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GCN1, a Translational Activator of *GCN4* in *Saccharomyces cerevisiae*, Is Required for Phosphorylation of Eukaryotic Translation Initiation Factor 2 by Protein Kinase GCN2

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Phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2α) by the protein kinase GCN2 mediates increased translation of the transcriptional activator *GCN4* in amino acid-starved yeast cells. We show that this key phosphorylation event and the attendant translational induction of *GCN4* are dependent on the product of a previously uncharacterized gene, *GCN1*. Inactivation of *GCN1* did not affect the level of eIF-2α phosphorylation when mammalian eIF-2α kinases were expressed in yeast cells in place of GCN2, arguing against an involvement of GCN1 in dephosphorylation of eIF-2α. In addition, while GCN1 is required in vivo for phosphorylation of eIF-2α by GCN2, cell extracts from *gcn1Δ* strains contained wild-type levels of GCN2 eIF-2α-kinase activity. On the basis of these results, we propose that GCN1 is not needed for GCN2 kinase activity per se but is required for in vivo activation of GCN2 in response to the starvation signal, uncharged tRNA. GCN1 encodes a protein of 297 kDa with an 88-kDa region that is highly similar in sequence to translation elongation factor 3 identified in several fungal species. This sequence similarity raises the possibility that GCN1 interacts with ribosomes or tRNA molecules and functions in conjunction with GCN2 in monitoring uncharged tRNA levels during the process of translation elongation.

Starvation for an amino acid or the presence of a defective aminoacyl-tRNA synthetase in the yeast *Saccharomyces cerevisiae* leads to increased transcription of more than 30 genes encoding amino acid biosynthetic enzymes in 10 different pathways. This response, called general amino acid control (reviewed in reference 35), is mediated by the transcriptional activator GCN4 that binds to promoter regions of the coregulated structural genes subject to the general control. Expression of *GCN4* itself is regulated by the availability of amino acids, but at the level of translation initiation. When amino acids are abundant, four short upstream open reading frames (uORFs) in the leader of *GCN4* mRNA restrict the flow of scanning ribosomes to the *GCN4* initiation codon. Under starvation conditions, *trans*-acting positive factors encoded by *GCN2* and *GCN3* allow ribosomes to bypass the most inhibitory of the uORFs and initiate translation at the *GCN4* AUG codon, thus increasing the level of GCN4 protein in the cell (1, 35).

GCN2 is a protein kinase that stimulates *GCN4* translation by phosphorylating the α subunit of eukaryotic translation initiation factor 2 (eIF-2α; encoded by *SUI2*) on the serine residue at position 51 (20, 75). In mammalian cells, phosphorylation of eIF-2α on serine 51 inhibits general translation initiation by impairing the guanine nucleotide exchange factor for eIF-2, known as eIF-2B. GDP-GTP exchange on eIF-2 catalyzed by eIF-2B is essential for translation because only the GTP-bound form of eIF-2 can deliver initiator $tRNA^{Met}$ to small ribosomal subunits (32). By analogy with mammalian systems, it was proposed that eIF-2α phosphorylation by GCN2 in *S. cerevisiae* would decrease the level of eIF-2 activity in the cell. As a result, ribosomes which had translated the first uORF and resumed scanning would not rebind initiator $tRNA^{Met}$ until after bypassing the remaining uORFs, allowing them to reinitiate translation at *GCN4* instead (1, 20). This hypothesis can explain why reduced-function mutations in essential subunits of eIF-2 (encoded by *SUI2* [13], *SUI3* [23], and *GCD11* [30]) or eIF-2B (encoded by *GCD1*, *GCD2*, *GCD6*, and *GCD7* [4, 5, 11, 12]), all of which should diminish eIF-2 activity, lead to high-level *GCN4* translation independent of GCN2 and amino acid availability (4, 35, 76). GCN3 is a nonessential subunit of eIF-2B that is believed to mediate the inhibitory effect of phosphorylated eIF-2 on the guanine nucleotide exchange activity of eIF-2B (19, 29, 31, 56).

GCN2 contains a large region adjacent to the protein kinase domain that is similar in sequence to histidyl-tRNA synthetases (HisRSs) and is required in vivo for phosphorylation of eIF-2α under starvation conditions (20, 74, 75). Mutations in the HisRS-like region of GCN2 which lead to constitutive activation of GCN2 kinase function and alter amino acids conserved among class II aminoacyl-tRNA synthetases have been identified (56). These findings, plus the fact that uncharged tRNA is a more direct signal than amino acid limitation for derepression of *GCN4* (reviewed in reference 35), led to the hypothesis that the HisRS-related segment of GCN2 is a regulatory region involved in the recognition of uncharged tRNA and activation of GCN2 kinase function under starvation conditions.

In this report, we investigate the role of a previously uncharacterized gene known as *GCN1* in the general control pathway. We have cloned this gene and found that it encodes a protein with a predicted molecular mass of nearly 300 kDa containing an 88-kDa segment that is very similar in sequence to yeast translation elongation factor 3 (EF-3) (22, 50, 54, 60, 77). We show that GCN1 is a translational activator of *GCN4* because loss of *GCN1* function renders *GCN4* nonderepressible and because its ability to stimulate *GCN4* expression is completely dependent on the uORFs in

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GCN4 mRNA. Our experiments indicate that GCN1 is required in vivo for the phosphorylation of eIF-2α on serine 51 catalyzed by GCN2 under conditions of amino acid starvation. GCN1 does not appear to act by inhibiting an eIF-2α phosphatase nor by stimulating *GCN2* expression. In addition, GCN2 protein isolated from *gcn1*Δ extracts specifically phosphorylates purified eIF-2 in vitro, indicating that GCN1 is not required for the ability of GCN2 to bind and phosphorylate its physiological substrate. These results, coupled with the intriguing finding that GCN1 shows sequence similarity with a factor that plays an essential role in translational elongation, possibly by escorting tRNA substrates onto and off of the ribosome (7, 69), suggest that GCN1 is involved in sensing uncharged tRNA and stimulating the kinase activity of GCN2 in amino acid-starved cells.

MATERIALS AND METHODS

Plasmids. Plasmids p664, p665, and p666 were isolated by selecting for complementation of the 3-amino-1,2,4-triazole (3-AT; Fluka Chemical Corp.)-sensitive phenotype of the *gcn1-1* allele after transformation (37) of strain H1169 with a genomic library (57) containing 15- to 30-kb fragments from a partial *Sau*3A digest cloned into the *Bam*HI site of YCp50 (51). These three plasmids have related restriction enzyme digestion patterns, but since p665 gave the strongest complementation of *gcn1-1*, it was subjected to further study. Plasmid pLC13 was constructed by subcloning the *Sna*BI-*Sal*I fragment of p665 into *Sma*I- and *Sal*I-digested pRS316 (64). Plasmid pLC15 was constructed by digesting pLC13 with *Kpn*I and religating it. Plasmid pLC17 was constructed by digesting pLC13 with *Xho*I (in the polylinker) and *Nru*I, treating it with Klenow fragment to create blunt ends, and religating the result. pLC12 was constructed by subcloning the 5.5-kb *Sal*I-*Sac*II fragment from p665 into *Sal*I- and *Sac*II-digested pRS316. pLC20 was constructed in two steps: first, the *Xho*I site in pLC13 was filled in with Klenow fragment, creating plasmid pLC16, which was then digested with *Not*I (in the polylinker) and *Pfl*MI, filled in with Klenow fragment, and religated. pLC27 was constructed by digesting pLC13 with *Eam*I105I in the presence of ethidium bromide at 50 μg/ml (52). Linearized plasmid was subjected to electrophoresis through 1% low-melting point agarose, gel purified, treated with Klenow fragment, and religated. Clones which had lost the *Eam*I105I site in the genomic *GCN1* sequence (as opposed to losing the *Eam*I105I site in the ampicillin resistance gene) were isolated. Destruction of the *Eam*I105I site in the *GCN1* sequence was confirmed by double digests with *Eam*I105I and various restriction endonucleases.

Plasmid pLC23, used to obtain the null allele of *GCN1*, was constructed in two steps: (i) the 1.1-kb *Bgl*II-*Sal*I fragment from the 3′ end of the *GCN1* gene was cloned into the *Bam*HI-*Sal*I sites of plasmid pNKY51 (2), creating plasmid pLC19, and (ii) the 2.4-kb *Bgl*II fragment from sequences immediately upstream of the *GCN1* coding sequence was cloned into the *Bgl*II site of plasmid pLC19 so that the *GCN1* coding strand was in the same orientation as the *Bgl*II-*Sal*I fragment. The integrating plasmid pLC1 was constructed by subcloning the 4.1-kb *Sal*I-*Xba*I fragment from p665 into *Sal*I- and *Xba*I-digested pRS305 (64). The integrating plasmid pLC3 was constructed by subcloning the 6.4-kb *Cla*I-*Sac*II fragment from p665 into pRS306 (64). Plasmids pLC1 and pLC3 were used to direct integration of *LEU2* and *URA3* markers, respectively, into the yeast genome at the *GCN1* locus. Plasmid pLC25, used for RNase protection experiments, was constructed by subcloning the 900-bp *Xba*I fragment of p665 into pRS305 in the orientation allowing transcription of antisense RNA from the *GCN1* sequences by T3 polymerase.

Plasmids p180, p226, and p227, which contain the wild-type GCN4 leader, the leader with only ORF4, and the leader with no uORFs, respectively, have been previously described (49). Plasmid p585 contains the wild-type *GCN2* gene on the low-copy-number *URA3*-containing plasmid YCp50 (75). Plasmids p630 and p644 contain the wild-type *GCN2* gene or the *gcn2-K559R* allele, respectively, on the high-copy-number *URA3* vector YEp24 (75). The various *GCN2*^{c} mutations employed in this study have been described elsewhere (56) and are designated with the wild-type amino acid (single-letter code) followed by its position relative to the N terminus and the substituting amino acid in the mutant allele. Plasmid p914 contains the *GCN2*^{c}*-E1537G* (also known as *GCN2*^{c}*-517*) allele, and plasmid p1056 contains the *GCN2*^{c}*-E537K,E1522K* (also known as *GCN2*^{c}*-516*) allele. Plasmids p1246 and p1247 were kindly provided by Glen Barber and Michael Katze (University of Washington, Seattle) and contain the wild-type double-stranded-RNA-dependent protein kinase (dsRNA-PK) gene and the mutated form of the gene carrying the K296R substitution, respectively, subcloned into the high-copy-number pEMBLyex4 vector (19). Plasmids p1420 and p1421 were generously supplied by Jane-Jane Chen (Massachusetts Institute of Technology) and contain the wild-type heme-regulated inhibitor (HRI) gene and the mutated form of the gene carrying the K199R substitution subcloned into the pEMBLyex4 vector (19).

Strains and genetic techniques. Table 1 lists the yeast strains used in this study. Strains H1048 and H1169 were constructed by tetrad analysis of several genetic crosses between *GCN* strains in our collection and a *gcn1-1* mutant kindly provided by Peter Niederberger and Ralf Hütter, scoring *gcn1-1* by sensitivity to 3-AT. Complementation of the *gcn1-1* mutation (62) was scored by the ability to grow on SD medium (63) supplemented with all amino acids at 0.1 mg/ml, except for leucine (present at 40 mM) and histidine (omitted), plus 3-AT at 30 mM. Analysis of the growth rate of strains containing the dsRNA-PK or the HRI protein kinase was carried out on the following media: 3-AT as described above; SGAL, identical to SD medium except containing 10% galactose instead of 2% glucose as the carbon source; SR, identical to SD medium except containing 2% raffinose instead of glucose; SR + 3-AT, SR medium containing 30 mM 3-AT. For growing strains in raffinose, saturated cultures grown in SD medium were diluted at least 1:50 into SR medium and cells were harvested 12 h after inoculation. Additional procedures for testing amino acid analog sensitivity have been described previously (36).

