A Ribosomal Protein Is Required for Translational Regulation of GCN4 mRNA

EVIDENCE FOR INVOLVEMENT OF THE RIBOSOME IN eIF2 RECYCLING

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In amino acid-starved yeast cells, inhibition of the guanine nucleotide exchange factor eIF2B by phosphorylated translation initiation factor 2 results in increased translation of GCN4 mRNA. We isolated a suppressor of a mutant eIF2B. The suppressor prevents efficient GCN4 mRNA translation due to inactivation of the small ribosomal subunit protein Rps31 and results in low amounts of mutant 40 S ribosomal subunits. Deletion of one of two genes encoding ribosomal protein Rps17 also reduces the amounts of 40 S subunits but does not suppress eIF2B mutations or prevent efficient GCN4 translation. Our findings show that Rps31-deficient ribosomes are altered in a way that decreases the eIF2B requirement and that the small ribosomal subunit mediates the effects of low eIF2B activity on cell viability and translational regulation in response to eIF2 phosphorylation.

In current models for protein synthesis, eIF2 associates with GTP and Met-tRNA to form a ternary complex that joins the small ribosomal subunit with other initiation factors. The preinitiation complex binds to the 5' end of mRNA and migrates downstream. Upon recognition of an AUG initiation codon eIF2-bound GTP is hydrolyzed, and eIF2 is discharged from the ribosome as an inactive eIF2-GDP complex that must be recycled by the guanine nucleotide exchange factor eIF2B.

Phosphorylation of the α subunit at Ser-51 converts eIF2 from an eIF2B substrate to an inhibitor. Mammalian eIF2α-GDP binds eIF2B with a high affinity, but the bound GDP cannot be released, leading to a reduction in the rate of ternary complex formation. Yeast eIF2 phosphorylation increases the affinity for eIF2B and inhibits the GDP release. Thus, the mechanism of translational inhibition by eIF2 phosphorylation is conserved between yeast and mammals. The eIF2 kinase Gcn2 becomes activated in yeast cells starved for amino acids. In contrast to the overall rate of protein synthesis, the translational efficiency of GCN4 mRNA increases in starved cells. Gcn4 protein stimulates transcription of over 40 genes that are required for amino acid biosynthesis or tRNA charging.

Translational regulation of GCN4 mRNA is mediated by four uORFs. The first uORF is the leuostin inhibitory and is required for the ability of ribosomes to bypass the translational barrier imposed by the remaining three uORFs. A large amount of data supports a scanning-reinitiation model in which ribosomes translate the first uORF but remain mRNA-bound thereafter. While moving further downstream, reinitiation at the inhibitory uORF4 precludes subsequent reinitiation at GCN4. In amino acid-starved cells, the reduction in ternary complex levels is thought to delay rebindining of ternary complexes to ribosomes scanning downstream of uORF1. Consequently, many ribosomes bypass the inhibitory uORF4 before acquiring a ternary complex, thus permitting recognition of the GCN4 start codon and translation of the GCN4 uORF5.

Many genes required for the starvation response in yeast were identified using genetic approaches. The β, γ, δ, and ε subunits of yeast eIF2B encoded by GCD7, GCD1, GCD2, and GCD6, respectively, were identified as mutations that mimic the effect of eIF2 phosphorylation on GCN4 expression. Mutations in the α subunit Gcn3 and in the Gcd7 and Gcd2 subunits of eIF2B can make eIF2B insensitive to eIF2α-GDP (8–11). To identify novel eIF2B interactions in vivo, revertants were selected from a yeast strain with a conditional lethal mutant eIF2B that causes constitutively high levels of GCN4 translation. A revertant was isolated that prevents both the lethality and efficient GCN4 translation. Suppression is due to a mutation in the small subunit ribosomal protein gene RPS31 (previous designations URI3 and RPS27A), providing evidence for an in vivo involvement of the ribosome in eIF2 recycling.

EXPERIMENTAL PROCEDURES

Plasmids and Nucleic Acid Manipulations—YCP50 is a low copy number shuttle vector carrying the URA3 gene as a selectable yeast marker and β-lactamase for selection in Escherichia coli (12). Sc4014 is a yeast-E. coli shuttle vector carrying the URA3 and GCD1 genes (13). The two gcd1 mutant alleles pB336 and pB337 were constructed by inserting an 8-mer (5'-CAGATCTG 3') or a 10-mer self-complementary oligonucleotide (5'-GAAGATCTCC 3') into the unique PvuII site near the amino terminus in the protein-coding region of the GCD1 gene of Sc4014. GCD4-lacZ and GCN4-lacZ constructs on the yeast-E. coli shuttle plasmids p164 and p227, with and without the four GCN4 upstream aCGTACGAGGATAAAAGA sequences, are constructs that contain the GCN4 mRNA leader and the first 54 codons of the GCN4 protein coding sequence fused in frame to the bacterial lacZ gene (6). The GCN4-lacZ and GCN4-lacZ constructs on plasmids p209 and pM99 contain as the sole upstream reading frame either wild-type uORF1 (p209; Ref. 6) or a hybrid reading frame with the coding region and 25 base pairs immediately following the uORF1 stop codon replaced by the corresponding ORF4 sequence (pM99; Ref. 14). Plasmids were propagated in E. coli JM109 (15). E. coli cells competent for transformation (16) were stored frozen at −70 °C until needed. Plasmid DNA was isolated...
Yeast strains were obtained from the following sources: A. G. H., Alan G. Hinnebusch, Bethesda; A. M. C., A. Mark Cigan, Bethesda; G. R. F., Gerald R. Fink, Cambridge, MA; D. F., Daniel Finley, R. H., Ralph Huetter, Zurich, Switzerland; T. D. D., Thomas Donahue, Bloomington, IN.

