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GCN20, a novel ATP binding cassette protein, and GCN1 reside in a complex that mediates activation of the eIF-2α kinase GCN2 in amino acid-starved cells

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GCN2 is a protein kinase that phosphorylates the α-subunit of translation initiation factor 2 (eIF-2) and thereby stimulates translation of *GCN4* mRNA in amino acid-starved cells. We isolated a null mutation in a previously unidentified gene, *GCN20*, that suppresses the growth-inhibitory effect of eIF-2α hyperphosphorylation catalyzed by mutationally activated forms of GCN2. The deletion of *GCN20* in otherwise wild-type strains impairs derepression of *GCN4* translation and reduces the level of eIF-2α phosphorylation *in vivo*, showing that GCN20 is a positive effector of GCN2 kinase function. In accordance with this conclusion, GCN20 was co-immunoprecipitated from cell extracts with GCN1, another factor required to activate GCN2, and the two proteins interacted in the yeast two-hybrid system. We conclude that GCN1 and GCN20 are components of a protein complex that couples the kinase activity of GCN2 to the availability of amino acids. GCN20 is a member of the ATP binding cassette (ABC) family of proteins and is closely related to ABC proteins identified in *Caenorhabditis elegans*, rice and humans, suggesting that the function of GCN20 may be conserved among diverse eukaryotic organisms.

Key words: ABC proteins/eIF-2α kinases/GCN2/GCN20/translation

Introduction

In *Saccharomyces cerevisiae*, starvation for an amino acid or purines leads to increased transcription of >30 genes encoding amino acid biosynthetic enzymes in numerous pathways (Rolfes and Hinnebusch, 1993; reviewed in Hinnebusch, 1992). This regulatory mechanism, known as general amino acid control, involves binding of the transcriptional activator protein GCN4 to the promoter region of each gene subject to the general control. The level of GCN4 protein increases under conditions of amino acid starvation as the result of increased translation of *GCN4* mRNA. The GCN4 protein thus produced activates transcription from its target genes and thereby increases the level of amino acid biosynthesis in nutrient-deprived cells.

Translation of *GCN4* mRNA is repressed under non-starvation conditions by four short open reading frames (uORFs) present in the mRNA leader that block the flow of scanning ribosomes to the *GCN4* start codon. *GCN4* expression increases in amino acid-deprived cells because many ribosomes which have translated the first uORF are able to scan past the remaining three uORFs in the leader (uORFs 2–4) and reinitiate further downstream at the *GCN4* coding sequences (Figure 1; Hinnebusch, 1992). The protein kinase GCN2 plays a key role in derepressing *GCN4* translation by phosphorylating the α-subunit of translation initiation factor-2 (eIF-2) on serine 51 (Dever *et al.*, 1992). eIF-2 delivers the initiator tRNA in a ternary complex with GTP to the 40S ribosomal subunit in one of the first steps in the translation initiation pathway (reviewed in Hershey, 1991; Merrick, 1992). There is strong genetic evidence that phosphorylation of eIF-2 by GCN2 stimulates *GCN4* translation by reducing the activity of the eIF-2B complex (Cigan *et al.*, 1993; Dever *et al.*, 1993; Vazquez de Aldana and Hinnebusch, 1994), which catalyzes the exchange of bound GDP for GTP on eIF-2 following each round of initiation. It has been proposed that the ensuing depletion of the active GTP-bound form of eIF-2 is responsible for allowing ribosomes that have translated uORF1 in the *GCN4* mRNA leader to scan past uORF4 before rebinding charged initiator $tRNA^{Met}$. As a consequence, these ribosomes would fail to reinitiate at uORFs 2–4 and would utilize the *GCN4* start site instead (Dever *et al.*, 1992).

Uncharged tRNA is thought to be an important signal in coupling the phosphorylation of eIF-2α by GCN2 to the availability of amino acids in the cell (Wek *et al.*, 1989). Mutants defective for an aminoacyl-tRNA synthetase show an elevated expression of genes under the control of *GCN4* without being starved for the cognate amino acid (Messenguy and Delforge, 1976; Niederberger *et al.*, 1983; Lanker *et al.*, 1992). In addition, the derepression of *GCN4*, observed in mutants containing a defective lysyl-tRNA synthetase, appears to be dependent on GCN2 function (Lanker *et al.*, 1992). Recently, we showed that increasing $tRNA^{His}$ levels in histidine-deprived cells stimulates *GCN4* translation in mutants containing a leaky *gcn2* mutation, in part, by increasing GCN2 kinase function (Vazquez de Aldana *et al.*, 1994). GCN2 contains a regulatory domain of ~530 amino acids adjacent to the kinase catalytic domain that is homologous to histidyl-tRNA synthetase (HisRS; Wek *et al.*, 1989). This HisRS-like domain is required for GCN2 to stimulate *GCN4* expression *in vivo*, but is dispensable for the autophosphorylation activity of the kinase observed *in vitro* (Wek *et al.*, 1990). It was proposed that the HisRS-related region of GCN2 monitors the aminoacylation levels of many different tRNAs and activates the adjacent protein kinase moiety in response to an accumulation of any uncharged tRNA (Wek *et al.*, 1989). It has not been possible, however, to activate GCN2 kinase function *in vitro* with uncharged tRNA, suggesting that additional factors are required for this process.

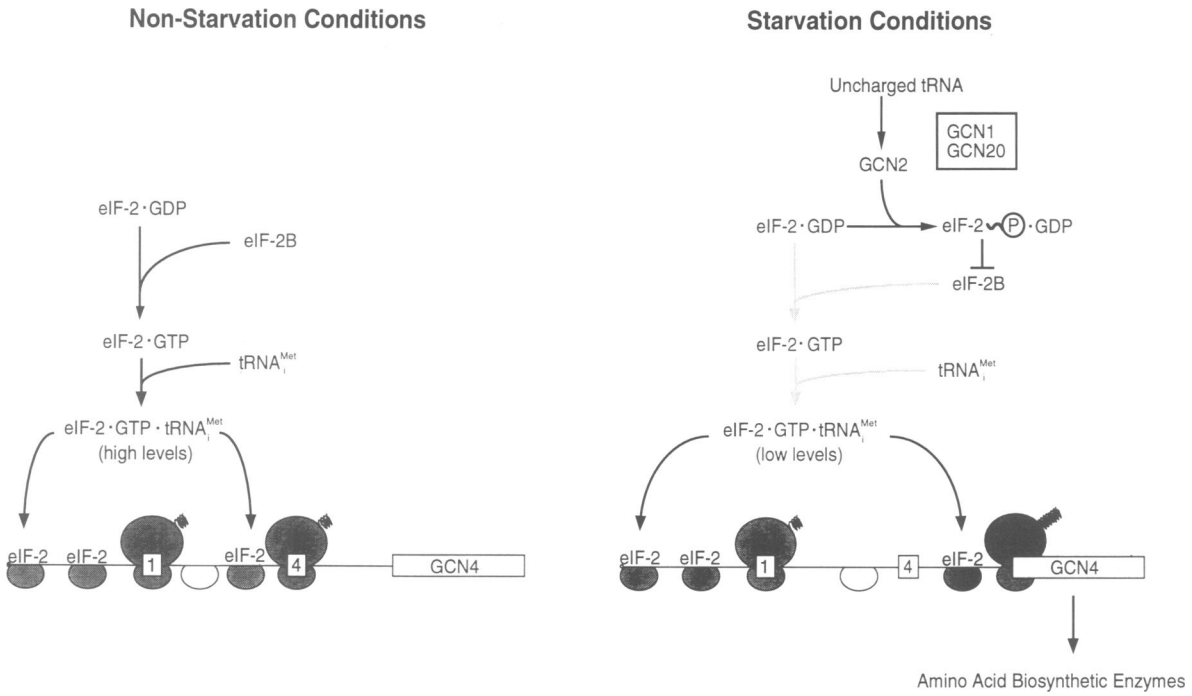


Fig. 1. A model for the translational control of yeast *GCN4* by phosphorylation of eIF-2α by the protein kinase GCN2. *GCN4* mRNA is shown with uORFs 1 and 4 and the *GCN4* coding sequences indicated as boxes. 40S ribosomal subunits are shaded when they are associated with the ternary complex composed of eIF-2, GTP and Met-tRNA$^{Met}_i$, and are thus competent to reinitiate translation; unshaded 40S subunits lack the ternary complex and, therefore, cannot reinitiate. Under non-starvation conditions, eIF-2·GDP is readily recycled to eIF-2·GTP by eIF-2B, leading to high levels of eIF-2·GTP and ternary complex formation. The ternary complexes thus formed reassemble with 40S ribosomes scanning downstream from uORF1, causing reinitiation to occur at uORF4. Under starvation conditions, uncharged tRNA accumulates and stimulates the activity of the protein kinase GCN2. GCN2 phosphorylates eIF-2α and the phosphorylated eIF-2 inhibits eIF-2B, reducing the recycling of eIF-2·GDP to eIF-2·GTP. The resulting low level of eIF-2·GTP and ternary complex formation diminishes the rate at which initiation complexes are reassembled on 40S subunits following translation of uORF1. Consequently, many 40S subunits scanning downstream from uORF1 are not competent to initiate at uORF4; these subunits acquire a ternary complex while scanning the interval between uORF4 and *GCN4* and reinitiate at the *GCN4* start site instead. As will be shown, GCN20 resides in a complex with GCN1 that is required for increased eIF-2α phosphorylation by GCN2 under conditions of amino acid deprivation.

GCN1 encodes a positive regulator of *GCN4* translation that is required *in vivo* for increased phosphorylation of eIF-2α catalyzed by GCN2 in response to amino acid starvation. Because it is not needed for GCN2 kinase function *in vitro*, it has been assumed that GCN1 functions in the activation of GCN2 by uncharged tRNA in amino acid-starved cells. GCN1 is a protein of 297 kDa containing a segment of ~200 residues related in sequence to fungal translation elongation factor 3 (EF-3; Marton *et al.*, 1993). EF-3 belongs to the ATP binding cassette (ABC) family of proteins that, with few exceptions, are components of membrane transporters. ABC transporters, found in both eukaryotes and prokaryotes, mediate the transport of a wide variety of molecules, including small ions, amino acids and peptides (Higgins, 1992). It has been proposed that EF-3 functions in translation elongation by stimulating the release of uncharged tRNA from the ribosomal E site (Triana *et al.*, 1993). We suggested previously that the sequence similarity between GCN1 and EF-3 might indicate that GCN1 facilitates the activation of GCN2 by uncharged tRNA during translation elongation (Marton *et al.*, 1993).

To identify additional factors involved in regulating GCN2 kinase activity, we isolated mutations that suppress the slow-growth phenotype conferred by a constitutively activated allele of *GCN2* (Vazquez de Aldana *et al.*, 1993; Vazquez de Aldana and Hinnebusch, 1994). Such *GCN2^c* alleles lead to hyperphosphorylation of eIF-2 under non-starvation conditions, inhibiting general translation initiation in addition to derepressing *GCN4* translation (Dever *et al.*, 1992; Ramirez *et al.*, 1992). In this report, we describe a suppressor of a *GCN2^c* mutation that maps in a previously unidentified gene called *GCN20*. This gene has been cloned and found to encode a new member of the ABC family of proteins. We show that GCN20 functions as a positive regulator of *GCN4* translation by stimulating the phosphorylation of eIF-2α by GCN2. In addition, strong genetic and biochemical evidence is presented indicating that GCN20 and GCN1 are associated in a protein complex. We propose that GCN1 and GCN20 are important components of the cellular machinery that couples the kinase activity of GCN2 to the availability of amino acids.