The *gcn1*Δ*::URA3* strain H2078 was constructed by transforming strain F113 to Ura$^+$ with the 6.7-kb *Spe*I-*Sal*I fragment from pLC23, containing a replacement of 7.3 kb of *GCN1* sequences (between the *Bgl*II sites at nucleotide [nt] −83 and at nt 7263; see Fig. 2) with the *URA3* gene flanked by a direct repeat of 1.1 kb from the *Salmonella hisG* gene (2). The *GCN1* deletion begins approximately at the start site of transcription and leaves approximately 1 kb of *GCN1* coding sequence at the C terminus (see Fig. 1). Ura$^+$ transformants were selected, and the occurrence of the desired gene replacement was confirmed by DNA blot hybridization analysis (see below). The direct repeats flanking the *URA3* gene provided a means to select for homologous recombination events that evict the *URA3* gene from

TABLE 1. Yeast strains

Strain	Genotype[a]	Source or reference
H3	*MATα ura3-52 leu2-3 leu2-112*	This study
H1048	*MATa leu2-3 leu2-112 gcn1-1*	This study
H1056	*MATa/MATα leu2-3/leu2-3 leu2-122/leu2-122 ura3-52/ura3-52*	This study
H1169	*MATa ura3-52 gcn1-1*	This study
H1515	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112*	A. M. Cigan
H1816	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2 LEU2) trp1::GCN4-lacZ TRP1 gcn2Δ*	20
H1817	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2-S51A LEU2) trp1::GCN4-lacZ TRP1 gcn2Δ*	20
H1896	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2 LEU2) trp1::GCN4-lacZ TRP1*	20
H1897	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2-S51A LEU2) trp1::GCN4-lacZ TRP1*	20
H1898	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2-S51D LEU2) trp1::GCN4-lacZ TRP1*	20
H2078	*MATa ino1 ura3-52 can1 gcn1Δ::URA3*	This study
H2079	*MATa ino1 ura3-52 can1 gcn1Δ*	This study
H2082	*MATa trp1Δ-63 ura3-52 leu2-3 leu2-112 gcn1Δ::URA3*	This study
H2084	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2 LEU2) trp1::GCN4-lacZ TRP1 gcn2Δ gcn1Δ::URA3*	This study
H2085	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2 LEU2) trp1::GCN4-lacZ TRP1 gcn2Δ gcn1Δ*	This study
H2086	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2-S51A LEU2) trp1::GCN4-lacZ TRP1 gcn2Δ gcn1Δ::URA3*	This study
H2087	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2-S51A LEU2) trp1::GCN4-lacZ TRP1 gcn2Δ gcn1Δ*	This study
H2088	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2 LEU2) trp1::GCN4-lacZ TRP1 gcn1Δ::URA3*	This study
H2089	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2 LEU2) trp1::GCN4-lacZ TRP1 gcn1Δ*	This study
H2090	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2-S51A LEU2) trp1::GCN4-lacZ TRP1 gcn1Δ::URA3*	This study
H2091	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2-S51A LEU2) trp1::GCN4-lacZ TRP1 gcn1Δ*	This study
H2092	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2-S51D LEU2) trp1::GCN4-lacZ TRP1 gcn1Δ::URA3*	This study
H2093	*MATa trp1-Δ63 ura3-52 leu2-3 leu2-112 sui2Δ (SUI2-S51D LEU2) trp1::GCN4-lacZ TRP1 gcn1Δ*	This study
F113	*MATa ino1 ura3-52 can1*	24

[a] The *SUI2* or *SUI2-S51A* alleles enclosed in parentheses are present on autonomously replicating single-copy-number (*CEN*) plasmids containing *LEU2*. Strains harboring *GCN4-lacZ* fusions contain the wild-type *GCN4* leader fused to *lacZ* (34) integrated between *trp1-Δ63* and *TRP1* (20).

the locus and create unmarked *gcn1Δ* alleles. Strain H2078 was replica plated to plates containing 5-fluoroorotic acid (PCR Inc., Gainesville, Fla.), and strain H2079 was isolated as a revertant resistant to 5-fluoroorotic acid (3). The desired homologous recombination event was confirmed by DNA blot hybridization of genomic DNA (see below). The same procedure was employed to generate several sets of isogenic strains containing either *gcn1Δ::URA3* or unmarked *gcn1* deletions. Thus, strains H1816, H1817, H1896, H1897, and H1898 were transformed with the 6.7-kb *Spe*I-*Sal*I fragment to construct *gcn1Δ::URA3* strains H2084, H2086, H2088, H2090, and H2092, respectively. These strains were then replica plated to 5-fluoroorotic acid plates to create the Ura$^-$ *gcn1Δ* strains H2085, H2087, H2089, H2091, and H2093, respectively.

Genetic demonstration that *GCN1* was cloned. To confirm that the genomic insert in plasmid p665 contained the *GCN1* gene, two different fragments of the insert were used to direct integration of nonreplicating *URA3* or *LEU2* plasmids to sites homologous to the cloned yeast DNA sequences. Plasmid pLC3 was linearized with *Mlu*I and used to transform the *ura3-52 gcn1-1* strain H1169 to Ura$^+$. Likewise, plasmid pLC1 linearized with *Mlu*I was used to transform the *leu2-3 leu2-112 gcn1-1* strain H1048 to Leu$^+$. Ura$^+$ and Leu$^+$ transformants, respectively, were selected and, as expected, were found to be 3-AT sensitive. Strain H1169 with the integrated pLC3 plasmid was mated to the *ura3-52 leu2-3 leu2-112 GCN1* strain H3. Diploids were isolated and sporulated. Sixteen tetrads were dissected, and in all cases the Ura$^+$ and 3-AT-sensitive phenotypes cosegregated 2$^+$:2$^-$. Strain H1048 with the integrated pLC1 plasmid was mated to strain H3. Diploids were isolated and sporulated, and 15 tetrads were dissected; in all cases the Leu$^+$ and 3-AT-sensitive phenotypes cosegregated. These results indicated that the integrated *URA3* and *LEU2* markers were tightly linked to *GCN1*, confirming that the genomic insert in p665 contains the authentic *GCN1* gene.

DNA blot hybridization of yeast transformants. To confirm the structure of gene replacements at *GCN1*, genomic DNA was isolated from transformants by the procedure of Davis et al. (18) as modified by Moehle and Hinnebusch (47). DNA was digested with *Eco*RI and subjected to electrophoresis on 1% agarose gels. DNA fragments were transferred to GeneScreen Plus membranes (NEN) by the capillary transfer method of Southern (67) as modified by Sambrook et al. (58). Filters were probed with the 2.4-kb *Bgl*II fragment from the *GCN1* promoter region radiolabeled by the random priming technique (58). The probe hybridized to a 3.0-kb fragment from parental strains, a 6.5-kb fragment from strains harboring the *gcn1Δ::URA3* gene replacement, and a 3.6-kb fragment from strains carrying the unmarked *gcn1Δ* deletion allele (data not shown).

Chromosomal mapping of *GCN1*. *GCN1* was mapped to chromosome VII by using a Chromo-Blot purchased from Clontech, as follows. Chromosomes were fractionated by clamped homogeneous electrical field electrophoresis, and a blot of that gel containing two identical lanes was probed with a radiolabeled 2.3-kb *Sac*II-*Sac*II fragment from pLC13, extending over the 5′ end of the gene and the first 2.2 kb of *GCN1* coding sequence (one of the *Sac*II sites is in the polylinker of pLC13). The same probe was hybridized to a set of filters containing an ordered lambda library of yeast

genomic DNA fragments obtained from Linda Riles and Maynard Olson, following the protocol provided by Riles and Olson (56a). The probe hybridized to three overlapping clones (numbers 5880, 5415, and 6012), one of which contains the *CDC55* gene, consistent with our assignment of *GCN1* to chromosome VII.

DNA sequence analysis. The sequence of the smallest fragment from p665 which complemented *gcn1-1* was determined by the dideoxy chain termination method (61) by Lofstrand Labs Ltd. (Gaithersburg, Md.). The results were confirmed by sequencing both strands at least twice. Sequences were analyzed by using the Genetics Computer Group sequence analysis software package (21). The symbol comparison table of Gribskov and Burgess (28), which is based on the Dayhoff PAM-250 matrix, was used in all sequence comparisons reported here except where indicated.

Comparisons of the GCN1 protein sequence were done by using FASTA with a word size of 2 to search GenBank and a word size of 1 to search the SWISSPROT data base (53). The PILEUP program (gap weight = 3, gap length = 0.1, and sequence comparison table GenRunData:PileUpPep.Cmp) was used to align the entire sequence of GCN1 with the three EF-3 proteins (see Fig. 4). The PRETTY program displayed the alignment with a consensus sequence using a plurality of 3.0. The consensus sequence was altered by hand as described in the legend to Fig. 4.

The BESTFIT program was used to determine the statistical significance of the alignments obtained from the FASTA or REPEATS program. This program aligns the first sequence in the alignment with a set of randomized sequences of the same length and composition as the second sequence and calculates the quality score for each of the alignments. Statistical significance is reported as the number of standard deviations by which the quality score of the actual alignment differs from the mean quality score derived from the randomized sequences. The number of randomizations employed was always at least 100.

The REPEATS program using a window of 65 and stringency of 40 was used to identify the internal repeat in *GCN1*. Two repeats were discovered initially. A 51-amino-acid region (amino acid positions 1624 to 1674) was found to be 41% identical and 61% similar to the region between 1887 and 1937. The statistical significance of this repeat was determined by using the BESTFIT program as described above. Its significance is greater than 13 standard deviations above the mean quality score of randomized sequences. A second repeat from 1385 to 1474 and 1701 to 1790 is 29% identical and 53% similar over these 90 amino acids and has a significance of 12 standard deviations above the mean. These repeats were combined to form the repeat described in Results. Finally, we noticed that the sequence RXXAAXXLXXLVXXXG or a very closely related sequence was repeated five times in *GCN1*, beginning at amino acids positions 1624, 1687, 1344, 1663, and 2312.

Analysis of *GCN1* expression and regulatory function. (i) Amino acid analog sensitivity. Plasmid-borne *GCN1* alleles were tested for complementation of *gcn1-1* for the inability to depress *HIS3* expression by measuring the growth rate of strain H1169 or F113 transformants replica plated to medium containing 30 mM 3-AT and excess (40 mM) leucine (36), as described above.

(ii) Assay of *GCN4-lacZ* fusions. β-Galactosidase assays were conducted as previously described (44). Strains F113 (*GCN1*) and H2079 (*gcn1*Δ) were transformed with plasmids bearing translational fusions between *GCN4* and *lacZ* containing mutations which remove various uORFs in the *GCN4* mRNA leader. Transformants were grown in SD medium (63) supplemented with 0.2 mM inositol. For repressing conditions, saturated cultures were diluted 1:50, grown at 30°C with rotary agitation at 275 rpm, 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 the addition of 3-AT to 10 mM.