| Strain | Genotype | Source |
|--------|----------|--------|
| A235   | MATa leu2 ura3-52 his4-303 su2-1 | T. D. D. |
| F2     | MATa gen2-10 | G. R. F. |
| F21    | MATa gen4-102 his1-29 | G. R. F. |
| F27    | MATa gen1-2 | G. R. F. |
| H1613  | MATa GCN2-516 HIS4-lacZ at ura3 leu2 ura3-52 iso1 | A. G. H. |
| H1989  | MATa gcd1-501 ura3-52 iso1 | A. G. H. |
| H472   | MATa gcd1-505 ura3-52 leu2 | A. G. H. |
| H1654  | MATa iso1 leu2 ura3-52 HIS4-lacZ rps17a::LEU2 | A. G. H. |
| H1649  | MATa gen3-10R4K HIS4-lacZ ura3-52 leu2 iso1 | A. G. H. |
| MC102  | MATa HIS4-lacZ ura3-52 trp1 gcd1::LEU2 <GCD1 TRP1> | A. C. M. |
| M76C   | MATa ade2-1 ura3-52 | This study |
| MT27B  | MATa rps31-10 ura3-52 leu2 | This study |
| MT27D  | MATa gcd1-505 ura3-52 ade2-1 | This study |
| MT28C  | MATa rps31-10 ura3-52 leu2 ade2-1 | This study |
| MT32A  | MATa gen6::LEU2 ura3-52 ade2-1 | This study |
| MT34A  | MATa gcd1-505 ura3-52 leu2 | This study |
| MT49B  | MATa gcd1-505 ura3-52 leu2 | This study |
| MT105D | MATa ura3-52 trp1 leu2 ade2-101 his3 | This study |
| MT106A | MATa ura3-52 trp1 leu2 ade2-101 his3 | This study |
| MT106B | MATa ura3-52 leu2 trp1 | This study |
| MT150C | MATa ura3-52 leu2 | This study |
| MT160D | MATa ura3-52 leu2 his3 | This study |
| M7A    | MATa gen3-10R4K rps31-10 leu2 ura3 <RPS31 URA3> | This study |
| M7B    | MATa gen3-10R4K rps31-10 leu2 ura3 | This study |
| M7C    | MATa gen3-10R4K rps31-10 leu2 ura3 <RPS31 URA3> | This study |
| M7D    | MATa gen3-10R4K rps31-10 leu2 ura3 | This study |
| M8A    | MATa gen3-10R4K rps31-10 leu2 ura3 <RPS31 URA3> | This study |
| M8B    | MATa gen3-10R4K rps31-10 leu2 ura3 | This study |
| M8C    | MATa gen3-10R4K rps31-10 leu2 ura3 <RPS31 URA3> | This study |
| M8D    | MATa gen3-10R4K rps31-10 leu2 ura3 | This study |
| M9A    | MATa gen3-10R4K rps31-10 leu2 ura3 <RPS31 URA3> | This study |
| M9B    | MATa gen3-10R4K rps31-10 leu2 ura3 | This study |
| M9C    | MATa GCN2-516 rps31-10 HIS4-lacZ leu2 ura3 <RPS31 URA3> | This study |
| M9D    | MATa GCN2-516 rps31-10 HIS4-lacZ leu2 ura3 | This study |
| M10C   | MATa sui2-1 rps31-10 ura3-52 leu2 <RPS31 URA3> | This study |
| M10D   | MATa sui2-1 rps31-10 ura3-52 leu2 | This study |
| M11A   | MATa sui2-1 rps31-10 ura3-52 leu2 <RPS31 URA3> | This study |
| M11B   | MATa sui2-1 rps31-10 ura3-52 leu2 | This study |
| M11C   | MATa sui2-1 rps31-10 ura3-52 leu2 <RPS31 URA3> | This study |
| M15C   | MATa gcd1-501 ura3-52 leu2 | This study |
| M16B   | MATa gcd2-1 ura3-52 | This study |
| M16C   | MATa gcd2-1 ura3-52 | This study |
| M16D   | MATa gcd1-501 rps31-10 leu2 ura3-52 | This study |
| M17A   | MATa gcd5-10 rps31-10 leu2 ura3-52 | This study |
| M17B   | MATa gcd2-1 rps31-10 leu2 ura3-52 | This study |
| M17C   | MATa gcd2-1 rps31-10 leu2 ura3-52 | This study |
| M17D   | MATa gcd2-1 rps31-10 leu2 ura3-52 | This study |
| M18A   | MATa gcd2-1 leu2 met8-1 | This study |
| M18B   | MATa gcd2-1 leu2 met8-1 | This study |
| M24C   | MATa URA3 at RPS21 ura3-52 leu2 | This study |
| M35C   | MATa rps31-10 ura3-52 leu2 | This study |
| M45D   | MATa rps31-10 ura3-52 leu2 | This study |
| M98    | MATa gcd1-505 rps31-10 ura3-52 leu2 | This study |
| RH770  | MATa gcd2-1 leu2-2 met8-1 | R. H. |
| SUB61  | MATa ura3-52 leu2 trp1 his3 lys2 | D. F. |
| SUB74  | MATa ubi1::URA3 ura3-52 leu2 trp1 his3 lys2 | D. F. |
| SUB121 | MATa rps31::HIS3 ura3-52 leu2 trp1 his3 lys2 | D. F. |
| SUB123 | MATa ubi1::TRP1 ura3-52 leu2 trp1 his3 lys2 | D. F. |

