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Truncated Protein Phosphatase GLC7 Restores Translational Activation of *GCN4* Expression in Yeast Mutants Defective for the eIF-2α Kinase GCN2

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GCN2 is a protein kinase in *Saccharomyces cerevisiae* that is required for increased expression of the transcriptional activator GCN4 in amino acid-starved cells. GCN2 stimulates GCN4 synthesis at the translational level by phosphorylating the α subunit of eukaryotic translation initiation factor 2 (eIF-2). We identified a truncated form of the *GLC7* gene, encoding the catalytic subunit of a type 1 protein phosphatase, by its ability to restore derepression of *GCN4* expression in a strain containing the partially defective *gcn2-507* allele. Genetic analysis suggests that the truncated *GLC7* allele has a dominant negative phenotype, reducing the level of native type 1 protein phosphatase activity in the cell. The truncated form of *GLC7* does not suppress the regulatory defect associated with a *gcn2* deletion or a mutation in the phosphorylation site of eIF-2α (Ser-51). In addition, the presence of multiple copies of wild-type *GLC7* impairs the derepression of *GCN4* that occurs in response to amino acid starvation or dominant-activating mutations in *GCN2*. These findings suggest that the phosphatase activity of GLC7 acts in opposition to the kinase activity of GCN2 in modulating the level of eIF-2α phosphorylation and the translational efficiency of *GCN4* mRNA. This conclusion is supported by biochemical studies showing that the truncated *GLC7* allele increases the level of eIF-2α phosphorylation in the *gcn2-507* mutant to a level approaching that seen in wild-type cells under starvation conditions. The truncated *GLC7* allele also leads to reduced glycogen accumulation, indicating that this protein phosphatase is involved in regulating diverse metabolic pathways in yeast cells.

Protein phosphorylation is a reversible modification that regulates many cellular processes, including cell division, protein synthesis, and glycogen metabolism. The phosphorylation state of a given protein is determined by the relative activities of protein kinases and protein phosphatases which recognize it as a substrate (reviewed in references 12, 15, and 23). Much effort is being directed toward identifying cellular kinases and phosphatases and understanding their regulatory mechanisms. In this report, we provide evidence that a protein phosphatase known as GLC7 functions in opposition to the GCN2 protein kinase in regulating the expression of amino acid biosynthetic genes in yeast cells.

In *Saccharomyces cerevisiae*, starvation for an amino acid, or a defective aminoacyl-tRNA synthetase, leads to increased transcription of over 30 genes encoding amino acid biosynthetic enzymes in several different pathways. This regulatory mechanism is known as general amino acid control (reviewed in reference 27). The immediate effector of the general control response is the GCN4 protein, a transcriptional activator that binds upstream of each amino acid biosynthetic gene that is subject to the general control. Expression of *GCN4* itself is regulated by amino acid availability, but this occurs at the level of translation initiation. Four short upstream open reading frames (uORFs) in the leader of *GCN4* mRNA prevent translation initiation at the *GCN4* start codon under nonstarvation conditions. In amino acid-starved cells, the inhibitory effect of these uORFs is overcome to permit increased translation of *GCN4* protein-coding sequences. The GCN4 protein thus produced activates transcription from its target genes (27).

The protein kinase GCN2 is required to stimulate (derepress) *GCN4* expression in response to amino acid starvation. GCN2 phosphorylates the α subunit of eukaryotic initiation factor 2 (eIF-2) in amino acid-starved yeast cells (14). In mammalian cells, phosphorylation of eIF-2α diminishes its activity because the phosphorylated species of the protein sequesters a second initiation factor called eIF-2B in an inactive form. eIF-2B is a multisubunit protein that catalyzes the exchange of GDP for GTP on eIF-2 after each round of translation initiation. Thus, impairing the exchange activity of eIF-2B leads to a reduction in the level of active eIF-2 available for translation initiation. Because eIF-2 is more abundant than eIF-2B, phosphorylation of only a fraction of eIF-2α is sufficient to inhibit all the eIF-2B activity in the cell (25).

GCN2 contains unique sequence homology to the mammalian eIF-2α kinases DAI (37) and HCR (6), suggesting that these three proteins constitute a subfamily of closely related serine/threonine kinases (6, 29, 44). We recently presented strong evidence (14) that GCN2 phosphorylates the identical residue in eIF-2α, serine 51, that is modified by DAI and HCR in higher eukaryotes. Substitution of serine 51 by alanine abolishes phosphorylation of eIF-2α by GCN2 in vivo and in vitro and impairs increased translation of *GCN4* mRNA in amino acid-starved cells (14). By analogy with mammalian systems, we proposed that phosphorylation of eIF-2α by GCN2 would lead to inactivation of the yeast equivalent of eIF-2B and the consequent inhibition of GDP-GTP exchange on eIF-2. This reduction in eIF-2 activity

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under starvation conditions would allow ribosomes which have translated the first uORF in the leader (uORF1) and resumed scanning to ignore the start codons at the remaining uORFs 2, 3, and 4 and reinitiate translation at *GCN4* instead (1, 14).

Uncharged tRNA is thought to play an important role in coupling the phosphorylation of eIF-2α by GCN2 to the availability of amino acids, because mutants defective for an aminoacyl-tRNA synthetase show elevated expression of genes regulated by GCN4 without being starved for the cognate amino acid (36, 40). Regulation of GCN2 kinase activity involves a region of about 530 amino acids adjacent to the kinase catalytic domain that is homologous to histidyl-tRNA synthetases from *S. cerevisiae*, humans, and *Escherichia coli* (54). This domain is absolutely required for the ability of GCN2 to stimulate *GCN4* expression in vivo, but is dispensable for the autophosphorylation activity of GCN2 observed in vitro (55). Given that aminoacyl-tRNA synthetases distinguish between charged and uncharged forms of tRNA, we proposed that the histidyl-tRNA synthetase-related region is a regulatory domain in GCN2 that monitors the levels of uncharged tRNA and activates the adjacent protein kinase moiety in amino acid-starved cells (54). This hypothesis is in accord with the isolation of a *GCN2*^{c} mutation mapping in a region immediately adjacent to the histidyl-tRNA synthetase-related domain that leads to constitutive activation of GCN2 regulatory function without altering the steady-state level of the protein (55). As would be expected if GCN2 is the immediate sensor of amino acid starvation, this and other *GCN2*^{c} alleles mapping in the protein kinase domain are largely dependent on two other positive effectors in the general control system, GCN1 and GCN3, for eliciting derepression of *GCN4* expression (24).

To identify additional regulatory factors that interact with the GCN2 protein kinase, we screened a yeast genomic library for genes that in high copy number would suppress the low constitutive *GCN4* expression associated with the partially defective *gcn2-507* mutation. One such suppressor that was isolated is a truncated form of a previously identified gene known as *DIS2S1* (41) or *GLC7* (18), encoding a type 1 protein phosphatase. Our genetic analysis suggests that the suppressor allele reduces the level of GLC7 protein phosphatase activity in the cell, thereby compensating for the reduced protein kinase activity of the *gcn2-507* product. In accord with this explanation, overexpression of wild-type *GLC7* impaired the derepression of *GCN4* expression, mimicking a *gcn2* mutation. In addition, biochemical analysis revealed that the truncated *GLC7* suppressor allele leads to increased phosphorylation of eIF-2α in a *gcn2-507* mutant under conditions of amino acid starvation. The GLC7 protein phosphatase also functions in glycogen metabolism (18, 43), and strains containing the truncated *GLC7* allele in high copy number have reduced glycogen levels. Thus, our results demonstrate that the GLC7 protein phosphatase recognizes substrates in at least two distinct metabolic pathways in yeast cells.