(iii) Analysis of *GCN1*, *HIS4*, *PYK1*, and Ty1 mRNA abundance. Isogenic strains F113 (*GCN1*) and H2078 (*gcn1*Δ::*URA3*) were grown under repressing and derepressing conditions as described above, except that strain F113 was also supplemented with 0.2 mM uracil. Total RNA was extracted and subjected to formaldehyde-agarose gel electrophoresis and blot hybridization analysis as described previously (36). *GCN1*, *HIS4*, *PYK1*, and Ty1 mRNAs were probed, respectively, with the 1.1-kb *GCN1* *Sac*II-*Mlu*I fragment of pLC13, the 2.8-kb *HIS4* *Eco*RI fragment from pR5 (25), the *Hin*dIII-digested *PYK1* plasmid pFR2 (40), and the 1.3-kb *Hin*dIII-*Eco*RI fragment from pBD6-456 containing the Ty1 element (obtained from Tom Burkett, Frederick Cancer Research and Development Center, Frederick, Md.).

Isoelectric focusing gels. Saturated cultures of the strains to be analyzed were diluted 1:35 and grown in SD medium (supplemented with 0.2 mM uracil where appropriate) for 6 h at 30°C with shaking. For derepressing conditions, 1 h prior to harvesting, cultures were supplemented with 3-AT to 10 mM. Preparation of the extracts, vertical-slab gel isoelectric focusing, and detection of eIF-2α by immunoblot analysis were carried out as described by Dever et al. (20). For experiments employing the HRI kinase instead of GCN2, saturated cultures grown in SD medium were diluted 1:50 in SR medium and harvested in logarithmic phase 12 h later. Cells were harvested, and extracts were analyzed as described above.

In vitro protein kinase assays. The method of Wek et al. (75) with the following modifications was used for the preparation of extracts and analysis of the GCN2 kinase activity. Saturated cultures of the strains to be analyzed were diluted 1:100 and grown under repressing or derepressing conditions as described above for β-galactosidase assays. To analyze GCN2 autophosphorylation, 400 μg of protein extract was incubated overnight with GCN2 antiserum. Immune complexes were collected by centrifugation, washed three times with TN buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM sodium molybdate, 1 mM dithiothreitol plus detergents, and additional protease and phosphatase inhibitors according to the description of Wek et al. [75]), once in TN buffer without detergents, and twice in KIN buffer (25 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 50 mM NaCl, and 1 mM dithiothreitol plus the protease and phosphatase inhibitors present in TN buffer). In assays containing rabbit eIF-2 as the substrate, the purified eIF-2 was preincubated with 20 μM cold ATP for 9 min at 30°C before the addition of 10 μCi of [γ-^{32}P]ATP. The eIF-2 was kindly provided by William C. Merrick (Case Western Reserve University, Cleveland, Ohio).

Nucleotide sequence accession number. The GenBank accession number for *GCN1* is L12467.

RESULTS

Characterization of the *GCN1* gene. The *gcn1-1* mutant was isolated on the basis of causing increased sensitivity to an inhibitor of tryptophan biosynthesis and was shown to

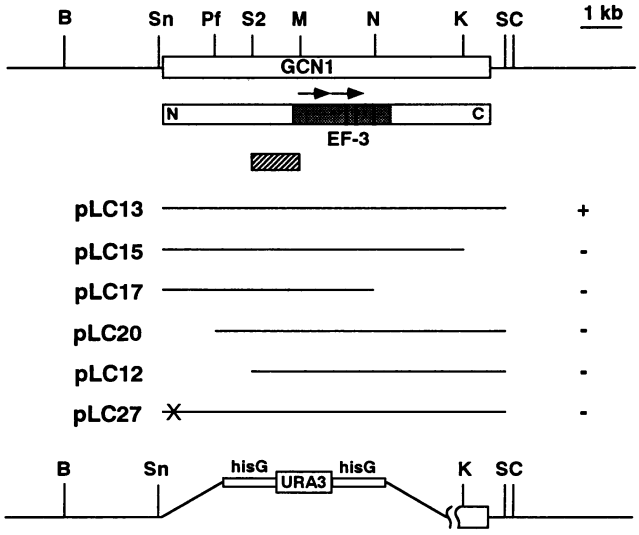

FIG. 1. Functional map of *GCN1*. The top line represents genomic DNA containing the *GCN1* gene, with the open box designating the GCN1 protein-coding sequence oriented 5′ to 3′. Immediately below it is a representation of the GCN1 protein, including amino (N) and carboxy (C) termini. The region with homology to EF-3 is indicated by shading. Within this region, vertical bars indicate the positions corresponding to the bipartite nucleotide-binding motifs in the homologous portions of EF-3. The region containing the second part of the C-proximal nucleotide-binding motif is absent in GCN1; in addition, the conserved amino acids of these motifs are absent in GCN1. The internal repeat of GCN1 is indicated by the two arrows. The hatched box below represents the stretch of DNA used as a probe in the RNA hybridization experiments. To the right are indicated the complementation activities of various partial deletion alleles of *GCN1* carried on the indicated plasmids: +, wild-type growth after replica plating to medium containing 30 mM 3-AT; −, very poor growth under the same conditions, indistinguishable from that given by vector alone. The position of a frameshift mutation in pLC27 at the *Eam*I105I site within the GCN1 coding sequence is indicated (X). The extent of the deletion in the *gcn1Δ::URA3* allele constructed in pLC23 is shown by the portion of the *GCN1* gene replaced by the *URA3* gene flanked by *hisG* repeats. Letter designations for restriction sites: B, *Bam*HI; Sn, *Sna*BI; Pf, *Pfl*MI; S2, *Sac*II; M, *Mlu*I; N, *Nru*I; K, *Kpn*; C, *Cla*I; S, *Sal*I.

prevent derepression of enzymes subject to the general control in response to an amino acid starvation (62). The *GCN1* gene was cloned from a yeast plasmid genomic library (57) by complementation of the 3-AT-sensitive phenotype conferred by the *gcn1-1* mutation in strain H1169. Three complementing plasmids (p664, p665, and p666) with related restriction endonuclease digestion patterns were isolated. Plasmid p665, which contains a ca. 15-kb insert, gave the strongest complementation of *gcn1-1*. Fragments from the putative *GCN1* genomic insert in p665 were subcloned in a nonreplicating *URA3* plasmid and used to direct plasmid integration to the homologous sequences in the yeast genome. Genetic analysis revealed that the integrated *URA3* marker in these transformants was tightly linked to *gcn1-1*, confirming that the plasmids contain the authentic *GCN1* gene (see Materials and Methods for details).

The boundaries of the *GCN1* complementation unit were determined by deleting portions of the genomic insert in p665 and subcloning various restriction fragments from the insert into the low-copy-number plasmid pRS316 and then by testing the resulting constructs for complementation of the 3-AT-sensitive phenotype of *gcn1-1*. An 8.4-kb *Sna*BI-*Sal*I fragment from the *GCN1* region contained in plasmid pLC13 was found to fully complement the 3-AT sensitivity of strain H1169 (Fig. 1). Deletion from pLC13 of the 1.4-kb *Sna*BI-*Pfl*MI fragment (generating pLC20) or the 895-bp *Kpn*I-*Sal*I fragment (generating pLC15) completely eliminated complementation of *gcn1-1* (Fig. 1). These results suggested that the *GCN1* complementation unit extended over most of the 8.4-kb *Sna*BI-*Sal*I fragment.

The DNA sequence of this fragment was determined and found to contain a large ORF of 8,019 bp, beginning at a methionine codon located only 124 bp downstream of the *Sna*BI site (Fig. 2). The smallest 5′ and 3′ deletions shown in Fig. 1 which destroyed the complementing activity of the 8.4-kb *Sna*BI-*Sal*I fragment removed coding sequences from the ends of this ORF, indicating that the *GCN1* genetic complementation unit is coextensive with the ORF. To test whether the methionine codon present 124 bp downstream of the *Sna*BI site is the *GCN1* initiation codon, we introduced a +1 frameshift mutation at the *Eam*I105I site located about 195 bp downstream of this ATG triplet in plasmid pLC13, creating pLC27. As expected if the *GCN1* ORF begins upstream of this site, the mutation in pLC27 abolished the complementing ability of the plasmid-borne sequences (Fig. 1). This finding, plus the fact that the 124 bp upstream of the putative initiator methionine codon contains at least four stop codons in each of the reading frames, led us to conclude that the ATG triplet at position +1 in the *GCN1* sequence (Fig. 2) is the initiation codon. Our results predict that *GCN1* encodes a protein 2,672 amino acids in length with a molecular mass of 296,695 Da.

We used RNA blot hybridization analysis to determine whether *GCN1* mRNA is large enough to encode the predicted ORF of 8,019 bp. Using a 1.2-kb *Sac*II-*Mlu*I fragment from within the *GCN1* ORF as the hybridization probe (Fig. 1), we detected a single transcript of ca. 8.5 kb (Fig. 3). In addition, the approximate 5′ end of *GCN1* mRNA was localized by RNase protection mapping to a region ca. 70 nt upstream of the ATG codon at +1 in the *GCN1* sequence (44a). The results shown in Fig. 3 also indicate that the abundance of *GCN1* mRNA does not increase significantly under amino acid starvation conditions when transcription of genes subject to the general control is being stimulated by GCN4. Relative to the levels of *PYK1* mRNA (encoding pyruvate kinase) and Ty1 RNA examined as unregulated controls, little or no increase in *GCN1* mRNA abundance was observed in response to histidine starvation of the wild-type *GCN1* strain, whereas *HIS4* mRNA showed the expected derepression under these conditions (24). In accord with the idea that GCN1 is a positive effector in the general control system, deletion of *GCN1* substantially reduced the derepression of *HIS4* mRNA in response to starvation (Fig. 3). The residual *HIS4* derepression observed in the *gcn1Δ::URA3* strain is consistent with our previous results (31), indicating the existence of a GCN4-independent component of HIS4 regulation by amino acid levels.

We constructed a deletion of *GCN1* in several haploid yeast strains that replaces 7.3 kb of the 8.02-kb ORF with the *URA3* gene. In each case, the replacement of *GCN1* with the *gcn1Δ::URA3* allele was confirmed by DNA blot hybridization analysis. As expected, deletion of *GCN1* in these strains led to a 3-AT-sensitive phenotype that could not be complemented by crossing to a *gcn1-1* strain.