Manipulation of Yeast Cells—The genotypes of yeast strains are listed in Table I. SUB61, SUB74, SUB121, and SUB123 are isogenic strains (18). M98 is the isogenic revertant of strain H427 containing the rps31-10 suppressor allele. MT27B, MT27D, and MT28C are meiotic segregants of diploid cells formed by crossing M98 with MT6C. M16B, M16C, M17B, M17C, M17D, M18A, and M18B are meiotic segregants of diploid cells obtained from crossing MT27B with RH770. MT150C is a RPS31-derived strain from our strain collection. M15C, M16D, and M17A were meiotic.

from E. coli by the alkaline lysis method (17). Total RNA was extracted from yeast cells and separated electrophoretically on a 0.8% agarose gel. Amounts of RNA were adjusted by ethidium bromide-induced fluorescence of ribosomal RNA under UV illumination. The ratios of rRNA were evaluated by computer analysis of the fluorescence intensity of individual bands using the program ImageQuaNT (Molecular Dynamics). rRNA ratios of rps31 strains were averaged and compared with the average ratios of 4 RPS31 strains, with a standard deviation of 15% between the individual determinations. RNA was transferred to a nylon membrane by capillary blotting. A 3.5-kilobase pair EcoRI restriction fragment from p227 DNA that contained GCN4-lacZ fusion sequences was isolated using the Prep A Gene DNA purification matrix kit (Bio-Rad). DNA (40 ng) was labeled with [α-32P]dCTP using an oligo-labeling kit (Amersham Pharmacia Biotech, Freiburg, Germany). Labeled DNA was separated from unincorporated nucleotides on 1-mL Sephadex G-25 columns and then hybridized to GeneScreen Plus membranes according to the manufacturer's recommendations (DuPont, Bad Homburg, Germany). Blots were washed at 62 °C in 300 mM NaCl, 40 mM sodium citrate, and 35 mM sodium dodecyl sulfate, pH 7, followed by autoradiography.
RPS31-type mutant M98, a high temperature-resistant revertant had a Csm phenotype in cold-sensitive segregants of a diploid formed by crossing H469 with MT27B. The presence of the gcd1-505 allele was confirmed by non-complementation of the temperature sensitivity phenotype of the gcd1-505 allele. For the construction of gcd1-505 rps17a::LEU2 double mutants, the sl2-1 phenotype of M98 was transformed into M18B, and (ii) by reappearance of the gcd2-1 mutation, the allelic status of gcd2-1 was confirmed by non-complementation of the Slg phenotype in the resulting diploids indicates the presence of gcd1-505 in the gcd2-1 strain. As expected, these double mutants grew slowly at 30 °C, and exhibited sensitivity to 3-AT. All three phenotypes were recessive when diploids were formed with GCD1 strains of the opposite mating type and complemented in diploids formed by crossing MT27B with strains P2, F21, P27, and MT22A, containing the mutant alleles gcn2-101, gcd2-103, gcd1-12, and gcd2-1LEU2, respectively, suggesting that the suppressor was not an allele of these GCN genes. Meiotic segregants that exhibit the Slg, Csm, and 3-AT-sensitive phenotypes of the rps1-10 mutation were obtained from a diploid formed between a sup mutant and a GCD1 strain. The three phenotypes of the revertant, slow growth, cold sensitivity, and suppression of gcd1 temperature sensitivity, co-segregated in genetic crosses and are thereby due to a single extragenic mutation, a conclusion that was confirmed subsequently by transformation with the wild-type RPS31 gene. Recycling and Genetic Characterization of the Suppressor—The rps1-10 allele was isolated as a suppressor of the Tsm lethal phenotype of the gcd1-505 allele in strain H472. The Tsm phenotype of H472 is due to a mutation in GCD1, since transformation of the strain with Sc4015 plasmid DNA, a derivative of the URA3 vector YCP50 carrying the cloned GCD1 gene, restored growth at high temperatures (13). Transformation with control constructs pB336 and pB337, containing frameshift mutations in the GCD1 coding region, did not complement the Tsm phenotype of H472. For the construction of the temperature-resistant revertant strain H472 seven single colonies on solid YPD medium at 25 °C. Over 1000 single colonies were then transferred individually to solid YPEG medium. To allow formation of revertant colonies the cells were incubated for 2 weeks at 37 °C. A single revertant colony from each original colony was then transferred to solid YPD medium and grown for 2 days at 37 °C. Two YPD replicas were prepared from each original plate and incubated at 20 and at 37 °C, respectively. After 3 days, growth of colonies at low and high temperature was compared. M98, a high temperature-resistant revertant had a Csm phenotype, grew slowly at 30 °C, and exhibited sensitivity to 3-AT. All three phenotypes were recessive when diploids were formed with GCD1 strains of the opposite mating type and complemented in diploids formed by crossing MT27B with strains P2, F21, P27, and MT22A, containing the mutant alleles gcn2-101, gcd2-103, gcd1-12, and gcd2-1LEU2, respectively, suggesting that the suppressor was not an allele of these GCN genes. Meiotic segregants that exhibit the Slg, Csm, and 3-AT-sensitive phenotypes of the rps1-10 mutation were obtained from a diploid formed between a sup mutant and a GCD1 strain. The three phenotypes of the revertant, slow growth, cold sensitivity, and suppression of gcd1 temperature sensitivity, co-segregated in genetic crosses and are thereby due to a single extragenic mutation, a conclusion that was confirmed subsequently by transformation with the wild-type RPS31 gene.

