Guanine Nucleotide Exchange Factor for Eukaryotic Translation Initiation Factor 2 in \textit{Saccharomyces cerevisiae}: Interactions between the Essential Subunits GCD2, GCD6, and GCD7 and the Regulatory Subunit GCN3

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Phosphorylation of eukaryotic translation initiation factor 2 (eIF-2) in amino acid-starved cells of the yeast \textit{Saccharomyces cerevisiae} reduces general protein synthesis but specifically stimulates translation of \textit{GCN4} mRNA. This regulatory mechanism is dependent on the nonessential GCN3 protein and multiple essential proteins encoded by GCN genes. Previous genetic and biochemical experiments led to the conclusion that GCD1, GCD2, and GCN3 are components of the GCD complex, recently shown to be the yeast equivalent of the mammalian guanine nucleotide exchange factor for eIF-2, known as eIF-2B. In this report, we identify new constituents of the GCD-eIF-2B complex and probe interactions between its different subunits. Biochemical evidence is presented that GCN3 is an integral component of the GCD-eIF-2B complex that, while dispensable, can be mutagenically altered to have a substantial inhibitory effect on general translation initiation. The amino acid sequence changes for three gcn2 mutations have been determined, and we describe several examples of mutual suppression involving the gcn2 mutations and particular alleles of GCN3. These allele-specific interactions have led us to propose that GCN3 and GCD2 directly interact in the GCD-eIF-2B complex. Genetic evidence that GCD6 and GCD7 encode additional subunits of the GCD-eIF-2B complex was provided by the fact that reduced-function mutations in these genes are lethal in strains deleted for GCN3, the same interaction described previously for mutations in \textit{GCD1} and \textit{GCD2}. Biochemical experiments showing that GCD6 and GCD7 copurify and communoprecipitate with GCD1, GCD2, GCN3, and subunits of eIF-2 have confirmed that GCD6 and GCD7 are subunits of the GCD-eIF-2B complex. The fact that all five subunits of yeast eIF-2B were first identified as translational regulators of \textit{GCN4} strongly suggests that regulation of guanine nucleotide exchange on eIF-2 is a key control point for translation in yeast cells just as in mammalian cells.

Starvation of the budding yeast \textit{Saccharomyces cerevisiae} for one or more amino acids leads to elevated transcription of more than 30 genes involved in amino acid biosynthesis. This response (general amino acid control) is mediated by an increase in the levels of the transcriptional activator protein GCN4 (reviewed in reference 25). The increase in GCN4 synthesis is coupled to amino acid starvation by a unique translational regulatory mechanism involving phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2α) by the protein kinase GCN2 (10; reviewed in reference 29). In mammalian cells, phosphorylation of eIF-2α on the serine residue at position 51 inhibits translation in response to stress conditions, which include viral infection, amino acid starvation, and heat shock (reviewed in references 23 and 34). Recent results also implicate this translational mechanism in mammalian cell cycle control (32).

According to our current model, expression of \textit{GCN4} in \textit{S. cerevisiae} is linked to the activity of eIF-2 by short upstream open reading frames (uORFs) in the \textit{GCN4} mRNA leader (1, 10). It is thought that ribosomes scanning from the 5′ end of \textit{GCN4} mRNA translate the first uORF (uORF1) and resume scanning. Under conditions of sufficient eIF-2 activity, these ribosomes reinitiate translation at one of the remaining uORFs (uORF2 to uORF4) before reaching \textit{GCN4}. Unlike uORF1, translation of these downstream uORFs does not allow subsequent reinitiation at the \textit{GCN4} AUG codon; therefore, \textit{GCN4} expression is repressed. When eIF-2 activity is reduced by mutations or by phosphorylation under conditions of amino acid starvation, many ribosomes scanning downstream from uORF1 cannot reinitiate at uORF2 to uORF4 and instead reinitiate further downstream at the \textit{GCN4} start codon. Thus, a decrease in the efficiency of reinitiation leads to an increase in \textit{GCN4} translation.

