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Mutations Activating the Yeast eIF-2α Kinase GCN2: Isolation of Alleles Altering the Domain Related to Histidyl-tRNA Synthetases

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The protein kinase GCN2 stimulates expression of the yeast transcriptional activator GCN4 at the translational level by phosphorylating the α subunit of translation initiation factor 2 (eIF-2α) in amino acid-starved cells. Phosphorylation of eIF-2α reduces its activity, allowing ribosomes to bypass short open reading frames present in the *GCN4* mRNA leader and initiate translation at the *GCN4* start codon. We describe here 17 dominant *GCN2* mutations that lead to derepression of *GCN4* expression in the absence of amino acid starvation. Seven of these *GCN2*^{c} alleles map in the protein kinase moiety, and two in this group alter the presumed ATP-binding domain, suggesting that ATP binding is a regulated aspect of GCN2 function. Six *GCN2*^{c} alleles map in a region related to histidyl-tRNA synthetases, and two in this group alter a sequence motif conserved among class II aminoacyl-tRNA synthetases that directly interacts with the acceptor stem of tRNA. These results support the idea that GCN2 kinase function is activated under starvation conditions by binding uncharged tRNA to the domain related to histidyl-tRNA synthetase. The remaining *GCN2*^{c} alleles map at the extreme C terminus, a domain required for ribosome association of the protein. Representative mutations in each domain were shown to depend on the phosphorylation site in eIF-2α for their effects on *GCN4* expression and to increase the level of eIF-2α phosphorylation in the absence of amino acid starvation. Synthetic *GCN2*^{c} double mutations show greater derepression of *GCN4* expression than the parental single mutations, and they have a slow-growth phenotype that we attribute to inhibition of general translation initiation. The phenotypes of the *GCN2*^{c} alleles are dependent on *GCN1* and *GCN3*, indicating that these two positive regulators of *GCN4* expression mediate the inhibitory effects on translation initiation associated with activation of the yeast eIF-2α kinase GCN2.

Phosphorylation of the α subunit of translation initiation factor 2 (eIF-2α) in mammalian cells leads to inhibition of protein synthesis at the initiation step. Two different mammalian (eIF-2α) kinases have been identified: the double-stranded-RNA-activated inhibitor of translation (DAI) that is activated in response to viral infections and the heme-controlled repressor (HCR) that is activated in reticulocytes by heme deficiency (for a review, see reference 26). Both kinases phosphorylate eIF-2α on the serine residue at position 51 (Ser-51) (9, 47). The phosphorylation of mammalian eIF-2α inhibits translation initiation by impairing the conversion of eIF-2-GDP to eIF-2-GTP at the completion of each initiation cycle, a reaction carried out by the guanine nucleotide exchange factor eIF-2B. Only the GTP-bound form of eIF-2 is able to form a ternary complex with the initiator $tRNA^{Met}$ and catalyze new rounds of translation initiation (41).

GCN2 is an eIF-2α kinase that plays a central role in the regulation of amino acid biosynthesis in the yeast *Saccharomyces cerevisiae*. Amino acid starvation of this yeast leads to increased synthesis of GCN4, a transcriptional activator of numerous genes encoding enzymes involved in amino acid biosynthesis. The kinase activity of GCN2 is required for increased synthesis of the GCN4 protein under conditions of amino acid starvation (50, 59). GCN2 stimulates *GCN4* expression at the translational level by overcoming the inhibitory effects of short open reading frames (uORFs) present in the mRNA leader which prevent initiation at the *GCN4* AUG codon under nonstarvation conditions (27, 42, 55). According to a recently proposed model (1), ribosomes scanning from the 5′ end of *GCN4* mRNA will translate the first uORF encountered (uORF1) and resume scanning. Under nonstarvation conditions, these ribosomes will reinitiate translation at the second, third, or fourth uORFs and be unable to reinitiate again at *GCN4*. However, when cells are starved for an amino acid, ribosomes scanning downstream from uORF1 will ignore the start codons at uORF2 to uORF4 and reinitiate at *GCN4* instead.

Dever et al. (12) have shown that GCN2 phosphorylates eIF-2α in yeast cells in response to amino acid starvation. Substitution of Ser-51 in yeast eIF-2α with alanine abolished this phosphorylation reaction and also impaired derepression of *GCN4* translation in amino acid-starved cells. From these results, it was proposed that phosphorylation of eIF-2α by GCN2 reduces the concentration of eIF-2-GTP-Met-$tRNA_i^{Met}$ ternary complexes by inhibiting the recycling of eIF-2-GDP to eIF-2-GTP by the yeast equivalent of eIF-2B. As a consequence, many of the ribosomes which have translated uORF1 and resumed scanning would not acquire

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the ternary complex needed for reinitiation until after scanning past uORF2, uORF3, and uORF4. These ribosomes would bind the ternary complex in the uORF4-*GCN4* interval and reinitiate translation at *GCN4* instead.

Mutations affecting the α and β subunits of eIF-2 that lead to constitutive derepression of *GCN4* translation independently of GCN2 have been reported elsewhere (5, 61). Presumably, these mutations impair eIF-2 function and thus mimic the inhibitory effect on translation elicited by the phosphorylation of eIF-2α by GCN2 in amino acid-starved cells. Mutations in multiple *GCD* genes also cause constitutive derepression of *GCN4* expression (29) and lead to a reduction in the efficiency of general translation initiation (7, 17, 57). From these results and from the fact that GCD1 and GCD2 are present in a high-molecular-weight complex that contains a fraction of eIF-2, it was proposed that GCD1 and GCD2 are subunits of the yeast equivalent of eIF-2B (7). According to this model, mutations affecting either eIF-2 or the subunits of its recycling factor eIF-2B (GCD1 or GCD2) would stimulate *GCN4* translation by diminishing eIF-2 activity. GCN3 is another component of the putative eIF-2B complex in *S. cerevisiae* (7); however, inactivation of this protein prevents derepression of *GCN4* translation (24). The latter findings suggest that GCN3 mediates the inhibitory effect of eIF-2α phosphorylation on the ability of the eIF-2B complex to recycle eIF-2 (7). GCN1 is a third regulatory factor required for an increase in expression of *GCN4* in response to amino acid starvation (55) whose mode of action is not yet known.

It is believed that GCN2 kinase function is stimulated by the accumulation of uncharged tRNA in amino acid-starved cells, because nonlethal mutations in aminoacyl-tRNA synthetases lead to derepression of the general control system in nutrient-rich medium (38, 44). No evidence for increased expression of the GCN2 protein under starvation conditions has been obtained (60); therefore, an increase in the biochemical activity of the kinase moiety or increased access to the substrate eIF-2α is the most probable mechanism for stimulating GCN2 function in response to starvation. A large portion of the C-terminal half of GCN2 is closely related in sequence to histidyl-tRNA synthetases (HisRSs) from *S. cerevisiae*, humans, and *Escherichia coli*. This sequence similarity suggests a model for coupling GCN2 protein kinase activity to amino acid levels, in which uncharged tRNA that accumulates in starved cells would bind to the HisRS-related domain and stimulate the adjacent protein kinase moiety (59).

In an effort to map in detail regions of GCN2 that are involved in regulating its kinase activity, we isolated and characterized a large number of *GCN2*^{c} mutations that lead to derepression of *GCN4* expression in the absence of amino acid starvation. Ten *GCN2* alleles that alter different residues in the protein kinase domain or at the extreme C terminus of the protein were obtained. Consideration of the mutations affecting the kinase domain in the context of the recently described X-ray crystal structure of murine cyclic AMP-dependent protein kinase (cAPK; 34) suggests that ATP binding may be rate limiting for GCN2 kinase function. Given the high degree of sequence similarity among GCN2, DAI, and HCR, the *GCN2*^{c} mutations mapping in the kinase domain may provide important clues about residues involved in regulating the mammalian eIF-2α kinases. On the basis of our hypothesis that the HisRS-related sequences of GCN2 have a regulatory function (59), we expected to isolate *GCN2*^{c} alleles affecting this domain. We report here six *GCN2*^{c} alleles mapping in the HisRS-related region.

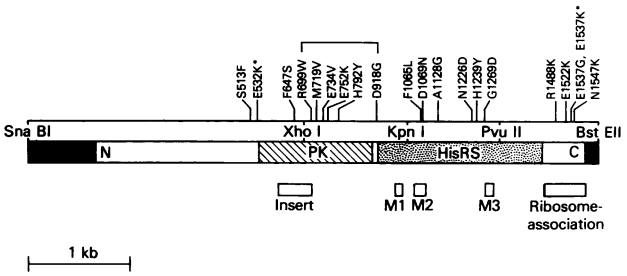


FIG. 1. The locations of *GCN2*^{c} mutations affecting different domains of the protein. The chromosomal *GCN2* gene is depicted with protein-coding sequences shown by the open box reading from the N terminus (N) to the C terminus (C). The following different segments of the protein are identified by shading: the protein kinase domain (PK) and the HisRS-related region (HisRS). The locations of restriction sites used for subcloning fragments from the mutagenized plasmids are indicated above the schematic, as are the positions of the *GCN2*^{c} mutations. Each of the latter is identified by the wild-type amino acid, its position in the polypeptide chain (numbered from the N terminus), and the substituting amino acid. The two *GCN2*^{c} alleles that were isolated previously (60) and not obtained in the present study are marked with asterisks. The R699W and D918G substitutions are connected by a line because they occur in the same *GCN2*^{c} allele; it is not known whether both substitutions are required for the mutant phenotype. Below the schematics are shown the locations of the large insert present in the three known eIF-2α kinases and the conserved sequence motifs (M) 1, 2, and 3 characteristic of class II aminoacyl-tRNA synthetases (see text for details).