MATERIALS AND METHODS

Yeast strains. Genotypes of yeast strains used in this study are described in Table 1. The *gcn2::LEU2* deletion allele in H1472 was introduced by transforming H1438 to Leu$^+$ by using the 4.1-kb *Bam*HI fragment of plasmid p500 (55). The *gcn2::URA3* deletion allele in H1473 was introduced by transforming H1438 to Ura$^+$ by using the 3.2-kb *Sna*BI-*Bgl*II fragment of p638 (24). The *gcn3::LEU2* deletion allele in H1471 was introduced by transforming H1438 to Leu$^+$ by using the 4.1-kb *Nru*I-*Bgl*I fragment from Ep308 (24). The *gcn3::URA3* allele in strain H1470 was introduced by transforming H1438 to Ura$^+$ by using the 4.1-kb *Eco*RI-*Bam*HI fragment from plasmid Ep310. H1149 was crossed with JC782-24D (*MAT***a** *glc7-1 lys2-1 met4 ura3-52 leu2-3 leu2-112*), and a Leu$^+$ Glc$^-$ meiotic segregant was selected and designated Y25 (Table 1). Y29 and Y46 (Table 1) are meiotic segregants from a cross between Y25 and H1438. To integrate the *GCN4-lacZ* fusion at the *TRP1* locus, strain H1515 (*MAT***a** *ura3-52 leu2-3 leu2-112 trp1Δ63*) was transformed to Trp$^+$ with plasmid p1108, yielding strain H1642 (14). Strain H1642 was crossed with Y22 (*MAT*α *gcn2-507 lys1-1 his1-29 leu2-3 leu2-112 ino1*), and the Ura$^+$ meiotic segregants Y31 and Y32 containing the *GCN4-lacZ* fusion were selected.

Strain Y27 was constructed from H1333 by a previously described two-step gene replacement procedure used to introduce new alleles at *GCN2* (24). Briefly, H1333 was transformed to Leu$^+$ by the integrating plasmid p724 digested with *Sna*BI to direct integration to the *GCN2* locus, and a Ura$^-$ Leu$^-$ derivative of this transformant was selected on 5-fluoro-orotic acid medium (3). Strains H1414, H1608, H1609, and H1613 were constructed similarly by using plasmids p636, p1064, p1065, and p1067, respectively, containing different *GCN2*^{c} alleles (Table 1). The *GCN2*^{c}*-501* allele has been described previously (55) and contains a substitution of glutamate 532 by lysine (E532K). We noted previously that when two *GCN2*^{c} mutations are combined in a single gene, this leads to a constitutively derepressed phenotype greater in degree than that seen with either mutation alone (55). Other *GCN2*^{c} alleles analyzed in Table 4 also contain multiple amino acid substitutions: *GCN2*^{c}*-516* (E532K plus E1522K), *GCN2*^{c}*-513* (M719V plus E1537G), and *GCN2*^{c}*-514* (R699W plus D918G plus E1537G). These mutations were isolated by the same procedures described previously for *GCN2*^{c}*-501* (55), and the complete description of these and other *GCN2*^{c} mutations will be presented separately (44a). Strains Y64 and Y65 were constructed from Y27 (Table 1) by using a plasmid-shuffling technique described by Dever et al. (14). Strains Y66 and Y67 were similarly constructed from H1374 (*MAT*α *gcn2-K559V ura3-52 leu2-3 leu2-112 ino1 HIS4-lacZ* [24]).

Isolation and characterization of *glc7-Δ209-312* allele as a high-copy-number suppressor of the regulatory defect in a *gcn2-507* mutant. Strain H1450 (Table 1) is unable to grow on synthetic dextrose (SD) medium (49) supplemented with 10 mM 3-aminotriazole (3-AT), an inhibitor of histidine biosynthesis, because of the inability to derepress histidine biosynthetic enzymes conferred by the *gcn2-507* mutation (54). The leaky *his1-29* mutation enhances 3-AT sensitivity in this and other *gcn* mutants. H1450 was transformed (33) with a yeast chromosomal library (39) constructed in the high-copy-number *LEU2*-containing plasmid YEp13, and the transformants were screened by replica plating to SD agar medium containing 10 mM 3-AT (28). Transformant clones that gave confluent growth on 3-AT medium after incubation for 2 to 3 days at 30°C were examined for their dependence on *GCN3* function to express this 3-AT^r phenotype. Ura$^-$ derivatives that lack the *GCN3*-containing plasmid Ep69 were isolated from each 3-AT^r transformant by growth on 5-fluoro-orotic acid medium; the resulting Ura$^-$ strains were then compared with the corresponding parental transformants for resistance to 3-AT. Those strains showing significantly poorer growth on 3-AT medium after the loss of Ep69 were chosen for further study. Plasmids were recovered from the 5-fluoro-orotic acid-resistant derivatives of these transformants (30) and introduced into the set of isogenic strains H1438, H1473,

TABLE 1. Genotypes of yeast strains used in this study

Strain	Genotype	Source
H1149	*MATα gcn2::LEU2 ura3-52 leu2-3 leu2-112 inol HIS4-lacZ*	54
H1333	*MATα gcn2::URA3 ura3-52 leu2-3 leu2-112 inol HIS4-lacZ*	24
H1374	*MATα gcn2-K559V ura3-52 leu2-3 leu2-112 inol HIS4-lacZ*	24
H1402	*MATα ura3-52 leu2-3 leu2-112 inol HIS4-lacZ*	24
H1414	*MATα GCN2ᶜ-501 ura3-52 leu2-3 leu2-112 inol HIS4-lacZ*	24
H1438	*MATα gcn2-507 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1446	*MATa gcn2-507 gcn3-101 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1450	Strain H1446 transformed with Ep69 (*GCN3 URA3 CEN4*)	This study
H1470	*MATα gcn2-507 gcn3::URA3 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1471	*MATα gcn2-507 gcn3::LEU2 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1472	*MATα gcn2::LEU2 his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1473	*MATα gcn2::URA3 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1486	*MATα his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	This study
H1608	*MATα GCN2ᶜ-513 ura3-52 leu2-3 leu2-112 inol HIS4-lacZ*	This study
H1609	*MATα GCN2ᶜ-514 ura3-52 leu2-3 leu2-112 inol HIS4-LacZ*	This study
H1613	*MATα GCN2ᶜ-516 ura3-52 leu2-3 leu2-112 inol HIS4-LacZ*	This study
Y3	*MATα gcn2::LEU2 ura3-52 leu2-3 leu2-112 inol HIS4-lacZ* pAH15 [*GCN2 LEU2*]	This study
Y25	*MATa gcn2::LEU2 glc7-1 lys2-1 ura3-52 leu2-3 leu2-112 inol*	This study
Y27	*MATα gcn2-507 ura3-52 leu2-3 leu2-112 inol HIS4-lacZ*	This study
Y29	*Mata gcn2-507 glc7-1 ura3-52 leu2-3 leu2-112 inol*	This study
Y31	*MATα lys1-1 ura3-52 leu2-3 leu2-112 inol GCN4-lacZ*	This study
Y32	*MATa gcn2-507 lys1-1 ura3-52 leu2-3 leu2-112 inol GCN4-lacZ*	This study
Y46	*MATα gcn2-507 lys2-1 ura3-52 leu2-3 leu2-112*	This study
Y64	*MATα gcn2-507 ura3-52 leu2-3 leu2-112 inol sui2Δ HIS4-lacZ* p1097 [*SUI2 LEU2*]	This study
Y65	*MATα gcn2-507 ura3-52 leu2-3 leu2-112 inol sui2Δ HIS4-lacZ* p1098 [*SUI2-S51A LEU2*]	This study
Y66	*MATα gcn2-K559V ura3-52 leu2-3 leu2-112 inol sui2Δ HIS4-lacZ* p1097 [*SUI2 LEU2*]	This study
Y67	*MATα gcn2-K559V ura3-52 leu2-3 leu2-112 inol sui2Δ HIS4-lacZ* p1098 [*SUI2-S51A LEU2*]	This study
JC746Dip/1840	*MATa/MATα ura3-52/ura3-52 leu2-3,-112/leu2-3,-112 can1-100/can1-100 trp1/trp1 his3-11,-15/his3-11,-15 GLC7/glc7::HIS3*	9a

and H1470 to verify that the recovered plasmids suppress the 3-AT^s phenotype of *gcn2-507* in a *GCN3*-dependent manner and to analyze suppression of a *GCN2* deletion (see Results). Plasmid pA26 was one of several plasmids obtained that confers *GCN3*-dependent suppression of the 3-AT^s phenotype of *gcn2-507* but not that of *gcn2::URA3*.