The principal role of eIF-2 in translation initiation is to deliver charged initiator tRNA\(^{Met}\) to the ribosome (for reviews of eukaryotic translation initiation, see references 39 and 44). Analogous to elongation factor 1 in eukaryotes and elongation factor Tu in prokaryotes, the activity of eIF-2 is regulated by a cycle of GTP binding and hydrolysis. eIF-2 forms a ternary complex with GTP and tRNA\(^{Met}\) and subsequently binds to the 40S ribosomal subunit. Upon recognition of the AUG start codon, the GTP is hydrolyzed and eIF-2-GDP is released. In mammalian cells, a second initiation factor, known as eIF-2B, is required to promote GDP release and rebinding of GTP by eIF-2 (31). Phos-
phorylation of the α subunit of eIF-2 on serine 51 inhibits the guanine nucleotide exchange activity of eIF-2B (36, 47, 49), decreasing the level of eIF-2 · GTP and thereby inhibiting translation initiation.

In S. cerevisiae, replacement of serine 51 of eIF-2α with alanine abolishes phosphorylation of eIF-2 by GCN2 and prevents the increase in GCN4 translation that normally accompanies amino acid starvation (10). By analogy with mammalian systems, phosphorylation of eIF-2α in yeast cells is thought to diminish the rate of guanine nucleotide exchange on eIF-2 catalyzed by eIF-2B. The resulting decrease in levels of eIF-2 · GTP · Met-tRNA^Met ternary complexes would stimulate translation of GCN4 by the mechanism described above. Because GCN4 expression is sensitive to the level of eIF-2 activity (10, 53), mutations that affect the ability of eIF-2B to recycle eIF-2 · GDP to eIF-2 · GTP should also impair translational control of GCN4. Several lines of evidence indicate that previously identified trans-acting regulators of GCN4 are subunits of the yeast equivalent of mammalian eIF-2B. Three such factors, GCD1, GCD2, and GCN3, were found to be components of a high-molecular-weight complex (the GCD complex) that resembles mammalian eIF-2B in being associated with a fraction of the eIF-2 in the cell (6). Recent biochemical experiments indicate that this GCD complex has the guanine nucleotide exchange activity characteristic of mammalian eIF-2B (5). In accord with the idea that a reduction in eIF-2B activity is responsible for increased translation of GCN4, reduced-function mutations in GCD1 and GCD2 lead to high-level GCN4 translation, independent of GCN2 kinase function (25).

Our recent analysis of the GCD6 and GCD7 genes led us to propose that they encode additional subunits of the yeast GCD-eIF-2B complex (4). Reduced-function mutations in either gene lead to constitutive derepression of GCN4 translation independently of GCN2, and deletion of each gene is lethal. Analysis of the cloned genes revealed significant amino acid sequence similarities between GCD7 and the GCN3 and GCD2 subunits of the GCD-eIF-2B complex and between GCD6 and GCD1. In addition, we discovered strong sequence similarity between GCD6 and the largest subunit of rabbit eIF-2B (4). We chose to interpret these mutant phenotypes and sequence similarities as evidence that GCD6 and GCD7 are integral subunits of the GCD-eIF-2B complex. An alternative possibility is that the sequence relatedness between the proteins reflects redundancy of function and that multiple GCD complexes which contain exclusively GCD2, GCD7, or GCN3 and either GCD1 or GCD6 could exist.

In this report, we provide genetic and biochemical evidence that GCD6, GCD7, and GCN3 occur largely, if not entirely, as subunits of the same complexes which contain GCD1, GCD2, and a fraction of eIF-2. We demonstrate that the dispensable subunit GCN3 can be mutated to have strong effects on general protein synthesis in addition to affecting the translational control of GCN4. Finally, we describe several cases of mutual suppression involving mutations in GCN3, GCD2, and GCD7. These genetic interactions, combined with the sequence similarities among GCN3, GCD2, and GCD7, lead us to suggest that these three related proteins interact with one another in the GCD-eIF-2B complex and play an important role in its regulation by phosphorylated eIF-2.