We observed that transformants containing *gcn1Δ::URA3* were obtained at essentially the same frequencies in haploid and diploid strains, suggesting that deletion of *GCN1* has no

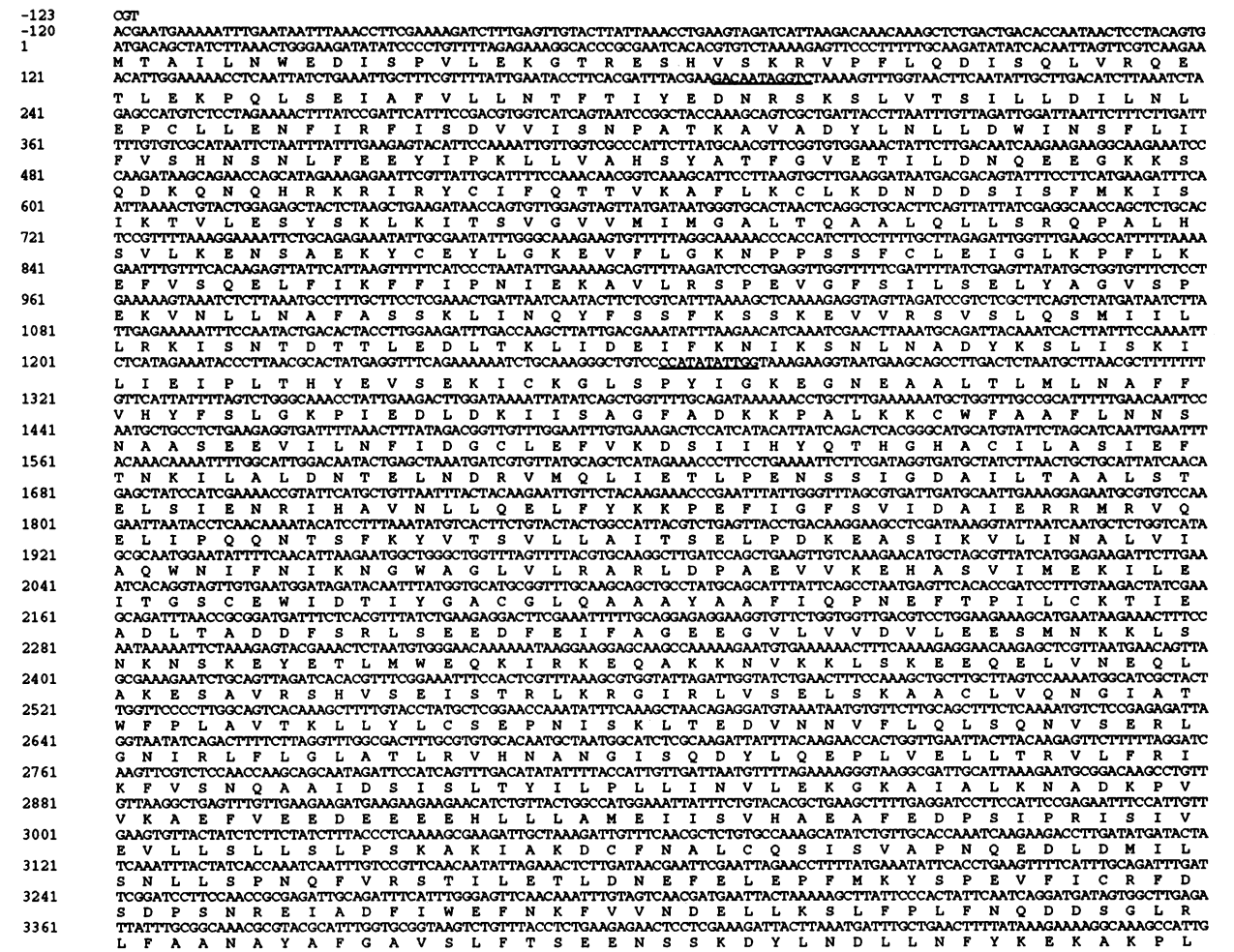

FIG. 2. Nucleotide sequence and deduced amino acid sequence of *GCN1*. The sequence begins at the *Sna*BI site 123 nt upstream of the presumed initiator ATG of the GCN1 protein sequence and extends to the *Sal*I site 521 nt downstream of the TGA stop codon. The numbers on the left and right are nucleotide and codon positions, respectively, numbered from the first methionine at +1. The *Eam*I105I site at nt 192 (the site of the frameshift mutation in plasmid pLC27) is underlined. Sites denoting the endpoints of deletions in plasmids pLC15 (the *Pfl*MI site at nt 1267) and pLC20 (the *Kpn*I site at nt 7424) are also underlined. The shaded region indicates the portion of GCN1 that is homologous to the EF-3 proteins. The internal repeat of GCN1 is also underlined.

effect on cell viability under nonstarvation conditions. To test directly whether *GCN1* is required for growth under nonstarvation conditions, we deleted the *GCN1* gene from one homolog and replaced it with the *URA3* gene in the *ura3-52/ura3-52* diploid strain H1056, as described in Materials and Methods. The strain harboring the *gcn1*Δ::*URA3* gene replacement was sporulated; in 29 tetrads we observed $2^+:2^-$ segregation for both Ura prototrophy and 3-AT sensitivity, and all Ura^+ spores were 3-AT sensitive. This finding indicates that the *gcn1*Δ strains were viable and 3-AT sensitive. In addition, we saw no effect on growth rate under nonstarvation conditions when *gcn1*Δ strains were transformed with a plasmid bearing *GCN1* (data not shown). On the basis of these results, we conclude that *GCN1* is a nonessential gene which is required for wild-type growth only when cells are starved for an amino acid.

GCN1 was localized to chromosome VII in the vicinity of the *CDC55* gene by hybridizing a radiolabeled *GCN1* probe to an ordered lambda library of yeast genomic DNA fragments (data not shown; see Materials and Methods).

Sequence similarity between GCN1 and a translation elongation factor present in fungi. A search of the GenBank data base revealed that a portion of the GCN1 protein of more than 800 amino acids is highly similar in sequence to a protein identified in several fungal species known as EF-3. In *S. cerevisiae*, EF-3 is a protein of 116 kDa with ribosome-dependent ATPase and GTPase activities that is essential for translation elongation in vitro (16, 65, 66). At present, EF-3 has no obvious counterpart in the mammalian or wheat germ translational apparatus. Biochemical analysis of the *S. cerevisiae* EF-3 protein has led to the proposal that EF-3 stimulates EF-1α-dependent aminoacyl-tRNA binding to the ribosomal A site during elongation (8, 38). More recent studies suggest that EF-3 promotes translation elongation by stimulating the release of deacylated tRNA from the E site (69). This function appears to require the ribosome-dependent nucleoside triphosphatase (NTPase) activity of EF-3, which is influenced by deacylated tRNA (58a, 69).

Figure 4 shows a sequence alignment between 808 residues (residues 1330 to 2137) of GCN1 and the homologous sequences of EF-3 from *S. cerevisiae* (54, 60), *Candida albicans* (22, 50), and *Pneumocystis carinii* (77). The three

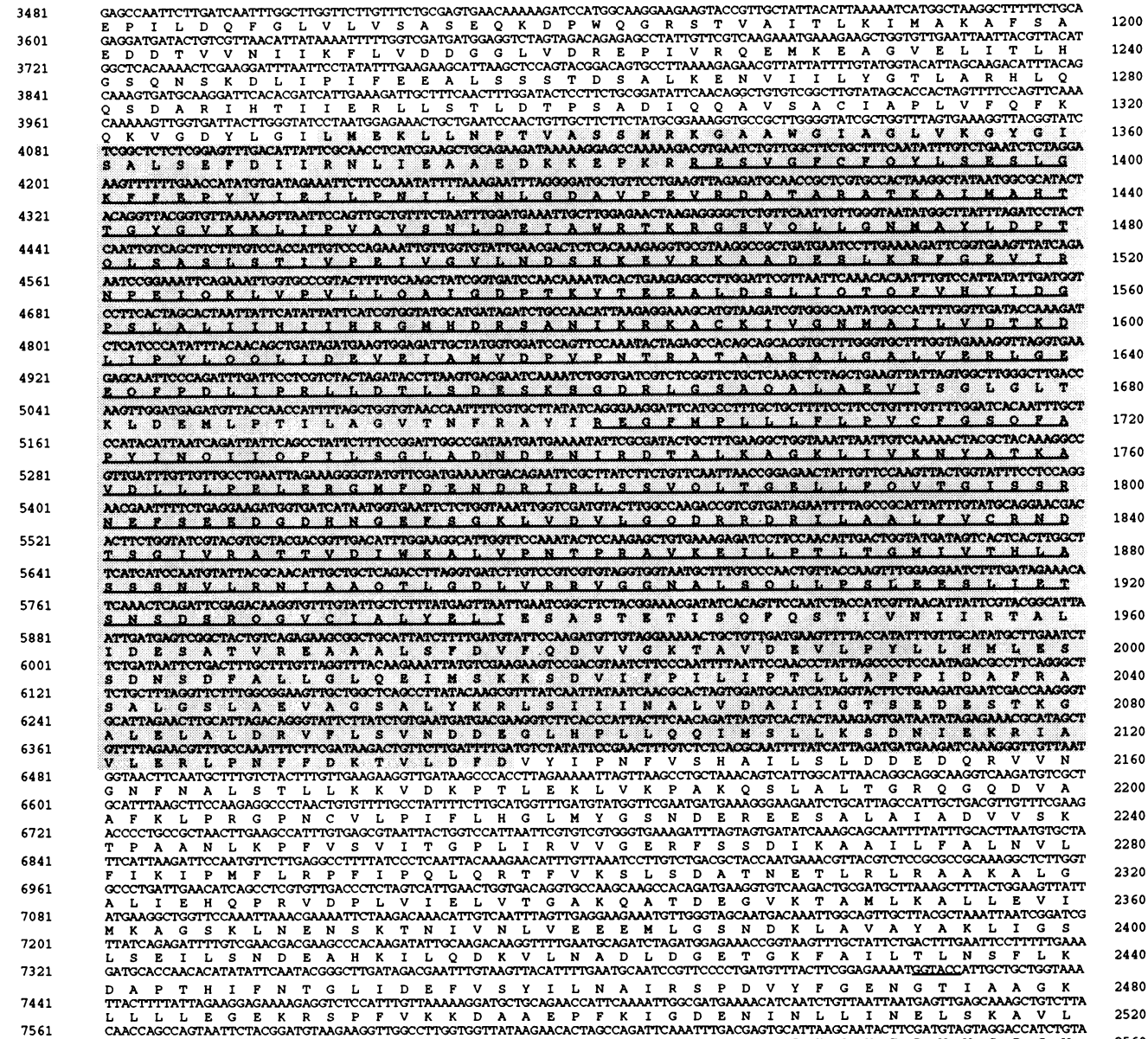

FIG. 2—*Continued.*

EF-3 genes are extremely similar, with the *S. cerevisiae* protein sequence showing 77% identity and 84% similarity to the *C. albicans* protein and 58% identity and 72% similarity to EF-3 of *P. carinii*. All three EF-3 proteins possess identical amino acids at 48% of the positions. Among these invariant residues, GCN1 contains the identical amino acid or a conservative replacement 25 and 48% of the time, respectively. A comparison of GCN1 with the *S. cerevisiae* EF-3 protein indicated that the proteins are 21% identical and 48% similar over a region of 855 amino acids (representing greater than 80% of the EF-3 sequence). The statistical significance of this similarity was tested by determining the quality scores (a value incorporating similarities and gaps in sequence alignments) between the homologous segments in GCN1 and *S. cerevisiae* EF-3 with 100 randomized sequences of the same composition and length (see Materials and Methods). The quality score for the alignment of the two segments from GCN1 and EF-3 was 13.6 standard deviations above the average score for the randomized sequences, indicating a very high level of statistical significance for this

