4*, and Δ*gcn2* isogenic strains grown in repressing (R) or derepressing (D) conditions. (B) Transcripts were examined in equal amounts of total RNA isolated from Δ*gcn2* strain H1149 transformed with different plasmid-borne *GCN2* alleles as indicated. The wild-type (WT) strain contained the low-copy-number *GCN2* plasmid p585. All lanes in panel B are from the same autoradiographic exposure following hybridization with each of the three probes. Panel A was from a separate experiment. Strain H1149 in panel B appears to have a somewhat higher basal level of *HIS4* mRNA than the strains analyzed in panel A.

in this transformant (see below). Thus, a large increase in GCN2 protein levels can produce significant derepression of genes subject to the general control in the absence of amino acid starvation. In addition, Driscoll-Penn et al. (9) reported that *GCN2* mRNA levels increase under starvation conditions in a GCN4-dependent fashion. The requirement for GCN4 to stimulate transcription of *GCN2* was attributed to the presence of a potential GCN4-binding site in the 5′ noncoding DNA at *GCN2* (35). These observations led to the idea that increasing *GCN2* expression is an important mechanism for elevating GCN2 protein kinase activity in response to amino acid starvation.

In contrast to the aforementioned results, we observed no increase in the level of *GCN2* mRNA in wild-type cells under conditions of histidine starvation, in which *HIS4* mRNA is derepressed (Fig. 4A). The abundance of *GCN3* mRNA, encoding another positive regulator of *GCN4* and *HIS4* expression, was similarly unchanged by histidine starvation. Expression of *GCN3* mRNA was shown previously to be constant under these conditions relative to both rRNA and pyruvate kinase mRNA (14). As expected, deletions of *GCN4* and *GCN2* in isogenic strains led to lower *HIS4* mRNA levels in histidine-starved cells. By contrast, *GCN2* transcript levels were actually elevated fivefold in response to starvation in the Δ*gcn4* mutant (Fig. 4A). Our results are inconsistent with the idea that GCN4 protein is required for efficient transcription of *GCN2*.

Transformants of a Δ*gcn2* strain containing *GCN2* on low-copy-number plasmid p722 exhibited a *GCN2* mRNA level fourfold higher than that seen in wild-type strains (Fig. 4B). This unexpected elevation in the *GCN2* mRNA level could arise if multiple copies of p722 are present per cell or from the fortuitous occurrence of a transcriptional enhancer of *GCN2* in the vector sequences. In any case, the moderate increase in *GCN2* mRNA abundance detected in the p722 transformant did not lead to increased expression of *HIS4* mRNA (Fig. 4B) or HIS4-LacZ enzyme activity (data not shown) under nonstarvation conditions. Apparently, much higher *GCN2* expression, such as that achieved by a large increase in *GCN2* gene dosage, appears to be needed to bypass the normal mechanism for activating GCN2 regulatory function in amino acid-starved cells.

The rate of GCN2 protein synthesis was also examined by pulse-labeling wild-type cells with [^{35}S]methionine for 20 min after steady-state growth in repressing or derepressing conditions. GCN2 protein was immunoprecipitated from labeled cell extracts and analyzed by SDS-PAGE. As an internal control for derepression, HIS4-LacZ fusion protein was immunoprecipitated from the same extracts by using monoclonal antibodies against β-galactosidase. Radiolabeled GCN2 and HIS4-LacZ proteins were stable for several hours during a chase with nonradioactive methionine (data not shown); therefore, the amounts of [^{35}S]methionine incorporated into these proteins during the 20-min pulse should be proportional to their rates of synthesis. As expected, the rate of HIS4-LacZ protein synthesis increased greatly in response to histidine starvation. By contrast, GCN2 protein synthesis increased only slightly under starvation conditions (Fig. 5A), and even this modest increase was not observed in separate experiments. Also as expected, derepression of HIS4-LacZ protein synthesis under starvation conditions was impaired in an isogenic *gcn2-K559V* strain; however, synthesis of the *gcn2-K559V* product itself was unaffected by the mutation (Fig. 5A). The latter result is additional evidence against the idea that GCN4 stimulates *GCN2* expression for the following reason: because the *gcn2-K559V* mutation impairs derepression of *GCN4* (Table 1), it should also reduce GCN2 protein synthesis under starvation conditions.