Three of these mutations map near sequence motifs conserved among class II aminoacyl-tRNA synthetases which make up the conserved catalytic core that binds the 3′ end of tRNA. The nature of these mutations lends additional support to the idea that the HisRS-like domain of GCN2 monitors amino acid abundance by interacting with uncharged tRNA.

MATERIALS AND METHODS

Isolation of *GCN2*^{c} alleles. Plasmid p585 (60), a low-copy-number *URA3* plasmid containing the wild-type *GCN2* gene, was subjected to UV irradiation according to a protocol kindly provided by T. N. Davis (University of Washington, Seattle, Wash.). Following irradiation, plasmid DNA was used to transform the *E. coli uvrA* mutant strain AB1886 bearing plasmid pGW249 that enhances error-prone repair mutagenesis (21). Plasmid DNA was prepared from approximately 18,000 pooled *E. coli* transformants and introduced into yeast strain H1149 by the method of Ito et al. (31). Approximately 85,000 Ura$^+$ yeast transformants were screened by replica plating to minimal medium supplemented with the necessary nutrients and containing 0.5 mM 5-fluorotryptophan (5-FT) (45). About 500 5-FT-resistant (5-FT^r) yeast transformants were identified and pooled. Total DNA was isolated from this pool of yeast transformants and used to obtain approximately 2,400 transformants of *E. coli* DH5α. Plasmid DNA was isolated from a pool of these *E. coli* transformants, yielding a large plasmid-borne collection of presumptive *GCN2*^{c} alleles. Four different segments of the *GCN2* gene (2.8-kb *Sna*BI-*Xho*I fragment, 1.01-kb *Xho*I-*Kpn*I fragment, 0.89-kb *Kpn*I-*Pvu*II fragment, and 0.95-kb *Pvu*II-*Bst*EII fragment; Fig. 1) were isolated from the pool of mutant plasmids and inserted en masse into p722 (60), another low-copy-number *URA3* plasmid contain-

TABLE 1. Yeast strains employed in this study

Strain	Genotype	Source or reference
H4	*MATα leu2-3,112 ura3-52*	24
H19	*MATα leu2-3,112 gcn4-101*	30
H384	*MATα his1-29 ura3-52 gcn4Δ-103 (HIS4-lacZ ura3-52)*[a]	42
H1374	*MATα leu2-3,112, ura3-52 ino1-1 gcn2-K559V (HIS4-lacZ ura3-52)*[a]	23
H1345	*MATa ura3-52 his1-29 leu2-3,112 gcn4::LEU2*	P. F. Miller
H1513	*MATα ura3-52 trp1Δ-63 leu2-3,112 ino1-13*	A. M. Cigan
H1149	*MATα gcn2::LEU2 inol ura3-52 leu2-3,112 (HIS4-lacZ ura3-52)*[a]	60
H1402	*MATα inol ura3-52 leu2-3,112 (HIS4-lacZ ura3-52)*[a]	23
H1427	*MATα leu2-3,112 ura3-52 his1-29 gcn2::LEU2 gcn4::LEU2*	P. F. Miller
H1466	*MATα gcn2-505 gcn3::LEU2 inol ura3-52 leu2-3,112 (HIS4-lacZ ura3-52)*[a]	23
H1506	*MATa gcn2::LEU2 gcn1-1 ura3-52 leu2-3,112 (HIS4-lacZ ura3-52)*[a]	23
H1608	*MATα inol ura3-52 leu2-3,112 GCN2^c-M719V-E1537G (HIS4-lacZ ura3-52)*[a]	This study
H1609	*MATα inol ura3-52 leu2-3,112 GCN2^c-R699W-D918G-E1537G (HIS4-lacZ ura3-52)*[a]	This study
H1611	*MATα inol ura3-52 leu2-3,112 GCN2^c-E532K-E1537G (HIS4-lacZ ura3-52)*[a]	This study
H1613	*MATα inol ura3-52 leu2-3,112 GCN2^c-E532K-E1522K (HIS4-lacZ ura3-52)*[a]	This study
H1665	*MATa leu2-3,112 ura3-52 his1-29 gcn4::LEU2 trp1Δ-63*	C. M. Grant
H1685	*MATα inol ura3-52 leu2-3,112 GCN2^c-M719V-E1522K (HIS4-lacZ ura3-52)*[a]	This study
H1698	*MATα gcn2-505 gcn4::LEU2 his1-29 inol ura3-52 leu2-3,112 (HIS4-lacZ ura3-52)*[a]	C. M. Drysdale
H1816	*MATa gcn2Δ sui2Δ trp1-Δ63 ura3-52 leu2-3,112 (GCN4-lacZ TRP1)*[b] *(SUI2 LEU2)*[c]	12
H1817	*MATa gcn2Δ sui2Δ trp1-Δ63 ura3-52 leu2-3,112 (GCN4-lacZ TRP1)*[b] *(SUI2-S51A LEU2)*[c]	12
MY10	*MATa leu2-3,112 ura3-52 gcn4::LEU2*	P. F. Miller
MY31	*MATα leu2-3,112 ura3-52 his1-29 gcn4-103*	P. F. Miller

[a] The *HIS4-lacZ* fusion containing 670 bp of 5′ noncoding DNA described in Lucchini et al. (36) is integrated between two copies of *ura3-52*.
[b] The *GCN4-lacZ* fusion containing all four uORFs described in Hinnebusch (28) is integrated between *trp1-Δ63* and *TRP1* (12).
[c] The *SUI2* or *SUI2-S51A* alleles are present on autonomously replicating single-copy-number (*CEN*) plasmids containing *LEU2*.

ing *GCN2*. A total of 1,000 to 2,500 *E. coli* transformants were pooled from each subcloning experiment, producing four sets of subcloned plasmids, each containing *GCN2^c* mutations restricted to a particular segment of the gene. Plasmid DNA isolated from each of the four subcloned pools was introduced into yeast strain H1149, and approximately 1,200 yeast transformants obtained with each of the pools were screened for resistance to 0.5 mM 5-FT and 0.25 mM triazolealanine (TRA) (63). The results indicated that roughly two-thirds of the original pool of presumptive *GCN2^c*-containing plasmids gave rise to a subcloned derivative that conferred both 5-FT^r and TRA resistance (TRA^r) phenotypes when reintroduced into H1149. About 50% of the subcloned plasmids that conferred a mutant phenotype contained the *Xho*I-*Kpn*I fragment, 20% contained the *Sna*BI-*Xho*I fragment, 20% contained the *Pvu*II-*Bst*EII fragment, and 10% contained the *Kpn*I-*Pvu*II fragment derived from the mutagenized pool. Approximately 75 transformants showing the greatest resistance to 5-FT and TRA were chosen, and the *GCN2*-containing plasmids were isolated from total DNA isolated from each member of this group. After reintroduction of the isolated plasmids into strain H1149, nearly all were found to confer 5-FT^r and TRA^r phenotypes and to produce derepression of *HIS4-lacZ* expression under nonstarvation conditions compared with the parental plasmid p722. The 29 plasmids conferring the greatest derepression of *HIS4-lacZ* expression were subjected to DNA sequence analysis. For each mutation, we determined the sequence (52) of the entire DNA fragment derived from the original pool of mutagenized plasmids. Each plasmid that we analyzed contained only one or two base changes at a single location in the subcloned region (with the exception of the *GCN2^c-R699W-D918G* double mutation [see Table 2]). Seventeen unique mutations diagrammed in Fig. 1 and listed in Table 2 were identified among the collection of 29 sequenced plasmids; thus, several of the mutations were isolated more than once.

Construction of yeast strains. The yeast strains employed are listed in Table 1. The derivations of strains not described previously will be given below. Strain H1698 was an ascospore clone derived from a cross between H1665 (*MATa leu2-3,112 ura3-52 his1-29 gcn4::LEU2 trp1Δ-63*) and H1374 (23) (*MATα leu2-3,112 ura3-52 ino1-1 gcn2-505 HIS4-lacZ*), as a Leu$^+$ 3-aminotriazole-sensitive (3-AT^s) segregant in a tetrad containing two 3-AT^s and two 3-AT^r spores. The presence of the *gcn2-K559V* mutation in this strain was verified by complementation testing with *gcn2-1* tester strains. Strain H1665 was a Leu$^+$ AT^s ascospore segregant derived from a cross between H1345 (*MATa ura3-52 his1-29 leu2-3,112 gcn4::LEU2*) and H1513 (*MATα ura3-52 trp1Δ-63 leu2-3,112 ino1-13*). Strain H1345 was an ascospore segregant from a cross between MY10 (*MATa leu2-3,112 ura3-52 gcn4::LEU2*) and MY31 (*MATα leu2-3,112 ura3-52 his1-29 gcn4-103*). MY31 was an ascospore segregant derived from a cross between H384 (42) and H4 (24). MY10 was derived from H4 by transformation to Leu$^+$ AT^s with the ca. 4.4-kb *Pst*I-*Pvu*II fragment isolated from plasmid pM12 (see below), thereby replacing the *GCN4* gene with the *gcn4::LEU2* allele carried on pM12. The presence of the *gcn4::LEU2* allele in MY10 was confirmed by showing 2 Leu$^+$:2 Leu$^-$ and 0 AT^r:4 AT^s segregation in tetrads derived from a cross between MY10 and strain H19 (*MATα leu2-3,112 gcn4-101*) (30).