Results

Isolation and characterization of the GCN20 gene

The *gcn20-501* mutation was isolated (Vazquez de Aldana and Hinnebusch, 1994) as a recessive suppressor of the slow-growth phenotype caused by *GCN2^c-E532K, E1522K*, a hyperactivated allele of *GCN2*. Derepression of *GCN4* expression is required for resistance to 3-amino-triazole (3-AT), an inhibitor of histidine biosynthesis. Because they fail to derepress *GCN4*, strains carrying a

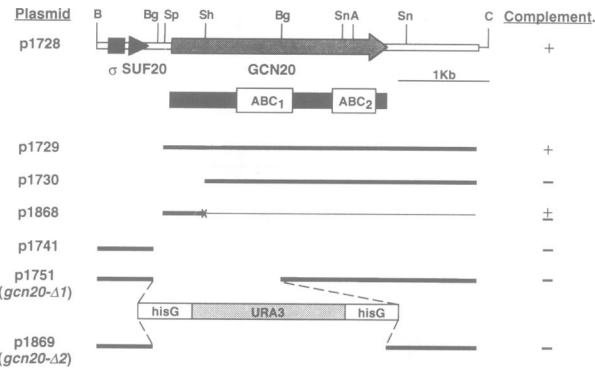


Fig. 2. Physical and functional maps of *GCN20*. At the top is a map of the *GCN20* region containing a sigma element (black square), *SUF20* (black triangle) and *GCN20*, with arrows indicating the 5′ to 3′ orientation of the coding sequences. Immediately below is a schematic representation of GCN20 protein, with the two conserved repeats common to members of the ABC family of proteins shown as boxes labeled ABC_1 and ABC_2. Deletion constructs used to test *GCN20*-complementing activity are indicated, with lines designating the sequences present in each plasmid. The X in plasmid p1868 indicates the position where a frame shift mutation was created, generating an in-frame stop codon five amino acids further downstream. To the right are indicated the complementing activities of the different constructs in *gcn20-501* strain H2566: (+) wild-type growth on plates containing 30 mM 3-AT; (±) weak growth on plates containing 30 mM 3-AT; (–) no growth on the same plates. The extent of the deletion in the *gcn20-Δ1* and *gcn20-Δ2* alleles is indicated by the portion of *GCN20* that is replaced with *URA3* flanked by *hisG* repeats. Restriction sites: A, *Aat*II; B, *Bam*HI; Bg, *Bgl*II; C, *Cla*I (vector sequences); Sh, *Sph*I; Sn, *Sna*BI; Sp, *Spe*I.

mutation in any of the known *GCN* genes are sensitive to 3-AT (3-AT^S; Hinnebusch, 1992). Fast-growing revertants containing *GCN2^c-E532K,E1522K* and the suppressor *gcn20-501* showed wild-type resistance to 3-AT (3-AT^R), indicating a relatively high level of *GCN4* expression. To examine the phenotype of *gcn20-501* in the presence of wild-type *GCN2*, we crossed together strains H1933 *(GCN2^c-E532K,E1522K gcn20-501)* and F113 *(GCN2 GCN20)* and scored the growth of haploid progeny on medium containing 3-AT. All spores with the genotype *GCN2 gcn20-501* were 3-AT^S, suggesting a defect in derepression of *GCN4*; consequently, the suppressor was designated *gcn20-501*.

The wild-type *GCN20* gene was cloned by complementing the 3-AT^S phenotype of the *gcn20-501* mutation in *GCN2* strain H2566 using a yeast genomic library (Rose *et al.*, 1987). One plasmid we obtained (p1714) also complemented the suppressor phenotype of *gcn20-501* in *GCN2^c-E532K,E1522K* strain H1933, restoring the slow-growth phenotype characteristic of the GCN2^c kinase. A 3.5 kb *Cla*I–*Spe*I fragment isolated from the genomic insert in p1714 was shown to direct plasmid integration by homologous recombination to a genomic locus linked tightly to *GCN20*, confirming that the cloned DNA contained *GCN20* (see Materials and methods for details).

GCN20 was localized on the genomic insert in p1714 by testing various subcloned fragments for complementation of the 3-AT^S phenotype of a *gcn20-501 GCN2* strain and the Slg$^+$ phenotype of a *gcn20-501 GCN2^c-E532K,E1522K* strain. The 4 kb *Bam*HI–*Cla*I fragment in p1728 contained all the information necessary to complement both phenotypes (Figure 2). The nucleotide sequence of this region was determined and found to contain a single long ORF of 2259 bp, beginning at an ATG codon located 714 bp downstream of the *Bam*HI site in p1728. This ORF encodes a slightly acidic protein, 752 amino acids in length, with a predicted molecular weight of 85 132. Comparison of the DNA sequence of the *GCN20* region with sequences in Genbank revealed the presence of a sigma element and the previously reported tRNA^{Gly} gene *SUF20* (Mendenhall *et al.*, 1987; Figure 2). A plasmid containing the *GCN20* ORF but lacking *SUF20* (p1729) fully complemented *gcn20-501*, whereas p1741 containing only *SUF20* had no complementing activity (Figure 2). Additionally, we found that a frame shift introduced at the *Sph*I site that leads to termination of the *GCN20* ORF after only 118 amino acids (plasmid p1868, Figure 2), substantially decreased complementation of the 3-AT^S phenotype of a *gcn20-501* mutation.

The fact that *SUF20* is present just upstream of *GCN20* locates the gene on the right arm of chromosome VI, close to the centromere (Gaber *et al.*, 1983). We confirmed this result by hybridizing an internal fragment of *GCN20* to an ordered lambda library of yeast genomic DNA (Riles *et al.*, 1993; see Materials and methods).

GCN20 is a member of the ABC family of proteins

Analysis of the GCN20 protein sequence shows that it contains an internal repeat of ~200 amino acids (Figure 2) that is similar to a sequence found duplicated in members of the ABC family of proteins (Figure 3A). The most highly conserved sequences in each repeat include two short nucleotide binding motifs known as the Walker A and B motifs (Walker *et al.*, 1982; Higgins *et al.*, 1985; for a review see Higgins, 1992). Sequences matching the Walker A motif, encoding a glycine-rich loop (GX_4GKS/T), are found in GCN20 at amino acid positions 232–239 and 565–572. Matches to the Walker B motif, encoding a hydrophobic pocket thought to lie near the glycine-rich loop in the folded protein (Higgins, 1992), occur in GCN20 between residues 381–394 and 664–677. GCN20 also contains close matches to the signature sequence of the ABC family, LSGGQ (Higgins *et al.*, 1986), located immediately upstream of each Walker B motif (positions 368–380 and 651–663) (Figure 3A). It is worth noting that the spacing between the Walker A and B motifs differs significantly for the two repeats in GCN20, with separations of 142 and 92 amino acids for the N-proximal and N-distal copies, respectively. In most other ABC proteins, in contrast, the distances separating the A and B elements in the two repeats only vary between 100 and 120 amino acids (Higgins, 1992). An analysis of the GCN20 sequence using the algorithm of Kyte and Doolittle (1982) did not identify regions sufficiently hydrophobic to be considered potential membrane-spanning segments.

GCN20 is most closely related to ABC proteins of unknown function, identified recently by genome sequencing projects in *Caenorhabditis elegans* (Wilson *et al.*, 1994), *S.cerevisiae* (U18796) and *Escherichia coli* (U18997), and to a truncated ORF from *Pseudomonas aeruginosa* (Figure 3A). GCN20 and a predicted *C.elegans* protein that we designated ceGCN20-1 are 61% similar and 43% identical over their entire lengths of 752 and 712 amino acids, respectively. A second *C.elegans* sequence we identified (designated ceGCN20-2) is 60% similar and

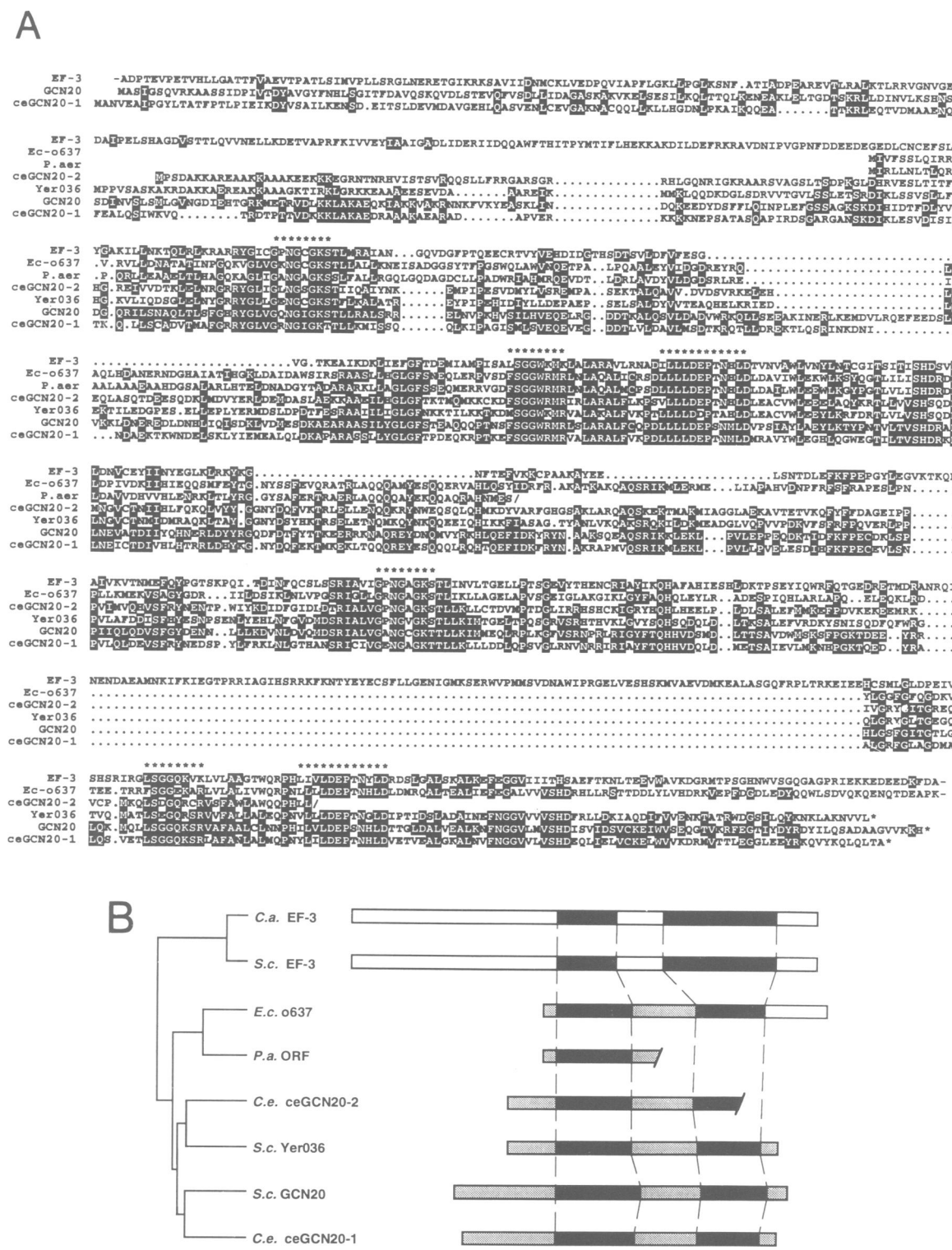


Fig. 3. Sequence alignment of GCN20 with closely related ABC proteins. (**A**) The complete amino acid sequence of GCN20 was aligned with *C.elegans* proteins ceGCN20-1 (accession number U10414) and ceGCN20-2 (U20783), *S.cerevisiae* EF-3 (J05197) and Yer036 (U18796), *E.coli* ORF-o637 (U18997) and the *P.aeruginosa* ORF (P.aer; M30145) using the program PILEUP. The sequence of *S.cerevisiae* EF-3 is shown between amino acids 214 and 1000, and the *E.coli* protein ORF-o637 between residues 1 and 534. Dots represent gaps in the sequences introduced to maximize alignment and slashes (/) indicate truncations in the sequence. The conserved Walker A and B motifs in each ABC protein are indicated by asterisks above the alignments; residues that are identical to the corresponding amino acid in *S.cerevisiae* GCN20 are indicated with black boxes. (**B**) Proposed relationship between GCN20 and homologous proteins in other organisms. The dendrogram was generated by the program PILEUP from the alignment of *S.cerevisiae* (S.c.) sequences GCN20, Yer036 and EF-3, *C.albicans* (C.a.) EF-3, *C.elegans* (C.e.) ceGCN20-1 and ceGCN20-2, *E.coli* (E.c.) ORF-o637 and the *P.aeruginosa* (P.a) ORF. EST sequences from rice (D23597) and human origin (T12459) are closely related to the GCN20/ceGCN20-1 branch, whereas the second rice clone (D15183) and the *A.thaliana* (T04469) genes are related to the Yer036/ceGCN20-2 branch. To the right, a linear representation of each protein sequence is shown, with black boxes representing ABC regions and gray boxes indicating sequence similarity among the last six proteins in regions flanking the ABCs.