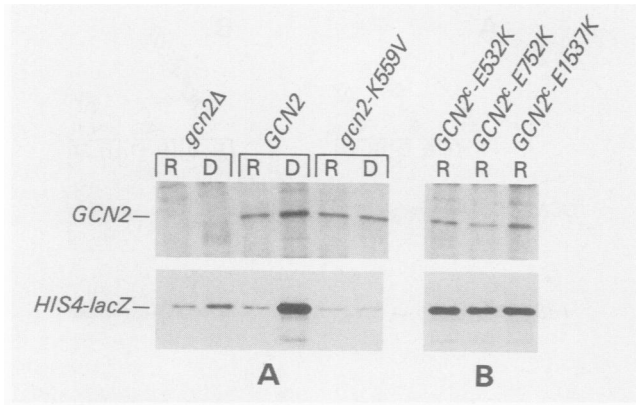

FIG. 5. Immunoprecipitation of GCN2 protein from [^{35}S]methionine-pulse-labeled total protein extracts. GCN2 was immunoprecipitated from protein extracts prepared from Δ*gcn2* strain H1149 transformed with different plasmid-borne *GCN2* alleles, grown in repressing (R) or derepressing (D) conditions. Just prior to harvesting, cells were pulse-labeled with [^{35}S]methionine. Samples containing equal amounts of radioactivity were immunoprecipitated and analyzed by 7.5% SDS-PAGE, followed by flourography. Each lane is designated by the *GCN2* allele present in the strain from which the extract was prepared; lane gcn2Δ corresponds to strain H1149 transformed with the vector YCp50. Radiolabeled GCN2 protein is indicated. Shown beneath are immunoprecipitations of HIS4-LacZ fusion protein from the same radiolabeled extracts. Panels A and B are taken from the same autoradiographic exposure of GCN2 or HIS4-LacZ immunoprecipitations.

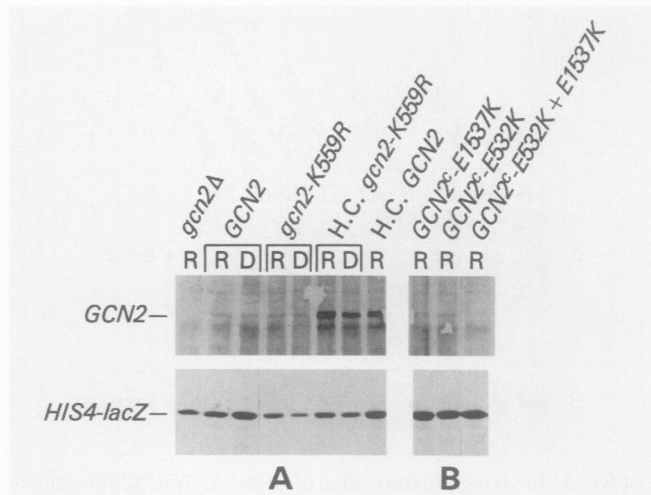

FIG. 6. Immunoblot analysis of GCN2 protein. Protein extracts were prepared from Δ*gcn2* strain H1149 transformed with different plasmid-borne *GCN2* alleles, grown in repressing (R) or derepressing (D) conditions. Samples containing equal amounts of total protein were separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with antiserum prepared against a TrpE-GCN2 fusion protein. Each lane is designated by the *GCN2* allele present in the strain from which the extract was prepared; lane gcn2Δ corresponds to strain H1149 transformed with the vector YCp50; H.C., high copy number. HIS4-LacZ fusion protein was analyzed in the same extracts with monoclonal antibody against β-galactosidase. Panels A and B were taken from the same immunoblot for GCN2 or HIS4-LacZ protein.