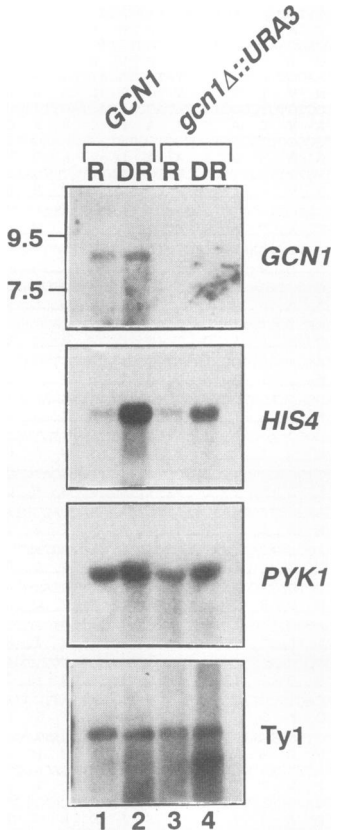

FIG. 3. RNA blot hybridization analysis of *GCN1* mRNA. Total RNA from isogenic yeast strains F113 (*GCN1*) and H2078 (*gcn1Δ::URA3*) was fractionated by 1% agarose-formaldehyde gel electrophoresis, transferred to GeneScreen Plus membranes, and hybridized with radiolabeled DNA probes for the indicated genes. (Exposures for the four probes were different.) R and DR, conditions repressing and derepressing for genes under general control, respectively, as described in Materials and Methods. The sizes (in kilobases) of RNA molecular weight markers (purchased from Bethesda Research Laboratories) are shown on the left of the *GCN1* panel.

sequence relatedness. The level of homology is even greater when the analysis is restricted to the N-terminal third of *S. cerevisiae* EF-3: GCN1 is 26% identical and 51% similar to EF-3 over a stretch of 317 amino acids (residues 1330 to 1641 of GCN1 and residues 14 to 326 in EF-3) in which the level of significance is 21 standard deviations over the mean. In addition, there appear to be smaller regions of even greater similarity. For example, there is a region of GCN1 (positions 1489 to 1609) that is 64% similar to the EF-3 genes and contains a segment of 25 amino acids in which all four proteins are 48% identical (GCN1 residues 1572 to 1596; Fig. 4).

These sequence comparisons suggest that this roughly 800-amino-acid segment of GCN1 has a structural feature or biochemical function in common with the fungal EF-3 gene products. EF-3 contains an internal repeat of approximately 200 amino acids, and each repeat contains a bipartite nucleotide-binding motif (54, 60) that is common to a number of NTPases involved in membrane transport and drug resistance (GXXGXGKS and LSGG[X_{16}]ILLLDEP[X_3]LD [33, 72]). Although the region of homology between GCN1 and EF-3 overlaps these nucleotide-binding sites, the invariant residues in these motifs are not present in GCN1 (Fig. 4), making it unlikely that the corresponding regions in GCN1 have nucleotide-binding properties. It is curious, however, that GCN1 contains a long internal repeat of approximately 290 amino acids which overlaps the region of sequence similarity with EF-3 but which is not coincident with the EF-3 repeats (Fig. 1 and 4). An alignment of these GCN1 repeats (at positions 1385 to 1674 and 1701 to 1937 in GCN1) revealed 28% identity and 52% similarity between the two, with a quality score 13 standard deviations above the mean.

GCN1 stimulates *GCN4* expression at the translational level. Under conditions of amino acid sufficiency, translation of *GCN4* mRNA is repressed by four uORFs in its mRNA leader. By using *GCN4-lacZ* fusions, it was shown that removal of all four uORFs leads to high constitutive *GCN4* expression, independent of amino acid levels and the positive regulators GCN2 and GCN3 (34, 48). The presence of uORF4 alone (counting from the 5′ end) is sufficient to fully repress *GCN4* translation, whereas uORF1 must be present upstream from uORF4 to overcome this repression and permit increased *GCN4* expression under conditions of amino acid starvation (49). It was shown previously that the low-level expression of amino acid biosynthetic enzymes associated with the *gcn1-1* mutation could be overcome by removal of all four uORFs from the *GCN4* mRNA leader (68). This finding was consistent with the idea that GCN1 is an activator of *GCN4* expression that functions by antagonizing the inhibitory effects of the uORFs on translation initiation at *GCN4*.

To obtain more direct evidence for this conclusion, we measured the expression of *GCN4-lacZ* fusions containing different combinations of the uORFs in isogenic wild-type *GCN1* and mutant *gcn1Δ* strains (constructed as described in Materials and Methods). In agreement with previous results, we observed a seven- to eightfold derepression of β-galactosidase activity when wild-type *GCN1* cells bearing the fusion construct containing all four uORFs were subjected to histidine starvation by the addition of 3-AT (derepressing conditions). Deletion of *GCN1* severely impaired the derepression of this construct, indicating that GCN1 is required for increased *GCN4* expression under starvation conditions (Fig. 5). As expected, in the wild-type *GCN1* strain, expression of the fusion lacking all four uORFs was very high and unregulated, whereas that containing only uORF4 showed diminished derepression and reduced expression under starvation conditions compared with the construct containing all four uORFs. Deletion of *GCN1* had little or no effect on the expression of these last two constructs (Fig. 5) that are defective for translational control in wild-type cells. These results indicate that GCN1 stimulates *GCN4* expression at the translational level by overcoming the inhibitory effects of the uORFs in the *GCN4* mRNA leader.

Deletion of *GCN1* eliminates the GCN2-dependent phosphorylation of eIF-2α in vivo. The GCN2 protein kinase is required to overcome the inhibitory effects of the uORFs on translation of *GCN4* under conditions of amino acid limitation. GCN2 stimulates *GCN4* expression by phosphorylation of eIF-2α on serine 51. We sought to determine whether GCN1 stimulates *GCN4* expression by promoting the phosphorylation of eIF-2α catalyzed by GCN2. To address this possibility, we examined the phosphorylation state of eIF-2α in isogenic *GCN1* and *gcn1Δ* strains using isoelectric-focusing gel electrophoresis to separate differentially phosphorylated forms of eIF-2α, followed by immunoblotting with eIF-2α antibodies to visualize the different isoforms. In agreement with the results of Dever et al. (20), the phosphor-

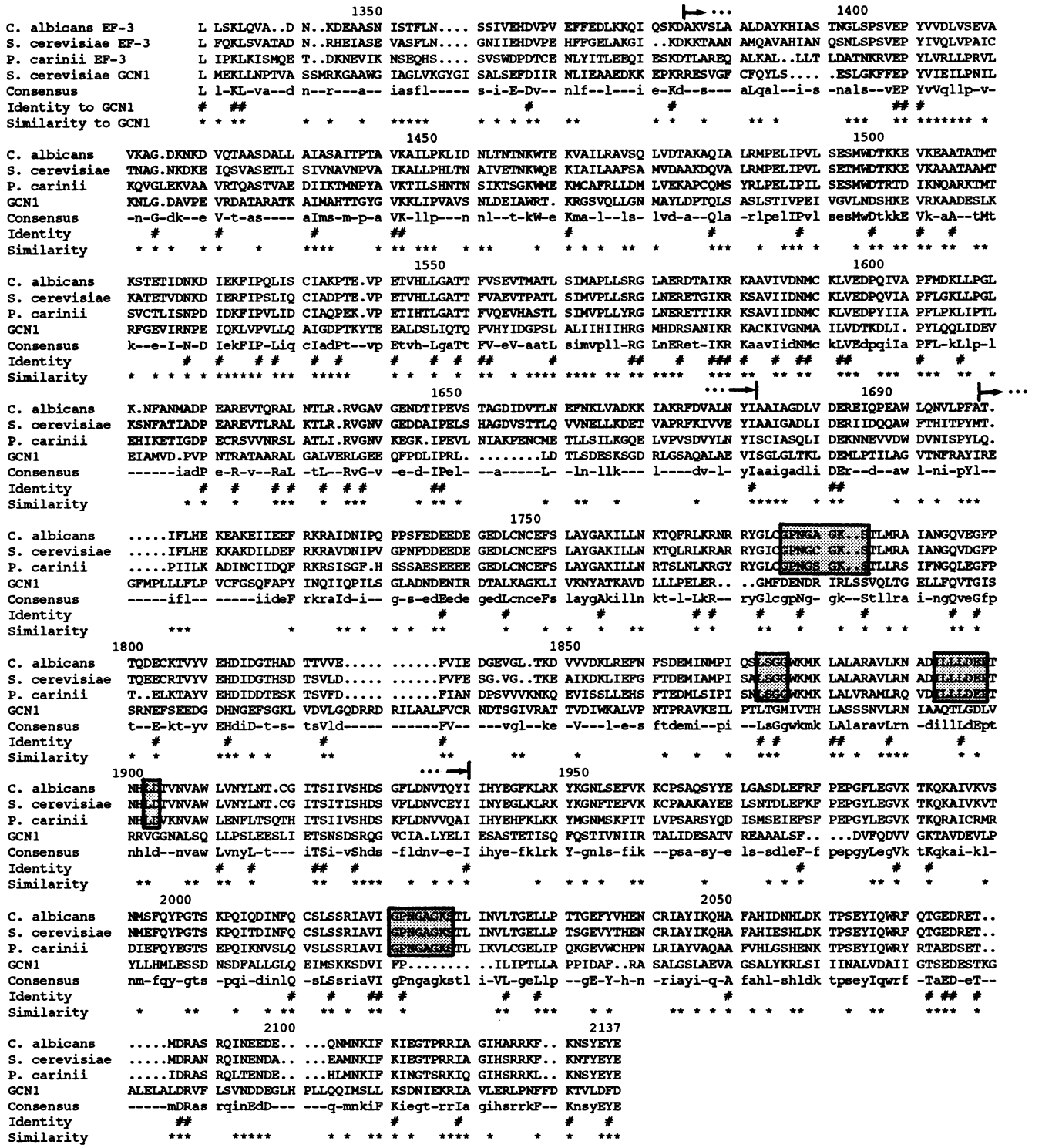

FIG. 4. Sequence alignment of a portion of GCN1 with EF-3 from three species of fungi. The BESTFIT program was used initially to align the GCN1 sequence with the sequence of EF-3 from *S. cerevisiae*. The boundaries of the homology determined there (GCN1 positions 1330 to 2137) were used with the PILEUP program to align all four sequences. The *C. albicans*, *S. cerevisiae*, and *P. carinii* sequences begin at the 17th, 14th, and 14th amino acid from their respective N termini. Dots indicate gaps introduced to maximize similarities. The line labeled Consensus marks the positions of amino acid identity or similarity among any three of the four proteins. A lowercase letter indicates sequence similarity, allowing for conservative replacements according to the criteria of Doolittle (26). An uppercase letter indicates identity or near identity; i.e., an uppercase letter is used if each pairwise combination of amino acids gives a score of greater than 1.0 by using the sequence comparison table of Gribskov and Burgess (28). The line marked Identity uses pound signs to indicate positions identical among all four of the aligned sequences. The line marked Similarity uses asterisks to indicate positions in which the GCN1 sequence shares the same amino acids or a conservative replacement with the Consensus line. The numbers above the lines represent the positions of the amino acids in the predicted GCN1 protein sequence, aligning the last digit with the amino acid. The regions in the EF-3 sequences corresponding to the NTP-binding motifs GXXGXGKS and LSGG(X_{16}) ILLLDEP(X_3)LD are shaded. The locations of the two GCN1 repeats (amino acids 1385 to 1674 and 1701 to 1937) are indicated by the arrows followed by three dots. Identity between all four proteins is 104/828 (12% of positions); similarities between any three of the four proteins is 528/828 (62%); GCN1 is part of this latter consensus sequence at 330/528 (63% of the positions). There exists a 1-amino-acid discrepancy between the predicted sequences of the *C. albicans* protein (22, 50). The *C. albicans* sequence of Myers et al. (50) was used in this figure.