36% identical to GCN20. The predicted protein from *S.cerevisiae* (designated Yer036p) is 61% similar and 39% identical to GCN20 over 610 amino acids, and the *E.coli* sequence ORF-o637 is 61% similar and 36% identical to GCN20 over 637 residues. We also found a truncated ORF from *P.aeruginosa* (Kato *et al.*, 1989) that is 60% similar and 42% identical to GCN20 over the known 261 amino acids of the bacterial protein. The similarity of GCN20 to this group of sequences is not restricted to the segments encompassing the Walker A and B motifs, but also involves the spacer regions between the motifs in each repeat and the sequences flanking the repeats, which are not generally conserved among ABC proteins (Higgins, 1992). GCN20 is also related to EF-3 proteins from *S.cerevisiae* (Qin *et al.*, 1990; Sandbaken *et al.*, 1990), *Candida albicans* (Colthurst *et al.*, 1991) and *Pneumocystis carinii* (Ypma-Wong *et al.*, 1992), although a high degree of similarity between GCN20 and the EF-3 proteins occurs only in the conserved ABC domains (Figure 3).

In an attempt to determine the relationships between the various ABC proteins that are closely related to GCN20, we used the program PILEUP (Devereux *et al.*, 1984) to construct the dendrogram shown in Figure 3B from the multiple sequence alignments shown in Figure 3A. The results of this analysis suggest that the predicted *C.elegans* protein ceGCN20-1 is most closely related to GCN20, whereas ceGCN20-2 is more similar to *S.cerevisiae* protein Yer036p than to any of the other six proteins. These four proteins appear to be more closely related to one another than to the bacterial proteins, and are even more distantly related to the EF-3 proteins. The pairs of proteins grouped together on separate branches of the dendrogram also exhibit similarities in overall size and the locations of the ABC repeats relative to the N- and C-termini of the proteins (Figure 3B). These structural similarities support the groupings shown in the dendrogram which are based on primary sequence differences. It is worth noting that GCN20 and ceGCN20-1 contain N-terminal domains preceding the first ABC that are similar in length and sequence, and that these domains are absent in the two bacterial proteins. Moreover, the *E.coli* protein contains a domain C-terminal to the second ABC that is unrelated to the C-terminal segments of the yeast and *C.elegans* proteins. The N- and C-terminal segments flanking the ABCs in EF-3 differ greatly in both length and sequence from the corresponding segments found in the other six proteins (Figure 3B). These comparisons suggest that, among the sequences shown in Figure 3A, ceGCN20-1 from *C.elegans* is the most likely candidate to be a functional homolog of GCN20.

By searching 'expressed sequence tags' (EST) databases, we also discovered portions of two predicted proteins from rice that are closely related to the second ABC motif of GCN20, and segments of two proteins, one from *Arabidopsis thaliana* and one from humans, that are related to the first ABC motif of GCN20. These EST sequences are more similar to GCN20 than they are to the bacterial proteins or the fungal EF-3 sequences. The *Arabidopsis* EST and one of the rice sequences are more closely related to yeast Yer036 than they are to GCN20, whereas the human and the other rice sequences are more similar to GCN20 than to Yer036 (see legend to Figure 3 for details). The presence of proteins highly similar to GCN20 in plants, worms and humans could indicate that some aspect of GCN20 function has been conserved throughout eukaryotic evolution.

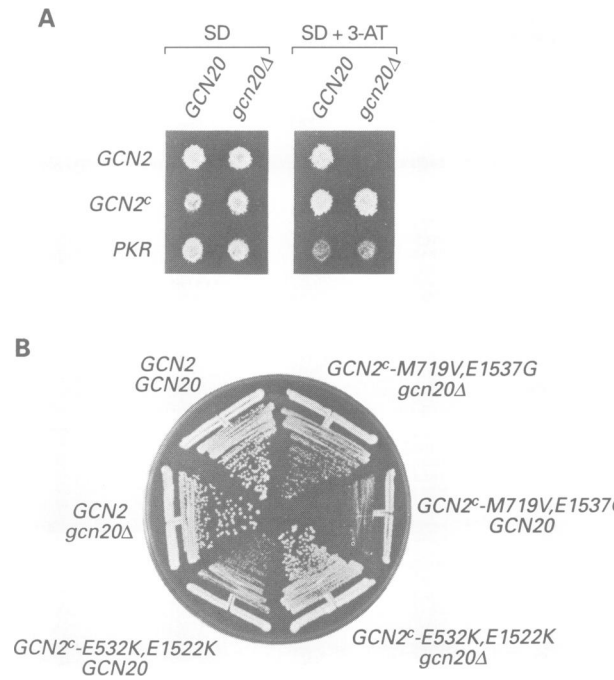


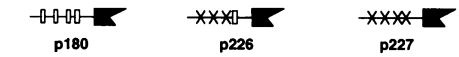
Fig. 4. Effects of deleting *GCN20* on general amino acid control and cellular growth rate in strains expressing different alleles of *GCN2* or human PKR. (**A**) Isogenic strains H2513 (*gcn2Δ gcn20Δ*) and H2511 (*GCN2 gcn20Δ*), transformed with low copy-number plasmids carrying wild-type *GCN2*, *GCN2^c-E532K,E1522K* (*GCN2^c*) or the human PKR gene under the control of the *GAL1* galactose-inducible promoter, were tested for their ability to derepress *HIS* genes subject to *GCN4* control. Patches of transformants were grown to confluence on SD plates, replica-plated to SD plates or to SD plates containing 10 mM 3-AT and incubated for 3 days at 30°C. (**B**) Strains carrying the indicated *GCN2* and *GCN20* alleles were streaked on SD plates containing minimal supplements and incubated for 3 days at 30°C.

GCN20 is a non-essential gene required for the derepression of GCN4 translation in amino acid-starved cells

To determine whether *GCN20* is necessary for cell viability, we constructed a heterozygous diploid strain in which one of the two *GCN20* alleles was replaced with the yeast *URA3* gene (Alani *et al.*, 1987; Figure 2). When this strain was sporulated and subjected to tetrad analysis, we found that all four haploid spores in each ascus were viable, indicating that *GCN20* is not an essential gene (see details in Materials and methods). Additionally, a deletion of *GCN20* was indistinguishable from the *gcn20-501* allele in impairing the general control response in strains containing wild-type *GCN2* (3-AT^s phenotype), and in suppressing the growth defect produced by *GCN2^c-E532K,E1522K* (Figure 4 and data not shown).

Expression of *GCN4* is derepressed at the translational level under conditions of amino acid deprivation, and this response is dependent on the positive regulators GCN1, GCN2 and GCN3 (Hinnebusch, 1992). The fact that the deletion of *GCN20* reversed the Sig$^-$ phenotype of a *GCN2^c* mutation (Figure 4B) strongly suggested that *GCN20* also functions as a positive regulator of *GCN4*

GCN4-lacZ expression (U)



	R	DR	R	DR	R	DR
W.T.	15	120	7	22	2300	2000
gcn2Δ	7	16	6	12	1700	2000
gcn20Δ	10	31	7	16	1700	2500

Fig. 5. Analysis of β-galactosidase expression from *GCN4–lacZ* fusions in *GCN20* and *gcn20Δ* strains. Isogenic strains F113 (W.T.), H2511 (*gcn2Δ*) and H2512 (*gcn20Δ*) were transformed with plasmids containing *GCN4–lacZ* fusions with the wild-type leader (p180) or two variants lacking either all four (p227) or the first three uORFs (p226), as indicated. Rectangles represent wild-type uORFs, 'X's indicate mutations that remove the ATG start codons of the corresponding uORFs. Cells were grown on SD medium with minimal supplements (repressing conditions, R) or on the same medium supplemented with 3-AT (derepressing conditions, DR), as described in Materials and methods. Listed in the table are the mean values obtained from three or four independent transformants. Individual measurements varied from the mean by ≤30%. U, units of β-galactosidase expressed as nanomoles of *o*-nitrophenyl-β-D-galactoside hydrolyzed per minute per milligram of protein.

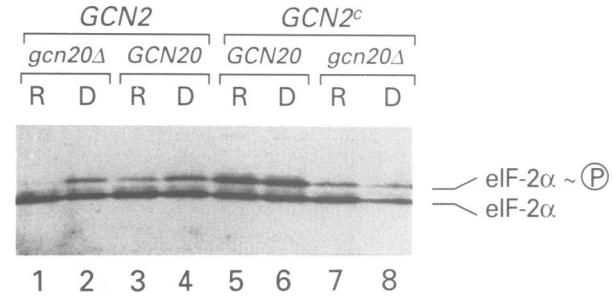


Fig. 6. Isoelectric focusing gel electrophoresis of eIF-2α phosphorylation in *GCN20* and *gcn20Δ* strains. Isogenic strains H1402 (*GCN2 GCN20*), H2563 (*GCN2 gcn20Δ*), H1613 (*GCN2c-E532K,E1522K GCN20*) and H2564 (*GCN2c-E532K,E1522K gcn20Δ*) were grown under non-starvation conditions (repressing, R) or under conditions of amino acid starvation (derepressing, D) and prepared for isoelectric focusing as described in Materials and methods. Proteins (20 μg per lane) were focused, transferred to nitrocellulose and subjected to immunoblot analysis using polyclonal antiserum specific for yeast eIF-2α. The P symbol denotes the position of eIF-2α phosphorylated on Ser51.

translation. To confirm this conclusion, we used *GCN4–lacZ* fusions with different combinations of uORFs to quantitate the effects of deleting *GCN20* on *GCN4* expression. As expected, we observed an 8-fold increase in expression of the wild-type *GCN4–lacZ* construct following histidine starvation of the wild-type strain, and the deletion of *GCN2* in this strain severely impaired the derepression response (Figure 5). The deletion of *GCN20* also reduced the level of *GCN4* expression under starvation conditions compared with the wild-type strain (31 versus 120 U), although the defect was less severe in the *gcn20Δ* strain than in the isogenic *gcn2Δ* strain. In agreement with previous results (Mueller and Hinnebusch, 1986), in the wild-type strain we observed high unregulated expression of a *GCN4–lacZ* fusion lacking all four uORFs, and low constitutive expression from the fusion containing uORF4 alone. The deletion of *GCN20* had little or no effect on the expression of these last two constructs; the same results were obtained in the *gcn2Δ* strain (Figure 5) and in strains lacking *GCN1* or *GCN3* (Hinnebusch, 1992). These data indicate that *GCN20* encodes a novel factor that stimulates *GCN4* expression at the translational level by overcoming the inhibitory effects of the uORFs in the *GCN4* mRNA leader. It appears that GCN20 is less critical for derepression of *GCN4* than is GCN1 (Marton *et al.*, 1993) or GCN2 (Figure 5), but it is comparable in this respect with GCN3 (Hinnebusch, 1992).