### MATERIALS AND METHODS

**Polysome profile analysis.** Preparation and gradient analysis of yeast polysomes were performed essentially as described previously (12) except that cultures were grown at 30°C in YEPD or SD medium (48) as indicated. Single-copy plasmids bearing GCN3 alleles were described previously (17). Immunoblot analysis of gradient fractions for the B subunit of eIF-2 was performed as described for eIF-2α (12) except that eIF-2B antibodies (6) were used. The Pet derivative of strain H1402 used as a control in the experiments was generated by growing H1402 in the presence of 10 μg of ethidium bromide per ml and screening for respiratory-deficient colonies on YEPG plates (48).

**Construction of plasmids used for marker rescue mapping of gcd2 mutations.** High-copy-number plasmids containing GCD2 or derivatives thereof were constructed as follows. A SalI-EagI fragment containing GCD2 was isolated from pCP46 (43) and inserted between the SalI and EagI sites of YEp24 (45), generating pCP57. gcd2 genes lacking internal portions of the ORF were generated by restriction enzyme digestion of pCP46 followed by religation in the presence of synthetic oligonucleotides designed to produce in-frame deletions. The deletion junctions were sequenced, and the deletion alleles were subcloned into YEp24 on SalI-EagI fragments as described for pCP57. The construction of these plasmids is summarized in Table 1.

**Cloning and nucleotide sequence analysis of gcd2 alleles.** (i) **gcd2-502.** A 450-bp fragment containing the 340-bp BamHI-NdeI region in GCD2 was amplified from genomic DNA of strain H63 isolated as described by Winston et al. (54) by using the polymerase chain reaction (PCR) and oligonucleotide primers 6 (5'-ATTATCTAGACGATACAGTCG TTAGAAC-3') and 7 (5'-AAAAGAATTCGAGAATAAGA CCTATCCATCGTCG-3'). The BamHI-NdeI fragment from the amplified sequence was inserted between the BamHI and NdeI sites of pCP46 to form plasmid pMF11. DNA sequencing of both strands of two independent isolates

<table>
<thead>
<tr>
<th>TABLE 1. Construction of plasmids for marker rescue mapping of GCD2 alleles*</th>
<th>Site used to create deletions</th>
<th>Oligonucleotide used to generate in-frame deletions</th>
<th>Nucleotides deleteda</th>
</tr>
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<tbody>
<tr>
<td>pCP46</td>
<td>pCP57</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>pCP53</td>
<td>pCP58</td>
<td>PstI-BamHI</td>
<td>5'-GATCCTGCA-3'</td>
</tr>
<tr>
<td>pCP54</td>
<td>pCP59</td>
<td>PstI-NdeI</td>
<td>5'-TATCGA-3'</td>
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<tr>
<td>pCP55</td>
<td>BamHI-EcoRI</td>
<td>5'-GATCCATCCAG-3'</td>
<td>940-1970</td>
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<tr>
<td>pCP56</td>
<td>NdeI-EcoRI</td>
<td>5'-AATTCTGGGATG-3'</td>
<td>1283-1970</td>
</tr>
</tbody>
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*See text for details.

b Numbering relative to the 5' end of GCD2 mRNA (42).
of the amplified BamHI-NdeI region revealed a single-point mutation (AGA to GGA) that changes arginine 334 to glycine in GCD2.

(ii) gcd2-503. The 450-bp fragment described above containing the 340-bp BamHI-NdeI region in GCD2 was PCR amplified from genomic DNA of strain H64, using primers 6 and 7 described above. The amplified fragment was digested with XbaI and EcoRI and cloned between the XbaI and EcoRI sites of pBLUESCRIPTsk to form plasmid pMF20. DNA sequencing of both strands of two independent isolates of the amplified BamHI-NdeI region revealed a single-point mutation (AGA to AGT) that changes arginine 334 to serine in GCD2.