As the Tsm phenotype of gcd1-505 cannot be observed in the presence of the rps1-10 mutation, the allelic status of GCD1 in cold-sensitive meiotic segregants was tested by formation of diploids with gcd1-505 Tsm mutant strains H472 or MT27D. Non-complementation of the Tsm phenotype in the resulting diploids indicates the presence of gcd1-505 in the rps1-10 strain. As expected, these double mutants exhibited the cold-sensitive and 3-AT-sensitive phenotypes of rps1-10 single mutants and displayed the Slg and Tsm phenotypes typical of gcd1-505 strains after transformation with plasmids carrying the wild-type RPS31 gene.

The presence of the gcd2-1 allele in rps1-10 gcd2-1 double mutant meiotic segregants was established in two ways as follows: (i) by non-complementation of the Slg phenotype of gcd2-1 in diploids derived from crosses of potential double mutants with gcd1-505 parents M18A or M18B, and (ii) by reappearance of the gcd2-1 phenotype in meiotic segregants of diploids derived from crosses to GCD strains. Meiotic segregants were considered to contain gcd2-1 if they exhibited an Slg phenotype, were resistant to amino acid starvation, and did not complement the Slg phenotype of either gcd2-1 strain M18A or M18B strains. M7, M7C, M8A, M8C, and M9B were meiotic segregants of a diploid obtained by crossing H1489 to M35C that have been transformed with the RPS31 containing plasmid pB272; strains M7A, M7C, M8A, and M9B were meiotic segregants of a diploid strain H1613 to the rps1-10 strains M35C and then to MT27B. M9C contains the RPS31 gene on the plasmid pB272 and is of unknown origin. The results of individual assays varied 30% or less within each triplicate set. To test resistance to amino acid analogs, freshly grown yeast cells were streaked radially on solid SD medium to which supplements were added to satisfy auxotrophic requirements. Solid plates were incubated for 2 weeks at 37 °C. A single revertant colony from each original colony was then transferred to solid YPD medium and grown for 2 days at 37 °C. Two YPD replicas were prepared from each original plate and incubated at 20 and at 37 °C, respectively. After 3 days, growth of colonies at low and high temperature was compared. M98, a high temperature-resistant revertant had a Csm phenotype, grew slowly at 30 °C, and exhibited sensitivity to 3-AT. All three phenotypes were recessive when diploids were formed with GCD1 strains of the opposite mating type and complemented in diploids formed by crossing MT27B with strains P2, F21, P27, and MT22A, containing the mutant alleles gcn2-101, gcd2-103, gcd1-12, and gcd2-1LEU2, respectively, suggesting that the suppressor was not an allele of these GCN genes. Meiotic segregants that exhibit the Slg, Csm, and 3-AT-sensitive phenotypes of the rps1-10 mutation were obtained from a diploid formed between a sup mutant and a GCD1 strain. The three phenotypes of the revertant, slow growth, cold sensitivity, and suppression of gcd1 temperature sensitivity, co-segregated in genetic crosses and are thereby due to a single extragenic mutation, a conclusion that was confirmed subsequently by transformation with the wild-type RPS31 gene.
plasmid pB303, both in the diploid heterozygous transformants and in haploidal meiotic segregants obtained by sporulating one such diploid, in agreement with the subsequent finding that the disrupted sequences are outside the translational reading frame that complemented the growth defects of the suppressor mutation. A haploid integrant M24C was then mated to the suppressor-containing strain T27B, and the resulting diploid was sporulated. In 10 out of 10 tetrads analyzed, each had two spores that were Ura- and cold-sensitive, showing that the integration site is closely linked to the suppressor locus. Therefore, the cloned DNA fragment is derived from the chromosomal region of the suppressor locus and most likely carries the wild-type suppressor allele.