Finally, we examined the steady-state level of GCN2 protein under different conditions by an immunoblot assay. Although GCN2 protein is difficult to detect by this technique, its abundance appeared to be similar under starvation and nonstarvation conditions in both wild-type and *gcn2-K559R* mutant strains (Fig. 6A). As expected, the protein was present in greater amounts in a strain containing *GCN2* on a high-copy-number plasmid and absent in a Δ*gcn2* strain. These results make it improbable that the GCN2 protein level increases under starvation conditions because of increased protein stability. In view of the results presented in Fig. 4 to 6, we conclude that modulation of GCN2 abundance is not an important mechanism for coupling GCN2 regulatory function to amino acid availability. Instead, it appears that GCN2 protein kinase function is regulated posttranslationally.

The data in Fig. 6A also exclude the possibility that the *gcn2-K559R* mutation destroys the positive regulatory function of GCN2 as the result of decreased protein stability rather than impaired protein kinase activity. The strain containing *gcn2-K559R* on a high-copy-number plasmid had a very high steady-state level of GCN2 protein compared with the strain containing *GCN2* in single copy; however, the former was completely defective for derepression of *HIS* gene expression (Table 1).

Deletions of sequences flanking the protein kinase domain abolish GCN2 function in vivo without destroying kinase activity in vitro. For several known protein kinases that resemble GCN2 in containing extensive additional sequences flanking the catalytic domain, it has been shown that removal of these flanking sequences results in an activated form of the kinase (see below). Homology with the catalytic domain of protein kinases occurs between GCN2 residues 530 and 910 (35, 41). We showed previously by deletion and insertion mutagenesis that *GCN2* coding sequences outside this domain are required for its positive regulatory function in vivo. For example, an insertion of two codons (Glu-Leu) between residues 1092 and 1093 in the HisRS-related domain destroys the ability of *GCN2* to complement a Δ*gcn2* strain for its derepression defect (41). However, the corresponding mutant protein autophosphorylated in the in vitro assay by an amount similar to that seen for wild-type GCN2 (data not shown). Thus, the *gcn2-1092EL* product is unable to function as a positive regulator in vivo even though its protein kinase moiety is functional in vitro.

Similar results were obtained for deletion alleles *gcn2-Δ15-421* and *gcn2-Δ1025-1590*, which lack most of the amino-terminal and carboxyl-terminal sequences flanking the *GCN2* kinase domain, respectively. Immunoblot analysis of transformants containing these deletion alleles on high-copy-number plasmids showed that truncated proteins of the expected molecular weights were present at levels comparable to those found in an isogenic strain containing *GCN2* on a high-copy-number plasmid (Fig. 7, bottom panel). Thus, the stabilities of the truncated proteins were similar to that of wild-type GCN2. In addition, both truncated proteins retained the ability to autophosphorylate in vitro (Fig. 7, top panel). However, even when present on a high-copy-number plasmid, neither deletion allele showed any complementation of a Δ*gcn2* strain for its derepression defect (Table 1). Although the level of autophosphorylation was reduced in complexes obtained from transformants containing the high-copy truncated genes versus high-copy wild-type *GCN2*, the level of autophosphorylation detected with the truncated proteins was still greater than that seen for an equivalent amount of extract obtained from a single-copy *GCN2* strain (Fig. 7, top). (Note also that the truncated GCN2 phosphoprotein levels would be reduced compared with wild-type GCN2 if the deleted sequences contain sites of auto-