Strain	Construct	GCN4-lacZ Expression (U) R	DR
GCN1	-□-□-□-□-□	14	100
	-X-X-X-X-□	1100	1000
	-X-X-X-□	9	26
gcn1Δ	-□-□-□-□-□	7	14
	-X-X-X-X-□	920	1000
	-X-X-X-□	8	16

FIG. 5. Analysis of β-galactosidase expression from *GCN4-lacZ* fusions in *GCN1* and *gcn1Δ* yeast strains. Strain F113 and its isogenic derivative H2079 were transformed with plasmids bearing *GCN4-lacZ* fusions with the wild-type leader or two variants of it lacking either all four or the first three uORFs, as indicated. (Boxes represent the upstream ORFs present in the GCN4 message, and an "X" indicates the presence of a mutation that alters the AUG codon of the ORF.) Cells were grown under repressing (R) or derepressing (DR) conditions as described in Materials and Methods. Values reported represent the mean values from three independent transformants and varied less than 30% from the individual measurements that were averaged, except for the derepressed values from the *gcn1* deletion strain, which all varied less than 84% from the mean. U, units of β-galactosidase expressed as nanomoles of *o*-nitrophenyl-β-D-galactoside hydrolyzed per minute per milligram of protein.

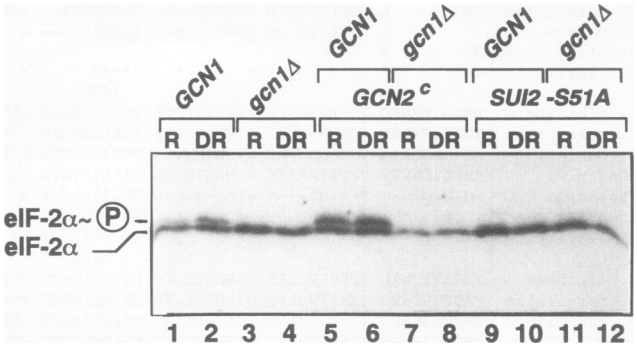

FIG. 6. Isoelectric focusing gel electrophoresis of eIF-2α phosphorylation in *GCN1* and *gcn1Δ* yeast strains expressing wild-type GCN2 or the GCN2^c-E1537G protein. Whole-cell extracts were prepared from strains grown under conditions in which the general control system is repressed (R) or derepressed (DR), as described in Materials and Methods. Total proteins were separated by isoelectric focusing on a vertical slab gel, and eIF-2α was detected by immunoblot analysis using polyclonal antiserum specific for this protein. The acidic end of the gel is at the top. The relevant genotypes of the strains from which the extracts were prepared are shown above the lanes. Lanes 1 and 2, strain H1896 *GCN1 SUI2 GCN2*; lanes 3 and 4, strain H2089 *gcn1Δ SUI2 GCN2*; lanes 5 and 6, strain H1816 *GCN1 SUI2 gcn2Δ* transformed with plasmid p914 bearing the *GCN2^c-E1537G* allele; lanes 7 and 8, strain H2085 *gcn1Δ SUI2 gcn2Δ* transformed with plasmid p914; lanes 9 and 10, strain H1897 *GCN1 SUI2-S51A GCN2*; lanes 11 and 12, strain H2091 *gcn1Δ SUI2-S51A GCN2*. The *SUI2-S51A* allele encodes eIF-2α containing alanine instead of serine at position 51. The ~P symbol denotes the location of eIF-2α that is phosphorylated on serine 51.

ylated form of eIF-2α increased in abundance when the wild-type *GCN1* strain was grown under histidine starvation conditions (Fig. 6, lanes 1 and 2). It was shown previously (20) that the appearance of this phosphorylated species is completely dependent on the GCN2 protein kinase and serine 51 in eIF-2α (Fig. 6, compare lanes 1 and 2 with lanes 9 and 10). Deletion of *GCN1* completely abolished the phosphorylated form of wild-type eIF-2α under both starvation and nonstarvation conditions (Fig. 6, lanes 3 and 4). The fact that the *gcn1Δ* deletion had no effect on the isoelectric point of the mutant form of eIF-2α in which serine 51 was substituted with alanine (Fig. 6, compare lanes 9 to 12) indicates that inactivation of *GCN1* prevents phosphorylation on serine 51 rather than affecting one of the sites of constitutive phosphorylation on eIF-2α in yeast cells (20).

We also examined the effect of the *gcn1Δ* mutation on the phosphorylation state of eIF-2α in the presence of a plasmid expressing the constitutively activated form of GCN2 protein kinase encoded by *GCN2^c-E1537G* (56). In the *GCN1* strain, this dominant *GCN2* allele led to hyperphosphorylation of eIF-2α in the absence of amino acid starvation; however, the phosphorylated form of eIF-2α was completely eliminated by deletion of *GCN1* (Fig. 6, compare lanes 7 and 8 with lanes 5 and 6). As would be expected from these results, the *gcn1Δ* allele overcomes the constitutive derepression of the general control system that is normally conferred by the *GCN2^c-E1537G* allele (44a). These findings suggested that GCN1 is required in vivo for the phosphorylation of eIF-2α on serine 51 by GCN2.

Evidence that GCN1 is specifically required for GCN2 kinase function rather than for negative regulation of an eIF-2α protein phosphatase. The fact that we did not observe eIF-2α phosphorylation in the *gcn1Δ* mutant could indicate that GCN1 prevents the dephosphorylation of eIF-2α on serine 51 by inhibiting a protein phosphatase instead of stimulating the protein kinase activity of GCN2. According to the former model, deletion of *GCN1* would activate the putative phosphatase and thereby eliminate the phosphorylated form of eIF-2α that is required for translational derepression of *GCN4*. We reasoned that if this hypothesis were correct, the *gcn1Δ* mutation should decrease the levels of phosphorylated eIF-2α even when the phosphorylation reaction is catalyzed by a heterologous eIF-2α kinase.

Two mammalian protein kinases that phosphorylate eIF-2α on serine 51 have been described: HRI (also known as HCR), which is activated in heme-depleted reticulocyte lysates, and dsRNA-PK (also known as p68, dsI, and DAI), which is activated by double-stranded RNA during viral infections (reviewed in reference 32). It was shown recently that both of these mammalian eIF-2α kinases will phosphorylate *S. cerevisiae* eIF-2α, specifically on serine 51, and substitute for GCN2 in stimulating *GCN4* translation when expressed in *gcn2Δ* yeast strains (19). In addition, overexpression of these mammalian kinases inhibits the growth of yeast cells (10, 19), mimicking the effects of genetically activated forms of GCN2 encoded by *GCN2^c* alleles (20, 56).

To determine whether GCN1 is required for hyperphosphorylation of eIF-2α by dsRNA-PK, we examined the effect of deleting *GCN1* on the growth rate of a *gcn2Δ* strain expressing dsRNA-PK under the control of a galactose-inducible promoter. In a wild-type *GCN1* strain, induction of dsRNA-PK by growth on galactose was nearly lethal, preventing colony formation from single cells (Fig. 7A). In agreement with previous results (10, 19), this lethality was overcome completely either by growing the strains on medium lacking galactose (in which *GAL* promoters are unin-

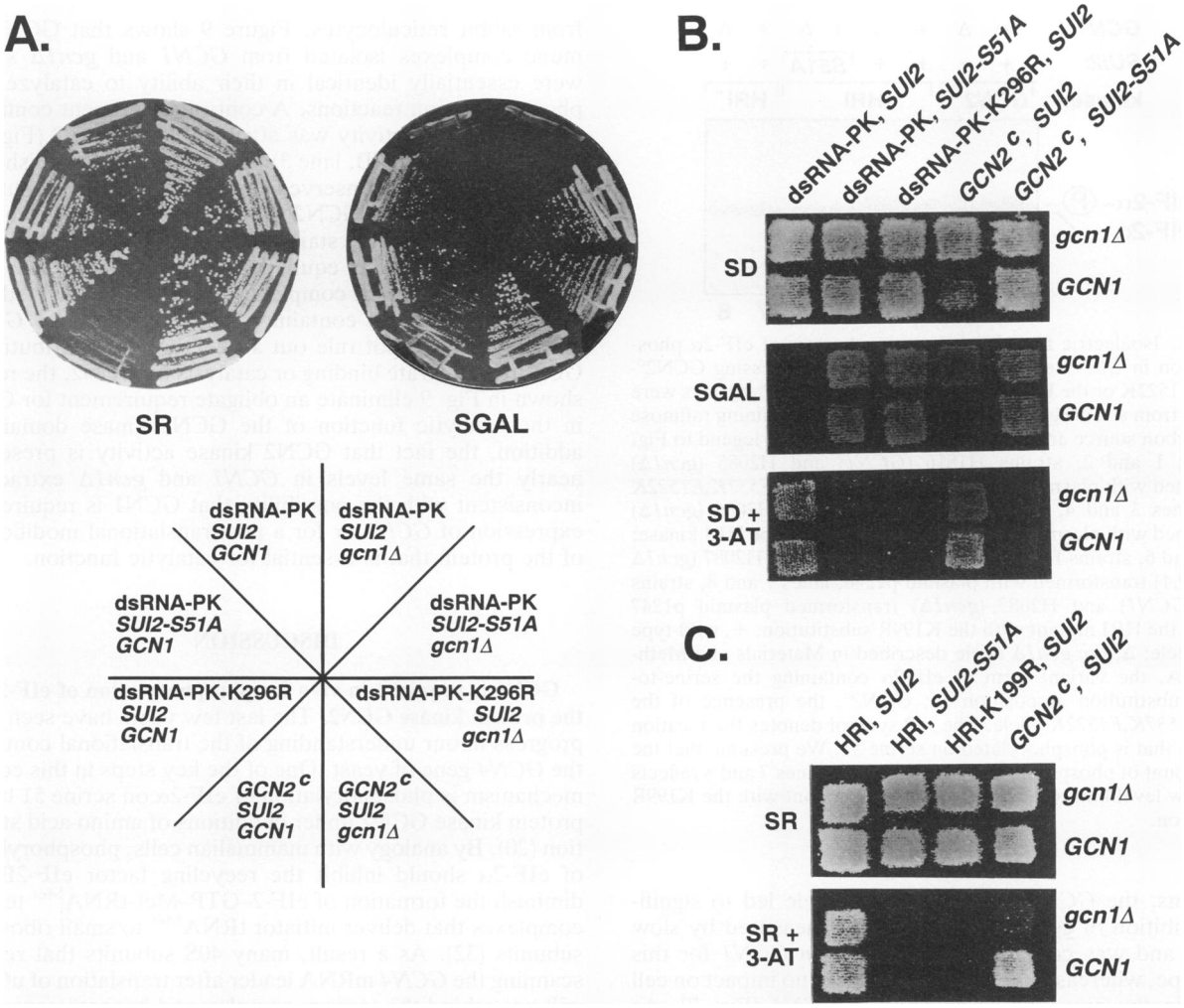

FIG. 7. Effects of deleting *GCN1* on amino acid analog sensitivity and colony size (growth rate) under nonstarvation and starvation conditions in *gcn2Δ* strains expressing dsRNA-PK, HRI, or the GCN2^c-E537K,E1522K protein kinase. Strains H1816 and H1817 and their isogenic derivatives H2086 and H2088 containing *gcn1Δ* were transformed with plasmids encoding wild-type dsRNA-PK (p1420), the dsRNA-PK mutant with the K296R substitution (p1421), wild-type HRI (p1246), the HRI mutant with the K199R substitution (p1247), or the GCN2^c-E537K,E1522K protein kinase. (A) Transformants were streaked on minimal medium containing raffinose (SR) or galactose (SGAL) as the carbon source, as indicated, and incubated at 30°C for 3 to 5 days. (B and C) Patches of transformants were grown to confluence on SD medium plates and replica plated to SD, SR, SGAL, SD + 3-AT, or SR + 3-AT medium. Plates were incubated at 30°C for 3 days.

duced), by mutating lysine 296 in the putative ATP-binding site of dsRNA-PK, or by mutating serine 51 in eIF-2α (Fig. 7). Deletion of *GCN1*, however, did not relieve the lethal effect of dsRNA-PK induction on galactose medium (Fig. 7A). This last result stands in contrast to our previous observation that deletion of *GCN1* eliminates the slow-growth phenotype associated with *GCN2^c* alleles (56), as shown in Fig. 7 for *GCN2^c-E537K,E1522K*.