The synthetic *GCN2^c* double mutations listed in Table 2 were used to replace a *gcn2::URA3* deletion allele in strain H1333 (isogenic to the *gcn2::LEU2* strain H1149) following the methods described previously (23), generating strains H1608, H1609, H1611, H1613, and H1685. This procedure involved integration of the *LEU2* plasmids p1064, p1065, p1066, p1067, and p1112, containing different *GCN2^c* double mutations (see below) at *gcn2::URA3*, followed by selection of Leu$^-$ Ura$^-$ segregants on medium containing 5-fluoroorotic acid (4). The resulting segregants were screened for the phenotypes characteristic of *GCN2^c* double mutations:

TABLE 2. *GCN2*^{c} mutations mapping in different domains leading to derepression of *HIS4-lacZ* expression and slow growth under nonstarvation conditions[a]

Plasmid	Location or type and name of plasmid-borne allele	Nucleotide change	*HIS4-lacZ* expression (U)[b]	Colony size[c]
	Protein kinase domain			
p1251	*GCN2*^{c}*-S513F*	C1564T	350	+++++
p1252	*GCN2*^{c}*-F647S*	T1966C	270	+++++
p913	*GCN2*^{c}*-R699W-D918G*	A2120G, *A2121T*, *G2778A*, and *A2780G*	530	++++
p912	*GCN2*^{c}*-M719V*	A2181G	670	++++
p1253	*GCN2*^{c}*-E734V*	A2227T	410	+++++
p1254	*GCN2*^{c}*-E752K*	G2280A	470	+++++
p1255	*GCN2*^{c}*-H792Y*	C2400T	540	+++++
	HisRS-related domain			
p1165	*GCN2*^{c}*-F1065L*	T3221A	320	+++++
p1161	*GCN2*^{c}*-D1069N*	G3231A	350	+++++
p1164	*GCN2*^{c}*-A1128G*	C3409G	410	++++
p1168	*GCN2*^{c}*-N1226D*	A3702G	280	+++++
p1167	*GCN2*^{c}*-H1239Y*	C3741T	260	+++++
p1169	*GCN2*^{c}*-G1269D*	G3832A and A3833T	400	+++++
	C-terminal domain			
p1256	*GCN2*^{c}*-R1488K*	G4489A	320	+++++
p915	*GCN2*^{c}*-E1522K*	G4590A	500	+++++
p914	*GCN2*^{c}*-E1537G*	A4636G	750	++++
p1257	*GCN2*^{c}*-N1547K*	T4667A	440	+++++
	Double mutations[d]			
p1055	*GCN2*^{c}*-M719V-E1522K*	SA	890	+++
p1056	*GCN2*^{c}*-E532K-E1522K*	SA	870	++
p1053	*GCN2*^{c}*-R699W-D918G -E1537G*	SA	980	++
p1052	*GCN2*^{c}*-M719V-E1537G*	SA	910	+
p1054	*GCN2*^{c}*-E532K-E1537G*	SA	1,100	+
	Controls			
p722	*GCN2*	None	150	+++++
p238	*GCN4*^{c}	NA	860	+++
p703	None	NA	120	+++++

[a] The plasmid-borne *GCN2* alleles are designated by the wild-type amino acid, its position in the amino acid sequence numbered from the N terminus, and the amino acid present at that position in the mutant protein, in that order. The nucleotide changes present in each *GCN2*^{c} allele are designated in the same way, numbered relative to the 5′ end of *GCN2* mRNA (50), as in Wek et al. (59). SA, same as above; NA, not applicable.

[b] Transformants of strain H1149 containing the plasmid-borne *GCN2* alleles shown on the left were grown to mid-exponential phase on minimal SD medium (54) containing only the required supplements. Total cell extracts were prepared and analyzed for the units of β-galactosidase activities present in each strain. Enzyme activities are given in units, defined as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside cleaved per minute per milligram of protein. The data given are the averages obtained from assays conducted on two to four independent transformants for each plasmid, the results of which differed from the average values by 30% or less.

[c] Colony size was assessed qualitatively 3 to 4 days after cells were plated on SD medium containing only the necessary supplements and after they were incubated at 30°C.

[d] These alleles were present at the *GCN2* chromosomal locus as the only copy of *GCN2* in strains H1685, H1613, H1609, H1607, and H1611 (Table 1), all of which are isogenic to H1149.

resistance to 3-AT, resistance to 5-FT, slow growth, and constitutive derepression of *HIS4-lacZ* expression.

Plasmid constructions. The plasmids in Table 2 containing synthetic *GCN2*^{c} double mutations were generated by replacing the 3.8-kb *Sna*BI-*Kpn*I fragments of p914 or p915 with the corresponding fragments from p912, p913, or p628 (*GCN2*^{c}*-E532K*) (60). DNA sequence analysis of the resulting plasmids was conducted to verify the presence of both *GCN2*^{c} mutations. The integrating *LEU2* plasmids used for the gene replacements of *gcn2::URA3* in H1333 with *GCN2*^{c} double mutations, p1112, p1067, p1065, p1064, and p1066, were constructed by insertion of the 6.6-kb *Xba*I-*Sal*I fragments from, respectively, p1055, p1056, p1053, p1052, and p1054 (Table 2) at the *Xba*I and *Sal*I sites of plasmid p627 (23).

The derivation of plasmid pM12 used to make the *gcn4::LEU2* disruption present in strain H1698 began with p292, a low-copy-number *URA3 GCN4* plasmid that was derived from p298 (62) by removal of the 3.0-kb *lacZ Bam*HI fragment. The 2.3-kb *Sac*II-*Xba*I fragment containing the *GCN4* mRNA leader and N-terminal one-half of the GCN4 protein-coding region was removed, and the ends were made flush by treatment with nuclease S1 and T4 DNA polymerase and modified by the addition of *Bgl*II linkers (37), creating pM11. The 2.8-kb *LEU2 Bgl*II fragment isolated from YEp13 (46) was inserted at the *Bgl*II site of pM11, thus creating pM12 (39a).

Analysis of *HIS4-lacZ* and *GCN4-lacZ* fusions. Assays were conducted as described previously (36) after strains were grown in SD medium (54) containing only the following

required supplements: 2 mM leucine, 0.5 mM isoleucine, 0.5 mM valine, and 0.2 mM inositol. Saturated cultures were diluted 1:50 and harvested in logarithmic phase after 6 h of growth.

Immunoblot analysis of steady-state GCN2 protein levels. Strains were grown as described above for assaying *lacZ* fusions, and total protein extracts were prepared as described elsewhere (60). Samples were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to nitrocellulose, and probed with polyclonal GCN2-specific antiserum, all as described previously (60).

Analysis of polysome distributions. Total yeast extracts were prepared and analyzed by velocity sedimentation on 7 to 47% sucrose gradients. The gradients were fractionated and scanned at 254 nm with an ISCO gradient collector, all as described previously (17).

Isoelectric-focusing gel electrophoretic analysis of eIF-2α phosphorylation. Yeast strains were grown under nonstarvation and starvation conditions as described above, chilled, and harvested by centrifugation. Cell extracts were prepared and analyzed by the vertical isoelectric-focusing gel electrophoresis system described by Dever et al. (12). After focusing, immunoblot analysis was carried out as described previously (7) with antiserum prepared against a TrpE-eIF-2α fusion protein (8).

RESULTS

***GCN2^c* mutations in three different domains of GCN2 lead to constitutive derepression of genes subject to general amino acid control.** Mutations that inactivate *GCN2* prevent the derepression of *GCN4* and amino acid biosynthetic genes under its control in response to amino acid starvation. Consequently, *gcn2* mutants exhibit increased sensitivity to 3-AT, a metabolic inhibitor of histidine biosynthesis (for a review, see reference 29). We have isolated a large number of dominant mutations in *GCN2* that lead to constitutive derepression of the general control system. These *GCN2^c* alleles were identified on the basis of conferring increased resistance to the amino acid analogs 5-FT and TRA, resulting from constitutive derepression of tryptophan and histidine biosynthetic enzymes, respectively (45, 63). The mutations were generated in vitro by UV irradiation of a plasmid-borne *GCN2* gene and identified by screening transformants of a *gcn2::LEU2* deletion strain containing the mutagenized plasmids for increased resistance to the analogs. Plasmids conferring this phenotype were isolated from *S. cerevisiae*, and the mutation responsible for the analog resistance was determined by DNA sequence analysis of the *GCN2* gene (see Materials and Methods).

With one exception (*GCN2^c-R699W-D918G*), the 17 *GCN2^c* mutations that we analyzed all make single amino acid substitutions in the GCN2 protein sequence (Fig. 1). Seven alleles affect the protein kinase domain, and one of these (*GCN2^c-E752K*) was isolated independently in an earlier study (60). The *GCN2^c-E532K* allele described previously (60) was not reisolated in the present work. Six of the *GCN2^c* alleles alter residues in the HisRS-related domain, and the remaining four alleles alter amino acids in the extreme C-terminal segment of GCN2, adjacent to the HisRS-related sequences. One of the latter group of alleles (*GCN2^c-E1537G*) alters the same amino acid that was changed by the single mutation that we isolated previously in the C-terminal domain (60); however, the allele described in the present work introduces a glycine instead of a lysine in place of Glu-1537 (Fig. 1). With the possible exception of the *GCN2^c-S513F* allele, none of the mutations that we sequenced affects the large N-terminal domain of GCN2, even though this region appears to be required for regulation of GCN2 kinase function, not for catalytic activity per se (60). It is possible that regulatory mutations affecting the N-terminal domain were present in our collection but were not analyzed because they have weaker phenotypes than the mutations listed in Table 2.