Restriction site mapping of the chromosomal DNA insert in pA26 was conducted by standard techniques (47). The boundaries of the pA26 suppressor gene were defined by using the Tn*10-lacZ-kanR-URA3* transposition system as previously described (31). Plasmids containing insertions in the yeast chromosomal DNA insert were introduced into strain H1438 (Table 1) by selection for Ura$^+$, and growth on 3-AT medium was scored to localize the suppressor gene (Fig. 1). The nucleotide sequence of the suppressor gene was determined by the dideoxy-chain termination technique (48) and found to be identical to a portion of the *DIS2S1* sequence reported previously (41), corresponding to amino acid residues 1 to 208. Presumably, this carboxyl-terminal truncation occurred during construction of the genomic library, which involved the insertion of restriction fragments from a partial *Sau*3AI digestion of genomic DNA at the *Bam*HI site of YEp13 (39). In pA26, a *Sau*3AI site at nucleotide 1148 relative to the start codon of *DIS2S1* is joined to the YEp13 *Bam*HI site, fusing the DIS2S1 protein sequence at amino acid 208 to pBR322 sequences. This fusion adds 16 amino acids encoded by pBR322 sequences (from position 379 to the TAG stop codon at nucleotide 325) to the truncated fragment of *DIS2S1* in pA26.

Plasmids. A 2.1-kb *Hin*dIII fragment containing *glc7-Δ209-312* was isolated from pA26 (one of the *Hin*dIII sites is located in YEp13 sequences, 345 bp downstream of the *Sau*3AI-*Bam*HI junction) and inserted into the *Hin*dIII site of the polylinker region of pRS316, a low-copy-number vector containing *CEN6*, *ARSH6*, and *URA3* (50). The resulting plasmid, p24-4, contains the 5′ end of the *glc7-Δ209-312* gene juxtaposed to the *Eco*RV site of the polylinker region; plasmid p24-2 contains the same *Hin*dIII fragment inserted in the opposite orientation, with the 5′ end of *glc7-Δ209-312* juxtaposed to the *Cla*I site of pRS316. The *glc7-Δ209-312* gene was removed from plasmid p24-2 by digestion at the *Xho*I and *Xma*I sites in the flanking polylinker sequences and inserted between the *Sal*I and *Xma*I sites of the high-copy-number *URA3* plasmid YEp24 (4), forming p27-1. To construct derivatives of these plasmids containing wild-type *GLC7*, a 1,860-bp *Bgl*II-*Bal*I fragment (positions 124 and 1984, respectively, relative to the *GLC7* initiation codon) was isolated from plasmid p1855, containing wild-type *GLC7* (9a), and inserted between the *Bgl*II and *Sal*I sites of p24-4, generating p28-1. (The *Sal*I site of p24-4 is in the polylinker and was end filled before ligation). The *GLC7* gene is 1,464 bp in length and includes a 525-bp intron after codon 59 (41). The *Bgl*II-*Bal*I fragment encoding *GLC7* does not include the histidine tRNA gene located 580 bp 3′ to the *GLC7* gene (pYB12 sequence in reference 13). A 2.5-kb *Xho*I-*Xma*I fragment containing the entire *GLC7* gene was isolated from p28-1 and inserted between the *Sal*I and *Xma*I sites of YEp24, producing p29-1.

Plasmid p724 is a yeast integrative plasmid containing *LEU2* and *gcn2-507*. Its construction began with the insertion of a 13-bp synthetic oligonucleotide at the *Sal*I site of YIp32 (42), reconstituting the *Sal*I site and introducing a new *Xba*I site to produce p627. An 8.2-kb *Xba*I-*Sal*I fragment containing the *gcn2-507* allele was isolated from plasmid p561 and inserted between the *Sal*I and *Xba*I sites of p627 to yield p724. Plasmid Ep310 containing the *gcn3::URA3* deletion allele was constructed as already described for Ep308

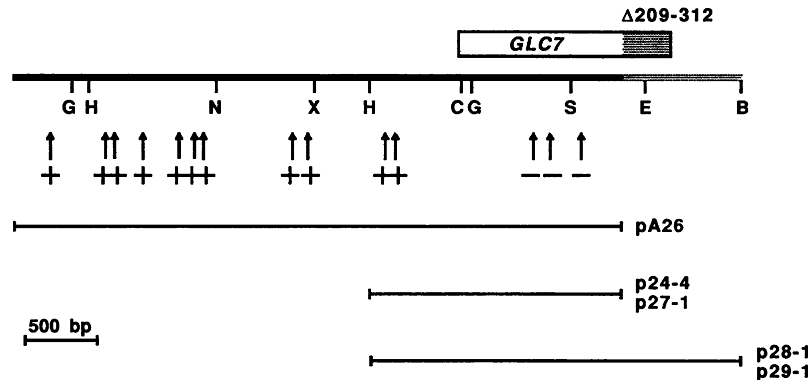

FIG. 1. Functional localization of the high-copy-number suppressor of *gcn2-507* on plasmid pA26, as defined by Tn*10-lacZ-kanR-URA3* insertional mutagenesis. The open box indicates the *GLC7* gene determined by nucleotide sequencing, including a 525-bp intron following codon 59, and the shaded region indicates the portion of *GLC7* that is absent in pA26, p24-4, and p27-1. The shaded portion of the restriction map indicates the carboxy-terminal region of *GLC7* and flanking sequences found in p28-1 and p29-1 that contain wild-type *GLC7*. H1438 (*gcn2-507*) was transformed with derivatives of pA26 containing Tn*10-lacZ-kanR-URA3* insertions and analyzed for suppression of the *gcn2-507* regulatory defect by measuring the growth of transformants on 3-AT medium. Locations of transposons are indicated by arrows below the restriction map along with their ability to suppress the *gcn2-507* defect for growth on 3-AT medium. + indicates confluent growth on 3-AT medium after 3 days; − indicates no growth. Bars below the restriction map indicate the extent of the *GLC7* region present in the corresponding plasmids. Restriction sites are designated as follows: B, *Bal*I; C, *Cla*I; G, *Bgl*II; H, *Hin*dIII; N, *Nru*I; S, *Sal*I; X, *Xho*I; E, *Eco*RI. Note that an additional *Hin*dIII site is located in the vector sequence of pA26, 350 bp to the right of the insert as shown in this diagram, that was used to construct p24-4 and p27-1 (Materials and Methods).

(24) except that the 1.1-kb *Hin*dIII *URA3* fragment was used to replace the 1.14-kb *GCN3 Hin*dIII fragment of Ep235.

Plasmid p180 contains a *GCN4-lacZ* translational fusion, with all four uORFs in the mRNA leader intact, on a low-copy-number *URA3* vector (26). Plasmid p226, derived from this plasmid, contains base substitutions in the ATG start codons of uORFs 1 to 3, leaving only the downstream uORF4 intact (38). Plasmid p1108 was constructed by inserting the *GCN4-lacZ* fusion from p180 into the *TRP1* integrating plasmid pRS304 (14). Plasmid p1045-541 contains the *RAS2-541* allele on the low-copy-number *URA3* plasmid YCp50. *RAS2-541* is a dominant-activated allele that lacks the Gly codon at position 19 (5a). Plasmid p585 (55) contains *GCN2* on YCp50, and pAH15 (28) contains *GCN2* on the high-copy-number *LEU2* plasmid YEp13.

Measurement of glycogen accumulation. Glycogen accumulation (the Glc phenotype) was measured qualitatively by inverting agar plates containing colonies of the strains of interest over I_2 crystals contained in a glass dish. Colonies develop a brown color with an intensity that is proportional to the glycogen content (7).

Assay of *HIS4-lacZ* and *GCN4-lacZ* fusions. Assays were conducted on cell extracts as described previously (35) after growing transformants in SD medium containing only the required supplements. For repressing conditions, saturated cultures were diluted 1:50 and harvested in the 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 the addition of 3-AT to 10 mM. Values shown 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.

Isoelectric focusing gel electrophoresis. Yeast strains were grown under repressing and derepressing conditions as described above, chilled, and harvested by centrifugation. Cell extracts were prepared and analyzed by using the vertical isoelectric focusing gel electrophoresis system described by Dever et al. (14). After focusing, immunoblot analysis was done as described previously (8), using antiserum prepared against a TrpE–eIF-2α fusion protein (9). Antigen-antibody complexes were detected by using ^{125}I-labeled protein A as described previously (14).