It could be argued that induction of dsRNA-PK on galactose medium leads to such high levels of eIF-2α phosphorylation that the hypothetical stimulation of an eIF-2α-phosphatase by inactivation of *GCN1* would not reduce the level of eIF-2α phosphorylation enough to overcome the toxicity of dsRNA-PK. In an effort to eliminate this possibility, we determined whether deletion of *GCN1* would reverse the effects on translation initiation associated with low-level expression of the mammalian eIF-2α kinases. In agreement with the results of Dever et al. (19), low-level expression of dsRNA-PK on glucose medium (which represses *GAL* promoters) or HRI on raffinose medium (which neither represses nor induces *GAL* promoters) had no effect on the cellular growth rate (Fig. 7B and C); however, it was sufficient to stimulate *GCN4* expression and confer resistance to 3-AT in a *gcn2Δ* strain. As shown in Fig. 7B and C, this 3-AT-resistant phenotype was completely dependent on conserved lysines in the catalytic domains of the mammalian eIF-2α kinases (lysine 296 in dsRNA-PK and lysine 199 in HRI) and on serine 51 in eIF-2α, indicative of translational derepression of *GCN4* by phosphorylation of eIF-2α. Importantly, the ability of dsRNA-PK or HRI to confer 3-AT resistance in the *gcn2Δ* strain was not detectably affected by inactivation of *GCN1*, whereas the significantly greater resistance to 3-AT conferred by the *GCN2^c-E1537G* allele (56) was diminished by the *gcn1Δ* allele (Fig. 7B and C).

We also used isoelectric-focusing gel electrophoresis analysis to measure the effect of a *gcn1Δ* allele on the level of eIF-2α phosphorylation catalyzed by low levels of HRI in strains grown on raffinose medium. Recall that under these

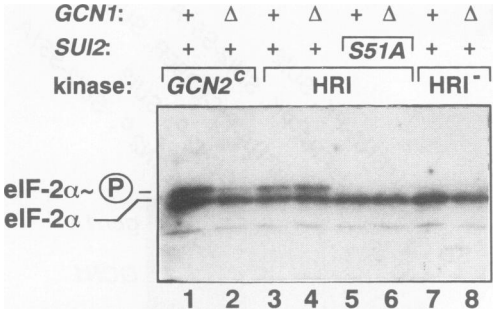

FIG. 8. Isoelectric focusing gel electrophoresis of eIF-2α phosphorylation in *GCN1* and *gcn1*Δ yeast strains expressing GCN2^c-E537K,E1522K or the HRI protein kinase. Whole-cell extracts were prepared from strains grown for 12 h in medium containing raffinose as the carbon source and analyzed as described in the legend to Fig. 6. Lanes 1 and 2, strains H1816 (*GCN1*) and H2085 (*gcn1*Δ) transformed with plasmid p1056 bearing the *GCN2^c-E537K,E1522K* allele; lanes 3 and 4, strains H1816 (*GCN1*) and H2085 (*gcn1*Δ) transformed with plasmid p1246 encoding the wild-type HRI kinase; lanes 5 and 6, strains H1817 (*GCN1 SUI2-S51A*) and H2087 (*gcn1*Δ *SUI2-S51A*) transformed with plasmid p1246; lanes 7 and 8, strains H1816 (*GCN1*) and H2085 (*gcn1*Δ) transformed plasmid p1247 encoding the HRI mutant with the K199R substitution. +, wild-type *GCN1* allele; Δ, the *gcn1*Δ allele described in Materials and Methods; S51A, the variant form of eIF-2α containing the serine-to-alanine substitution at position 51; *GCN2^c*, the presence of the *GCN2^c-E537K,E1522K* allele. The ~P symbol denotes the location of eIF-2α that is phosphorylated on serine 51. We presume that the trace amount of phosphorylated eIF-2α seen in lanes 7 and 8 reflects a very low level kinase activity for the HRI mutant with the K199R substitution.

conditions, the *GCN2^c-E537K,E1522K* allele led to significant inhibition of general translation (as measured by slow growth) and was completely dependent on *GCN1* for this phenotype, whereas expression of HRI had no impact on cell growth in the presence or absence of *GCN1* (Fig. 7). As shown in Fig. 8, the *gcn1*Δ mutation had no detectable effect on the level of eIF-2α phosphorylation catalyzed by HRI in raffinose-grown cells, whereas inactivation of *GCN1* greatly reduced eIF-2α phosphorylation by the *GCN2^c-E537K, E1522K* product (compare lanes 3 and 4 with lanes 1 and 2). The fact that GCN2 is dependent on GCN1 for the phosphorylation of eIF-2α in vivo whereas the same reaction catalyzed by dsRNA-PK or HRI is not detectably affected by deletion of *GCN1* strongly suggests that GCN1 is specifically required for GCN2 kinase function rather than for negative regulation of an eIF-2α phosphatase.

GCN2 protein isolated from cell extracts lacking GCN1 can phosphorylate eIF-2α in vitro. We next sought to determine whether *GCN1* is required for expression of *GCN2* or for some fundamental aspect of GCN2 kinase function, such as substrate binding or catalysis. It was shown previously that GCN2 protein immunoprecipitated from cell extracts can catalyze autophosphorylation (55, 75) as well as specific phosphorylation of eIF-2α purified from rabbit reticulocytes or from yeast (20). The level of GCN2 kinase activity detected in these assays is unaffected by *GCN2^c* mutations and by amino acid starvation of cells prior to extract preparation (75). To determine whether GCN2 protein kinase is present in an active form in *gcn1*Δ cells, we immunoprecipitated GCN2 from cell extracts prepared from isogenic *GCN1* and *gcn1*Δ strains and analyzed the resulting immune complexes for their ability to catalyze autophosphorylation of GCN2 and phosphorylation of eIF-2α purified from rabbit reticulocytes. Figure 9 shows that GCN2 immune complexes isolated from *GCN1* and *gcn1*Δ strains were essentially identical in their ability to catalyze both phosphorylation reactions. A control experiment confirmed that the kinase activity was attributable to GCN2 (Fig. 9A, lanes 6 and 7; Fig. 9B, lane 3), insofar as it was abolished by a mutation in the conserved lysine residue in the putative ATP-binding site of GCN2 (lysine 559 changed to an arginine). Coomassie blue staining of the polyacrylamide gel in Fig. 9B showed that equivalent amounts of GCN2 were present in the immune complexes isolated from the wild-type and Gcn1Δ strains containing high-copy-number *GCN2*. Although we cannot rule out a quantitative contribution of GCN1 to substrate binding or catalysis by GCN2, the results shown in Fig. 9 eliminate an obligate requirement for GCN1 in the catalytic function of the GCN2 kinase domain. In addition, the fact that GCN2 kinase activity is present at nearly the same levels in *GCN1* and *gcn1*Δ extracts is inconsistent with the possibility that GCN1 is required for expression of *GCN2* or for a posttranslational modification of the protein that is essential for catalytic function.

DISCUSSION

GCN1 is required in vivo for phosphorylation of eIF-2α by the protein kinase GCN2. The last few years have seen great progress in our understanding of the translational control of the *GCN4* gene of yeast. One of the key steps in this control mechanism is phosphorylation of eIF-2α on serine 51 by the protein kinase GCN2 under conditions of amino acid starvation (20). By analogy with mammalian cells, phosphorylation of eIF-2α should inhibit the recycling factor eIF-2B and diminish the formation of eIF-2–GTP–Met-tRNA$_i^{Met}$ ternary complexes that deliver initiator tRNA^{Met} to small ribosomal subunits (32). As a result, many 40S subunits that resume scanning the *GCN4* mRNA leader after translation of uORF1 will not rebind the ternary complex and become competent for reinitiation until after scanning past uORFs 2 to 4 but will do so before bypassing the *GCN4* start codon and reinitiate at *GCN4* instead (1, 20). The positive regulator *GCN3* encodes a nonessential subunit of eIF-2B that appears to be required only for the inhibition of eIF-2B function by the phosphorylated form of eIF-2α (11, 12, 19, 29, 56). It is thought that, in the absence of GCN3, no decrease in eIF-2 recycling occurs in response to phosphorylation of eIF-2α and consequently *GCN4* translation is not induced in response to amino acid starvation.

The *gcn1-1* mutation has the same phenotype as a deletion of *GCN2* or *GCN3*, impairing the derepression of genes encoding enzymes subject to general amino acid control (Fig. 3). The results presented here show directly that *GCN1* functions in conjunction with GCN2 and GCN3 in the translational derepression of *GCN4* expression in amino acid-starved cells (Fig. 5). Unlike GCN3, which mediates the inhibitory effect of phosphorylated eIF-2 on translation initiation (19), GCN1 is required in vivo for the phosphorylation of eIF-2α by GCN2 (Fig. 6 and 7). These findings are consistent with our previous genetic observations that the constitutive derepression of *GCN4* translation and low growth rates conferred by *GCN2^c* alleles are eliminated by the *gcn1-1* mutation (29, 56). The same phenotypes conferred by *gcn3^c* mutations were found to be unaffected by deletion of *GCN2* and by the *gcn1-1* allele (29). This result is in accord with the idea that GCN1 works in conjunction with GCN2 to catalyze eIF-2α phosphorylation, whereas GCN3