On the basis of their 5-FT^r and TRA^r phenotypes, the *GCN2^c* mutations are expected to produce derepression of tryptophan and histidine biosynthetic pathways in the absence of amino acid starvation. To test this assumption and to quantitate the degree of derepression associated with each mutation, we measured β-galactosidase expression levels from a chromosomal *HIS4-lacZ* fusion present in the *gcn2::LEU2* deletion strain in which the mutations were first identified. Transformants bearing wild-type *GCN2* (on plasmid p722) expressed about 150 U of β-galactosidase activity from the *HIS4-lacZ* fusion under nonstarvation conditions (Table 2). Each of the *GCN2^c* mutations led to higher levels of *HIS4-lacZ* expression under the same growth conditions, with increases ranging from a factor of 1.8 for the least-derepressing allele (*GCN2^c-F647S*) to 5.0 for the most-derepressing allele (*GCN2^c-E1537G*). The latter mutation produced a level of derepression similar to that given by a dominant constitutive allele of *GCN4* that lacks all four uORFs required for translational repression of *GCN4* under nonstarvation conditions (p238; Table 2). Thus, several of the *GCN2^c* mutations listed in Table 2 result in nearly complete activation of GCN2 regulatory function and derepression of the general control system.

Because many of the *GCN2^c* alleles did not lead to complete derepression of *GCN4* expression, we tested whether combining mutations mapping in the protein kinase domain with those affecting the C-terminal region would result in greater derepression than that which occurred with either single mutation alone. In fact, all five of the *GCN2^c* double mutations that we constructed led to complete derepression of *HIS4-lacZ* expression under nonstarvation conditions, at the same level given by the *GCN4^c* allele (Table 2). No further derepression of *HIS4-lacZ* expression was observed in strains containing these *GCN2^c* double mutations under conditions of histidine starvation in which the wild-type strain showed a fivefold derepression in the level of fusion enzyme activity (data not shown). We conclude that the *GCN2^c* mutations bypass the requirement for amino acid starvation for derepression of *GCN4* and amino acid biosynthetic genes subject to the general control.

The *GCN2^c* mutations do not increase the steady-state level of the GCN2 protein. It was shown previously that 20-fold overexpression of *GCN2* achieved by introducing the gene on a high-copy-number plasmid led to partial derepression of the general control system, comparable to that seen in several of the *GCN2^c* mutants described in Table 2 (58, 60). Therefore, it was important to determine whether any of the mutations that we isolated derepress the general control because they increase the abundance of the GCN2 protein. To address this question, we conducted immunoblot analysis on total protein extracts of the transformant strains analyzed in Table 2 containing *GCN2^c* or wild-type *GCN2* on a low-copy-number plasmid. The same parental strain transformed with *GCN2* on a high-copy-number plasmid was analyzed in parallel. The results shown in Fig. 2 indicate that while some of the *GCN2^c* mutations may lead to small increases in the steady-state level of the GCN2 protein, none

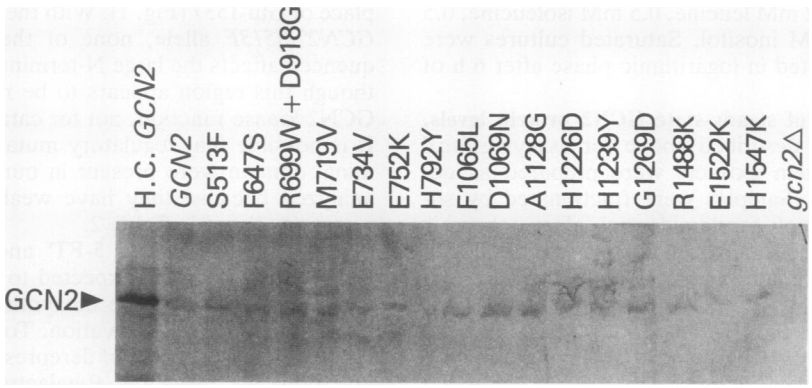


FIG. 2. Immunoblot analysis of GCN2 protein levels of *GCN2^c^* strains. Protein extracts were prepared from *gcn2::LEU2* deletion strain H1149 transformed with different plasmid-borne *GCN2^c^* alleles and grown to mid-logarithmic phase. Samples containing equal amounts of total protein were separated by SDS-PAGE (6.0% polyacrylamide), transferred to nitrocellulose, and probed with antiserum specific for GCN2. Each lane is labeled with the allele designation of the *GCN2* gene that is present on a low-copy-number plasmid in the strain from which the extract was prepared. H.C. *GCN2* corresponds to the high-copy-number wild-type *GCN2* plasmid p630 (60). The lane labeled *GCN2* designates the wild-type parental plasmid p722; *gcn2Δ* designates the strain transformed with the vector alone.

caused increases in the order of magnitude required for partial derepression of the general control system. The same result was obtained for the double *GCN2^c^*-alleles (data not shown). Therefore, we attribute the derepressed phenotype of these mutations to an increase in the kinase function of the mutant *GCN2* proteins under nonstarvation conditions.

Evidence that *GCN2^c^* mutations derepress the general control system by phosphorylation of translation initiation factor eIF-2α. Dever et al. (12) reported that the constitutive derepression of the general control system associated with the *GCN2^c^-M719V-E1537G* and *GCN2^c^-E752K-E1537G* double mutations was completely suppressed by substituting Ser-51, the presumed phosphorylation site in eIF-2α, with alanine. The data shown in Table 3 extend these findings to include representative *GCN2^c^* single mutations mapping in the protein kinase domain, the HisRs-related domain, or the C-terminal domain. The *GCN2^c^* mutations under consideration were introduced into isogenic *gcn2Δ* strains containing the *SUI2* gene (encoding wild-type eIF-2α) or the *SUI2-S51A* allele (encoding eIF-2α with Ser-51 substituted by alanine). These strains also contain a chromosomal *GCN4-lacZ* fusion with all four uORFs intact. In the *SUI2* strain, each of the *GCN2^c^* alleles led to increased expression of the *GCN4-lacZ* fusion relative to that seen in the *GCN2* strain, to an extent that roughly paralleled the derepressing effects of these mutations on *HIS4-lacZ* expression shown in Table 2. In contrast, the *GCN2^c^* mutations had no effect on *GCN4-lacZ* expression in the *SUI2-S51A* mutant (Table 3). These findings suggest that *GCN2^c^* mutations altering each of the three domains of GCN2 stimulate *GCN4* expression by increasing phosphorylation of eIF-2α on Ser-51.

To further substantiate this conclusion, we analyzed biochemically the effects of selected *GCN2^c^* mutations on the phosphorylation state of eIF-2α in vivo. Isoelectric-focusing gel electrophoresis was used to separate the phosphorylated and nonphosphorylated forms of eIF-2α, and immunoblotting with antibodies directed against eIF-2α was used to visualize the relative amounts of the two species present under different conditions. Consistent with the findings of Dever et al. (12), histidine starvation of the *gcn2::LEU2* strain transformed with a plasmid bearing wild-type *GCN2* led to an increase in the relative amount of the phosphorylated form of eIF-2α (Fig. 3). As expected, no phosphorylation under either growth condition in a transformant of this strain containing vector alone was observed (data not shown), since GCN2 is absolutely required for eIF-2α phosphorylation under these conditions (12). Transformants containing a *GCN2^c^* single mutation in the kinase domain (*GCN2^c^-M719V*), the HisRS-like domain (*GCN2^c^-A1128G*),

TABLE 3. Derepression of *GCN4-lacZ* expression under nonstarvation conditions in *GCN2^c^* mutants abolished by the *SUI2-S51A* mutation

Location or type and name of plasmid-borne allele	*GCN4-lacZ* expression (U) in the following strains[a]:	
	H1816 (*SUI2*)	H1817 (*SUI2-S51A*)
Protein kinase domain		
GCN2^c^-S513F	38	14
GCN2^c^-M719V	63	14
GCN2^c^-H792Y	65	15
HisRS-related domain		
GCN2^c^-D1069N	37	15
GCN2^c^-G1269D	50	15
C-terminal domain		
GCN2^c^-E1522K	49	15
GCN2^c^-E1537G	130	13
Double mutations		
GCN2^c^-M719V-E1522K	150	16
GCN2^c^-E532K-E1522K	150	21
GCN2^c^-R699W-D918G -E1537G	130	17
GCN2^c^-M719V-E1537G	170	18
GCN2^c^-E532K-E1537G	120	17
Wild-type *GCN2*	16	20

[a] Transformants of strain H1816 or H1817 containing the *GCN2* alleles shown on the left (borne on the plasmids listed in Table 2) were grown to mid-exponential phase on minimal medium containing only the required supplements. Total cell extracts were prepared and analyzed for the units of β-galactosidase activities present in each strain. Enzyme activities are given in units, defined as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside cleaved per minute per milligram of protein.

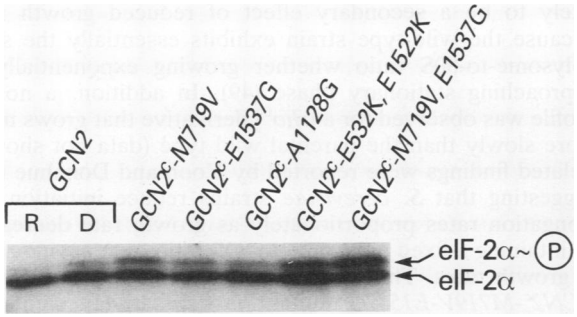


FIG. 3. *GCN2c* mutations affecting each of three domains lead to increased phosphorylation of eIF-2α in the absence of amino acid starvation. Protein extracts were prepared from transformants of *gcn2::LEU2* strain H1149 containing the indicated plasmid-borne *GCN2* alleles. The *GCN2* transformant was grown under nonstarvation (R) or histidine starvation (D) conditions; the transformants containing *GCN2c* alleles were grown under nonstarvation conditions. Samples containing approximately equal amounts 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 (12).