Deletion of GCN20 reduces phosphorylation of eIF-2α by GCN2 in vivo

We showed previously that the slow-growth phenotype of *GCN2c* alleles results from hyperphosphorylation of eIF-2α (Ramirez *et al.*, 1992). To determine whether the deletion of *GCN20* suppresses the growth defect of *GCN2c* alleles by decreasing eIF-2α phosphorylation, we used isoelectric focusing gel electrophoresis to measure the amounts of the two isoforms of eIF-2α that differ only by phosphorylation on Ser51. In accordance with previous results (Ramirez *et al.*, 1992), the *GCN2c-E532K,E1522K* allele led to relatively high levels of eIF-2α phosphorylation independent of amino acid availability (Figure 6, lanes 5 and 6). The deletion of *GCN20* clearly reduced the extent of eIF-2α phosphorylation in the *GCN2c* strain (Figure 6, lanes 5–8). As expected (Dever *et al.*, 1992), phosphorylation of eIF-2α increased in the wild-type *GCN2 GCN20* strain in response to histidine starvation (Figure 6, lanes 3 and 4). The deletion of *GCN20* in this last strain abolished eIF-2α phosphorylation under repressing conditions (lane 1), and consistently led to a small reduction in the extent of phosphorylation following amino acid starvation (Figure 6, lane 2). These data indicate that *GCN20* is required for the efficient phosphorylation of eIF-2α by both wild-type and mutationally activated forms of *GCN2*. The fact that the residual eIF-2α phosphorylation we observe in the *GCN2 gcn20Δ* strain (Figure 6, lane 2) leads to only modest derepression of *GCN4–lacZ* in this strain (Figure 5) is probably attributable to the fact that a threshold level of phosphorylation is required for any derepression to occur. Thus, a significant fraction of eIF-2α is phosphorylated even under conditions where *GCN4* expression is fully repressed (Dever *et al.*, 1992).

GCN20 is not required for eIF-2α phosphorylation by mammalian protein kinase RNA regulated (PKR) in yeast cells

We wished to determine whether phosphorylation of eIF-2α catalyzed by other *GCN2c* alleles, or by a heterologous mammalian eIF-2α kinase, was dependent on *GCN20*. To answer the first, we asked whether deletion of *GCN20* suppresses the slow-growth phenotypes of the *GCN2c-R699W,D918G,E1537G*, *GCN2c-M719V,E1537G* or *GCN2c-E532K,E1537K* alleles that produce higher levels of eIF-2α phosphorylation and more severe growth defects in otherwise wild-type strains than does *GCN2c-E532K, E1522K* (Ramirez *et al.*, 1992). We found that deleting *GCN20* reduced the growth defect associated with all three of these *GCN2c* alleles, although suppression was incomplete for the two most highly activated proteins that conferred the strongest growth defect in the *GCN20* strain

(GCN2^c-M719V,E1537G and GCN2^c-E532K,E1537K; Figure 4B and data not shown).

To determine whether *GCN20* is required specifically for GCN2 kinase function, we asked whether deleting *GCN20* would suppress the phenotypes associated with expression of the human eIF-2α kinase PKR (Meurs *et al.*, 1990) in yeast. High-level expression of the PKR gene from a galactose-inducible promoter in cells cultured on galactose medium severely impairs cell growth (Chong *et al.*, 1992; Dever *et al.*, 1993). Low-level expression of the same PKR construct in cells growing on glucose medium does not reduce the growth rate, but leads to partial induction of *GCN4* translation and increased 3-AT resistance in *gcn2*Δ strains (Dever *et al.*, 1993). Both consequences of expressing PKR in yeast are completely dependent on the phosphorylation site on eIF-2α at Ser51 (Chong *et al.*, 1992; Dever *et al.*, 1993). We found that the deletion of *GCN20* does not suppress the lethal effect of high-level PKR expression on galactose medium (data not shown). It could be argued, however, that with such a high level of eIF-2α kinase activity, the deletion of *GCN20* would not decrease phosphorylation enough to overcome the growth inhibition. In an effort to eliminate this possibility, we asked whether deleting *GCN20* would reverse the 3-AT^R phenotype conferred by low-level expression of PKR in a *gcn2*Δ strain. As shown in Figure 4A, the deletion of *GCN20* did not reduce the ability of PKR to derepress *GCN4* translation in a *gcn2*Δ strain grown on glucose medium, even though the level of eIF-2α phosphorylation under these conditions was insufficient to produce wild-type 3-AT resistance. The fact that GCN20 is required for eIF-2α phosphorylation in yeast when catalyzed by GCN2 but not by PKR strongly suggests that GCN20 is required specifically for GCN2 kinase function. This finding is inconsistent with the possibility that GCN20 is a negative effector of an eIF-2α phosphatase.

Deletion of GCN1 reduces the level of GCN20 protein

It was conceivable that *gcn20* mutations decrease GCN2 kinase function *in vivo* by lowering the expression of either *GCN2* or its positive effector *GCN1*. One way in which we addressed this possibility was by measuring the levels of *GCN2* and *GCN1* mRNAs in isogenic *GCN20* and *gcn20*Δ strains by RNA blot hybridization analysis. The results indicated that deleting *GCN20* did not decrease the levels of either *GCN2* or *GCN1* mRNA (data not shown). We also examined the expression of GCN1 protein by immunoblot analysis and, as shown in Figure 7A, deleting *GCN20* had no effect on the expression of GCN1 protein. In contrast, the deletion of *GCN1* reduced the steady-state level of GCN20 protein by ~4-fold. In control experiments we showed that the deletion of *GCN1* did not reduce the amount of GCD6 protein, a member of the eIF-2B complex (Cigan *et al.*, 1993) or the α subunit of eIF-2 (SUI2; Cigan *et al.*, 1989; Figure 7A).

Because there was no difference in the expression of *GCN20* mRNA between wild-type and *gcn1*Δ strains, we suspected that the decreased expression of GCN20 seen in the *gcn1*Δ strain resulted from instability of the protein in the absence of GCN1. To test this idea, we pulse–chased wild-type and *gcn1*Δ strains with radioactive amino

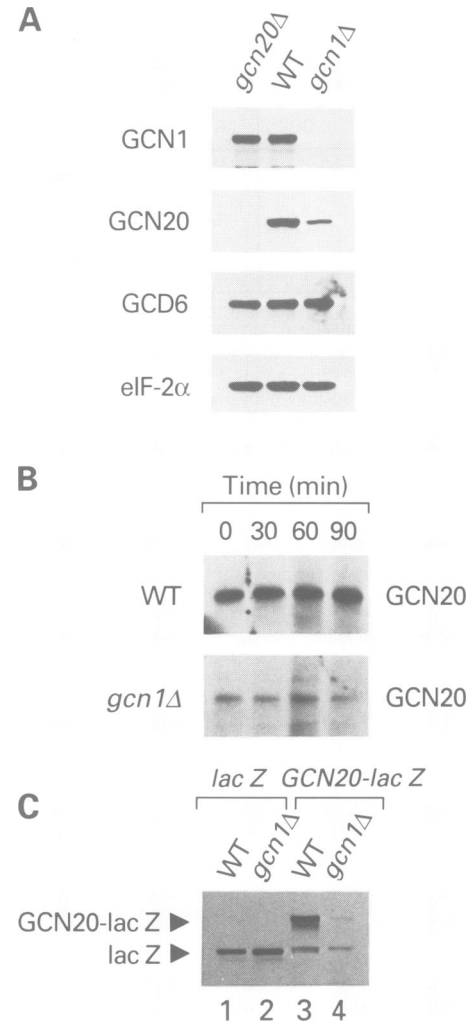


Fig. 7. Effects of deleting *GCN1* on GCN20 protein levels. (**A**) Immunoblot analysis of steady-state GCN20 protein levels. Cell extracts were prepared from isogenic strains F113 (WT), H2512 (*gcn20*Δ) and H2079 (*gcn1*Δ) grown to mid-logarithmic phase on SD medium with minimal supplements. Extracts were fractionated by 8% SDS–PAGE, transferred to nitrocellulose and incubated with specific antibodies against the indicated proteins. Immune complexes were visualized by chemiluminescence. (**B**) Pulse–chase analysis of the synthesis and stability of GCN20 protein. Four OD_{600} units of yeast cells (F113 or H2079) were pulse-labeled with Tran^{35}S-label in SD medium with minimal supplements for 10 min, and then chased for 30, 60 or 90 min by the addition of non-radioactive methionine and cysteine to a final concentration of 2 mM. Cells were broken with glass beads and immunoprecipitations were performed on aliquots of extracts containing equivalent amounts of radioactivity using antibodies against GCN20. Immune complexes were fractionated by 8% SDS–PAGE and the gels were dried and subjected to autoradiography. (**C**) Immunoblot analysis of steady-state GCN20–lacZ fusion protein levels. Isogenic strains F113 (WT, lanes 1 and 3) and H2079 (*gcn1*Δ, lanes 2 and 4) transformed with plasmids p1922 or p1924, containing *GCN20–lacZ* fusions, in which the first two (p1922) or the first 457 (p1924) codons of *GCN20* are fused to *lacZ*, were grown to mid-logarithmic phase on SD medium with minimal supplements. Cell extracts were fractionated by 8% SDS–PAGE, transferred to nitrocellulose and incubated with antibodies against β-galactosidase. Immune complexes were visualized by chemiluminescence.

acids and measured the amount of GCN20 protein immunoprecipitated from cell extracts using GCN20 antibodies. In the *GCN1* strain, we detected no turnover of

labeled GCN20 during a 90 min chase (Figure 7B). In the *gcn1Δ* strain, the relative amount of radioactivity incorporated into GCN20 during the 10 min pulse was reduced substantially; however, the residual labeled protein did not decay during the 90 min chase. (We verified that a control protein, GCD6, was labeled indistinguishably in wild-type and *gcn1Δ* strains; data not shown.) The reduction in the amount of radiolabeled GCN20 seen in the *gcn1Δ* versus the wild-type strain in Figure 7B is similar in magnitude to the decrease in the steady-state level of GCN20 measured by immunoblot analysis in Figure 7A. These results could indicate that *GCN20* expression is reduced at the translational level in cells lacking *GCN1*. However, in view of results presented below (indicating that GCN1 and GCN20 are components of the same protein complex), it seemed more likely that GCN20 is unstable in *gcn1Δ* cells and that most of the protein is degraded *in vivo* within minutes of its synthesis or after breaking the cells.

Strong support for the idea that deleting *GCN1* reduces the stability of GCN20 was provided by a comparison of *GCN20–lacZ* fusions containing either 457 residues or only the first two residues of GCN20 fused to the N-terminus of β-galactosidase. As shown in Figure 7C, the longer fusion protein accumulated to lower levels in the *gcn1Δ* mutant versus the *GCN1* strain, similar to what we observed for authentic GCN20 in these two strains. In contrast, expression of the fusion protein containing only two amino acids from GCN20 was essentially unaffected by deleting *GCN1* (Figure 7C). Because both fusions contain the same 5′ non-coding DNA and mRNA leader sequences from *GCN20*, these results strongly suggest that GCN20 protein is less stable in *gcn1Δ* mutants. The rapid degradation of GCN20 in *gcn1Δ* mutants implied by the results shown in Figure 7B has been observed for certain ribosomal proteins when overexpressed in yeast (Maicas *et al.*, 1988). The small fraction of GCN20 that is metabolically stable in the *gcn1Δ* strain might indicate the existence of a separate pool of GCN20 with a stability that is not dependent on GCN1.