(iii) gcd2-1. An 850-bp fragment containing the 685-bp NdeI-EcoRI region was PCR amplified from genomic DNA of strain H954, using primers 4 (5'-TAAAGATTCTTACCCCGACAAG-3') and 5 (5'-ACAAG GACAGTAAAGGTGCCAAG-3'). The amplified fragment was digested with EcoRI and cloned into the EcoRI site of pBLUESCRIPTsk for sequencing. The only clones obtained containing the amplified fragment had the 3' end of the fragment fused to the vector through the primer 5 used for amplification. DNA sequencing of both strands 3' to the NdeI site of two independent isolates of the amplified fragment revealed a single-point mutation (GAA to TAA) that changes glutamate 631 to a stop codon, creating a 21-amino-acid truncation of GCD2. This change destroys the EcoRI site in the 3' end of GCD2 that was being used to subclone the amplified fragments, thus accounting for the products that we recovered.

**Genetic methods and yeast strain constructions.** Standard techniques for growth, genetic analysis, and plasmid transformation of yeast strains were performed as described elsewhere (30, 48). Resistance to 3-aminotriazole (3-AT), an inhibitor of the H153 product (25), was determined by replica plating to SD medium containing 30 mM 3-AT as described previously (27). Strains having inducible or constitutively elevated GCN4 expression are resistant to 3-AT (3-AT') because GCN4 activates transcription of genes encoding histidine biosynthetic enzymes. gcn2 or gcn3 mutant strains are uninducible and thus sensitive to 3-AT (3-AT*). Resistance to 5-fluoro-DL-tryptophan (5-FT) was determined by replica plating to SD medium containing 0.5 mM 5-FT as described previously (53). Because 5-FT is toxic unless tryptophan levels are elevated but does not cause an amino acid starvation signal, only strains with constitutively elevated expression of GCN4 are resistant to this analog (5-FT); thus, wild-type and gcn strains are sensitive to 5-FT (5-FT*).

Yeast strains used in this study (Table 2) are congenic to the wild-type strain S288C. Strain ETY462 was constructed by replacement of the gcn3-101 allele in H1456 with the gcn3::LEU2 allele isolated from EP308 as described previously (17). gcd6-1 strain H1597 was generated from successive backcrosses of F222 (MATa gcd6-1 leu2-2) (40) to H750 (MATa gcn2::LEU2 leu2-3 leu2-112 ura3-52) or H751 (MATa gcn2::LEU2 leu2-3 leu2-112 ura3-52) as described previously for gcd6-1 strains H1916 and H1917 (4). gcn3-102 GCD6::URA3 strain H1724 was cloned from a cross between gcd3-102 strain H51 and GCD6::URA3 strain H1724. The URA3-marked allele of GCD6 in H1724 was generated by integration of URA3 GCD6 plasmid pPB68 at gcd6-1; H1724 was the Ura+ Gcd+ transformant from which the 5-fluoro-orotic acid-resistant (3) (Ura-) strains H1728 and H1730 were derived as described previously (4). gcn3::R104K strains H1499 and H1504 were generated as described for H1489 (17). H1724 was crossed to gcn3::R104K strain H1504 to produce H2114 and H2115. The URA3-marked allele of GCD7 was generated previously by integration of plasmid pJB87(GCD7 URA3) at GCD7 in strain H750 as described; the resulting strain H1834 was used for the genetic demonstration that GCD7 had been cloned (4). GCD7::URA3 strain H1834 was crossed to gcd3-102 strain H17 to generate H1839. H1834 was crossed to gcn3::R104K strain H1499 to generate H1840.

Double-mutant combinations to test the interactions between gcd6-1 and gcd7-201 and different alleles of GCN3 were generated and analyzed as follows.

(i) gcd6-1 gcn3A strains. Ura+ gcd6-1 strain H1597 was crossed to gcd3::URA3 strain ETY162, and 26 tetrads were analyzed. Phenotypes of slow growth, 3-AT*, and 5-FT were used to monitor segregation of the gcd6-1 allele; Ura* and 3-AT* phenotypes were used to identify the gcn3::URA3 allele. Many ascospore clones failed to grow or produced pinpoint colonies after 2 weeks. Nine tetrads had two viable colonies, 14 tetrads had three viable colonies, and 3 tetrads had four viable colonies. No viable gcd6-1 gcn3::URA3 double mutants were recovered, and the nonviable ascospores were predicted to contain both gcn3::URA3 and gcd6-1, as judged from the segregation of the phenotypes described above in the viable colonies. From these results, we concluded that gcd6-1 gcn3::URA3 double mutants are inviable.