**Subcloning and DNA Sequence Analysis**—For complementation analysis, pB272 (Fig. 3, construct 1) was cleaved with HindIII and then religated to delete the fragment between the HindIII site in YCP50 and the HindIII site within the yeast genomic insert (pB276, Fig. 3, construct 2). pB272 was cleaved with BamHI and EcoRI, and the larger fragment was circularized in the presence of the oligonucleotides 5’-GATGCCTGCAGCC 3’ and 5’-AATTCGGTCGAC 3’ (pB277, Fig. 3, construct 3). A BamHI fragment was deleted from the insert in pB272 by cleavage with BstEII and recircularization (pB301, Fig. 3, construct 4). Partial cleavage of pB272 with BgiII and religation deleted the BgiI fragment from the yeast DNA insert (pB281, Fig. 3, construct 5). The region between the XhoI site in the genomic insert and the SalI site of pB272 was deleted by cleavage with XhoI and partial cleavage with XbaI followed by ligation using a BglII-SalI linker (pB282, Fig. 3, construct 6). BamHI-HindIII restricted pB273 DNA was separated by gel electrophoresis, extracted, and then ligated with BamHI-HindIII-digested YCP50, yielding pB393 (Fig. 3, construct 7). pB301 was constructed by deleting the XhoI fragment carrying the URA3 gene from pB272. To construct pB303, pB301 was linearized with BamHI and a 1.1-kilobase pair BglII fragment from pVT-U carrying the URA3 gene was inserted (Fig. 3, construct 8).

To obtain a plasmid suitable for preparation of serial deletions, one of the two XhoI sites of pB272 was eliminated by formation of an XhoI-SalI hybrid site. To this end pB272 was cleaved with XhoI and SalI and the two large fragments ligated, resulting in pB282. pB282 was linearized with XhoI, and serial deletions were created by treatment with exonuclease Bal-31. The fragments were then ligated in the presence of BglII and religation produced pB303 (Fig. 3, construct 6). The plasmid was then cleaved with BgiI and religation deleted the BgiI fragment that lay between the XhoI and SalI sites, yielding pB307 (Fig. 3, construct 7). pB301 was constructed by deleting the XhoI fragment carrying the URA3 gene from pB272. To construct pB303, pB301 was linearized with BamHI and a 1.1-kilobase pair BglII fragment from pVT-U carrying the URA3 gene was inserted (Fig. 3, construct 8).

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**RESULTS**

**Isolation and Genetic Characterization of an Extragenic Suppressor of a Mutation in the γ Subunit of eIF2B**—A spontaneous revertant of the temperature-sensitive lethal gcd1-505 allele was isolated. Whereas gcd1-505 cells died at 37 °C, revertant cells were viable (Fig. 1, 37 °C). The revertant carried a suppressor mutation (sup) that was recessive and unlinked to gcd1 (see “Experimental Procedures”). The reduced growth rate of the revertant at 37 °C was not due to incomplete suppression of the gcd1 mutation, because the sup allele led to slow growth independently of the gcd1 allele (Fig. 1, 37 °C, gcd1 sup and sup). 30 °C was semi-permissive for both the gcd1 and the revertant cells. At low temperature, growth of the sup strains was severely inhibited (Fig. 1, 30 °C compared with 20 °C). The cold-sensitive phenotype of the sup allele indicates a defect in a function required for normal cell growth.

The gcd1-505 allele was originally isolated by selecting revertants of gcn2 gcn3 double mutants that were resistant to 3-AT (28). 3-AT causes histidine starvation by inhibiting the HIS3 gene product, which catalyzes the seventh step of histidine biosynthesis (29). In histidine-starved wild-type cells, increased translation of GCN4 mRNA leads to higher levels of Gcn4 protein, which in turn stimulates HIS3 transcription. This enables the cell to produce sufficient histidine in the presence of 3-AT to permit cell growth. gcn2 mutants cannot phosphorilate eIF2α, whereas gcn3 mutants contain a defective eIF2B α subunit that renders eIF2B insensitive to inhibition by eIF2α(P), thus preventing the increase in GCN4 translation (5). The gcd1-505 mutation restored high level GCN4 translation independently of Gcn2 and Gcn3 (28). To determine whether the sup allele alters GCN4 translation, we examined the 3-AT sensitivity. Compared with wild-type cells and gcd1 mutant cells, the sup allele conferred sensitivity to 3-AT and to the amino acid analogs ethionine (a methionine analog) and canavanine (an arginine analog), showing that the sup allele acts in a non-pathway specific manner (Table II, wild type, gcd1, and sup). This is typical for gcn mutations that prevent derepression of GCN4 and the multiple amino acid biosynthetic pathways under its control.

The amino acid analog-sensitive phenotype of the sup allele was epistatic to the analog-resistant phenotypes of mutations in the Gcd1, Gcd2, or Gcn3 subunits of eIF2B (Table II, compare growth in the presence and absence of the sup allele). The sup mutation also reversed the 3-AT-resistant phenotype of a dominant Gcn2* mutant that leads to constitutive high level eIF2 phosphorylation and GCN4 translation (GCN2*-516, Table II). These findings imply that the sup mutation prevents the induction of GCN4 translation when eIF2 recycling is reduced. Both cold-sensitive growth and suppression of amino acid analog-resistant phenotypes of eIF2B mutants are unique among the known gcn alleles, indicating a novel mode of action.

**To quantify the effects of the sup allele on GCN4-mediated growth:**
 transcribal activation of the HIS4 gene, we assayed a HIS4-lacZ fusion. As expected, enzyme activity increased upon starvation in wild-type cells, and in the presence of a constitutively active Gcn2 kinase high level expression occurred independently of starvation (Table III, HIS4-lacZ activities, wild type, and GCN2–516). Enzyme activity in the sup strain was low and was not increased when the cells were starved or in the presence of the constitutive Gcn2 kinase (Table III, HIS4-lacZ activities, sup and GCN2–516 sup). That the phenotype of the sup mutation is independent of Gcn2 kinase activity indicates that the suppressor blocks a step downstream of Gcn2.