The GCN20 and GCN1 proteins are members of a heteromeric protein complex

The observations that GCN1 and GCN20 each stimulate *GCN4* translation by increasing phosphorylation of eIF-2α by GCN2, and that GCN20 protein levels are reduced by deletion of *GCN1*, indicated a close functional relationship between these two proteins. To determine whether GCN1 and GCN20 physically interact, we asked whether GCN1 protein could be co-immunoprecipitated with GCN20. An epitope-tagged version of *GCN20* was constructed containing the coding sequences for the FLAG epitope (Hopp *et al.*, 1988) inserted after the last codon of *GCN20*. The tagged allele, *GCN20-FLAG*, was indistinguishable from wild-type *GCN20* in complementing the 3-AT^s phenotype of a *gcn20Δ* deletion (data not shown). Whole-cell extracts from strains bearing wild-type *GCN20* or *GCN20–FLAG* were immunoprecipitated using monoclonal antibodies against the FLAG epitope. As shown in Figure 8A, equal proportions of the GCN20 and GCN1 proteins present in the extracts were co-immunoprecipitated with anti-FLAG antibodies from the cells bearing *GCN20–FLAG*, whereas neither was immunoprecipitated from extracts prepared from wild-type *GCN20* cells. The identity of GCN1 in the immunoprecipitates was shown by the fact that it was not recovered from the isogenic *gcn1Δ GCN20–FLAG* strain (lane 4). These results strongly suggest that GCN1 and GCN20 are components of the same heteromeric protein complex.

In an effort to confirm this result, we asked whether a GCN20–lacZ fusion protein could be co-immunoprecipitated with GCN1. Extracts were prepared from *gcn20Δ* strains bearing one of the two *GCN20–lacZ* fusions described above, containing either all 457 amino acids or only the first two amino acids of GCN20 fused to the N-terminus of β-galactosidase. Extracts from these strains were immunoprecipitated with antibodies against GCN1 and the resulting immune complexes were probed with antibodies against β-galactosidase. As shown in Figure 8B, nearly all of the larger GCN20–lacZ fusion protein, but essentially none of the shorter fusion protein, was immunoprecipitated with GCN1 (Figure 8B, compare top and bottom panels in lanes 3 and 1). These results provide strong additional support for the idea that GCN1 and GCN20 are stably associated in a protein complex; moreover, they imply that the majority of GCN20 is present in complexes that also contain GCN1.

It was of interest to determine whether GCN1 and GCN20 are physically associated with GCN2. We tested this possibility by performing immunoprecipitations under the same conditions described above, except using polyclonal sera specific for GCN1 or GCN2 as the precipitating antibodies. GCN1 and GCN20 were detected in the immune complexes by immunoblot analysis, whereas GCN2 was identified by assaying its autokinase activity. In accordance with the results described above, antibodies against GCN1 immunoprecipitated all of the GCN1 and most of the GCN20 protein present in extracts prepared from the wild-type *GCN1 GCN20* strain (Figure 8C, α-GCN1, column labeled WT, panels labeled GCN1 and GCN20; compare pellet with supernatant). As expected, the deletion of *GCN1* eliminated the immunoprecipitation of GCN20 and also greatly reduced its abundance in the extracts. The immune complexes obtained using antibodies against GCN1 contained no detectable GCN2 kinase activity (Figure 8C, α-GCN1, panel labeled GCN2). GCN2 autokinase activity was immunoprecipitated using antibodies against GCN2 from all of the extracts except that derived from the *gcn2Δ* strain (Figure 8C, α-GCN2, panel labeled GCN2); however, none of the GCN1 or GCN20 protein co-immunoprecipitated with GCN2. We believe that the immunoprecipitations of GCN2 were quantitative because a second immunoprecipitation of the supernatants yielded no additional GCN2 kinase activity (data not shown). Thus, we could not detect a physical interaction between GCN2 and GCN1 or between GCN2 and GCN20 under conditions in which GCN1 and GCN20 were stably associated. The results in Figure 8C do indicate, however, that GCN20 is not required for the expression of GCN2 protein or for its autokinase activity *in vitro*, which is the same conclusion reached previously for GCN1 (Marton *et al.*, 1993).

Portions of the GCN1 and GCN20 proteins interact in the two-hybrid system

The association between GCN20 and GCN1 was also investigated using the yeast two-hybrid system (Fields

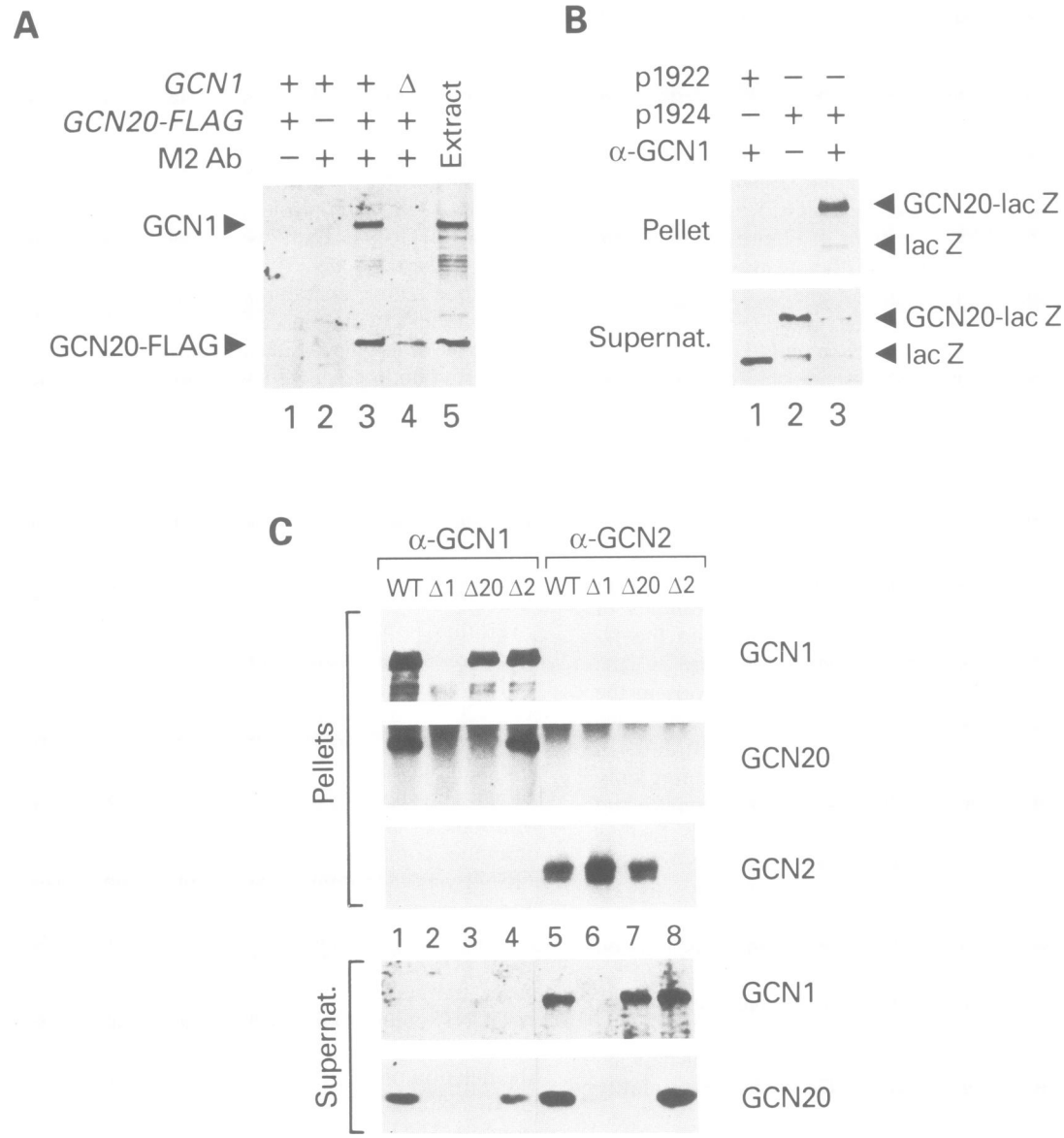


Fig. 8. Co-immunoprecipitation of GCN20 and GCN1. (**A**) Cell extracts prepared from strain H2563 (*gcn20-Δ2*) transformed with plasmid p1870 bearing *GCN20–FLAG* (lanes 1 and 3) or p1729 bearing *GCN20* (lane 2), or from the isogenic strain H2081(*gcn1Δ*) transformed with plasmid p1870 (lane 4), were immunoprecipitated with monoclonal antibodies against FLAG epitope (lanes 2–4). Immune complexes were separated by 8% SDS–PAGE, transferred to nitrocellulose and probed with polyclonal antibodies against GCN1 and GCN20. Immune complexes were visualized by chemiluminescence. Lanes 1–4, material immunoprecipitated from 100 μg of protein; lane 5, 20 μg of the starting extract from strain H2563 containing epitope-tagged GCN20. (**B**) 40 μg of protein extract from strain H2512 (*gcn20-Δ1*) transformed with plasmids p1922 (lane 1) or p1924 (lanes 2 and 3), containing, respectively, *lacZ* fused at the second codon on *GCN20* (lacZ) or at the 457th residue of *GCN20* (GCN20–lacZ), were immunoprecipitated with polyclonal antibodies against GCN1 (Pellet, lanes 1 and 3) or treated identically in the absence of antibodies (Pellet, lane 2). Proteins in the supernatant were precipitated by the addition of TCA to 5%. Samples were resolved by 8% SDS–PAGE, transferred to nitrocellulose and probed with monoclonal antibodies against β-galactosidase. Immune complexes were visualized as in (A). (**C**) 40 μg of protein extracts from isogenic strains F113 (W, lanes 1 and 5), H2079 (Δ1, lanes 2 and 6), H2512 (Δ20, lanes 3 and 7) or H2511 (Δ2, lanes 4 and 8) were immunoprecipitated with polyclonal antibodies against GCN1 (α-GCN1, lanes 1–4) or GCN2 (α-GCN2, lanes 5–8). Immune complexes were separated by 7.5% SDS–PAGE, transferred to nitrocellulose and probed with antibodies against GCN1 or GCN20, as indicated in the first two panels from the top. Immune complexes were visualized as in (A). GCN2 was identified by assaying its autokinase activity by incubating immune complexes with radiolabeled ATP and fractionating by 7.5% SDS–PAGE. The gels were fixed, dried and subjected to autoradiography (third panel from top labeled GCN2). Proteins remaining in the supernatants after collecting the immune complexes were precipitated by the addition of TCA to 5%, separated by 7.5% SDS–PAGE and probed with antibodies against GCN1 or GCN20, as indicated in the bottom two panels (supernatants). Immune complexes were visualized as in (A).

and Song, 1989). This method examines protein–protein interactions by assaying the ability of GAL4 fusion proteins to reconstitute GAL4-dependent transcription *in vivo*. The yeast strain we used contains two GAL4-dependent reporter genes, *GAL1–lacZ* and *GAL1–HIS3* fusions; thus, reconstituted GAL4 activation in this strain leads to an increased expression of β-galactosidase and an improved growth on medium containing 3-AT. We constructed plasmids encoding hybrid proteins containing either all 752 amino acids or only the N-terminal 118

Table I. Analysis of interactions between GCN1 and GCN20 using the yeast two-hybrid system

DNA binding domain fusion	Activation domain fusion	β-Galactosidase activity	Growth on 3-AT media
GCN1 (672–2672)	pACT II (no fusion)	0.4	–
pAS1-CYH2 (no fusion)	GCN20 (1–118)	0.9	–
GCN1 (672–2672)	GCN20 (1–118)	16	+
SNF1 (1–633)	SNF4 (1–322)	27	+

Strain Y190 was co-transformed with *TRP1* plasmids encoding the GAL4 DNA binding domain alone (pAS1-CYH2) or a fusion of this GAL4 domain to either the SNF1 protein or a portion of GCN1, and with *LEU2* plasmids encoding the GAL4 activation domain alone (pACTII) or a fusion of this GAL4 domain to the entire SNF4 protein or to a portion of GCN20. The amino acids from GCN1 or GCN20 present in each fusion protein are indicated in parentheses. For β-galactosidase assays, cells were grown, as described in Materials and methods, and mean values from three transformants are reported. Units of β-galactosidase activity are expressed as nanomoles of *o*-nitrophenyl β-D-galactopyranoside hydrolyzed per milligram of protein per 10 min interval. (+) Growth on minimal media containing 35 mM 3-AT; (–) little or no growth on the same plates.

co-expressed with the full-length GCN20–GAL4 fusion protein (data not shown). In contrast, co-expression of the GCN1–GAL4 fusion with the smaller GCN20–GAL4 fusion containing only the N-terminal 118 amino acids of GCN20 conferred 3-AT resistance and increased *GAL1–lacZ* expression. The amount of β-galactosidase activity produced in cells containing this last combination of fusion proteins is shown in Table I. Neither the *GCN1–GAL4* fusion nor the *GCN20–GAL4* fusion constructs elicited 3-AT resistance or β-galactosidase activity in combination with an empty vector control. These data confirm our conclusion that the GCN1 and GCN20 are components of a protein complex. They further demonstrate that the first 118 amino acids of GCN20 are sufficient for an interaction with a large C-terminal segment of GCN1.