RESULTS

Multiple copies of a truncated allele of *GLC7*, encoding a type 1 protein phosphatase, suppress the derepression defect in *gcn2-507* mutants. To identify factors that participate with GCN2 in regulating *GCN4* expression, we decided to isolate genes that in high copy number would suppress the regulatory defect associated with the *gcn2-507* mutation. This mutation, which inserts two amino acids in the HisRS-related domain of GCN2, results in reduced derepression of histidine biosynthetic genes subject to the general control during growth in the presence of 3-AT. (3-AT is an inhibitor of the histidine biosynthetic enzyme encoded by the *HIS3* gene.) The *gcn2-507* allele is leaky, allowing very limited growth in the presence of 3-AT at concentrations that completely inhibit the growth of *gcn2* deletion mutants (data not shown). To isolate suppressors of *gcn2-507*, we transformed strain H1450 (Table 1) with a genomic library constructed in a high-copy-number plasmid, and transformants were identified that showed improved growth on medium containing 3-AT compared with transformants of H1450 containing vector alone (Materials and Methods).

Plasmids were recovered from 3-AT-resistant (3-AT^r) transformants of H1450 and tested for their ability to confer this phenotype when introduced into three different isogenic strains: one containing *gcn2-507* (H1438), the second containing a *gcn2::URA3* deletion in place of *gcn2-507* (H1473), and the third containing a *gcn3::URA3* deletion in addition to the *gcn2-507* mutation (H1470). Plasmid pA26 is one of several plasmids we identified that suppresses the 3-AT-sensitive (3-AT^s) phenotype of *gcn2-507* in H1438, but fails to do so in both the *gcn2::URA3* strain H1473 and the *gcn3::URA3 gcn2-507* strain H1470. The inability of pA26 to suppress the 3-AT^s phenotype of *gcn2::URA3* suggests that

it contains a suppressor gene whose function depends on the low level of GCN2 activity provided by the *gcn2-507* allele. The requirement for *GCN3* in conferring 3-AT resistance suggests that the suppressor gene product functions via this downstream positive effector of *GCN4* expression in regulating histidine biosynthesis.

We localized the suppressor gene on pA26 by insertional mutagenesis to a 1.5-kb interval at the right end of the chromosomal insert (Fig. 1) (see Materials and Methods). Determination of the nucleotide sequence of this interval showed that it contains a portion of a previously described gene called *DIS2S1*, encoding a type 1 protein phosphatase (41). The *DIS2S1* gene is truncated in pA26, deleting amino acids 209 to 312 from the carboxyl terminus. *DIS2S1* was cloned on the basis of its sequence similarity with a type 1 protein phosphatase gene in *Schizosaccharomyces pombe* known as *dis2* (41). The assignment of its phosphatase function was based on the deduced amino acid sequence of DIS2S1, which is 81% identical to the catalytic subunit of rabbit skeletal muscle type 1 protein phosphatase (2). Recent work indicates that *DIS2S1* is identical to *GLC7* (18), whose product is involved in the regulation of glycogen metabolism in *S. cerevisiae*. Type 1 protein phosphatase activity is diminished in a *glc7-1* mutant compared with wild type, and this reduction impairs the activation of glycogen synthase, an enzyme required for glycogen biosynthesis (18, 20, 43). Henceforth, we refer to this gene as *GLC7*.

We wished to determine whether suppression of the *gcn2-507* mutation required both truncation and overexpression of the *GLC7* gene. A 2.1-kb *Hin*dIII fragment containing the truncated *glc7-Δ209-312* allele and adjacent vector sequences from pA26 was inserted into low-copy-number and high-copy-number plasmids to produce p24-4 and p27-1, respectively (Fig. 1). Similar plasmids containing the intact *GLC7* gene (p28-1 and p29-1, Fig. 1) were constructed from plasmid p1855, which was isolated previously by complementation of the glycogen-deficient phenotype (Glc⁻) of the *glc7-1* mutation (9a). Both sets of plasmids were introduced into the *gcn2-507 GLC7* strain Y27, and the transformants were tested for growth on 3-AT medium. The results shown in Fig. 2 indicate that the 2.1-kb *Hin*dIII fragment from pA26 bearing the *glc7-Δ209-312* allele conferred the suppressor phenotype when present on the high-copy-number plasmid p27-1; however, only weak suppression occurred when *glc7-Δ209-312* was introduced on the low-copy-number plasmid p24-4. As expected, p27-1 did not suppress the 3-AT^s phenotype associated with a *gcn2* deletion. Neither the low- nor the high-copy-number plasmid containing wild-type *GLC7*, p28-1 or p29-1, respectively, conferred any detectable growth on 3-AT medium in the *gcn2-507* mutant. Thus, suppression of the *gcn2-507* regulatory defect appears to require elevated expression of a truncated GLC7 protein. In fact, both plasmids that contain an intact *GLC7* gene led to reduced 3-AT resistance when introduced into a wild-type *GCN2* strain (Fig. 2), mimicking a *gcn2* mutation.

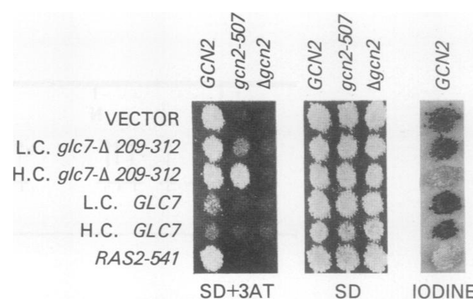

FIG. 2. Truncated and wild-type alleles of *GLC7* on high-copy-number plasmids alter the derepression of histidine biosynthetic genes subject to general amino acid control. Isogenic strains H1402 (*GCN2*), Y27 (*gcn2-507*), and H1149 (*Δgcn2*) transformed with different *GLC7* or *RAS2* alleles on low-copy-number (L.C.) or high-copy-number (H.C.) plasmids or with the vector YEp24 alone were replica plated to SD medium or SD medium supplemented with 3-AT (as indicated on the bottom) and incubated for 2 days at 30°C. On the far right are shown H1402 transformants containing the same plasmids tested for glycogen accumulation by iodine vapor staining after being grown to confluence on SD medium. Dark staining is indicative of glycogen accumulation.

To characterize suppression of the *gcn2-507* regulatory defect by a quantitative assay, we measured expression of a *HIS4-lacZ* fusion present in the same strains analyzed in Fig. 2. Increased transcription of *HIS4* under conditions of amino acid starvation requires the GCN4 protein, and increased synthesis of GCN4 is dependent on the phosphorylation of eIF-2α by GCN2 (14, 27). Thus, expression of the *HIS4-lacZ* fusion is indicative of the level of eIF-2α phosphorylation in the cell. As expected, the *gcn2-507* strain transformed with vector alone was defective for derepression of the *HIS4-lacZ* fusion in response to histidine starvation, compared with transformants of the *GCN2* strain containing vector alone (Table 2). The presence of *glc7-Δ209-312* on a high-copy-number plasmid in the *gcn2-507* strain led to threefold-greater *HIS4-lacZ* expression under starvation conditions than was seen with vector alone. However, suppression of *gcn2-507* was incomplete, as shown by the fact that the isogenic *GCN2* strain expressed HIS4-LacZ enzyme activity

TABLE 2. Effect of plasmid-borne *GLC7* alleles on the derepression of *HIS4-lacZ* expression in response to histidine starvation[a]

Plasmid	Plasmid-borne allele	HIS4-LacZ enzyme activity (U) in strains containing:					
		gcn2-507		*gcn2::LEU2*		*GCN2*	
		R	DR	R	DR	R	DR
YEp24	None (vector)	100	71	110	69	120	550
p27-1	*glc7-Δ209-312* (H.C.)	110	210	99	87	150	650
p24-4	*glc7-Δ209-312* (L.C.)	79	81	85	98	98	530
p29-1	*GLC7* (H.C.)	100	120	120	110	93	220
p28-1	*GLC7* (L.C.)	98	98	120	91	110	310

[a] β-Galactosidase activity was measured in extracts of isogenic strains grown under repressing (R, nonstarvation) or derepressing (DR, histidine starvation imposed by 3-AT) conditions as described in Materials and Methods. Strains assayed are transformants of H1402 (*GCN2*), Y27 (*gcn2-507*), or H1149 (*gcn2::LEU2*) bearing the designated *GLC7* allele on low-copy-number (L.C.) or high-copy-number (H.C.) plasmids. The results shown are averages of assays of from two to five independent transformants, and the individual measurements deviated from the average values shown here by 30% or less.

at a level two to three times higher than the *gcn2-507* strain bearing high-copy-number *glc7-Δ209-312*. As expected, the *glc7-Δ209-312* allele stimulated *HIS4-lacZ* expression in the *gcn2-507* mutant only when present in multiple copies, and it had no significant effect in high or low copy number when present in the *gcn2::LEU2* strain (Table 2). Finally, the presence of wild-type *GLC7* on low- or high-copy-number plasmids in the *GCN2* strain reduced HIS4-LacZ enzyme activity by a factor of two to three from that seen in transformants containing vector alone (Table 2).