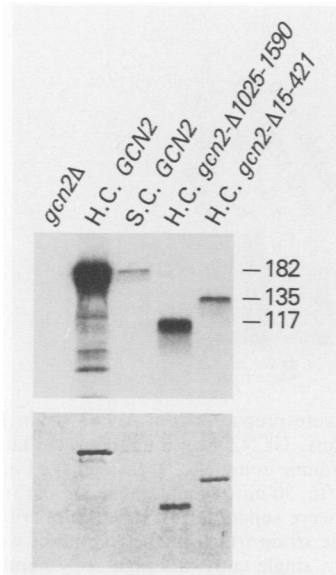

FIG. 7. Immune complex kinase assays and immunoblots of truncated GCN2 proteins. (Top) Immune complex kinase assays on transformants of Δ*gcn2* strain H1149 containing different plasmid-borne *GCN2* alleles, grown under repressing conditions. GCN2 was immunoprecipitated from samples of extracts containing equal amounts of total protein in the presence of detergents, and immune complexes were incubated in KIN buffer in the presence of [γ-^{32}P]ATP for 30 min. Phosphoproteins were separated by 7.5% SDS-PAGE, followed by autoradiography. Lanes are designated with the *GCN2* allele present in the transformant from which the extract was prepared; lane gcn2Δ corresponds to strain H1149 transformed with the vector YCp50; S.C. and H.C., single copy and high copy number, respectively. (Kinase assays for H1149 transformants containing high-copy-number *gcn2-Δ1025-1590* and *gcn2-Δ15-421* were also carried out with extracts of cells grown under derepressing conditions with results similar to those shown here.) Molecular weights (10^3) are shown. (Bottom) Immunoblot analysis of the protein extracts analyzed above. Samples were separated by 7.5% SDS-PAGE, transferred to nitrocellulose, and probed with antiserum against a TrpE-GCN2 fusion protein.

phosphorylation.) Thus, the inability of the truncated proteins to function as positive regulators in vivo does not appear to result simply from inactivation of the GCN2 catalytic domain.

Our results indicate that elimination of sequences flanking the GCN2 catalytic domain impairs rather than activates GCN2 regulatory function in vivo. These flanking sequences could be required in vivo for recognition of the correct protein substrate(s), for activation of GCN2 kinase activity above the basal level, or for some other function unrelated to protein kinase activity.

Constitutively derepressed *GCN2*(Con) alleles. To further define regulatory domains in GCN2, we isolated dominant mutations that led to constitutive activation of GCN2 positive regulatory function in vivo. The *GCN2* gene on a low-copy-number plasmid was mutagenized at random sites in vitro and introduced by transformation into a wild-type *GCN2* strain. The resulting transformants were screened for resistance to 0.5 mM 5-flourotryptophan (5-FT). At this concentration, 5-FT is toxic to wild-type cells but does not lead to derepression of enzymes subject to the general control. Mutations that lead to constitutive enzyme derepression overcome the toxicity of 5-FT (29), presumably through bypassing the starvation signal and increasing tryptophan biosynthesis, thereby reducing incorporation of 5-FT into proteins.

Three *GCN2*(Con) alleles were isolated that conferred elevated resistance to 5-FT. The 5-FT^r phenotype associated with each allele was shown to result from a single-base-pair substitution that changed a glutamate (GAA) to a lysine (AAA) codon. Two of the mutations were located in the protein kinase region. *GCN2*(Con)-*E532K* (glutamate at residue 532 replaced by lysine) mapped to the predicted ATP-binding domain, four residues upstream of the conserved amino acid sequence shown in Fig. 1; *GCN2*(Con)-*E752K* was a substitution in subdomain VI, as defined by Hanks et al. (13), that is also implicated in ATP binding (6). Based on sequence alignments, the *GCN2*(Con)-*E752K* mutation corresponds to position 152 of bovine cyclic AMP-dependent protein kinase (13). The third mutation, *GCN2*(Con)-*E1537K*, was located just downstream from the HisRS-related domain in the carboxyl-terminal portion of *GCN2* (Fig. 1).