Discussion

We have characterized a novel protein involved in the translational control of *GCN4* expression via phosphorylation of eIF-2. When yeast cells are deprived of amino acids or purines, the protein kinase GCN2 becomes activated and phosphorylates the α-subunit of eIF-2 on Ser51 (Dever *et al.*, 1992; Rolfes and Hinnebusch, 1993). There is strong genetic evidence that phosphorylated eIF-2 inhibits eIF-2B function and reduces the active GTP-bound form of eIF-2 in yeast cells (Cigan *et al.*, 1993; Dever *et al.*, 1993; Vazquez de Aldana and Hinnebusch, 1994), just as occurs in mammalian cells (Hershey, 1991). It is thought that the ensuing decrease in the abundance of eIF-2–GTP–Met-tRNA$^{Met}_i$ ternary complexes is what triggers increased translation of *GCN4* mRNA (Dever *et al.*, 1992). *GCN2^c* mutations increase the ability of GCN2 to phosphorylate eIF-2α, and thereby stimulate *GCN4* translation, in the absence of amino acid limitation (Wek *et al.*, 1990; Dever *et al.*, 1992; Ramirez *et al.*, 1992). The most potent *GCN2^c* alleles also reduce cellular growth by inhibiting general translation initiation (Dever *et al.*, 1992; Ramirez *et al.*, 1992). We isolated a suppressor mutation that overcomes the toxic effect of one such *GCN2^c* allele and showed that this mutation inactivates the *GCN20* gene. Our results indicate that wild-type GCN20 functions *in vivo* as a positive effector of the eIF-2α kinase activity of GCN2, and that GCN20 and GCN1 are components of a heteromeric protein complex.

The common phenotypes exhibited by *gcn20Δ* and *gcn1Δ* mutant strains were the first indication that these two proteins act at the same step in regulating *GCN4* expression. First, null mutations in *GCN20* impair the derepression of genes in the histidine biosynthetic pathway that are subject to general amino acid control. This phenotype results from the failure to derepress the translation of *GCN4* mRNA in response to amino acid limitation (Figures 4 and 5), just as occurs in *gcn1*, *gcn2* and *gcn3* mutants (Hinnebusch, 1992). Second, inactivation of *GCN20* abolishes the growth defect associated with *GCN2^c* alleles by reducing the extent of eIF-2α phosphorylation (Figure 6), which is the same mechanism established for *gcn1* suppressors of *GCN2^c* mutations (Marton *et al.*, 1993). Third, mutations in *GCN1* (Marton *et al.*, 1993) and *GCN20* specifically impair GCN2 kinase function, having no effect on the activity of the mammalian eIF-2α kinase PKR when this heterologous enzyme is expressed in yeast (Figure 4A). Neither GCN1 nor GCN20 are required for the expression of GCN2 or for its intrinsic kinase activity *in vitro* (Marton *et al.*, 1993; Figure 8C). Taken together, these findings indicate that GCN1 and GCN20 are required to link GCN2 kinase activity to the availability of amino acids *in vivo*.

We obtained strong biochemical and genetic evidence that GCN20 and GCN1 are physically associated in a complex. The reduction in the steady-state level of GCN20 protein observed in extracts of *gcn1Δ* mutants (Figure 7A) coupled with the absence of a discernible effect of deleting *GCN1* on *GCN20* mRNA levels, suggested that GCN20 protein is less stable in cells lacking GCN1. More conclusive evidence for this interpretation came from the fact that the steady-state level of the GCN20(1–457)–lacZ fusion protein was substantially reduced when *GCN1* was deleted, whereas the fusion containing only two N-terminal amino acids from GCN20 was expressed equally in *GCN1* and *gcn1Δ* strains (Figure 7C). Our interpretation of these results is that GCN1 and GCN20 are components of the same protein complex and that GCN20 is more susceptible to proteolysis when GCN1 is missing from the complex. Definitive biochemical evidence for a physical interaction between GCN1 and GCN20 was provided by co-immunoprecipitation of these two proteins from whole-cell extracts using polyclonal antibodies against GCN1 or monoclonal antibodies that recognize an epitope-tagged form of GCN20 (Figure 8). Genetic evidence for complex formation was provided by the observation that the N-terminal 118 amino acids of GCN20 and a large C-terminal fragment of GCN1 mediate protein–protein interactions *in vivo* detected using the yeast two-hybrid system (Table I). It is worth noting that this N-terminal segment of

GCN20 is sufficient for a partial activation of *GCN4* translation (Figure 2, plasmid p1868).

Models for GCN20–GCN1 function

An important clue about the function of the protein complex containing GCN20 and GCN1 is provided by the amino acid sequence of GCN20, which identifies it as a new member of the ABC family of proteins. The vast majority of ABC proteins are components of membrane transporters that utilize the energy of ATP hydrolysis to pump substrates against a concentration gradient. Each ABC transporter is relatively specific for a given substrate, and the variety of substrates transported is enormous (for reviews see Higgins, 1992; Fath and Kolter, 1993). The typical transporter consists of four membrane-associated domains, two of which are highly hydrophobic and contain six α-helical membrane-spanning segments apiece. These two domains form the pore through which the substrate crosses the membrane and appear to determine, in large part, the substrate specificity of the transporter. The other two domains are peripherally located on the cytoplasmic face of the membrane, bind ATP and couple ATP hydrolysis to the transport process. The peripheral domains are ~200 amino acids long and contain the Walker A and B motifs. In a given transporter protein, the peripheral domains are 30–50% identical to one another (Higgins, 1992)

GCN20 contains two pairs of Walker A–B motifs separated by ~100 amino acids, typical of the ATP binding domains of an ABC transporter (Figures 2 and 3). It seems to lack, however, regions hydrophobic enough to comprise a transmembrane domain. In most eukaryotic ABC transporters, the nucleotide binding domains are fused to the membrane-spanning domains in a single polypeptide chain. In prokaryotes, by contrast, the individual domains of the transporter are frequently expressed as separate polypeptides, or fused into multifunctional polypeptides in various combinations (Higgins, 1992). Thus, it is conceivable that GCN20 contains the two ATP binding domains and interacts with one or more transmembrane proteins in carrying out a transport function. GCN1 could be the transmembrane component of the transporter; alternatively, GCN1 could be associated with the transporter without being required for the transport function *per se*. A situation similar to this last possibility has been described for the PhoU protein of *E.coli*. PhoU is a peripheral membrane protein encoded in the same operon with the components of an ABC transporter of phosphorus-containing compounds. It is thought that PhoU functions primarily to couple transport to activation of phoB and phoR, proteins that comprise a two-component transcriptional regulatory module in the phosphate regulon (Parkinson and Kofoid, 1992; Wanner, 1993). It is interesting in this connection that the *Pseudomonas* ABC protein showing strong similarity to GCN20 (Figure 3) is encoded 0.5 kb downstream of the *algR2* gene, part of another two-component regulator that induces mucoid capsule production in response to high osmolarity in the lungs of patients afflicted with cystic fibrosis (Berry *et al.*, 1989).

By analogy with the *Pho* regulon of *E.coli*, perhaps GCN1 and GCN20 are components of an ABC transporter that couples amino acid transport to the activation of GCN2. The fact that inactivation of GCN1 or GCN20 confers sensitivity to 3-AT on minimal medium lacking all amino acid supplements seems incompatible with the idea that these proteins are involved in transporting amino acids across the plasma membrane to the cytoplasm. However, it might be possible to explain the 3-AT^s phenotype of *gcn1* and *gcn20* mutants if GCN1 and GCN20 are components of a system that transports amino acids from the cytoplasm to the vacuole. Several such vacuolar transporters have been characterized biochemically in yeast (Sato *et al.*, 1984). A defect in this hypothetical transporter in *gcn1Δ* or *gcn20Δ* mutants would lead to elevated cytoplasmic pools of amino acids and less uncharged tRNA for a given total amount of cellular amino acids. This would prevent activation of the GCN2^c kinases under non-starvation conditions if we also assume that GCN2^c proteins are hypersensitive to uncharged tRNA. In strains containing wild-type GCN2, deleting *GCN1* or *GCN20* would delay the activation of GCN2 when 3-AT is added to the medium until the histidine pool is nearly exhausted. At that point, it might be impossible to increase the synthesis of GCN4 protein and the amino acid biosynthetic enzymes under the general control.

Certain yeast vacuolar mutants that are defective in sequestering amino acids in the vacuole are hypersensitive to exogenous histidine and lysine, presumably because these amino acids are toxic when present at high levels in the cytoplasm. If GCN1 and GCN20 were components of a vacuolar amino acid transporter, inactivating these proteins might be expected to produce histidine or lysine sensitivity. We found that *gcn1Δ* and *gcn20Δ* mutants are more sensitive than the isogenic wild type to these amino acids; however, they are not more sensitive than an isogenic *gcn2Δ* strain (unpublished observations). Therefore, this phenotype is probably attributable to the impaired activation of GCN2 rather than to a defect in vacuolar transport of amino acids. Preliminary attempts to localize GCN1 by immunofluorescence experiments suggest that this protein is uniformly distributed throughout the cytoplasm (unpublished observations), another result which does not support a vacuolar transporter function for the GCN20–GCN1 complex.

Although the great majority of ABC proteins are associated with membrane transport events, a few are involved in functions ostensibly unrelated to transport (Higgins, 1992). UvrA is a cytoplasmic enzyme involved in DNA repair that contains two typical ABC domains separated by a zinc finger DNA binding domain, which hydrolyses ATP as part of its repair function (Higgins, 1992). EF-3 is a soluble protein that appears to stimulate EF-1α-dependent binding of aminoacyl-tRNA to the A site of the ribosome by promoting the release of the deacylated tRNA from the E site in a reaction requiring NTP hydrolysis (Triana *et al.*, 1993). There is also evidence that EF-3 contributes to the fidelity of translation by stimulating the binding of cognate aminoacyl-tRNAs to the A site at the expense of non-cognate aminoacyl-tRNAs (Uritani and Miyazaki, 1988). Thus, the identification of an ABC domain by sequence similarity does not necessarily indicate a role in membrane transport, and the nucleotide binding domains in some ABC proteins appear to couple ATP hydrolysis to other kinds of biological processes (Higgins, 1992). Thus, it is possible that GCN20 and

GCN1 are components of an ABC complex that has no role in membrane transport and which acts more directly to stimulate GCN2 kinase activity by uncharged tRNA.