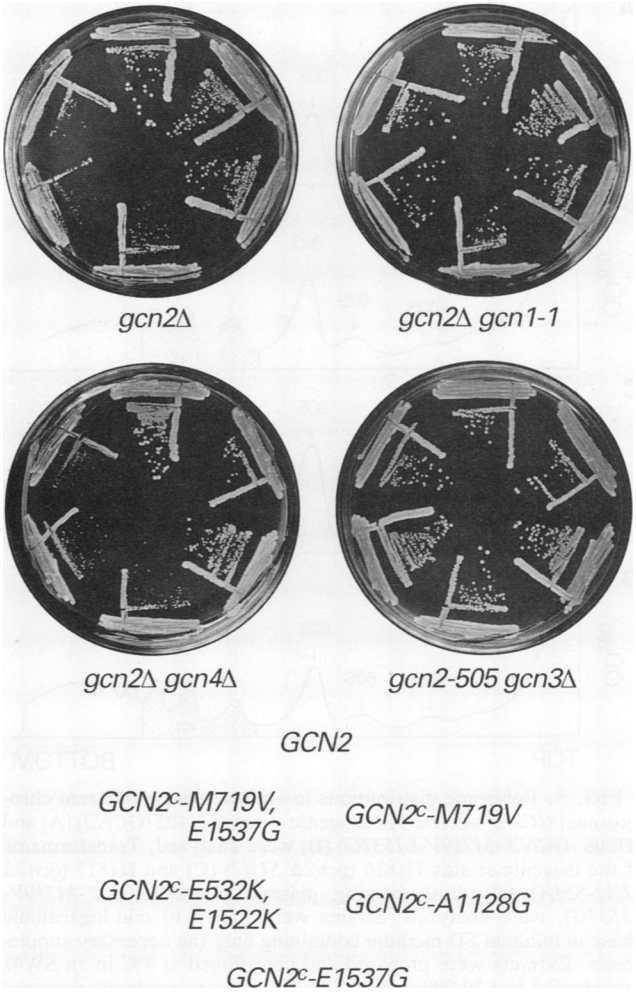


FIG. 4. Colony sizes of transformants bearing different *GCN2c* alleles in strains deleted for chromosomal *GCN2* and containing null mutations in *GCN1*, *GCN3*, or *GCN4*. Isogenic strains H1149 (*gcn2Δ*), H1506 (*gcn2Δ gcn1-1*), H1466 (*gcn2-505 gcn3Δ*), and H1427 (*gcn2Δ gcn4Δ*) were transformed with the low-copy-number *URA3* plasmids described in Table 2 bearing the *GCN2c* alleles listed at the bottom or with wild-type *GCN2*. These transformants were streaked on SD medium supplemented with the nutrients required by these strains (excluding uracil in order to select for the plasmid) and incubated at 30°C for 3 days. On each plate, strains were streaked in the same arrangement shown at the bottom for the list of *GCN2* allele names.

or the C-terminal domain (*GCN2c-E1537G*) or the double mutations *GCN2c-E532K-E1522K* and *GCN2c-M719V-E1537G* all showed increased phosphorylation relative to the *GCN2* transformant when grown under nonstarvation conditions. Thus, a mutation in any of the three domains appears to overcome the requirement for amino acid starvation in activating GCN2 protein kinase function. We found that the double *GCN2c* mutations led to higher steady-state levels of eIF-2α phosphorylation than those that occurred with the single mutations.

***GCN2c* mutations that fully derepress *GCN4* expression inhibit cell growth under nonstarvation conditions by impairing general translation initiation.** All five *GCN2c*-double mutations that we constructed led to a slow-growth (Slg$^-$) phenotype under nonstarvation conditions when introduced into the *gcn2::LEU2* strain described above. For example, the size of colonies produced from single cells on minimal medium was significantly smaller for the *GCN2c* double mutants than for the isogenic wild-type strain (Fig. 4). The strains containing the two most-derepressing single mutations that we isolated, *GCN2c-M719V* and *GCN2c-E1537G*, also show a modest Slg$^-$ phenotype (Fig. 4 and Table 2). These results suggest that extreme activation of GCN2 kinase function under nonstarvation conditions has a pleiotropic effect on cell growth and that the degree of this growth defect is directly correlated with derepression of the general control system. We also observed the Slg$^-$ phenotype of the *GCN2c*-double mutations in the *gcn4::LEU2 gcn2::LEU2* strain H1648 that lacks a functional GCN4 protein (Fig. 4), indicating that the growth defect does not result simply from overexpression of *GCN4*.

It was shown recently by Dever et al. (12) that the Slg$^-$ phenotype associated with the *GCN2c-E1537G-E532K* double mutation is eliminated by the substitution of Ser-51 in eIF-2α with alanine. We extended this observation to include all five *GCN2c* double mutations listed in Table 2 (data not shown). Because the Ser-51 substitution mutation abolishes phosphorylation of eIF-2α by GCN2, Dever et al. (12) proposed that the Slg$^-$ phenotype results from hyperphosphorylation of eIF-2α on Ser-51 and attendant inhibition of translation initiation, similar to what occurs in mammalian cells upon activation of the eIF-2α kinases DAI and HCR (26). To test this conclusion further, we examined the effects of the *GCN2c-E1537G-E532K* allele on translation initiation by comparing the distribution of total polyribosomes and 80S monosomes in wild-type and *GCN2c* strains. Velocity sedimentation in sucrose density gradients was used to separate polysomes, 80S ribosomes, and the 60S and 40S ribosomal subunits present in whole-cell extracts. Under the conditions of our experiments, excess free 60S and 40S subunits form inactive 80S couples (17, 41). Thus, the ratio of polysomes to 80S particles gives an indication of the rate of translation initiation: as the rate decreases, the fraction of subunits present in 80S couples should increase at the expense of

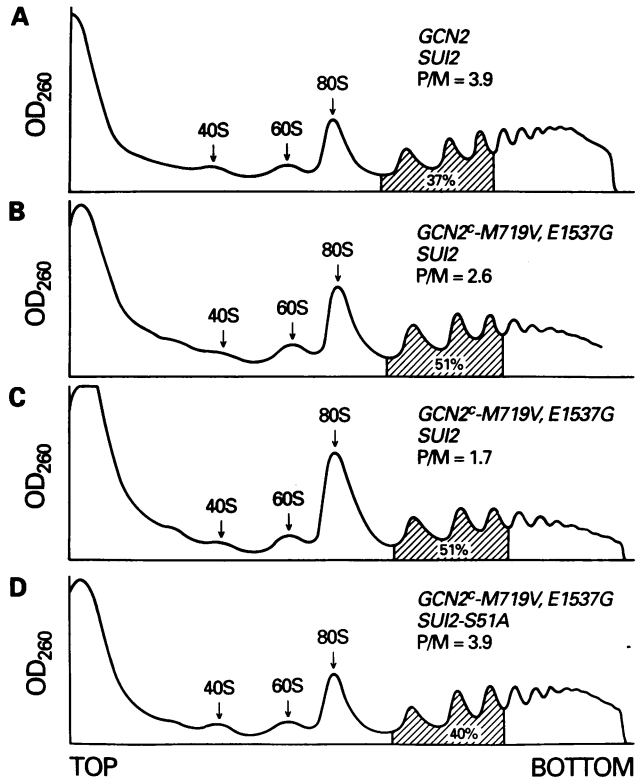


FIG. 5. Polysome distributions in strains bearing different chromosomal *GCN2^c^* alleles. The isogenic strains H1402 (*GCN2*) (A) and H1608 (*GCN2^c^-M719V-E1537G*) (B) were analyzed. Transformants of the isogenic strains H1816 (*gcn2Δ SUI2*) (C) and H1817 (*gcn2Δ SUI2-S51A*) (D), both bearing plasmid p1052 (*GCN2^c^-M719V-E1537G*), were analyzed. Strains were grown to mid-logarithmic phase in minimal SD medium containing only the necessary supplements. Extracts were prepared and centrifuged at 4°C in an SW41 rotor for 2.5 h at 39,000 rpm on 7 to 47% sucrose gradients prepared in a solution containing 50 mM Tris-acetate (pH 7.0), 50 mM NH_4Cl, 12 mM $MgCl_2$, and 1 mM dithiothreitol. Gradients were fractionated while being scanned at 260 nm, and the absorbance profiles are shown with the top of the gradient beginning on the left. The positions of 40S, 60S, and 80S ribosomal species are indicated. The ratios of polysomes (P) to monosomes (M) were calculated by measuring the areas under the 80S peak and the polysomes (up to the point where the absorbance tracing terminated in the experiment shown in panel B). The shaded areas give the percentages of total polysomes present in 2-mers, 3-mers, and 4-mers. OD_{260}, optical density at 260 nm.

polysomes. In addition, the average size of the polysomes should decrease when the rate of initiation is reduced. We consistently found that the ratio of polysomes to 80S particles was greater in the *GCN2* strain than in the isogenic *GCN2^c^-M719V-E1537G* mutant (e.g., 3.9 versus 2.6, respectively; Fig. 5A and B); in addition, the polysomes were larger in the wild-type strain than in the mutant (see legend to Fig. 5 for quantitation). To establish the significance of the alterations in the polysome profile and to verify that they originated from hyperphosphorylation of eIF-2α, we showed that the polysome-to-monosome ratio and the size distribution of polysomes in the *GCN2^c^-M719V-E1537G* mutant were both restored to the wild-type situation by substitution of Ser-51 in eIF-2α with alanine (Fig. 5C and D).