Each of the *GCN2*(Con) mutations led to elevated expression of GCN4-LacZ and HIS4-LacZ enzyme activity under nonstarvation conditions (Table 1). The *GCN4* mRNA levels in the *GCN2*(Con) transformants were unchanged compared with the *GCN2* transformant (data not shown), consistent with the elevated expression of *GCN4* in the *GCN2*(Con) transformants resulting from increased translational efficiency (17). Derepression in nonstarved cells was most pronounced for the *GCN2*(Con)-*E1537K* and *GCN2*(Con)-*E532K* alleles, with the *GCN2*(Con)-*E752K* allele showing only a two- to threefold increase in *HIS4*::*lacZ* and *GCN4*::*lacZ* expression under nonstarvation conditions compared with wild-type *GCN2*. Interestingly, when the *GCN2*(Con)-*E532K* and *GCN2*(Con)-*E1537K* mutations were combined, HIS4-LacZ and GCN4-LacZ enzyme activity under nonstarvation conditions was higher than that given by either mutation alone (Table 1). [In fact, the *GCN2*(Con)-*E532K*+*E1537K* allele generated *HIS4*::*lacZ* expression greater than that observed in an isogenic strain containing a *GCN4*(Con) allele that lacked all four uORFs in the mRNA leader required for translational repression of *GCN4*. The basis for this unexpectedly high *HIS4*::*lacZ* expression is under investigation.]

One possible explanation for the derepressed phenotype of the *GCN2*(Con) mutations could be increased abundance of GCN2 protein, since extreme overexpression of wild-type *GCN2* can derepress *HIS4* expression (Table 1). At odds with this possibility, the steady-state level of *GCN2*(Con)-*E532K* mRNA was similar to that of wild-type *GCN2* under repressing and derepressing conditions (Fig. 4B). Moreover, the rates of synthesis and steady-state amounts of proteins encoded by the three *GCN2*(Con) alleles containing single-amino-acid substitutions did not differ significantly from those of wild-type GCN2 (Fig. 5B and 6B). In fact, the double mutant *GCN2*(Con)-*E532K*+*E1537K* protein level was actually lower than that of wild-type GCN2. Therefore, the derepressed phenotype of the *GCN2*(Con) alleles does not result from increased expression or stability of GCN2 protein.

The constitutively derepressed phenotype of the *GCN2* (Con) alleles suggests that these mutations lead to elevated GCN2 protein kinase activity; however, we detected no obvious alteration in the efficiency of in vitro autophosphorylation for any of the *GCN2*(Con) mutant proteins compared with wild-type GCN2 when immune complexes were prepared in the absence of detergent (Fig. 8A). (The reduced autophosphorylation of the double mutant can be explained

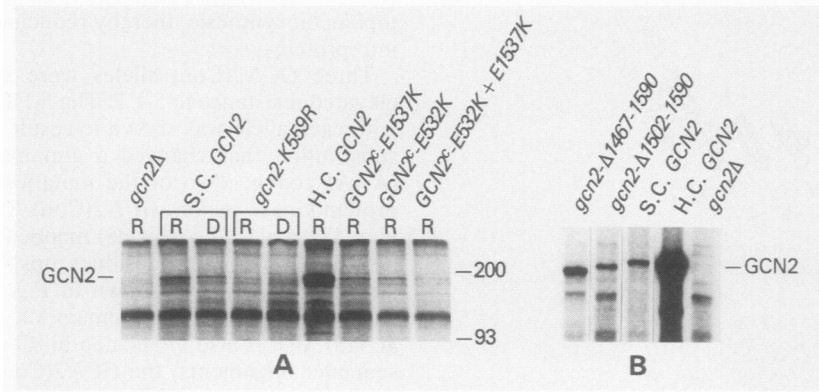

FIG. 8. Immune complex kinase assays on GCN2(Con) proteins. Protein extracts were prepared from Δ*gcn2* strain H1149 containing plasmid-borne alleles of *GCN2*, grown in repressing (R) or derepressing (D) conditions. GCN2 was immunoprecipitated from samples containing equal amounts of total protein extracted in the absence of detergents, and immune complexes were analyzed in the kinase assay for reaction times between 2.5 and 30 min. (The results shown here correspond to 30-min incubations; similar relative levels of autophosphorylation were observed for shorter incubations as well.) Phosphoproteins were separated by 10% (A) or 6% (B) SDS-PAGE, followed by autoradiography. Each lane is designated by the *GCN2* allele present in the strain from which the extract was prepared; lane gcn2Δ corresponds to strain H1149 transformed with the vector YCp50; S.C. and H.C., single copy and high copy number, respectively. Molecular weights (10^3) are shown to the right of panel A.