Much of the GCN2 protein in cell extracts can be found stably associated with ribosomes, and the extreme C-terminal segment of GCN2 is required for both ribosome binding and activation of GCN2 kinase function *in vivo* (Ramirez *et al.*, 1991). Thus, one simple idea is that GCN1 and GCN20 could be required to anchor GCN2 to the ribosome. At odds with this hypothesis, we found that the association of GCN2 with polysomes and ribosomal subunits in velocity sedimentation experiments was unaffected by deleting *GCN1* or *GCN20* (unpublished observations). A more complicated model is prompted by the sequence similarity between the ABC domains of GCN20 and EF-3 (Figure 3A) and by the fact that GCN1 also shows strong sequence similarity to EF-3 in a region N-terminal to the ABC domains in EF-3 (Marton *et al.*, 1993). Previously, we proposed that the binding of uncharged tRNA to the HisRS-like regulatory domain in GCN2 is what triggers the activation of GCN2 kinase function (Wek *et al.*, 1989, 1990). The ribosome association of GCN2 prompted the idea that GCN2 is activated by uncharged tRNA that binds to the ribosomal A site during translation elongation in amino acid-starved cells (Ramirez *et al.*, 1991). Based on their sequence similarities with EF-3, it could be imagined that GCN1 and GCN20 facilitate the interaction between uncharged tRNA bound at the A site and the HisRS-related domain of GCN2. This activity could be akin to the proposed function of EF-3 in stimulating the release of uncharged tRNA from the ribosomal E site. A related scenario envisages the GCN1–GCN20 complex shuttling uncharged tRNA from aminoacyl-tRNA synthetases to the HisRS-like domain of GCN2. Thus far, we have not detected any interactions between GCN1 or GCN20 and polysomes (unpublished observations), nor any physical association between these proteins and GCN2 (Figure 8). Of course, these interactions may occur *in vivo* but are disrupted under the conditions of our *in vitro* experiments. Perhaps genetic experiments aimed at detecting additional factors that interact with GCN1 or GCN20 will shed more light on how these proteins regulate GCN2 kinase function.

It is intriguing that the first 117 amino acids of GCN20, a segment completely N-terminal to the ABC domains of the protein, are sufficient for interaction with GCN1 in the two-hybrid assay (Table I) and for partial activation of *GCN4* translation in a *gcn20*Δ strain (Figure 2). The latter result may indicate that ATP binding and hydrolysis are not absolutely required for the function of GCN20 in activating GCN2, and that its critical function involves only the N-terminal domain which anchors GCN20 to GCN1. Alternatively, the GCN20 homolog Yer036 (Figure 3B) may be able to substitute for the ABC domains of GCN20 in the presence of the N-terminal 117 residues of GCN20.

We have identified proteins from diverse eukaryotic organisms that are closely related to GCN20. The *C.elegans* protein that we designated ceGCN20-1 is 43% identical to GCN20 over the entire length of both proteins, and 50–55% identical if we consider only the conserved ABC domains. The alignment of these two sequences requires the introduction of only four small gaps in the N-terminal halves of the proteins, and there are numerous clusters of identical amino acids in regions flanking the two segments containing the Walker motifs. The EST sequence of human origin and one of the two rice cDNAs we identified contain blocks of amino acids that are conserved with GCN20 and ceGCN20-1 but not present in the other ABC proteins shown in Figure 3 that are highly similar to GCN20. These sequence similarities could indicate that GCN20, ceGCN20-1 and the closely-related human and rice EST sequences define a subfamily of proteins with a biological function that is conserved among yeast, plants and animals. If so, higher eukaryotes may employ a mechanism for coupling the rate of translation initiation to the level of uncharged tRNA, similar to that identified in *S.cerevisiae* involving GCN2 and its positive effectors GCN1 and GCN20.

Materials and methods

Plasmid constructions

Plasmids p1713, p1714, p1715 and p1716 were isolated by complementation of the 3-AT-sensitive phenotype of the *gcn20-501* mutant after transformation (Ito *et al.*, 1983) with a genomic library (Rose *et al.*, 1987), and all four contain a 10 kb insert of yeast genomic DNA in the *Bam*HI site of YCp50 (Parent *et al.*, 1985). Plasmids p1711 and p1712 appear to contain the same genomic DNA fragment inserted in the vector in the opposite orientation. The smallest complementing region was mapped to a 4 kb *Bam*HI–*Cla*I fragment that was inserted in vector pRS316 (Sikorski and Hieter, 1989) to generate plasmid p1728 (Figure 2). The *Cla*I end of this fragment is located in pBR322 sequences adjoining the *GCN20* region in p1714. Plasmid p1729 was constructed by the digestion of p1728 with *Spe*I and recircularization of the product, thereby removing the *SUF20* gene. p1730 and p1741 were constructed by digesting p1728 with *Bam*HI–*Sph*I or *Cla*I–*Bgl*II, respectively, filling the ends with T4 DNA polymerase and religating the products. Plasmid p1742 was constructed by inserting the 3.5 kb *Cla*I–*Spe*I fragment of p1729 into the *Cla*I–*Nhe*I sites of YIp5 (Struhl *et al.*, 1979).

Plasmid p1751, used to generate the *GCN20* deletion allele *gcn20-Δ1*, was constructed by inserting a 5 kb *Hin*dIII fragment obtained from plasmid p1722 (one of the *Hin*dIII sites was in the vector) into the *Hin*dIII site of an SK^+ plasmid (Stratagene) modified to remove the *Sac*I–*Sma*I region of the multiple cloning site. The 1.3 kb *Bgl*II fragment containing *GCN20* (from position −132 to 1159) of the resulting plasmid, p1750, was replaced with the 3.8 kb *Bam*HI–*Bgl*II fragment of plasmid PNKY51 containing *URA3* flanked by *hisG* direct repeats (Alani *et al.*, 1987), to generate plasmid p1751. Plasmid p1869, used to produce the deletion allele *gcn20-Δ2*, was derived from p1750, and contains the same *hisG::URA3::hisG* fragment described above replacing the *GCN20* coding sequence. Plasmid p1869 was constructed by PCR amplification of a 780 bp fragment of the 3′ non-coding region of *GCN20* (starting at the TGA stop codon) using oligonucleotide primers that generated *Bgl*II and *Xho*I sites, followed by insertion of this fragment into the corresponding sites in p1750 (Figure 2).

Plasmid p1867 includes a *GCN20* allele containing novel *Bam*HI and *Hin*dIII sites located, respectively, upstream and downstream of the coding sequence, and was constructed in two steps. First, the GCN20 sequence from −10 to +2274 (relative to the ATG start codon) was PCR-amplified using Vent Polymerase (New England Biolabs) and oligonucleotide primers that introduced the novel *Bam*HI and *Hin*dIII restriction sites at the desired positions. The amplified fragment was inserted between the corresponding sites in vector pRS316, generating plasmid p1866. Second, the GCN20 promoter region (between positions −161 and −11) was amplified with oligonucleotide primers that generated *Xba*I and *Bam*HI ends, and the resulting fragment was inserted upstream of the *GCN20* coding region in p1866, to yield p1867. This plasmid fully complemented the null mutation *gcn20-Δ2*. Plasmid p1868 was derived from p1867 by digestion with *Sph*I, treatment with T4 DNA polymerase and ligation of the product. This treatment removed 5 bp and produced a frameshift in the *GCN20* coding sequence after codon 117, that leads to termination at a TGA triplet located five codons further downstream. To construct an epitope-tagged allele of *GCN20*, coding sequences for the FLAG antigen (DYKDDDDK in single-letter code;

Hopp *et al.*, 1988) were fused to the last sense codon of *GCN20*. An *Aat*II–*Hin*dIII fragment was PCR-amplified using a 3′ oligonucleotide primer that contained the desired addition to the GCN20 coding sequence, and the amplified fragment was used to replace the corresponding fragment of plasmid p1867, generating plasmid p1870. The amplified region was sequenced to confirm the absence of unwanted mutations.

Plasmid p1922 contains a *GCN20–lacZ* fusion in which the *lacZ* sequence was fused in-frame to the second codon of *GCN20*. To construct this plasmid, the *GCN20* sequence from −161 to +5 was PCR-amplified using oligonucleotide primers that generated *Not*I and *Xba*I sites at the ends of the fragment which was inserted between the corresponding sites in p1633, a derivative of pRS316 containing *lacZ* on a *Bam*HI fragment. The ATG start codon of *lacZ* is adjacent to the *Sac*I site of the polylinker in p1633. Plasmid p1924 contains a *GCN20–lacZ* fusion in which the *lacZ* was fused in-frame to codon 457 of *GCN20*. To construct this plasmid, the *GCN20* sequence from −161 to +1372 was PCR-amplified with oligonucleotide primers that generate *Not*I and *Bam*HI sites at the ends of the fragment, which was then inserted between the same sites in plasmid p1923, a derivative of p1633 in which the *Bam*HI site close to the 3′ end of *lacZ* was destroyed by partial digestion and end-filling with T4 DNA polymerase.

Plasmids pSE1111, pSE1112, pAS1-CYH2 and pACT II were obtained from S.Elledge (Baylor College of Medicine, Houston, TX) and have been described (Durfee *et al.*, 1993). pSE1111 is a *LEU2* vector encoding a fusion between the GAL4 activation domain and the SNF4 protein expressed under the control of the *ADH1* promoter. pSE1112 is a *TRP1* vector encoding a fusion between the DNA binding domain of GAL4 and the SNF1 protein expressed from the same promoter. Plasmid p1809, containing the *GAL4–GCN1* fusion, was constructed by subcloning the 6.3 kb *Nhe*I–*Sal*I fragment of *GCN1* from plasmid p1413 (formerly called pLC13; Marton *et al.*, 1993) into the *Nde*I site of pAS1-CYH2. Plasmid p1824, encoding a GAL4–GCN20 fusion containing the activation domain of GAL4 and full-length GCN20, was constructed by digesting plasmid p1867 with *Bam*HI, treating with the Klenow fragment of DNA polymerase I, inactivating the enzyme, digesting with *Sal*I and ligating the resulting fragment to pACTII which had been digested with *Nco*I, treated with Klenow fragment to fill in the *Nco*I site and then digested with *Xho*I. Plasmid p1825 was constructed similarly, except that plasmid p1868, containing a frame shift mutation at the *Sph*I site in *GCN20*, was used instead of p1867.

Plasmids p180, p226 and p227, containing respectively the wild-type *GCN4* mRNA leader, the leader with only ORF4, and the leader with no uORFs, have been described previously (Mueller and Hinnebusch, 1986). Plasmids derived from the vector pRS316 containing *GCN2^c-E532K,E1522K* (p1056), *GCN2^c-R699W,D918G,E1537K* (p1053), and *GCN2^c-M719V,E1537G* (p1052) have been described (Ramirez *et al.*, 1992), as were plasmids p1420 and p1421, encoding respectively wild-type and the catalytically inactive mutant PKR, both under the control of a galactose-inducible promoter (Dever *et al.*, 1993). Plasmids p1416 (previously called pLC23; Marton *et al.*, 1993) and p1144 (Dever *et al.*, 1992) were used to produce unmarked deletion alleles of *GCN1* or *GCN2*, respectively, in the appropriate yeast strains.