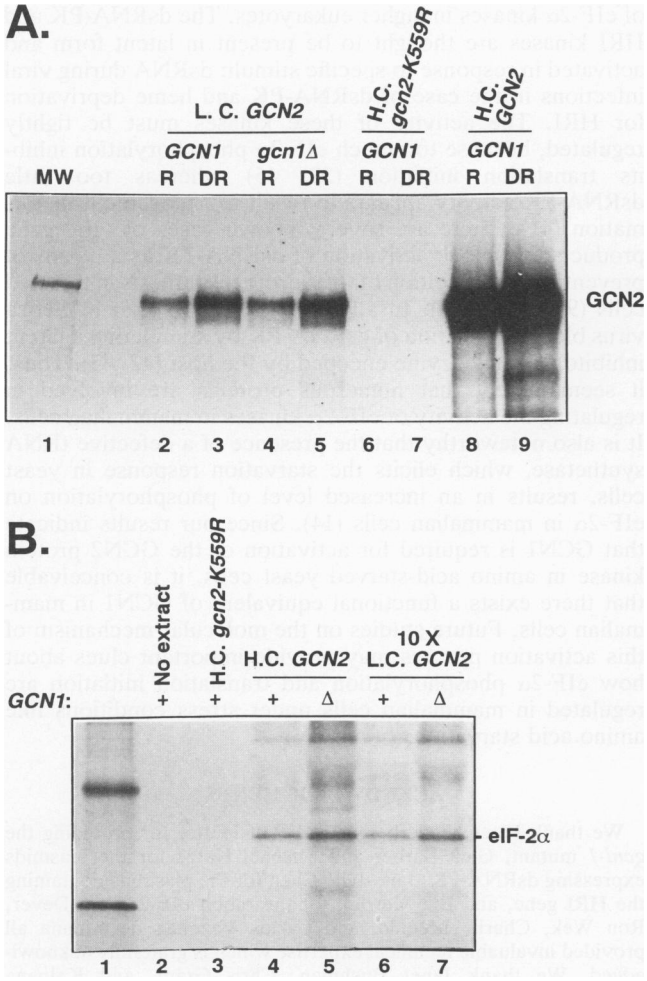

FIG. 9. In vitro analysis of GCN2 kinase activity from strains containing wild-type *GCN1* or the *gcn1* deletion allele. Strains were grown under repressing (R) or derepressing (DR) conditions and harvested as described in Materials and Methods. (A) GCN2 autophosphorylation assay. GCN2 was immunoprecipitated from samples containing equal amounts (400 μg) of total protein, and immune complexes were incubated in KIN buffer in the presence of [γ-^{32}P]ATP for 20 min. Radiolabeled samples were analyzed by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis followed by autoradiography. Lane 1, 200-kDa molecular weight marker; lanes 2 to 5, strain H1816 (*GCN1*) or H2085 (*gcn1*Δ) transformed with plasmid p585, a low-copy-number (L.C.) plasmid containing wild-type *GCN2*; lanes 6 and 7, strain H1816 (*GCN1*) transformed with p644, a high-copy-number (H.C.) plasmid containing the *gcn2-K559R* allele; lanes 8 and 9, strain H1816 (*GCN1*) transformed with H.C. plasmid p630 containing wild-type *GCN2*. (B) Phosphorylation of rabbit eIF-2α. Conducted as for panel A, except that all reactions contained 1 μg of purified rabbit eIF-2α and immune complexes contained GCN2, Gcn2-K559R, or no yeast proteins (lane 2). For reactions in lanes 2 to 7, the eIF-2 was incubated at 50°C for 9 min prior to the addition of GCN2 to reduce background phosphorylation by endogenous kinases in the eIF-2 preparation. Lane 1, 43- and 30-kDa molecular weight markers; lane 2, control reaction in which the yeast extract was omitted; lane 3, strain H1816 (*GCN1*) transformed with H.C. plasmid p644 containing *gcn2-K559R*; lanes 4 and 5, strain H1816 (*GCN1*) or H2085 (*gcn1*Δ) transformed with H.C. plasmid p630 containing *GCN2*; lanes 6 and 7, strain H1816 (*GCN1*) or H2085 (*gcn1*Δ) transformed with L.C. plasmid p585 containing *GCN2* by using 10 times as much extract (4 mg) as in lanes 5 and 6. The apparent differences in levels of GCN2 kinase activity between repressing and derepressing conditions in panel A were not observed reproducibly, in agreement with previous results (75), nor were the slightly higher levels seen in the *gcn1*Δ strain in panel B.

is required to transduce the phosphorylation signal to the translational machinery.

It was conceivable that GCN1 stimulated eIF-2α phosphorylation and the ensuing translational derepression of *GCN4* by negative regulation of an eIF-2α phosphatase such as GLC7 (73). This hypothesis is very unlikely in view of our finding that the level of phosphorylation of eIF-2α by the mammalian protein kinases HRI and dsRNA-PK is independent of *GCN1* over a wide range of kinase function (Fig. 7 and 8). Our observation that GCN2 kinase activity in cell extracts is not detectably diminished by deletion of *GCN1* (Fig. 9) indicates that GCN1 is not required for *GCN2* expression. It is also inconsistent with an obligate requirement for GCN1 in the catalytic function of the GCN2 kinase domain. However, the level of GCN2 kinase activity in our in vitro assays is unaffected by *GCN2^c* mutations and by amino acid starvation (75), indicating that the in vivo control mechanisms that couple GCN2 function to amino acid availability do not operate under these in vitro conditions. Therefore, the fact that GCN1 does not affect GCN2 kinase activity in vitro does not eliminate the possibility that GCN1 functions in vivo to stimulate substrate binding or catalysis by GCN2, either in a constitutive fashion or in response to amino acid starvation.

One possibility is that GCN1 mediates the stimulatory effect of uncharged tRNA on GCN2 kinase function, perhaps working in conjunction with the HisRS-related region in GCN2. This hypothesis is ostensibly at odds with the fact that *GCN2^c* mutations which lead to high-level GCN2 kinase function in the absence of amino acid starvation (20, 56) do not overcome the requirement for GCN1 in eIF-2α phosphorylation by GCN2 (Fig. 6 and 8). However, it is possible that the *GCN2^c* mutations simply make GCN2 hypersensitive to uncharged tRNA and that GCN1 is still required to mediate the stimulatory effect of uncharged tRNA on GCN2 kinase function. Alternatively, GCN1 could be required for proper localization of GCN2 with the translational machinery, providing it with access to the substrate eIF-2α or with the activator uncharged tRNA. The latter hypothesis provides a straightforward explanation for the nearly complete dependence of the phenotypes of *GCN2^c* mutations on *GCN1* function. It is noteworthy that in *GCN2^c-E1537G* cells, phosphorylation of eIF-2α was completely abolished by deletion of *GCN1* (Fig. 6), whereas in *GCN2^c-E537K, E1522K* cells, low-level phosphorylation still occurred in the absence of *GCN1* (Fig. 8). This observation is consistent with the fact that *GCN2^c-E537K,E1522K* leads to greater activation of GCN2 function than does *GCN2^c-E1537G* (56) and with the fact that GCN1 is not required for GCN2 catalytic function per se (Fig. 9). Apparently, the mutations in *GCN2^c-E537K,E1522K* render GCN2 kinase activity partially independent of the stimulatory function of GCN1 in vivo.

Sequence similarity between GCN1 and EF-3 suggests an interaction between GCN1 and the ribosome or tRNA. The sequence of *GCN1* was obtained in anticipation that it would provide insight into the role of GCN1 in general amino acid control. *GCN1* encodes an unusually large polypeptide with a predicted molecular weight of nearly 300,000. A stretch of ca. 88,000 Da in the central one-third of the GCN1 protein

shows a high degree of sequence similarity with EF-3. EF-3 was first identified as a biochemical activity indispensable for the elongation phase of protein synthesis in a cell-free translation system prepared from *S. cerevisiae* (65, 66). The EF-3 activity was purified and shown to reside in a single polypeptide of ca. 125,000 Da (16). Recent studies suggest that EF-3 stimulates the EF-1α-dependent binding of aminoacyl-tRNA to the A site of the ribosome by promoting release of deacylated tRNA from the E site, dependent on NTP hydrolysis (69). There is also evidence that EF-3 contributes to the fidelity of translation (59, 60) by stimulating binding of cognate aminoacyl-tRNAs at the expense of noncognate aminoacyl-tRNAs (38, 70). Although EF-3 has not been identified in plants and animals, this may not represent a fundamental difference between the mechanism of protein synthesis in fungi and higher eukaryotes. EF-3 possesses ATPase and GTPase activities that are dependent on the presence of yeast ribosomes (38, 71), raising the possibility that EF-3 represents a solubilized ribosomal protein that hydrolyzes NTPs only when bound to its proper location on ribosomes. Mammalian and plant ribosomes contain ATPase and GTPase activities that remain associated with the ribosome even when subjected to a high-salt wash, which could indicate that an equivalent of fungal EF-3 is present in these organisms and behaves like an integral ribosomal protein. Consistent with this latter hypothesis, antibodies against EF-3 were reported to cross-react with ribosomal components from brine shrimp, slime mold, and rat liver (46) as well as fission yeast (15).

Cloning and characterization of the genes encoding EF-3 from *S. cerevisiae* (54, 60) and two other fungi (22, 50, 77) revealed that EF-3 from each of these species contains two bipartite NTP-binding sites in the context of a large internal repeat of greater than 200 amino acids (54, 60, 77). These putative NTP-binding motifs have been shown to be involved in the ATPase and GTPase activities of EF-3 (7). It is curious that GCN1 does not possess the conserved and invariant residues comprising the bipartite NTP-binding sites of EF-3 but nonetheless possesses a large internal repeat of several hundred amino acids that encompasses one of the internal repeats in EF-3 containing the NTP-binding sites (Fig. 1). The repeats in GCN1 may represent two domains with similar functions, but these functions probably do not involve NTP binding and hydrolysis.

The fact that EF-3 stimulates the EF-1α-dependent binding of cognate aminoacyl-tRNAs to the ribosomal A site raises the possibility that the segment in GCN1 similar in sequence to EF-3 is involved in detecting uncharged tRNA in amino acid-starved cells. We showed previously that GCN2 interacts with ribosomes and suggested that its HisRS-like segment would be required to detect uncharged tRNA and activate the adjacent protein kinase moiety of GCN2 (55). In view of our present finding that GCN1 is required for GCN2 kinase activity in vivo, it could be suggested that GCN1 mediates an interaction between GCN2 and the ribosomal A site by virtue of its EF-3-like region. It is noteworthy that a positive effector of the general control response in bacteria, the RelA protein, is also associated with the ribosomal A site and produces the alarmone guanosine tetraphosphate (ppGpp) in response to nonenzymatic binding of uncharged tRNA to the ribosome (6, 27). Perhaps this efficient mechanism for detecting starvation for a single amino acid at the level of uncharged tRNA on the ribosome is similar between eukaryotes and prokaryotes.

Our results are also interesting in relation to the regulation of eIF-2α kinases in higher eukaryotes. The dsRNA-PK and HRI kinases are thought to be present in latent form and activated in response to specific stimuli: dsRNA during viral infections in the case of dsRNA-PK and heme deprivation for HRI. The activity of these kinases must be tightly regulated, because too much eIF-2α phosphorylation inhibits translation initiation (32, 45) whereas too little dsRNA-PK activity appears to result in oncogenic transformation (41). There are several known cases of viral gene products that block activation of dsRNA-PK as a means of preventing the inhibition of total protein synthesis in infected cells (9, 17, 39, 45). In addition, it appears that influenza virus blocks activation of dsRNA-PK by stimulating a latent inhibitor of the enzyme encoded by the host (42, 43). Thus, it seems likely that numerous proteins are involved in regulating the activity of eIF-2α kinases in mammalian cells. It is also noteworthy that the presence of a defective tRNA synthetase, which elicits the starvation response in yeast cells, results in an increased level of phosphorylation on eIF-2α in mammalian cells (14). Since our results indicate that GCN1 is required for activation of the GCN2 protein kinase in amino acid-starved yeast cells, it is conceivable that there exists a functional equivalent of GCN1 in mammalian cells. Future studies on the molecular mechanism of this activation process may provide important clues about how eIF-2α phosphorylation and translation initiation are regulated in mammalian cells under stress conditions like amino acid starvation.

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