The aberrant polysome distribution seen in the *GCN2^c^-M719V-E1537G* mutant containing wild-type eIF-2α is unlikely to be a secondary effect of reduced growth rate, because the wild-type strain exhibits essentially the same polysome-to-80S ratio whether growing exponentially or approaching stationary phase (49). In addition, a normal profile was observed for a [*rho*^{0}] derivative that grows much more slowly than the parental wild type (data not shown). Related findings were reported by Yoon and Donahue (64), suggesting that *S. cerevisiae* strains reduce initiation and elongation rates proportionately as growth rate decreases, maintaining a fixed polysome-to-80S ratio over a wide range of growth rates. The aberrant profile observed here for the *GCN2^c^-M719V-E1537G* mutant is qualitatively similar to that associated with temperature-sensitive lethal mutations in yeast translation initiation factors, although 80S accumulation is more pronounced in such mutants when cell division is completely arrested by incubation at the lethal temperature (7, 17, 25). We conclude that constitutive activation of GCN2 kinase function by the most-derepressing *GCN2^c^* mutations inhibits translation initiation to a degree that diminishes, but does not abolish, cell growth and division under nonstarvation conditions.

The effects of *GCN2^c^* mutations on the general control system and cellular growth rate are dependent on the positive regulators GCN1 and GCN3. Recessive mutations in *GCN1* or *GCN3* (including deletions of *GCN3*) have the same phenotype as a deletion of *GCN2*, impairing derepression of *GCN4* and the structural genes under its control (29). Constitutively derepressing alleles of *GCN3* that increase *GCN4* expression independently of *GCN1* and *GCN2* were identified elsewhere (23), suggesting that GCN3 functions downstream from these other positive effectors in the general control pathway. This conclusion was supported by the observation that GCN3 exists in a high-molecular-weight complex with several *GCD* gene products and a fraction of the eIF-2 present in the cell (7). GCN1 might also function downstream from GCN2, since it is required to mediate the effects of eIF-2α phosphorylation on translation initiation (23). Alternatively, GCN1 could be required for recognition of eIF-2α as the substrate or for activation of GCN2 catalytic function by uncharged tRNA in amino acid-starved cells. Therefore, it was of interest to determine whether any of the newly isolated *GCN2^c^* mutations, particularly those mapping in the HisRS region, bypass the requirement for *GCN1* in derepressing the general control system.

To accomplish this goal, we introduced each of the plasmid-borne *GCN2^c^* mutations, wild-type *GCN2*, or the *GCN4^c^* allele described above into the *gcn2::LEU2 gcn1-1* double mutant H1506 and measured expression of the *HIS4-lacZ* fusion present in this strain under nonstarvation conditions. The results listed in Table 4 (column 2) show that the *GCN4^c^* allele led to high-level expression of the *HIS4-lacZ* fusion in the *gcn2 gcn1* double mutant, as expected from the fact that elimination of the uORFs bypasses the requirement for *GCN1* in translational activation of *GCN4* expression (55). However, relative to wild-type *GCN2*, none of the *GCN2^c^* single mutations led to significant derepression of *HIS4-lacZ* expression in the *gcn1-1* mutant. In addition, the *GCN2^c^* and *GCN2* transformants of this strain showed identical sensitivity to 3-AT, indicative of nonderepressible expression of the general control system (data not shown). Essentially the same results with the *gcn2 gcn3* double mutant H1466 were obtained (Table 4, column 3), verifying that GCN3 is required for the derepression of the general control system elicited by each of the *GCN2^c^* alleles. Finally, none of the *GCN2^c^* alleles increased *HIS4-lacZ* expression when introduced into the *gcn2 gcn4* double

TABLE 4. Derepression of *HIS4-lacZ* expression under nonstarvation conditions in *GCN2*^{c} mutants dependent on *GCN1*, *GCN3*, and *GCN4*

Location or type and name of plasmid-borne allele	*HIS4-lacZ* expression (U) in the following strains[a]:			
	H1149 (*gcn2::LEU2*)	H1506 (*gcn2::LEU2 gcn1-1*[b])	H1466 (*gcn2-505*[c] *gcn3::LEU2*)	H1698 (*gcn2-505*[c] *gcn4::LEU2*)
Protein kinase domain				
GCN2^c*-S513F*	350	130	120	94
GCN2^c*-F647S*	270	120	140	69
GCN2^c*-R699W-D918G*	530	120	140	96
GCN2^c*-M719V*	670	150	130	75
GCN2^c*-E734V*	410	130	150	93
GCN2^c*-E752K*	470	150	130	97
GCN2^c*-H792Y*	540	130	180	92
HisRS-related domain				
GCN2^c*-F1065L*	320	110	140	88
GCN2^c*-D1069N*	350	130	150	95
GCN2^c*-A1128G*	410	110	130	82
GCN2^c*-N1226D*	280	150	130	100
GCN2^c*-H1239Y*	260	110	120	81
GCN2^c*-G1269D*	400	110	150	91
C-terminal domain				
GCN2^c*-R1488K*	320	110	140	81
GCN2^c*-E1522K*	500	100	150	96
GCN2^c*-E1537G*	750	110	170	98
GCN2^c*-N1547K*	440	110	130	78
Controls				
GCN2	150	120	120	85
GCN4^c	860	920	1,150	1,570
None	120	90	140	92

[a] Transformants of the indicated strains containing the *GCN2* alleles shown on the left (borne on the plasmids listed in Table 2) were grown to mid-exponential phase on minimal SD medium (54) containing only the required supplements. Total cell extracts were prepared and analyzed for the units of β-galactosidase activities present in each strain. Enzyme activities are given in units, defined as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside cleaved per minute per milligram of protein.

[b] *gcn1-1* was isolated in vivo (53), and the nature of the lesion has not been determined.

[c] *gcn2-505* (59) contains a substitution of Lys-559 with valine. Lys-559 corresponds to the invariant lysine present in the ATP-binding domain of eukaryotic protein kinases (22, 34); as expected, this substitution is nearly indistinguishable from a deletion of *GCN2* for impairment of derepression of the general control system (60).

mutant H1698 (Table 4, column 4), confirming that the enzyme derepression associated with each of these mutations is mediated by GCN4.

We also investigated whether *GCN1* and *GCN3* are required for the Slg$^-$ phenotype of the *GCN2*^{c} double mutations, which we attributed above to inhibition of general translation initiation by phosphorylation of eIF-2α. As shown in Fig. 4, the Slg$^-$ phenotype of the *GCN2*^{c} double mutations that we analyzed was eliminated by inactivation of *GCN1* or *GCN3*. This finding, combined with the results shown in Table 4, leads us to conclude that GCN1 and GCN3 are both required for the inhibition of translation initiation resulting from hyperactivation of GCN2 kinase function. On the basis of our results, GCN1 could function with GCN3 to mediate the effects of eIF-2α phosphorylation on the translational machinery or it could be required for the phosphorylation reaction itself. If the latter is true, the fact that none of our *GCN2*^{c} mutations allows GCN2 to regulate protein synthesis in the absence of GCN1 seems more consistent with a model in which GCN1 is required for catalytic activity per se rather than required solely for the activation of kinase function under starvation conditions. However, we cannot rule out the possibility that all of our *GCN2*^{c} mutations make GCN2 hypersensitive to low levels of uncharged tRNA and that sensing this signal is still completely dependent on GCN1.

DISCUSSION

We have used a genetic approach to identify amino acids in GCN2 that are involved in regulating its protein kinase activity. Regions flanking the protein kinase domain are required for high-level GCN2 function under starvation conditions, since internal deletions and two-codon insertions in these sequences eliminate the ability of GCN2 to derepress *GCN4* expression. Because these mutations do not impair the in vitro autophosphorylation activity of GCN2, the regions flanking the kinase moiety of GCN2 are thought to function primarily as positive regulatory domains (49, 60). The presence of a large segment in GCN2 related in sequence to HisRSs suggests a mechanism for coupling protein kinase activity to amino acid levels, whereby uncharged tRNA present in starved cells would bind to the HisRS-related domain and stimulate the adjacent kinase moiety (59). Here, we have described six *GCN2*^{c} mutations that alter single amino acids in the HisRS-related sequences of GCN2, providing new evidence in favor of this model. We also obtained four mutations in the extreme C-terminal

segment of the protein, underscoring the importance of this region in regulating GCN2 kinase function. All of the mutations appear to stimulate GCN2 kinase function rather than increase the abundance of the protein. The most-derepressing alleles in each domain were found to be absolutely dependent on Ser-51 in eIF-2α for activation of *GCN4* translation, indicating that each mutation enhances the ability of GCN2 to phosphorylate eIF-2α under nonstarvation conditions when levels of uncharged tRNA are low.

All of the *GCN2^c^* alleles require GCN1 and GCN3 for their inhibitory effects on general protein synthesis and for translational derepression of *GCN4*. This observation is consistent with the idea that GCN3 is a regulatory subunit of eIF-2B in *S. cerevisiae*, since this protein would be required to mediate the effects of eIF-2α hyperphosphorylation associated with any *GCN2^c^* allele. In the case of GCN1, whose function in the general control system is unknown, it was possible that certain *GCN2^c^* alleles would bypass the requirement for GCN1 in the derepression of *GCN4*. For example, if GCN1 was involved in recognition of uncharged tRNA, *GCN2^c^* mutations in the HisRS-related domain might mimic the stimulatory effect of the GCN1 protein on GCN2 kinase function in the absence of uncharged tRNA. The fact that the *gcn1-1* mutation suppresses all of the *GCN2^c^* alleles is consistent with several alternative possibilities for the role of GCN1 in the regulatory mechanism. There could be an obligate requirement for GCN1 in the catalytic activity of the GCN2 kinase domain; alternatively, GCN1 could be required for interaction with the substrate eIF-2α or an activator like uncharged tRNA. The fact that the *gcn1-1* mutation overcomes the Slg^−^ phenotype of the *GCN2^c^* mutations, which we attribute to inhibition of general protein synthesis, allows us to dismiss a different sort of model in which GCN1 would stimulate the transcription of *GCN4* or the ability of the GCN4 protein to activate transcription of genes encoding amino acid biosynthetic enzymes.