Yeast strains and genetic techniques

Table II lists the yeast strains used in this study. H2566 (*GCN2 gcn20-501*) was obtained as a 3-AT^s segregant of a cross between strains H1933 (*GCN2^c-E532K,E1522K gcn20-501*) and F113 (*GCN2 GCN20*). Strains H2512, H2563 and H2564 were constructed by transforming to Ura$^+$ strain F113 using plasmid p1751 digested with *Eco*RI and *Xba*I, and strains H1402 and H1613 using plasmid p1869 digested with *Eco*RI and *Xho*I. Ura$^+$ transformants were replica-printed to plates containing 5-fluoro-orotic acid (5-FOA) to obtain revertants resistant to 5-FOA (Boeke *et al.*, 1987) in which the *URA3* gene was evicted, resulting in Ura$^-$ strains containing the desired deletion alleles. H2511 and H2513 were constructed by the introduction of unmarked *gcn2* deletion alleles into F113 and H2512, respectively, utilizing plasmid p1144 as described previously (Dever *et al.*, 1992).

Genetic demonstration that GCN20 was cloned

Integrating plasmid p1742, linearized with *Bgl*II, was used to transform the *GCN2^c-E532K,E1522K GCN20* strain H1691 to Ura$^+$, thereby generating a *GCN20::URA3* marked allele. Two independent transformants were crossed with the *GCN2^c-E532K,E1522K gcn20-501* strain H1933 and the resulting diploids were sporulated and analyzed for segregation of the Ura and slow-growth (Slg) phenotypes. All 12 tetrads dissected were parental ditypes, containing only Ura$^+$Slg$^-$ and Ura$^-$Slg$^+$ spores, indicating that the DNA insert in p1742 had directed plasmid integration to a locus closely linked to *gcn20-501*.

Chromosomal mapping of GCN20

The chromosomal location of the *GCN20* gene was confirmed by hybridization of the radiolabeled 1.8 kb *Spe*I–*Sna*BI fragment from plasmid p1729 to a filter containing chromosomes fractionated by clamped homogeneous electrical field electrophoresis, purchased from Clontech, and to a set of filters containing an ordered lambda library of yeast genomic fragments obtained from Linda Riles and Maynard Olson (Riles *et al.*, 1993). The probe hybridized to chromosome VI and to two overlapping clones (numbers 4231 and 4233) which contain the gene *SUP11*, a tRNA^{Tyr} gene that maps on chromosome VI and is tightly linked to *SUF20*, the tRNA^{Gly} gene found immediately upstream of *GCN20*.

Deletion of the chromosomal GCN20 gene

Plasmid p1751 digested with *Eco*RI and *Xba*I was used to transform to Ura$^+$ a diploid strain obtained by crossing H3 and F113. The deletion of one of the two copies of the *GCN20* gene was confirmed by DNA blot hybridization analysis. The appropriate Ura$^+$ diploids were sporulated and subjected to tetrad analysis. All tetrads contained four viable spores with the same growth rates on nutrient-rich (YPD) medium, in which uracil prototrophy and 3-AT sensitivity co-segregated 2$^+$:2$^-$.

DNA sequencing and computer analysis

The sequence of the DNA fragment present in plasmid p1728 was determined with the Sequenase kit (US Biochemical Corp.) using a set of unidirectional deletions generated in plasmid p1729 digested with *Sac*I and *Spe*I using the Erase-a-base system (Promega). Specific oligonucleotide primers were used to sequence the complementary strand. Sequencing of the *C.elegans* clone cel22e4, that was kindly provided by Dr R.K.Waterston (St Louis, MO), was conducted using specific oligonucleotide primers. Sequences were analyzed using the Genetics Computer Group sequence analysis software package (Devereux *et al.*, 1984). Similarity searches of sequences in GenBank or in the 'expressed sequence tags' database (Dbest) were conducted using the BLAST service (Altschul *et al.*, 1990). The accession numbers for the sequences reported in this paper are U19971 (GCN20) and U20783 (ceGCN20-2).

Yeast two-hybrid system

GAL4 fusion constructs were introduced into strain Y190 (obtained from S.Elledge, Baylor College of Medicine) by standard techniques (Schiestl *et al.*, 1993). The expression of β-galactosidase activity in the transformants was detected initially using the colony filter lift assay described by Durfee *et al.* (1993). 3-AT resistance of the same transformants was assayed on SD medium supplemented with 0.15 mM adenine and 35 mM 3-AT.

Analysis of β-galactosidase activities

Quantitative enzyme assays were conducted as described previously (Lucchini *et al.*, 1984) after strains were grown on SD medium containing minimal supplements. For repressing conditions, saturated cultures were diluted 1:50 and grown for 6 h to mid-logarithmic phase. For derepressing conditions, cultures were grown as indicated for 2 h and then for 6 h after the addition of 3-AT to 10 mM. For the two-hybrid assays, saturated cultures of transformants of strain Y190 were diluted to 0.1 OD_{600} and grown for 10 h in SD medium supplemented with 0.4 mM adenine and 0.3 mM histidine.

Isoelectric focusing gel electrophoresis of eIF-2α

Growth of yeast strains, preparation of samples, vertical slab gel isoelectric focusing and detection of eIF-2α by immunoblot analysis using antiserum against yeast eIF-2α were carried out as described by Dever *et al.* (1992), except that the detection of antigen–antibody complexes was performed using the Enhanced Chemiluminescence system (Amersham) following the vendor's instructions.

Preparation of GCN20- and GCN1-specific antisera

Protein fusions were constructed between the *E.coli trpE* gene and *GCN20* or *GCN1* using the pATH vector system (Koerner *et al.*, 1991). Nucleotides 4–1162 of *GCN20* were PCR-amplified using oligonucleotide primers that generated *Sal*I and *Cla*I ends and inserted between the *Sal*I and *Cla*I sites of pATH3, in-frame with *trpE*. The resulting plasmid, p1749, encodes a trpE–GCN20 fusion protein containing 386 amino acids of GCN20, from amino acid 2 to 387. Two different portions of the *GCN1* gene were used to generate protein fusions: a 3.3 kb *Hin*dIII

Table II. Yeast strains used in this study

Strain	Genotype	Source or reference
F113	*MATa ino1 ura3-52 can1*	Donahue *et al.* (1983)
H3	*MATα ura3-52 leu2-3 leu2-112 can1*	Marton *et al.* (1993)
H1402	*MATα ura3-52 leu2-3 leu2-112 ino1 (HIS4–lacZ)*	Hannig *et al.* (1990)
H1613	*MATα ura3-52 leu2-3 leu2-112 ino1 GCN2^c-E532K,E1522K (HIS4–lacZ)*	Ramirez *et al.* (1992)
H1691	*MATa ura3-52 ino1 GCN2^c-E532K,E1522K (HIS4–lacZ)*	Vazquez de Aldana and Hinnebusch (1994)
H1933	*MATα ura3-52 leu2-3 leu2-112 ino1 GCN2^c-E532K,E1522K gcn20-501 (HIS4–lacZ)*	Vazquez de Aldana and Hinnebusch (1994)
H2079	*MATa ino1 ura3-52 can1 gcn1Δ*	Marton *et al.* (1993)
H2081	*MATα ura3-52 leu2-3 leu2-112 ino1 gcn1Δ (HIS4–lacZ)*	Marton *et al.* (1993)
H2511	*MATa ino1 ura3-52 can1 gcn2Δ*	this study
H2512	*MATa ino1 ura3-52 can1 gcn20-Δ1*	this study
H2513	*MATa ino1 ura3-52 can1 gcn2Δ gcn20-Δ1*	this study
H2563	*MATα ura3-52 leu2-3 leu2-112 ino1 gcn20-Δ2 (HIS4–lacZ)*	this study
H2564	*MATα ura3-52 leu2-3 leu2-112 ino1 GCN2^c-E532K,E1522K gcn20-Δ2 (HIS4–lacZ)*	this study
H2566	*MATα ura3-52 leu2-3 leu2-112 GCN2 gcn20-501*	this study
Y190	*MATa ade2-101 gal4-Δ gal80-Δ his3-200 leu2-3 leu2-112 trp1-Δ901 ura3-52 LYS2::GAL1–HIS3 URA3::GAL1–lacZ*	Harper *et al.* (1993)

fragment and a 1.6 kb *Bam*HI fragment were inserted between the corresponding restriction sites of pATH2, generating plasmids p1798 and p1799 respectively. These plasmids encode trpE–GCN1 fusion protein containing amino acids 1106–2202 and 1118–1617 of GCN1, respectively.

Plasmids p1749, p1798 and p1799 were introduced into *E.coli* strain HB101, and the trpE fusion proteins were induced and purified as described (Koerner *et al.*, 1991). Two New Zealand White rabbits (CV1316 and CV1317) were injected with 0.4 mg of the trpE–GCN20 fusion protein and boosted once at 4 week and, subsequently, at 2 week intervals by Hazelton Laboratories. For the trpE–GCN1 fusions, six New Zealand White rabbits (HL1402, HL1403 and HL1404 for the *Hin*dIII construct; HL1405, HL1406 and HL1407 for the *Bam*HI fusion) were injected with 1 mg of the fusion protein and boosted at 4 week intervals by the same laboratory.

Pulse–chase labeling of GCN20 protein

Immunoprecipitations of whole-cell extracts prepared from yeast cells after pulse labeling with Tran^{35}S-label (ICN Radiochemicals) were performed as described previously (Kölling and Hollenberg, 1994) with minor modifications. Briefly, cells grown overnight in SD minimal medium were labeled by adding 25 μCi of Tran^{35}S-label/OD_{600} cells. After 10 min, a concentrated chase solution (100 mM ammonium sulfate, 0.3% cysteine, 0.4% methionine) was diluted 100-fold into the culture. Aliquots of 1 OD_{600} of cells were removed at the appropriate time intervals, washed in cold 10 mM NaN_3 and resuspended in lysis buffer (0.3 M sorbitol, 10 mM Tris, pH 7.5, 0.1% NaN_3) containing protease inhibitors (1 μM pepstatin, 1 μM leupeptin, 0.15 μM aprotinin and 100 μM phenylmethylsulfonyl fluoride). Cells were lysed by vortexing with glass beads for 2 min, mixed with one volume of 2× Laemmli's sample buffer (Laemmli, 1970) and boiled for 4 min. Immunoprecipitations of the cleared lysates were performed according to Kölling and Hollenberg (1994).

Immunoprecipitation of complexes containing GCN20 and GCN1

Cells grown to early exponential phase were broken by vortexing with glass beads in 20 mM Tris–HCl (pH 7.5), 100 mM NaCl and 1 mM $MgCl_2$ (TNM buffer) supplemented with the protease inhibitors described above. Cell debris was removed by centrifugation in an Eppendorf microcentrifuge and the protein concentration in the supernatants was determined by the Bradford (1976) assay.

To immunoprecipitate GCN20 and GCN1, protein samples (40 μg) were diluted to a final volume of 0.4 ml of TNM buffer containing 0.25% Triton X-100 and 0.125% sodium deoxycholate, and the appropriate antibody [polyclonal antibodies against GCN1 or GCN2 or monoclonal antibody M2 (IBI) against the FLAG epitope] was added and incubated on ice for 2 h. Immune complexes were collected using protein A–Sepharose CL-4B beads and washed twice with TNM supplemented with detergents and then twice with TNM alone. The beads were suspended in 50 μl of Laemmli's sample buffer (Laemmli, 1970), boiled for 4 min, and analyzed by 7.5 or 8.0% SDS–PAGE. Proteins were transferred to nitrocellulose (Towbin *et al.*, 1979) and detected using rabbit polyclonal antibodies against GCN20 (at 1:1000 dilution) or GCN1 (diluted 1:1000). GCN2 was detected by assaying GCN2 autokinase activity in immune complexes obtained under the conditions described above for GCN1 or GCN20, all as described previously (Wek *et al.*, 1990).

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