The levels of *HIS4-lacZ* expression observed under starvation conditions in strains containing different plasmid-borne *GLC7* alleles (Table 2) are generally in good agreement with the ability of these strains to grow on starvation medium containing 3-AT (Fig. 2). One unexpected finding in Table 2 was that high-copy-number *GLC7* led to a small increase in *HIS4-lacZ* expression under starvation conditions, compared with vector alone, in both the *gcn2-507* and *gcn2::LEU2* strains. These latter findings may indicate that overexpression of GLC7 can increase *GCN4* expression by a GCN2-independent pathway in *gcn2* mutants, even though it impairs the ability of GCN2 to stimulate *GCN4* expression in otherwise wild-type cells. A second discrepancy was that low-copy-number *GLC7* in the *GCN2* strain gave higher *HIS4-lacZ* expression than did high-copy-number *glc7-Δ209-312* in the *gcn2-507* strain (Table 2); however, the former strain was more sensitive than the latter to 3-AT (Fig. 2). This discrepancy might be explained by our observation that additional copies of *GLC7* have a toxic effect on cellular growth (data not shown) that may be particularly evident on 3-AT medium.

Taken together, the results in Fig. 2 and Table 2 suggest that overexpression of the *glc7-Δ209-312* allele has a dominant negative phenotype, leading to reduced GLC7 protein phosphatase activity. Presumably, this effect partially compensates for reduced GCN2 protein kinase activity in *gcn2-507* mutants and restores the derepression of histidine biosynthetic genes under *GCN4* control. Overexpression of wild-type *GLC7* appears to have the opposite effect, limiting derepression of the general control system by reversing the effects of GCN2-mediated phosphorylation of eIF-2α in amino acid-starved cells.

Wild-type *GLC7* on a high-copy-number plasmid overcomes constitutive derepression of *HIS4* expression in *GCN2*ᶜ mutants. We have previously described dominant *GCN2*ᶜ mutations that lead to derepression of *GCN4* expression in the absence of amino acid starvation (55). This derepressed phenotype was subsequently attributed to constitutive activation of GCN2 kinase function because eIF-2α phosphorylation was found to be greatly increased in *GCN2*ᶜ mutants grown under nonstarvation conditions (14). We wished to determine whether overexpression of wild-type *GLC7* would reverse the derepression of the general control system that is elicited by the activating *GCN2*ᶜ mutations. As shown in Table 3 (columns labeled Vector alone), the *GCN2*ᶜ mutations we analyzed lead to derepression of *HIS4-lacZ* expression at levels fourfold to eightfold higher than in an isogenic *GCN2* strain under nonstarvation conditions. When the *GCN2*ᶜ*-501* mutant H1414 was transformed with *GLC7* on a high-copy-number plasmid, HIS4-LacZ enzyme activity was reduced to about 50% of that seen in the same strain transformed with vector alone, under both starvation and nonstarvation conditions. Similar reductions in *HIS4-lacZ* expression were observed in the other *GCN2*ᶜ strains containing high-copy-number *GLC7*, except for the most derepressed mutant H1608, which showed a reduction of only 25%. The latter finding suggests that a threshold of GCN2 activation exists beyond which derepression of the general control system cannot be reversed by overexpression of GLC7. Introduction of *GLC7* on a low-copy-number plasmid also decreased *HIS4-lacZ* expression in the *GCN2*ᶜ mutants, but to a lesser extent than that seen with high-copy-number *GLC7*. These results support the notion that the protein kinase GCN2 and the protein phosphatase GLC7 have opposite effects on the general control system. The simplest way to explain these opposing functions is to propose that GLC7 reduces the phosphorylation state of the substrate of GCN2, eIF-2α, and thereby diminishes the translational efficiency of *GCN4* mRNA.

TABLE 3. Multiple copies of wild-type *GLC7* lower *HIS4-lacZ* expression in constitutively derepressed *GCN2*ᶜ mutants[a]

Strain	Relevant genotype	HIS4-LacZ enzyme activity (U) in strains containing:					
		Vector alone		H.C. *GLC7*		L.C. *GLC7*	
		R	DR	R	DR	R	DR
H1402	*GCN2*	120	550	93	220	110	310
H1149	*gcn2::LEU2*	110	69	120	92	120	91
Y3	H.C. *GCN2*	490	540	210	260	400	360
H1414	*GCN2*ᶜ*-501*	510	510	280	280	330	330
H1613	*GCN2*ᶜ*-516*	660	750	280	300	380	390
H1609	*GCN2*ᶜ*-514*	700	850	370	310	630	600
H1608	*GCN2*ᶜ*-513*	930	940	670	710	790	700

[a] β-Galactosidase activity was measured in extracts prepared from an isogenic set of strains transformed with vector alone (YEp24) or with low-copy-number (L.C.) and high-copy-number (H.C.) plasmids p28.1 and p29.1, respectively, containing wild-type *GLC7*. Strains were grown under repressing (R, nonstarvation) or derepressing (DR, histidine starvation imposed by 3-AT) conditions. The high-copy-number *GCN2* plasmid in strain Y3 is pAH15. The results shown are averages of assays of from two to five independent transformants, and the individual measurements deviated from the average values shown here by 30% or less.

Truncated *glc7-Δ209-312* allele stimulates *GCN4* expression at the translational level by increasing the phosphorylation of eIF-2α in a *gcn2-507* mutant. To test our assumption that alterations in *GLC7* function perturb the levels of histidine biosynthetic enzymes by altering the synthesis of GCN4 protein, we measured expression of a *GCN4-lacZ* fusion in *gcn2-507* and *GCN2* strains containing different plasmid-borne *GLC7* alleles. Compared with vector alone, the *glc7-Δ209-312* allele on a high-copy-number plasmid led to threefold-higher expression of the *GCN4-lacZ* fusion in the *gcn2-507* mutant grown under histidine starvation conditions (Table 4). This increased expression of *GCN4* can account for the partial derepression of histidine biosynthetic enzymes that occurs in *gcn2-507* cells containing the truncated *GLC7* gene (Table 2). Conversely, wild-type *GLC7* on a high-copy-number plasmid decreased expression of the *GCN4-lacZ* fusion by about 50% in the *GCN2* strain under starvation conditions, explaining the inability of such transformants to derepress *HIS4* expression efficiently and to grow on medium containing 3-AT.

Translational control of *GCN4* expression is mediated by short uORFs present in the leader of *GCN4* mRNA (1, 27). uORF4 alone is sufficient to repress *GCN4* expression to the low level seen under nonstarvation conditions when all four uORFs are present, whereas uORF1 is required in starved cells to overcome the inhibitory effect of uORF4 and permit efficient translation initiation at *GCN4*. Therefore, mutations that eliminate the start codons of uORFs 1 to 3, leaving only uORF4 intact, considerably diminish derepression of *GCN4*

TABLE 4. Effect of plasmid-borne *GLC7* alleles on the derepression of *GCN4-lacZ* expression in response to histidine starvation[a]

Plasmid	Plasmid-borne allele	GCN4-LacZ enzyme activity (U) in strains containing:			
		gcn2-507		*GCN2*	
		R	DR	R	DR
YEp24	None (vector)	14	30	21	130
p27-1	*glc7-Δ209-312* (H.C.)	32	96	34	180
p24-4	*glc7-Δ209-312* (L.C.)	13	28	16	130
p29-1	*GLC7* (H.C.)	34	60	21	69
p28-1	*GLC7* (L.C.)	20	47	26	96

[a] β-Galactosidase activity was measured in extracts of strains Y31 (*GCN2*) and Y32 (*gcn2-507*) transformed with the designated *GLC7* allele on low-copy-number (L.C.) or high-copy-number (H.C.) plasmids and grown under repressing (R, nonstarvation) or derepressing (DR, histidine starvation imposed by 3-AT) conditions. The results shown are averages of assays of from two to five independent transformants, and the individual measurements deviated from the average values shown here by 30% or less.