***GCN2^c^* mutations mapping at the extreme C terminus of the protein.** GCN2 is associated with ribosomes, and this interaction is completely abolished by the removal of residues 1467 to 1590 (49), an interval encompassing all four of the *GCN2^c^* mutations at the C terminus that we isolated. It has been suggested that the ribosome association of GCN2 could provide it with access to eIF-2 or uncharged tRNA during the course of translation. Because the *GCN2^c^-E1537K* allele did not appear to affect the localization of GCN2 with ribosomes (49), this is probably not the sole function of the C terminus. We have found that two sequence intervals in the C terminus of GCN2 (residues 1481 to 1498 and 1518 to 1541) are predicted to form α-helices in which all of the charged residues are present on one face of the helix. It is interesting that α-helices of this type have been identified in accessory segments of several yeast aminoacyl-tRNA synthetases (35, 40). The fact that three of the four *GCN2^c^* mutations in the C terminus map in the predicted helical segments may indicate that such structures have an important role in the activation of GCN2 kinase function.

Proposed locations of *GCN2^c^* mutations in the three-dimensional structure of the protein kinase domain. The catalytic domains of all eukaryotic protein kinases have 11 subdomains in common, each containing highly conserved or invariant residues, most of which appear to have important functions in ATP binding or catalysis (22). The conservation of these subdomains suggests that the three-dimensional structure reported for the catalytic domain of murine cAPK can serve as a structural paradigm for all other protein kinases. This structure consists of two lobes separated by a deep cleft that is the site of catalysis (Fig. 6). The smaller N-terminal lobe contains the binding site for ATP; the larger lobe is involved primarily in peptide binding and catalysis. Many kinases have inserts located between conserved subdomains that can be accommodated in the cAPK model as loops extending from the surface of the two-lobed structure (34).

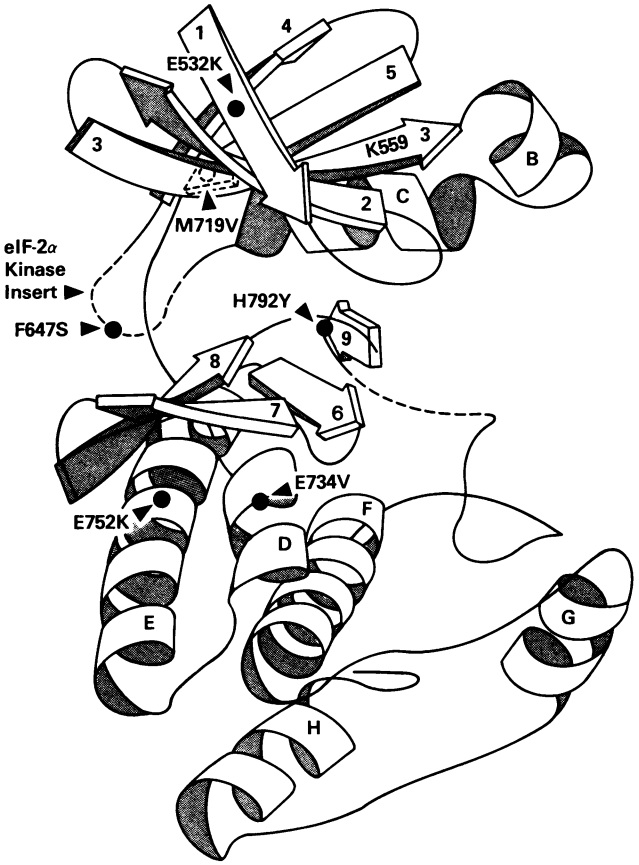


FIG. 6. Ribbon diagram of the conserved catalytic core of cAPK showing the proposed positions of *GCN2^c^* amino acid substitutions in the protein kinase domain of GCN2. The schematic of the X-ray crystal structure of the cAPK catalytic domain is adapted from that found in Knighton et al. (34), with β-strands shown as arrows (numbered from 1 to 9) and α-helices depicted as coils (numbered from A to H). The locations of inserts in the catalytic core predicted for the eIF-2α kinases are shown as dotted lines; the one that is labeled eIF-2α kinase insert is the larger of the two inserts and that which is most unique to this group of kinases. The postulated positions of residues altered by the *GCN2^c^* substitutions are indicated by black dots and labeled by the allele designations. These positions were obtained from the locations of the corresponding residues in cAPK (in the sequence alignments of Fig. 7) and from the positions of the various β-strands and α-helices in the structure of cAPK given by Knighton et al. (34).

In order to locate different residues in the GCN2 kinase domain in the predicted three-dimensional structure of its catalytic core, we aligned the sequence of GCN2 with that of murine cAPK (Fig. 7). The catalytic domains of human (39) and mouse (16) DAI and of HCR (6) were included in these alignments in an attempt to detect sequence motifs unique to the three eIF-2α kinases. On the basis of our alignment, the catalytic core of GCN2 shows 36 and 42% sequence identity with HCR and human DAI, respectively, similar to the 37%

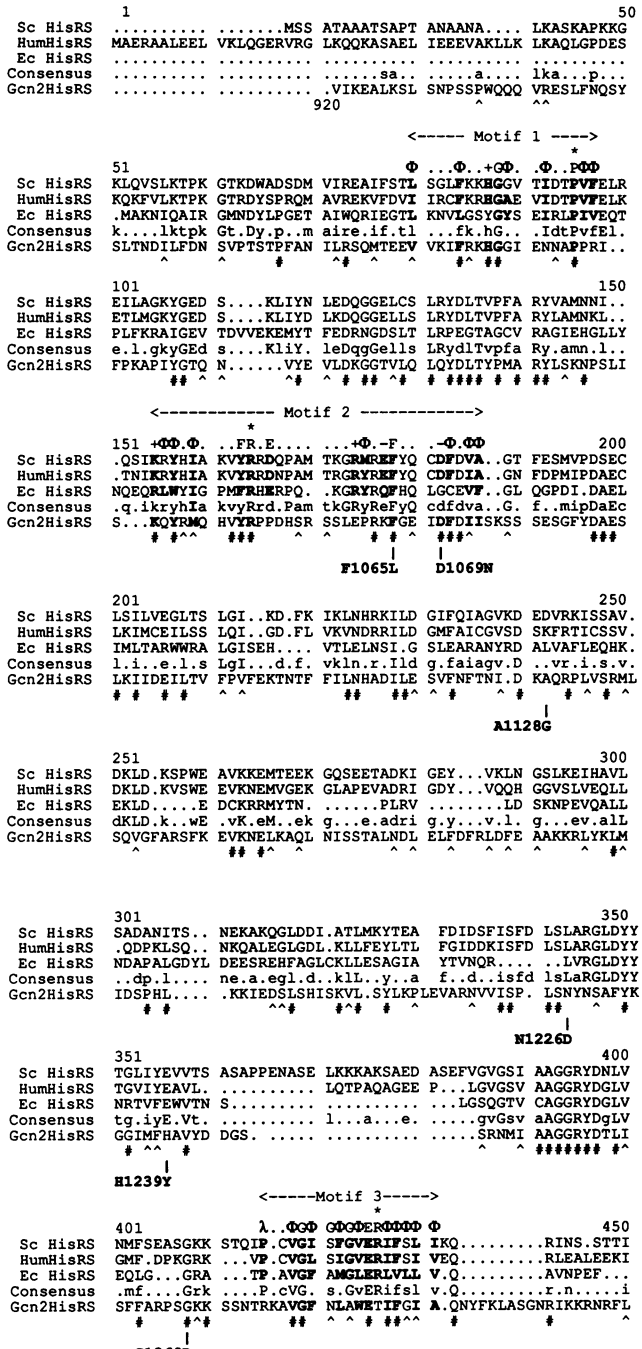
FIG. 7. Alignments of the protein kinase domains of the eIF-2α kinases GCN2, HCR, and human and mouse DAI with the catalytic subunit of murine cAPK. The alignments were constructed from pairwise- and multiple-sequence alignments obtained with, respectively, the BestFit and PileUp programs (13) with some manual adjustments made in regions of limited similarity. The amino acid positions in each protein are indicated on the far right of each line of sequence. Sequence identities are boxed. Dots indicate the positions of gaps in the alignment; asterisks indicate nearly invariant residues present in all serine-threonine protein kinases; filled arrowheads indicate residues present in GCN2, human (h) DAI, mouse (m) DAI, and HCR that occur in no more than 5 distinct protein kinases in the collection of 75 serine-threonine protein kinases aligned by Hanks and Quinn (22). The locations of amino acid substitutions made by the *GCN2*^{c} mutations mapping in this domain are indicated above the cAPK sequence.

identity that exists between human DAI and HCR. As expected, the human and mouse DAI sequences are more closely related, showing about 60% identity (16). By comparison, cAPK shows only 33, 25, and 30% identity with GCN2, HCR, and human DAI, respectively; in addition, a variety of other protein kinase sequences that we aligned (data not shown) are even less related (by ca. 5%) to GCN2, HCR, or human DAI than is cAPK. A feature unique to the four eIF-2α kinases is the presence of an insert between the residues that correspond to positions 104 to 105 in cAPK. On the basis of our alignment, these inserts are expected to occur in a loop located between α-helix C and β-strand 4 that would emanate from the base of the small lobe and extend into the back of the cleft between the two lobes (Fig. 6). The inserts in GCN2 and HCR are quite large (ca. 135 and 115 residues, respectively) compared with those present in human DAI (ca. 35 residues) and mouse DAI (ca. 20 residues). These inserts contain short regions of sequence similarity with one another (Fig. 7). A second insert present in GCN2, HCR, and the two DAIs is predicted to occur between residues corresponding to positions 192 to 193 in cAPK. These latter inserts are expected to increase the size of the large loop found between β-strand 9 and α-helix F that is important for binding the helical portion of the peptide substrate of cAPK (33, 34).