by its lower protein level, as shown in Fig. 6B.) However, when immune complexes were prepared in the presence of detergents, we failed to detect any *GCN2*(Con)-*E532K* phosphoprotein and observed reduced amounts of *GCN2*(Con)-*E1537K* phosphoprotein compared with similarly prepared wild-type GCN2. Presumably, these altered GCN2 proteins are more susceptible to denaturation by detergents than wild-type GCN2. Of course, these results do not eliminate the possibility that the *GCN2*(Con) mutations increase phosphorylation of the correct substrate(s) or lower the requirement for activation of protein kinase activity in vivo.

Positive role of the carboxyl-terminal region in GCN2 function. The *GCN2*(Con)-*E1537K* substitution in the carboxyl-terminus of GCN2 could lead to constitutive activation of protein kinase activity by impairing a negative regulatory domain. If so, deletion of the carboxyl terminus should produce the same phenotype as the *GCN2*(Con)-*E1537K* point mutation. However, two deletions that eliminated the carboxyl terminus of GCN2, leaving the entire HisRS-related domain intact (*gcn2-Δ1467-1590* and *gcn2-Δ1502-1590*, (Fig. 1), led to a complete loss of GCN2 regulatory function in vivo (Table 1). This loss of function did not result from instability of the truncated GCN2 proteins, as their steady-state amounts (data not shown) and in vitro autophosphorylation levels were indistinguishable from those of wild-type GCN2 (Fig. 8B). These findings suggest that the carboxyl-terminus of GCN2 plays a positive role in the regulation of GCN2-mediated phosphorylation in vivo and that the *GCN2*(Con)-*E1537K* mutation causes the stimulatory effect of this region to be exerted in the absence of amino acid starvation.

DISCUSSION

Starvation of *S. cerevisiae* for any amino acid activates a multistep pathway that leads to increased expression of the transcriptional activator GCN4 and consequent derepression of amino acid-biosynthetic genes under GCN4 control. Increased *GCN4* expression under starvation conditions occurs at the translational level and requires the positive regulatory protein GCN2. GCN2 contains a domain homologous to the catalytic subunit of eucaryotic protein kinases. We have shown that substitution of a conserved lysine residue in the presumed ATP-binding site of GCN2 impairs both autophosphorylation activity in vitro and the ability to stimulate *GCN4* expression in vivo (Fig. 2, Table 1). Based on these results, we conclude that GCN2 functions as a protein kinase in derepressing *GCN4* expression under conditions of amino acid starvation.

A large increase in *GCN2* expression resulting from elevated *GCN2* gene dosage leads to derepression of genes under general control in the absence of starvation (Table 1). This finding suggested that increased *GCN2* expression might be required for derepression of *GCN4*. However, our results indicate that GCN2-mediated derepression under starvation conditions occurs without any significant increase in *GCN2* mRNA and protein levels (Fig. 4A and 6A). In addition, a moderate increase in *GCN2* mRNA levels, such as the fourfold elevation in transformants containing plasmid p722, does not lead to any increase in *HIS4* expression under nonstarvation conditions (Fig. 4B). Therefore, we conclude that GCN2 positive regulatory function is controlled by amino acid availability through a posttranslational mechanism.