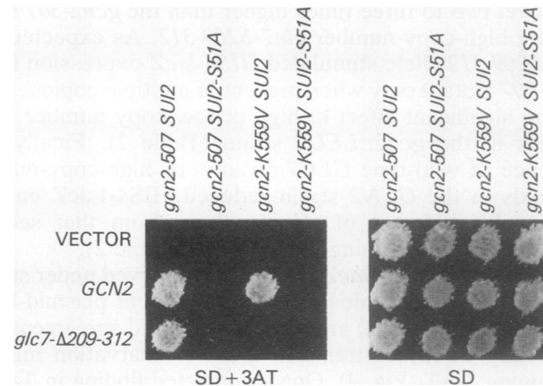

FIG. 3. Suppression of the derepression defect of *gcn2-507* by *glc7-Δ209-312* requires the phosphorylation site on eIF-2α, serine 51. The α subunit of eIF-2 is encoded by the *SUI2* gene (9). Isogenic strains Y64 (*gcn2-507 SUI2*), Y65 (*gcn2-507 SUI2-S51A*), Y66 (*gcn2-K559V SUI2*), and Y67 (*gcn2-K559V SUI2-S51A*) were transformed with YEp24 (vector), p585 (*GCN2*), or p27.1 (*glc7-Δ209-312*). Strains were replica plated to SD medium or SD medium supplemented with 3-AT and incubated for 2 days at 30°C.

expression in amino acid-starved cells. To address whether high-copy-number *glc7-Δ209-312* stimulates *GCN4* expression in *gcn2-507* mutants at the translational level, we compared expression from *GCN4-lacZ* fusions containing the wild-type mRNA leader or uORF4 alone (Table 5). In accord with results discussed above, transformants containing high-copy-number *glc7-Δ209-312* expressed the wild-type fusion under starvation conditions at a level ca. 2.5-fold higher than that seen with vector alone. By contrast, high-copy-number *glc7-Δ209-312* increased expression of the fusion containing uORF4 alone by only about 40% (Table 5). Similar results were obtained when a *GCN4-lacZ* fusion containing no uORFs was introduced into the *gcn2-507* transformants shown in Table 4 bearing high-copy-number *glc7-Δ209-312* on plasmid p27-1 or vector alone. Because it lacks all four uORFs, this *GCN4-lacZ* construct produces fusion enzyme activity at very high constitutive levels (38). In contrast to the threefold-higher expression given by the wild-type *GCN4-lacZ* fusion in the strain bearing *glc7-Δ209-312* (Table 4), expression from the construct lacking all four uORFs was only 60% higher in the presence of *glc7-Δ209-312* (1,500 U) versus the vector alone (930 U). The dependence of high-copy-number *glc7-Δ209-312* on multiple uORFs in the *GCN4* mRNA leader for efficient suppression of the *gcn2-507* regulatory defect suggests that reducing *GLC7* function stimulates *GCN4* expression at the translational level. The 50% increase in expression seen for the fusions containing uORF4 alone or no uORFs in the presence of *glc7-Δ209-312* may indicate that impairing *GLC7* function also leads to a small increase in *GCN4* transcription.

TABLE 5. Evidence that the *glc7-Δ209-312* allele in high copy number increases *GCN4* expression at the translational level in a *gcn2-507* mutant[a]

Plasmid	Plasmid-borne allele	GCN4-LacZ enzyme activity (U) in transformants containing:			
		p180 (uORFs 1–4)		p226 (uORF 4)	
		R	DR	R	DR
YEp13	None (vector)	8	18	7	17
pA26	*glc7-Δ209-312*	20	46	11	24

[a] β-Galactosidase activity was measured in extracts of Y46 transformed with p180 or p226 harboring *GCN4-lacZ* fusions containing all four uORF1s or uORF4 alone, respectively, in the *GCN4* mRNA leader. These strains were also transformed with the high-copy-number plasmid pA26 containing *glc7-Δ209-312* or with vector alone, as designated. Assays were done on extracts from cells grown under repressing (R, nonstarvation) or derepressing (DR, histidine starvation imposed by 3-AT) conditions. The results shown are averages of assays of from two to five independent transformants, and the individual measurements deviated from the average values shown here by 30% or less.

Dever et al. (14) showed that phosphorylation of the α subunit of eIF-2 by GCN2 is required for increased translation of *GCN4* mRNA in amino acid-starved cells. This phosphorylation event is thought to occur on serine 51 of eIF-2α because substitution of this amino acid with alanine completely abolished phosphorylation of the protein and impaired derepression of *GCN4* expression in amino acid-starved cells. If the *glc7-Δ209-312* allele stimulates *GCN4* translation by increasing the level of eIF-2α phosphorylation, then suppression of the 3-AT^s phenotype of *gcn2-507* should require serine 51 of eIF-2α.

To test this prediction, we introduced the *glc7-Δ209-312* allele on a high-copy-number plasmid into different *gcn2* mutants expressing wild-type eIF-2α (*SUI2*) or the mutant form containing an alanine residue at position 51 (*SUI2-S51A*). The *gcn2-K559V* allele, containing a valine substitution of the invariant lysine in the GCN2 kinase domain (lysine 559), is completely defective for GCN2 regulatory function (3-AT^s) (55). To illustrate the requirement for serine 51 in eIF-2α for derepression of the general control system, we showed that introduction of the *GCN2* gene on a plasmid complemented the 3-AT^s phenotype of the *gcn2-K559V* mutation in the *SUI2* strain but not in the isogenic strain containing the *SUI2-S51A* allele (Fig. 3). As expected, high-copy-number *glc7-Δ209-312* suppressed the 3-AT^s phenotype in the *gcn2-507* strain but not in the *gcn2-K559V* mutant. Importantly, the substitution of serine 51 for alanine in eIF-2α completely eliminated the ability of *glc7-Δ209-312* to suppress the derepression defect of *gcn2-507* (Fig. 3). These observations were confirmed by measurements of *HIS4-lacZ* expression in the same strains. In the *gcn2-507 SUI2* mutant, the presence of high-copy-number *glc7-Δ209-312* doubled HIS4-LacZ enzyme activity under starvation

TABLE 6. Suppression of the derepression defect in *gcn2-507* cells by *glc7-Δ209-312* requires serine 51 in eIF-2α[a]

Plasmid	Plasmid-borne allele	HIS4-LacZ enzyme activity (U) in strains containing:			
		SUI2-S51A		*SUI2*	
		R	DR	R	DR
YEp24	None (vector)	130	100	110	100
p27.1	*glc7-Δ209-312*	110	110	95	210
p585	*GCN2*	110	96	110	370

[a] β-Galactosidase activity was measured in extracts of isogenic strains Y65 (*gcn2-507 SUI2-S51A*) and Y64 (*gcn2-507 SUI2*) transformed with the designated plasmid-borne alleles. Assays were done on extracts from cells grown under repressing (R, nonstarvation) or derepressing (DR, histidine starvation imposed by 3-AT) conditions. The results shown are averages of assays of two independent transformants, and the individual measurements deviated from the average values shown here by 30% or less.

conditions but had no effect on *HIS4-lacZ* expression in the isogenic strain containing the *SUI2-S51A* allele (Table 6).