To test whether a residual Gcd1 function was required in sup cells, sup gcd1 disruption mutants were constructed in which the sole functional copy of the GCD1 gene was provided on an autonomously replicating plasmid. During growth on non-selective medium, the plasmid was stably maintained, whereas in the presence of the genomic GCD1 allele, the plasmid was readily lost. We concluded that the plasmid was stably maintained because the GCD1 gene remains essential for growth in sup strains. Thus the suppressor mutation overcomes a partial loss but not the complete absence of GCD1 function.

The Sup Mutation Prevents Efficient GCN4 mRNA Translation—To determine whether the sup mutation impairs GCN4 expression, we assayed a GCN4-lacZ fusion containing all four uORFs in the mRNA leader. In accordance with previous results, a 9-fold increase in expression was observed for this fusion in starved wild-type cells (Table III, wild-type GCN4-lacZ activity; Ref. 30). In addition, gcd1 and gcd2 mutants showed constitutively derepressed expression of this fusion protein (Table III, gcd1–505; gcd2–1; Ref. 28). Expression was very low in sup mutants, even in the presence of the gcd mutations (Table III, sup, gcd1–505 sup, gcd2–1 sup, compare also rps31Δ::HIS3). Therefore, similar to previously characterized gen mutations, the sup allele impairs derepression of GCN4 expression in amino acid-starved cells (31). However, it is unique in preventing GCN4 expression in mutants with reduced eIF2B activity.

In known gen mutants reduced GCN4 mRNA translation is dependent on four uORFs (6). This was also the case for the sup mutants (Table III, GCN4-lacZ activities). To confirm that in sup mutants the low enzyme activities did not arise from inefficient transcription or mRNA instability, we analyzed the steady-state levels and sizes of authentic GCN4 and GCN4-lacZ fusion mRNAs. The size of GCN4 mRNA appeared unchanged in all strains tested (Fig. 2). As observed previously, there were some variations in mRNA levels, but under starvation conditions, GCN4 mRNA levels in all sup strains were 50% or higher than the levels seen in the wild-type strain (Fig. 2, right; see Ref. 31). Thus, the over 10-fold lower GCN4 expression in sup strains under starvation conditions cannot be explained by decreases in GCN4 mRNA levels. We conclude that the suppressor decreases GCN4 expression at the translational level by an uORF-dependent mechanism.

The Sup Mutation Prevents Ribosomes from Bypassing uORFs 2–4 and Reinitiating Translation at GCN4—To bypass uORFs 2–4 and reach the GCN4 start codon under starvation conditions, ribosomes must first translate uORF1 and resume scanning (6). GCN4 mRNA translation in sup mutants might be low due to poor recognition of the uORF1 initiation codon, causing ribosomes to migrate further downstream and translate the inhibitory uORFs 2, 3, or 4 instead. Because reinitiation at the GCN4 open reading frame is inefficient following translation of these latter uORFs, a failure to recognize uORF1 could explain the low GCN4 expression levels (6). Replacement of sequences following the uORF1 stop codon with the corresponding sequences from uORF4 (hybrid uORF1/4) leads to a drastic reduction in GCN4 expression that was attributed to efficient initiation at the uORF1 AUG codon coupled with failure to resume scanning following termination at uORF1 (14). To determine by what mechanism the suppressor mutation prevents efficient GCN4 translation, expression from mutant uORF constructs were tested in the sup strain. The hybrid uORF1/4 was equally inhibitory in the wild-type strain and in the sup strain, indicating that in both strains ribosomes efficiently initiate translation at uORF1 (Table III, GCN4-lacZ and GCN4-lacZ activities).

It was also possible that the sup mutation would prevent a resumption of scanning following uORF1. However, in the presence of uORF1 alone, GCN4 was translated very efficiently in wild-type and sup strains alike, suggesting that reinitiation following uORF1 translation is not impaired (Table III, compare GCN4-lacZ and GCN4-lacZ activities). Therefore, in sup mutant cells, we detected no change in the ability of ribosomes to recognize and translate uORF1 and to resume scanning following termination at uORF1. This implies that, in sup cells, the ribosomes cannot bypass the start codons of the inhibitory uORFs 2–4 downstream of uORF1 (Table III, compare GCN4-lacZ and GCN4-lacZ activities). This is the same defect in translational control in gen1, gen2, and gen3 mutants.