Consistent with this conclusion, the dominant constitutive mutations that we isolated mapped in the protein-coding sequences and activated GCN2 regulatory function without increasing the abundance of GCN2 protein. Consequently, these mutations appear to increase the biochemical activity of GCN2 protein in the absence of amino acid starvation. We have not demonstrated that the *GCN2*(Con) mutations elevate GCN2-mediated phosphorylation events in vivo; however, this seems likely given that the *GCN2*(Con)-*E532K* and *GCN2*(Con)-*E752K* mutations mapped in the protein kinase domain, near regions implicated previously in ATP binding. Perhaps these two mutations reduce the requirement for amino acid starvation for efficient ATP binding. An example of this type of regulatory mechanism is provided by the double-stranded RNA-dependent protein kinase, which is thought to require its activating ligand, double-stranded RNA, for ATP binding (1). It is also of interest that two mutations that activate the *Schizosaccharomyces pombe cdc2*-encoded protein kinase affect the same two kinase

subdomains altered by the *GCN2*(Con)-*E532K* and *GCN2*(Con)-*E752K* mutations (2, 12).

GCN2 kinase activity in immune complexes. We observed little difference in the efficiency of GCN2 autophosphorylation in vitro whether the protein was isolated from cells grown under starvation or nonstarvation conditions (Fig. 2). Likewise, the *GCN2*(Con) mutations had no significant effect on in vitro autophosphorylation activity (Fig. 8A). For several reasons, these results are not inconsistent with the idea that GCN2-mediated phosphorylation increases under starvation conditions in wild-type cells or is constitutively elevated in *GCN2*(Con) mutants. First, it is uncertain whether GCN2 autophosphorylation even occurs in vivo, since the Lys-559 substitution did not reduce the level of GCN2 phosphorylation seen in vivo (Fig. 3), and the postulated stimulation of GCN2 kinase activity could be restricted to particular exogenous substrates. Two commonly used substrates for protein kinases, casein and histones, were examined for phosphorylation by GCN2 in immune complexes; however, low-level unregulated phosphorylation was observed in both cases (unpublished observations). Second, increased kinase activity in vivo may depend on a specific ligand, e.g., uncharged tRNA, or an additional subunit that is not present in GCN2 immune complexes. Thus far, attempts to stimulate GCN2 autophosphorylation activity in vitro by uncharged tRNA have been unsuccessful. Third, regulation of GCN2-mediated phosphorylation in vivo could involve altering the cellular location of GCN2 and thus its access to a substrate(s), rather than modulating its catalytic activity. Finally, the wild-type activity or specificity of GCN2 may be affected by its presence in immune complexes. If so, a demonstration of regulated GCN2 kinase activity in vitro will require purification of the protein. There are related instances in which mutations that affect the transforming potential of an oncogene-encoded protein kinase do not alter the level of autophosphorylation seen in immune complex kinase assays (16, 34).

Regulatory domains in GCN2 and other protein kinases. Eucaryotic protein kinases contain a homologous catalytic domain coupled with a nonconserved regulatory domain present either in the same polypeptide chain or in a separate subunit (reviewed in references 10, 25, 40, and 46). Characterization of oncogenes whose products represent truncations of protein kinases involved in growth control has yielded important information about the role of different domains in the regulation of receptor function. For example, the v-*fms* oncogene represents a carboxy-terminal truncation of the colony-stimulating factor-1 (CSF-1) receptor. Restoration of the truncated sequences from the c-*fms* proto-oncogene drastically reduces the transformation activity of v-*fms*, suggesting that the carboxy-terminal region of the CSF-1 receptor has a negative regulatory function (34). Similar examples are provided by the *src* and *raf* genes, in which deletion of sequences flanking the protein kinase catalytic domain at the carboxyl- or amino-terminus, respectively, is sufficient for oncogenic activation of the gene products (23, 30, 39). The transforming activity of these oncogenes is thought to result from deletion of a negative regulatory domain, leading to inappropriate phosphorylation of substrates that control cell proliferation.