By comparing the sequence of GCN2 with all other known protein kinases, Hanks and Quinn (22) suggested that GCN2 was not closely related to any other kinase but forms its own branch on the phylogenetic tree proposed for these enzymes. However, the 36 to 42% sequence identity found here among GCN2, human DAI, and HCR is characteristic of kinases considered to be members of the same subfamily (22). On the basis of this high degree of sequence similarity and of the identical positions of inserts predicted by our alignment, we propose that the eIF-2α kinases define a new branch cluster on the protein kinase phylogenetic tree. There are 11 positions in the kinase domains of these enzymes in which the same amino acid occurs in GCN2, the two DAIs, and HCR but is absent in the majority of other protein kinases (marked with arrowheads in Fig. 7). These conserved residues, as well as the characteristic inserts present in GCN2, DAI, and HCR, may be important for recognition of the substrate eIF-2α or in a regulatory mechanism unique to eIF-2α kinases.

Using the sequence alignment between GCN2 and cAPK shown in Fig. 7, we localized *GCN2*^{c} mutations in the predicted structure of the GCN2 kinase domain (Fig. 6). The *GCN2*^{c}*-E532K* and *GCN2*^{c}*-M719V* mutations are expected to alter residues in the N-terminal lobe that binds ATP. The *GCN2*^{c}*-M719V* substitution is of particular interest because it is immediately adjacent to the only two consecutive residues that are uniquely conserved among GCN2, the two DAIs, and HCR (Ile-Gln at positions 717 and 718 in GCN2; Fig. 7). Similarly, the *GCN2*^{c}*-E532K* substitution occurs at a position that is a glutamate or an aspartate in the four eIF-2α kinases (Fig. 7), but it is rarely an acidic residue in other kinases. The residue corresponding to Glu-532 of GCN2 in the cAPK catalytic domain (Arg-45) is thought to be in close proximity to the ATP-binding site (for a review, see reference 34). Interestingly, there is evidence that ATP binding to DAI is stimulated by double-stranded RNA, the activating ligand for this eIF-2α kinase (3, 19). Perhaps a similar mechanism operates for GCN2 involving uncharged tRNA as an activator. In this view, the *GCN2*^{c}*-E532K* and *GCN2*^{c}*-M719F* mutations would stimulate ATP binding to the GCN2 kinase domain at low concentrations of uncharged tRNA. In the case of *GCN2*^{c}*-E532K*, introduction of a lysine in place of glutamate might provide an additional stabilizing interaction between the ATP-binding domain and one of the negatively charged phosphate groups of ATP.

The *GCN2*^{c}*-F647S* allele substitutes an amino acid in the large insert region unique to eIF-2α kinases. The *GCN2*^{c}-



H792Y allele is predicted to alter a residue at the C terminus of β-strand 9. Both of these alterations are thought to occur near the cleft between the two lobes (Fig. 7). Because of their proximity to the active site, these mutations could also stimulate ATP binding. Alternatively, they might influence catalysis or positioning of the substrate in a way that mimics or bypasses the need for kinase activation. One of the latter two mechanisms probably applies to the *GCN2^c-E752K* and *GCN2^c-E734V* alleles that we predict will substitute residues in helices E and D, respectively, in the C-terminal lobe of the GCN2 kinase domain (Fig. 6). We consider it noteworthy that several of the *GCN2^c* mutations affecting the kinase domain alter residues or map to inserts that are idiosyncratic to the three eIF-2α kinases. Thus, our findings may provide important clues about sites in the catalytic domains of DAI or HCR that function in regulating the activity of these protein kinases in mammalian cells.

Predicted effects of *GCN2^c* alleles on the structure of the HisRS-like domain. All class II aminoacyl-tRNA synthetases share three relatively short sequence motifs (motifs 1 to 3; 11, 15) and the X-ray crystal structures of the class II enzymes seryl-tRNA synthetase (SerRS) (10) and aspartyl-tRNA synthetase (AspRS) (51) reveal a common structural core consisting of an eight-stranded antiparallel β-sheet that forms the catalytic domain of these two enzymes. Sequence motifs 2 and 3 are present in structurally homologous elements of the catalytic domains of SerRS and AspRS (10, 11, 15, 51). Motif 2 can be seen to interact closely with the acceptor stem of tRNA (where the amino acid is attached) in the tRNA^{Asp}-AspRS complex (51). It has been suggested that motif 3 sequences contribute to ATP binding (2, 51) and formation of the aminoacyladenylate (20, 32).

Motifs 1 to 3 have been identified in the correct order and with typical spacing (15) in HisRS from humans, *S. cerevisiae*, and *E. coli*, strongly suggesting that HisRS is a class II enzyme (11). GCN2 shows about 19% sequence identity with HisRS from *S. cerevisiae* and humans over approximately 500 residues, which is nearly as great as the similarity

FIG. 8. Sequence alignments of the HisRS-related region of GCN2 and the predicted catalytic domains of HisRS from *S. cerevisiae* (43), humans (48, 56), and *E. coli* (18). The alignments were constructed by using the PileUp program (13), with some manual adjustments made in regions of limited similarity. The amino acid positions of selected residues in the complete sequence of *S. cerevisiae* HisRS and GCN2 are indicated, respectively, above and below the sequence alignments. Dots indicate the positions of gaps in the sequence of that protein relative to those of the other aligned sequences. A consensus sequence (shown on the fourth line) derived from the three authentic HisRS sequences is indicated by an uppercase letter when all three sequences contain the same residue at that position, a lowercase letter when that amino acid is present in two of the three sequences, and a dot when all three sequences are different. Correspondence between the sequence of the GCN2 HisRS-like domain and the consensus sequence is indicated below the GCN2 sequence with # indicating a position of identity between GCN2 and the consensus sequence and ^ indicating a conservative replacement (14) in GCN2 with respect to the consensus sequence. Residues in the alignments shown in boldface type conform to the consensus sequences of motifs 1, 2, or 3 (15) that are indicated above the sequence alignments at the appropriate positions. Φ, the hydrophobic residues F, Y, W, I, M, V, L, and A; +, positively charged residues H, R, and K; −, negatively charged residues D, E, N and Q; λ, the small amino acids P, G, S, and T. The only invariant residues in these motifs are indicated by asterisks. The locations of the *GCN2^c* substitutions in the GCN2 HisRS-related domain are indicated in boldface type beneath the sequence alignments.

between *E. coli* HisRS and the yeast and human enzymes (ca. 23%) (59) (Fig. 8). It is noteworthy that the highest degree of similarity between GCN2 and the three HisRS sequences occurs in the region predicted to form the eight-stranded antiparallel β-sheet. In the roughly 360 amino acids stretching from motif 1 to motif 3, GCN2 contains the identical residue or a conservative replacement (14) at 56% of the 230 positions at which the same amino acid occurs in two or all three of the HisRS sequences that we aligned (Fig. 8). Two stretches of particularly high similarity contain motifs 2 and 3 and the residues immediately adjacent on the N-terminal side of each motif that are uniquely conserved among HisRS enzymes (11, 48). The extreme N- and C-terminal portions of the HisRS sequences, which are thought to have accessory functions specific for histidine (11), are less well conserved in GCN2 (Fig. 8).

The high degree of sequence conservation between GCN2 and the HisRS enzymes in the vicinity of motifs 2 and 3 suggests that GCN2 possesses much of the antiparallel β-sheet that contains the binding site for the 3′ end of tRNA. This suggestion is consistent with our hypothesis that GCN2 senses amino acid limitation by interacting with uncharged tRNA. Motif 2 forms a loop in AspRS that directly interacts with the 3′ end of the acceptor stem of $tRNA^{Asp}$ and this loop is of variable length and sequence in different class II enzymes (11, 15). GCN2 appears to contain the two highly conserved β-strands that would flank the loop formed by motif 2 but diverges from the HisRS sequences in the sequence of the loop itself (Fig. 8). Perhaps this divergence between GCN2 and authentic HisRS sequences is related to the proposed ability of GCN2 to interact with the 3′ end of multiple tRNAs (59). Our model does not require that the HisRS-like domain of GCN2 binds ATP or amino acids but requires only that it contains a binding site for uncharged tRNA that can distinguish between charged and uncharged forms. This could explain why motif 3 is more degenerate in GCN2 than in most class II synthetases (11, 15), in lacking the single invariant arginine residue in the motif (Fig. 8).

Two of the *GCN2^c^* alleles described in this report are of particular interest because they make conservative substitutions of amino acids in motif 2 (*GCN2^c^-F1065L* and *GCN2^c^-D1069N*) and thus are expected to alter (but not disrupt) the portion of the β-sheet that directly interacts with the acceptor stem of tRNA (11). Perhaps these two mutations increase the affinity of the HisRS-like domain for uncharged tRNA and thereby permit activation of GCN2 kinase function at lower concentrations of uncharged tRNA than are required in wild-type cells. The *GCN2^c^-G1269D* mutation affects a residue located immediately N terminal to motif 3. The remaining three *GCN2^c^* mutations in the HisRS-like domain (*GCN2^c^-A1128G*, *GCN2^c^-N1226D*, and *GCN2^c^-H1239Y*) affect residues located between motifs 2 and 3. On the basis of sequence alignments of class II synthetases (11), the last two mutations are expected to alter the antiparallel β-sheet at a location removed from motif 2 by several β-strands. However, because of their proximity to the active site, all three of these mutations could also modify an interaction with the 3′ end of tRNA. Testing our model for kinase activation will require physical and biochemical studies on the binding of tRNA by GCN2. The various *GCN2^c^* mutations described here should be very important tools in this undertaking.

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