The Sup Mutation Is a Null Allele of RPS31—Two plasmids were isolated from a yeast genomic plasmid library that complemented the cold-sensitive and suppressor phenotypes of the sup mutation, restoring the temperature-sensitive phenotype conferred by gcd1–505 in revertant cells. Restriction analysis of the genomic inserts revealed that these two plasmids contain overlapping fragments from the same genomic locus. The smallest fragment required for complementation carried the coding sequence of the RPS31 gene, encoding the yeast homolog of the mammalian small subunit ribosomal protein S27a fused to ubiquitin (Fig. 3, construct 7; see Refs. 18 and 32). An integrating plasmid was constructed bearing an insertion of the URA3 gene downstream of RPS31 (Fig. 3, construct 8). The plasmid was integrated into the genome at the RPS31 locus by homologous recombination. No meiotic recombination events were observed between the sup allele and the URA3 gene from the integrated plasmid. These findings prove that the sup allele
The suppressor prevents efficient GCN4 translation independent of Gcd1 and Gcd2 function but dependent on inhibitory uORFs in GCN4 mRNA

Yeast transformants were grown under non-starvation conditions (N) and starvation conditions (S), and β-galactosidase activities were determined from cell extracts. The strains used are: wild type, MT150C, gcd1–505, H472, sup, MT27B; gcd1–505 sup, M98. The average result is shown of strains with the same genotype tested individually as follows: gcd2–1, M16B and M16C, gcd2–1 sup, M17B, M17C and M17D. rps31::HIS3, SUB121. GCN4-lacZ, GCN4′-lacZ, GCN4′1/4-lacZ and GCN4′1/4-lacZ, and an uORF1/uORF4 hybrid (pM99), respectively.

### Table III

| Relevant genotype | N | S |
|-------------------|---|---|
| Wild type         | 290 | 1400 |
| GCN2′-516         | 1200 | 1800 |
| sup               | 90  | 140 |
| GCN2′-516 sup     | 110 | 120 |

| Relevant genotype | GCN4-lacZ<sub>a</sub> | GCN4′-lacZ<sub>a</sub> |
|-------------------|-------------------|-------------------|
| Wild type         | N                | S                |
| gcd1–505          | 10               | 90               |
| sup               | 7                | 6                |
| gcd1–505 sup      | 6                | 6                |
| gcd2–1            | 120              | 140              |
| gcd2–1 sup        | 12               | 11               |
| rps31::HIS3       | 8                | 12               |

| Relevant genotype | GCN4′1/4-lacZ<sub>a</sub> | GCN4′1/4-lacZ<sub>a</sub> |
|-------------------|-------------------|-------------------|
| Wild type         | N                | S                |
| sup               | 230              | 410              |
| sup               | 250              | 330              |

<sup>a</sup> Enzyme activity (lacZ units).

is genetically linked to RPS31.

The phenotypes of an rps31 disruption allele and the sup allele were indistinguishable with respect to GCN4 mRNA translation, amino acid analog sensitivity, suppression of gcd1–505, and cold-sensitive growth (Table IV, wild-type compared with sup and rps31::HIS3 phenotypes, Table III rps31::HIS3). In addition, sup and rps31::HIS3 alleles do not complement, whereas both are complemented by plasmid carrying the SUP gene (Table IV, sup rps31::HIS3 and <SUP>). Therefore, the suppressor of gcd1–505 is an rps31 allele, and henceforth we refer to it as the rps31-10 allele. The growth defect of rps31 mutants is due to the mutant ribosome and not due to reduced ubiquitin levels, since the growth defect can be partially compensated by additionally deleting one of the ubiquitin large ribosomal subunit genes (18). No significant effects on amino acid analog sensitivity were observed when strains were disrupted in either of the two genes, UBI1 or UBI2, encoding a ubiquitin-larg subunit ribosomal fusion protein, showing that the observed effect of rps31 alleles is specific to this ubiquitin fusion protein (Table IV, abi1::TRP1 and abi2::URA3).

Deletion of the RPS31 gene results in cold-sensitive cell growth, inefficient processing of 20 S to 18 S RNA, and low amounts of small ribosomal subunits (18). Similarly, all rps31-10 strains contained reduced amounts of small ribosomal RNA (Fig. 2, left). Analysis of free ribosomal subunits and 80 S monosomes revealed a deficiency in free 40 S subunits and an accumulation of free 60 S subunits in the extract from an rps31-10 strain when compared with an RPS31 wild-type cell extract (Fig. 4A compared with Fig. 4B). Mixing the two extracts before centrifugation confirmed that the differences between the rps31 mutant and wild-type profiles were not an artifact of the gradient (Fig. 4C). The ribosomal profile of the rps31-10 mutant is the same as that described for a rps31

![Fig. 2. Steady-state levels of GCN4 and GCN4-lacZ mRNA.](image-url)
 Phenotypes of yeast strains containing mutations in ubiquitin-ribosomal protein encoding genes

| Relevant genotype | Growth at 20 °C | 3-AT resistance | Ethionine resistance | Canavanine resistance |
|------------------|----------------|-----------------|----------------------|----------------------|
| Wild type        | + + +          | ND              | +                    | +                    |
| sup              | + + +          | ND              | +                    | +                    |
| rps31::HIS3      | + + +          | ND              | +                    | +                    |
| sup <SUP>        | + + +          | ND              | +                    | +                    |
| rps31::HIS3 <SUP> | + + +          | ND              | +                    | +                    |
| sup gcd1–505     | + + +          | ND              | +                    | +                    |
| rps31::HIS3 gcd1–505 | + + +         | ND              | +                    | +                    |
| rps31::HIS3 sup  | + + +          | ND              | +                    | +                    |
| ub1::TRP1        | + + +          | ND              | +                    | +                    |
| ub2::URA3        | + + +          | ND              | +                    | +                    |

Fig. 4. Cells carrying the suppressor allele have low amounts of small ribosomal subunits. Extracts of yeast cells were centrifuged on sucrose gradients, and the UV absorption profile was determined. A, extract from M53C yeast cells transformed with the suppressor wild-type (wt) gene (p532b). B, suppressor (rps31) mutant yeast extract from M53C. C, mixture of yeast extract used in A and B. Direction of sedimentation is from left to right.

deletion mutant (Fig. 4 compared with Fig. 4 in Ref. 18). Thus, by all phenotypic criteria, it appears that rps31–10 is a loss of function allele.