Unlike the regulatory domains mentioned above, sequences flanking the GCN2 kinase moiety appear to be positive-acting, since their removal impairs GCN2 function in vivo without eliminating protein kinase activity in vitro. Given that accumulation of uncharged tRNA triggers derepression of enzymes under general control (19), the HisRS-related domain in GCN2 is an obvious candidate for a sensor of uncharged tRNA that modulates kinase activity according to amino acid availability. Uncharged tRNA might function as a ligand that binds to the HisRS-related domain, elicting a conformational change in the protein that stimulates the adjacent protein kinase moiety. In this view, the carboxyl-terminus of GCN2 located downstream from the HisRS-like sequences could function to mediate the stimulatory effect of tRNA binding on kinase activity. Accordingly, the *GCN2*(Con)-*E1537K* mutation that maps in the carboxyl-terminus would cause constitutive activation of GCN2 function by reducing the requirement for tRNA binding needed to stimulate kinase activity. An alternative explanation is that the carboxyl-terminal functions independently of the HisRS-related sequences to facilitate interaction with the correct protein substrate(s), in which case the *GCN2*(Con)-*E1537K* mutation would increase the access or affinity of GCN2 for its substrate(s). An example of positive regulatory domains of this sort is provided by the Cdc2 protein kinase of *S. pombe*, in which complex formation between the *cdc13*-encoded cyclin and the Cdc2 protein enhances catalytic activity for particular substrates and is required for localization of the protein kinase complex in the cell nucleus (3). The kinase activity of the Cdc2 homolog in *S. cerevisiae*, known as CDC28, also appears to be stimulated by interaction with one or more positive regulatory proteins (43).

Constitutive expression of *GCN2*. We found that steady-state expression of *GCN2* mRNA and protein was very similar in repressed and derepressed growth conditions (Fig. 4A and 6A). However, we did observe elevated *GCN2* transcript levels in amino acid-starved Δ*gcn4* cells, a result that could indicate the existence of a GCN4-independent mechanism for increasing *GCN2* expression under severe starvation conditions. Together, our results appear to contradict a previous study showing that *GCN2* transcript levels increase in a GCN4-dependent manner when cultures are shifted from amino acid-complete to amino acid starvation medium (9). It was subsequently reported that GCN4 protein binds in vitro to *GCN2* 5′ noncoding DNA containing a potential GCN4-binding site (35). Based on these findings, it was concluded that derepression of *GCN4* leads to increased transcription of *GCN2*, forming a positive regulatory loop that amplifies *GCN4* expression (35). One possible explanation for the discrepancies between our findings and those of the previous studies could be differences in the repressing conditions used. Our nonstarvation medium consists of minimal medium (SD) containing only those amino acids needed to satisfy the nutritional requirements of the strains, whereas the nonstarvation medium used in the previous studies contained all 20 amino acids (9). Derepression of some genes subject to general control, including *HIS4*, occurs rapidly in a shift from amino acid-complete to unsupplemented minimal medium, and this derepression response is independent of certain *GCN* gene products needed for sustained derepression in response to severe starvation achieved by using 3-AT (9, 18). Thus, a shift from amino acid-complete to minimal medium containing 3-AT may represent two superimposed derepression phenomena that are mechanistically distinct (18). Another possible problem is uncontrolled differences in the genetic backgrounds of the mutant and wild-type strains being compared. We attempted to eliminate this problem by analyzing isogenic wild-type and Δ*gcn4* strains constructed by transformation techniques.

Much effort is now being devoted to understanding the structure-function relationships among the diverse members of the protein kinase family. The *GCN2* gene is well suited to

a genetic approach to elucidating protein kinase function for two reasons. First, the gene is completely dispensable, being required only under conditions of amino acid starvation. Thus, any *GCN2* mutation can be analyzed in vivo as the sole source of GCN2 protein without the complicating effects of reduced growth rate under normal culture conditions. Second, convenient screens and selections exist to identify mutants that are either defective or constitutively activated for *GCN2* function. These features should aid in determining the precise sequence requirements for catalytic function and substrate recognition by GCN2 protein kinase and in understanding how these parameters are modulated by amino acid availability.

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