The results of the previous experiments strongly suggested that the *glc7-Δ209-312* allele overcomes the derepression defect in *gcn2-507* cells by increasing the level of eIF-2α phosphorylation. To test this prediction biochemically, we used isoelectric focusing gel electrophoresis to separate the phosphorylated and nonphosphorylated forms of eIF-2α and visualized the relative amounts of the two species present under different conditions by immunoblotting with antibodies directed against eIF-2α. Consistent with the findings of Dever et al. (14), starvation of a *GCN2* strain transformed with vector alone increased the relative amount of the phosphorylated form of eIF-2α (Fig. 4). (Recall that phosphorylation of only a portion of the eIF-2α is sufficient to substantially reduce the efficiency of translation initiation [14, 25].) No increase in phosphorylation was observed in the isogenic *gcn2::LEU2* deletion strain (Fig. 4), nor does it occur in strains expressing eIF-2α with serine 51 substituted by alanine (14). In the *gcn2-507* mutant transformed with vector alone, phosphorylation of eIF-2α under starvation conditions was reduced from the level seen in the *GCN2* strain, consistent with the impaired ability of the *gcn2-507* product to mediate derepression of *GCN4* translation. Introduction of *glc7-Δ209-312* on a high-copy-number plasmid restored eIF-2α phosphorylation in the *gcn2-507* strain under

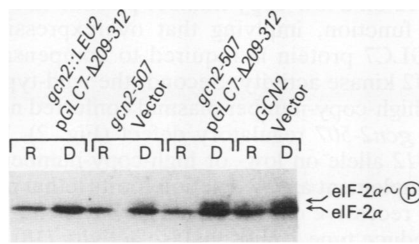

FIG. 4. High-copy-number *glc7-Δ209-312* stimulates phosphorylation of eIF-2α in amino acid-starved *gcn2-507* cells. Protein extracts were prepared from isogenic strains H1149 (*gcn2::LEU2*), Y27 (*gcn2-507*), and H1402 (*GCN2*) transformed with high-copy-number plasmid p27.1 bearing *glc7-Δ209-312* or with vector YEp24 alone, grown under nonstarvation (R) or histidine starvation (D) conditions. Samples containing 20 μg of protein were separated by isoelectric focusing on a vertical slab gel, and eIF-2α was detected by immunoblot analysis with antiserum raised against a TrpE–eIF-2α fusion protein. The more acidic hyperphosphorylated form of eIF-2α focuses above the less phosphorylated species (14).

starvation conditions to a level similar to that seen in the isogenic *GCN2* strain (Fig. 4). Densitometric analysis of this experiment indicated that high-copy-number *glc7-Δ209-312* increased the proportion of eIF-2α that is phosphorylated in the *gcn2-507* mutant under starvation conditions by about a factor of two. Very similar findings were obtained in several independent experiments. We conclude that the *glc7-Δ209-312* allele suppresses the *gcn2-507* mutation by increasing the steady-state level of eIF-2α phosphorylation, compensating for a reduction in the protein kinase activity of GCN2.

Truncated *glc7-Δ209-312* allele does not complement a chromosomal deletion of *GLC7*. The results presented thus far suggest that the *glc7-Δ209-312* allele is defective for GLC7 function and has a dominant negative phenotype when overexpressed. Additional support for this idea is provided by the fact that the *glc7-Δ209-312* allele does not complement the lethality associated with a chromosomal deletion of *GLC7* (10, 18). The *glc7::HIS3* allele contains the *HIS3* gene inserted in place of 480 bp of *GLC7* coding sequences (9a). This allele was used to replace one copy of the wild-type *GLC7* gene in a diploid strain, producing JC746Dip/1840 (*his3-11,-15/his3-11,-15 GLC7/glc7::HIS3 ura3-52/ura3-52* [Table 1]). After sporulation of this strain, all 15 tetrads that we dissected contained only two viable spores, all of which were phenotypically His^- (histidine auxotrophs) and Glc^+ (normal glycogen levels). By contrast, when wild-type *GLC7* was introduced into JC746Dip/1840 on the low-copy-number *URA3* plasmid p28.1 before sporulation, 9 tetrads that we dissected had two viable spores, 11 had three viable spores, and 6 had four viable spores. All viable spores were Glc^+, and all 24 His^+ (*glc7::HIS3*) spores (present only in tetrads with three or four viable spores) were also Ura^+, indicating that they contain plasmid p28.1. These results indicate that the *glc7::HIS3* disruption is lethal in a haploid strain and that this lethality can be overcome by the *GLC7* gene present on p28.1. Similar results were obtained with *GLC7* on the high-copy-number plasmid p29.1.

To then address whether the truncated allele *glc7-Δ209-312* could complement the lethality associated with *glc7::HIS3*, we introduced *glc7-Δ209-312* on a low- or high-copy-number plasmid (p24.4 or p27.1, respectively) into JC746Dip/1840 and sporulated the resulting diploid transformants. In 20 tetrads dissected for each transformant, all viable spores were Glc^+ His^-, suggesting that the plasmid-borne *glc7-Δ209-312* allele cannot provide sufficient GLC7 function for viability in *glc7::HIS3* spores. (Numerous viable spores isolated from these latter dissections were Ura^+, indicating that p24-4 and p27.1 were not lost during sporulation.)

Chromosomal *glc7-1* mutation suppresses *gcn2-507* defect in derepression of histidine biosynthetic genes. Another indication that reduced GLC7 function is responsible for restoring derepression of the general control system in *gcn2-507* mutants came from the fact that the recessive allele *glc7-1* suppresses the Gcn^- phenotype of *gcn2-507*. The *glc7-1* mutation was isolated on the basis of lowering the steady-state levels of glycogen (43, 43a) (Fig. 5). This reduction in glycogen accumulation appears to result from diminished activation of glycogen synthase, which requires the type 1 protein phosphatase activity of GLC7 (18, 43). To determine whether the *glc7-1* allele suppresses the *gcn2-507* regulatory defect, we analyzed tetrads from a diploid produced by a cross between strains Y25 (*glc7-1 gcn2::LEU2 leu2*) and H1438 (*gcn2-507 leu2*). All 11 *gcn2-507 glc7-1* spores we obtained showed suppression of the Gcn^- phenotype of *gcn2-507*, being capable of growth on medium containing 3-AT, whereas none of 13 *gcn2-507 GLC7* spores could grow

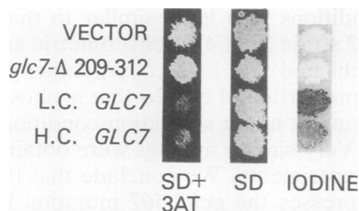

FIG. 5. *glc7-1* allele suppresses the *gcn2-507* regulatory defect and leads to reduced glycogen accumulation. Strain Y27 (*glc7-1 gcn2-507*) transformed with different *GLC7* alleles on low-copy-number (L.C.) or high-copy-number (H.C.) plasmids or with vector YEp24 alone was replica plated to SD medium or SD medium supplemented with 3-AT and incubated for 3 days at 30°C. On the far right, the same set of transformants were grown on SD medium and tested for glycogen accumulation by iodine vapor staining. Dark staining indicates glycogen accumulation.

in the presence of 3-AT. In addition, the *glc7-1* allele was dependent on the residual GCN2 function expressed by the *gcn2-507* allele, since all 12 *gcn2::LEU2 glc7-1* spores we analyzed were 3-AT^s. As expected, all 12 *gun2::LEU2 GLC7* spores failed to grow on 3-AT plates. Therefore, *glc7-1* suppresses the inability of *gcn2-507* to promote derepression of histidine biosynthetic enzymes, and as noted above for *glc7-Δ209-312*, this suppression requires at least a low level of GCN2 function.

One *gcn2-507 glc7-1* haploid strain (Y29) resulting from the tetrad analysis just described was transformed with plasmids containing wild-type *GLC7* or vector alone and examined for the ability to grow on 3-AT medium and for glycogen accumulation. Transformants of Y29 containing *GLC7* on low- or high-copy-number plasmids were unable to grow on 3-AT medium and accumulated high levels of glycogen compared with transformants of the same strain containing vector alone (Fig. 5). Thus, the *glc7-1* mutation is recessive to wild-type *GLC7* for both phenotypes. Introduction of the *glc7-Δ209-312* allele on a high-copy-number plasmid into Y29 failed to restore glycogen accumulation, indicating that this allele is defective for the function of GLC7 involved in glycogen accumulation (Fig. 5). Moreover, results shown in Fig. 2 indicate that the *glc7-Δ209-312* allele is dominant for its Glc$^-$ phenotype: introduction of this allele on a high-copy-number plasmid into a wild-type *GLC7* strain leads to reduced glycogen accumulation compared with that seen in transformants of the same strain containing vector alone.