Specific Suppression of eIF2B Mutations by Inactivation of Rps31—To analyze the specificity of the suppression, we examined the interaction of rps31–10 with a temperature-sensitive mutation in the a subunit of eIF2 (sui2–1), which has a Ged phenotype (33). The rps31–10 allele did not suppress the temperature-sensitive phenotype of sui2–1, suggesting that rps31 alleles do not generally overcome the growth defects associated with mutations that reduce eIF2B activity (Table V; sui2–1 rps31–10 compared with wild type and sui2–1). To determine whether the reduced amount of 40 S subunits or the absence or irregularity in these tetrads and was therefore not included. rps31::HIS3 sup, diploid cells formed by a cross SUB121 × MT28C; ub1::TRP1, SUB123; ub2::URA3, SUB74. SUB61, SUB74, and SUB123 were deficient in histidine biosynthesis and could therefore not be tested for 3-AT resistance. ND, not determined.

TABLE V

| Relevant genotype | 20 °C | 30 °C | 37 °C |
|------------------|------|------|------|
| Wild type        | + + +| +    | ND   |
| sui2–1           | +    | +    | +    |
| sui2–1 rps31–10  | +    | +    | +    |
| gcd1–505         | +    | +    | +    |
| rps31–10         | +    | +    | +    |
| gcd1–505 rps31–10| -    | +    | +    |
| gcd1–505 rps31–10 <SUP> | +    | +    | +    |
| rps17a::LEU2     | +    | +    | +    |
| gcd1–505 rps17a::LEU2 | +    | +    | +    |

Yeast was grown on solid YPD medium at the temperatures indicated until the wild-type strain colony diameter was 2 mm (++). Yeast strains employed were as follows: wild type, MT150C; sui2–1, M10C, M11A and M11C; sui2–1 rps31–10, M10D, M11B, and M45D; gcd1–505, H472; rps31–10, MT27B; gcd1–505 rps31–10, M98; gcd1–505 rps31–10 <SUP> | M98 transformed with pB272; rps17::LEU2, H1654; gcd1–505rps17::LEU2, see “Experimental Procedures.”

DISCUSSION

eIF2B-mediated recycling of eIF2 occurs independently of the ribosome and is a prerequisite for ternary complex formation, for the eIF2 association with the ribosome, and for the subsequent mRNA binding step (1). It is difficult to explain with this model how low levels of an altered ribosomal subunit could reduce the eIF2B requirement for cell survival and translational regulation. It has been suggested that Gcn4 expression is regulated by a translation-reinitiation mechanism. In this view, the properties of the initiation complex and the choice of initiation codon used depends not only on the presence of the ternary complex but also on the behavior of the mRNA-bound ribosome after GTP hydrolysis or in the absence of the ternary complex. We propose that eIF2B is critically required for such an additional, mRNA-bound ribosome-dependent eIF2 recycling step that is essential not only for the regulation of Gcn4 translation but also for viability. In this model, the critical step (Fig. 5, step b) blocked by eIF2 phosphorylation is the recharging of mRNA-bound small ribosomal subunits with ternary complex. In accord with our results, rps31 mutants would circumvent this block without influencing the rate of eIF2 recycling, possibly by facilitating the release of eIF2-GDP or by reducing the speed of ribosomal subunits lacking ternary complex, thereby reducing the scanning distance of reinitiating ribosomes required to rebind a ternary complex.

This model predicts that mRNA-bound small ribosomal subunits without a ternary complex can occur on mRNAs other than Gcn4, and since gcn4 mutations do not rescue the lethality of gcd1 mutations, translation reinitiation on mRNAs other than Gcn4 mRNA must be detrimental to cell survival, rather than the overall lower initiation rate. In the classical initiation pathway the consequence of eIF2B inhibition is not an accumulation of mRNA-bound initiation complexes, since the ternary complex is a prerequisite for mRNA binding of the initiation complex (35–37). gcd2–505 mutants accumulate small ribosomal subunits bound to polysomal mRNA, as expected if the recharging of ternary complex is delayed for mRNA-bound ribosomes that have hydrolyzed the GTP without formation of an elongating ribosome (27).

Several studies on mammalian systems suggest a ribosome-associated function of eIF2B. Under conditions leading to eIF2 phosphorylation, an accumulation of 48 S initiation complexes has been observed by several investigators (38–42) or accumulation of eIF2 on ribosomes that apparently result from aber-
Our results demonstrate the in vivo relevance of an eIF2α-mediated inhibition of eIF2B in blocking a ribosome-dependent function late in the initiation pathway, supporting a reinitiation mechanism for GCN4 translation. We have shown that this ribosome-associated function is critical for the effects of reduced eIF2B activity on GCN4 translation and on cell viability.

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A Ribosomal Protein Is Required for Translational Regulation of GCN4 mRNA: EVIDENCE FOR INVOLVEMENT OF THE RIBOSOME IN eIF2 RECYCLING

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