Finally, we wished to rule out the possibility that suppression of the *gcn2-507* regulatory defect by the *glc7* mutations is an indirect consequence of altered glycogen metabolism, rather than resulting from a specific involvement of GLC7 in general amino acid control. Toward this end, we examined the effect of the *RAS2-541* allele on histidine biosynthesis. As shown in Fig. 2, introduction of this dominant *RAS2* allele on a low-copy-number plasmid into a *GLC7* strain resembles the *glc7-Δ209-312* mutation in reducing the steady-state levels of glycogen. Presumably, this occurs because of abnormally high levels of cyclic AMP and the attendant activation of cyclic AMP-dependent protein kinase, which is thought to regulate glycogen metabolic enzymes in yeast cells (5, 22, 52, 53). Although strains transformed with *RAS2-541* showed reduced glycogen levels, they differed from Glc7$^-$ strains in showing no suppression of the *gcn2-507* regulatory defect, as judged by the inability of *gcn2-507 RAS2-541* strains to grow on 3-AT medium (Fig. 2). We conclude that suppression of *gcn2-507* in Glc7$^-$ mutants is not a consequence of a defect in glycogen metabolism and most probably results instead from the fact that the GLC7 protein phosphatase and the GCN2 protein kinase share eIF-2α as a substrate. Our findings also suggest that GLC7 recognizes at least two different substrates involved in distinct metabolic pathways: eIF-2α in the case of general amino acid control, and glycogen synthase in the case of glycogen metabolism.

DISCUSSION

Role of the protein phosphatase GLC7 in opposing the stimulatory effect of the protein kinase GCN2 on translation of *GCN4*. The catalytic subunits of the major protein phosphatase activities in mammalian cell extracts have been assigned to four principal classes defined by in vitro criteria, including substrate specificity, sensitivity to inhibitors, and divalent cation requirements (reviewed in reference 12). We used a genetic approach to show that a yeast type 1 protein phosphatase encoded by the *GLC7* gene functions in vivo in the regulation of amino acid biosynthetic genes mediated by the transcriptional activator protein GCN4. Our results indicate that GLC7 acts in opposition to GCN2, a protein kinase required for increased translation of *GCN4* mRNA in amino acid-starved cells.

We isolated a truncated form of the *GLC7* gene on the basis of its ability in multi-copy number to overcome the defective derepression of *GCN4* expression in *gcn2-507* mutants. The suppressor function of *glc7-Δ209-312* requires the low-level GCN2 kinase activity provided by *gcn2-507*, as no suppression occurred in a *gcn2* deletion strain (Fig. 2). Measurements of *HIS4-lacZ* and *GCN4-lacZ* expression indicated that overexpression of the *glc7-Δ209-312* allele partially restored derepression of the general control system in *gcn2-507* mutants under conditions of histidine starvation (Tables 2 and 4). The restoration of *GCN4-lacZ* derepression was dependent on the presence of multiple uORFs in the *GCN4* mRNA leader (Table 5), suggesting that GLC7 regulates *GCN4* expression at the translational level. This latter conclusion is supported by the fact that suppression of *gcn2-507* required serine 51 in eIF-2α (Fig. 3 and Table 6), the site of phosphorylation by GCN2 that elicits the general control response (14).

Several lines of genetic evidence suggest that overexpression of the *glc7-Δ209-312* product suppresses the *gcn2-507* regulatory defect by interfering with the chromosomally encoded GLC7 phosphatase activity. First, the presence of *glc7-Δ209-312* on a low-copy-number plasmid had very little suppressor function, implying that overexpression of the truncated GLC7 protein is required to compensate for reduced GCN2 kinase activity. Second, the wild-type allele of *GLC7* on a high-copy-number plasmid conferred no suppression of the *gcn2-507* regulatory defect (Fig. 2). Third, the *glc7-Δ209-312* allele on low- or high-copy-number plasmids failed to complement a *glc7* deletion for its lethal phenotype. Fourth, the recessive chromosomal *glc7-1* allele, which was shown to reduce type 1 phosphatase activity (18) and lower glycogen accumulation, mimicked the high-copy-number *glc7-Δ209-312* allele in suppressing the derepression defect in *gcn2-507* cells; likewise, high-copy-number *glc7-Δ209-312* led to reduced glycogen accumulation in the presence of wild-type chromosomal *GLC7* (Fig. 2 and 5). To account for the dominant negative phenotype of the *glc7-Δ209-312* allele, we propose that deletion of 104 C-terminal amino acids from GLC7 inactivates its catalytic activity but allows the protein to compete with wild-type GLC7 for substrates, positive regulatory subunits (12), or binding sites in the cell.

The fact that *glc7-Δ209-312* stimulates *GCN4* expression at the translational level, and requires a low level of GCN2 kinase function and serine 51 on eIF-2α to do so, strongly suggests that reducing GLC7 phosphatase activity leads to an increase in eIF-2α phosphorylation. Direct measurements of the phosphorylation state of eIF-2α by isoelectric focusing gel electrophoresis supported this interpretation: phosphorylation was barely detectable in the *gcn2-507* mutant and increased to nearly wild-type levels in the presence of multicopy *glc7-Δ209-312* (Fig. 4). Additional support for the idea that GLC7 phosphatase activity reduces the phosphorylation state of eIF-2α comes from the fact that increasing the dosage of wild-type *GLC7* impaired derepression of *GCN4* expression in an otherwise wild-type strain (Fig. 2), mimicking the phenotype of kinase-defective *gcn2* mutations. Moreover, high-copy-number *GLC7* reversed the constitutive derepression of *HIS4-lacZ* expression conferred by activated *GCN2*^{c} alleles (Table 3). Taken together, these results strongly suggest that the protein kinase activity of GCN2 and the protein phosphatase activity of GLC7 are opposing functions that regulate *GCN4* translation by altering the phosphorylation state of eIF-2α.

The simplest explanation for our findings is that eIF-2α is an in vivo substrate of the protein phosphatase GLC7. It is noteworthy in this regard that rabbit reticulocyte protein phosphatases type 1 and 2A efficiently dephosphorylate the α subunit of eIF-2 (32, 45). A significant portion of the type 1 activity was pelleted with the ribosomes during high-speed centrifugation of cell extracts (19), and addition of type 1 protein phosphatase inhibitor 2 to rabbit reticulocyte lysates increased the phosphorylation of eIF-2α concomitant with an inhibition of protein synthesis (16, 45). This inhibition was reversed by the addition of purified eIF-2. These results suggest that type 1 protein phosphatase stimulates translation in rabbit reticulocytes by dephosphorylation of eIF-2α. However, the type 1 enzyme can also dephosphorylate the eIF-2α protein kinase DAI isolated from rabbit reticulocytes (51), and autophosphorylation of DAI is thought to be required for its activation (21). Thus, type 1 protein phosphatase could indirectly promote dephosphorylation of eIF-2α by preventing the activation of DAI kinase function. It is not known whether the phosphorylation state of GCN2 similarly affects its protein kinase activity (55).

Involvement of GLC7 protein phosphatase in glycogen metabolism. The chromosomal mutation *glc7-1* (43) and the *glc7-Δ209-312* allele in high copy number both lead to reduced steady-state levels of glycogen (Fig. 2 and 5), indicating that GLC7 is involved in glycogen metabolism in addition to its role in *GCN4* translational control. Glycogen metabolism in mammalian systems is regulated by protein phosphorylation, with the glycogenolytic enzymes being activated and glycogen synthase being inhibited by phosphorylation (11, 12). Several lines of evidence suggest that type 1 protein phosphatase plays a major role in this regulatory mechanism (12). It has been suggested that GLC7 is a glycogen synthase phosphatase in *S. cerevisiae* because much more of the glycogen synthase is present in the inactive (D) form in the *glc7-1* mutant than occurs in wild-type cells (43). This result is consistent with the idea that GLC7 dephosphorylates glycogen synthase, since the *glc7-1* mutation would lead to increased phosphorylation of glycogen synthase and thereby inhibit the activity of this enzyme.

Unlike GCN2, which is essential for growth only under amino acid starvation conditions (46), a deletion of *GLC7* is unconditionally lethal (10, 18). The essential function of GLC7 is not likely to be involved in regulating glycogen synthesis because disruptions in both known genes encoding glycogen synthase lead to a viable mutant devoid of glycogen (17). Thus, it appears that the GLC7 protein phosphatase performs additional functions, at least one of which is essential under normal growth conditions. For example, a type 1 protein phosphatase in the fission yeast *S. pombe* performs an essential function in chromosome disjunction during mitosis (34, 41). Additional experiments are required to identify target proteins of the GLC7 phosphatase in *S. cerevisiae* and to probe yeast cells for the involvement of regulatory subunits of the kind characterized in mammalian systems.

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