(ii) gcd7-201 gcn3A strains. Ura+ gcd7-201 strain H2042 was crossed to gcd3::URA3 strain ETY162, and 29 tetrads were analyzed. Similar to the results obtained with gcd6-1, many ascospore clones failed to grow or produced pinpoint colonies after 2 weeks. Nine tetrads had two viable colonies, 15 tetrads had three viable colonies, and 4 tetrads had four viable colonies. The nonviable ascospores were predicted to contain both gcn3::URA3 and gcd7-201, as judged from the phenotypes of the viable colonies. From these results, we concluded that gcd7-201 gcn3::URA3 double mutants are inviable.

(iii) gcd6-1 gcn3-102 strains. gcd6-1 GNC3 strain H1728 was crossed to GCD6::URA3 gcn3-102 strain H1883, and 12 complete tetrads were analyzed. The presence of gcd6-1 was inferred on the basis of uracil auxotrophy, and the presence of gcn3-102 was determined by complementation tests with gcn3A strains H741 and H742. We observed no differences between the gcd6-1 GNC3 and the gcd6-1 gcn3-102 colonies for growth rate, 3-AT*, or 5-FT* phenotype. From these results, we concluded that gcd6-1 is epistatic to gcn3-102.

(iv) gcd7-201 gcn3-102 strains. gcd7-201 GNCN3 strain H1603 was crossed to GCD7::URA3 gcn3-102 strain H1839, and 21 complete tetrads were analyzed. The gcd7-201 colonies were identified on the basis of uracil auxotrophy, and the location of gcn3-102 was determined as described above. We observed three distinct spore colony sizes in these tetrads. The class containing large colonies was composed of wild-type GCD7::URA3 GCN3 and double-mutant gcd7-201 gcn3-102 ascospore clones. It was remarkable that the double-mutant colonies grew as well as did the wild-type colonies, since gcd7-201 typically causes a significant slow-growth phenotype. The wild-type colonies were more resistant to 3-AT than were the double-mutant colonies. The class of medium-size colonies was composed of GCD7::URA3 gcn3-102 ascospore clones, which grew as well as the previous class when streaked for single colonies on YEPD. Possibly, these gcn3 spores were slow to germinate because of amino acid imbalances or deficiencies that would otherwise have been corrected by the general amino
acid control pathway; growth defects on rich media have not previously been observed in gcn3 strains. As expected, these gcn3-102 colonies were 3-AT−. Finally, the class containing the smallest colonies was composed of gcd7-201 GCN3 ascorpore clones. These strains retained the slow-growth phenotype when streaked for single colonies on YEPD and were resistant to 3-AT and 5-FT, as expected for gcd7-201 mutants. These results indicate that gcn3-102 fully suppresses the slow-growth phenotype of gcd7-201 and that gcd7-201 partially suppresses the 3-AT sensitivity associated with gcn3-102.

(v) gcd6-1 gcn3Δ-R104K strains. gcd6-1 strain H1728 was crossed to GCD6::URA3 gcn3Δ-R104K strains H2114 and H2115, and a total of 28 tetrads were analyzed. Many ascospores were inviable; pinpoint colonies were not produced in this cross. The inviable ascospores were predicted to contain both gcd6-1 and gcn3Δ-R104K, indicating that gcd6-1 gcn3Δ-R104K double mutants are inviable.

(vi) gcd7-201 gcn3Δ-R104K strains. gcd7-201 strain H1603 was crossed to GCD7::URA3 gcn3Δ-R104K strain H1840, and 22 tetrads were analyzed. As with gcd6-1, many of the ascospores were inviable, and these were predicted to contain both gcd7-201 and gcn3Δ-R104K. This finding indicates that gcd7-201 gcn3Δ-R104K double mutants are also inviable.

**Assays of GCN4-lacZ expression.** The GCN4-lacZ fusion present on the low-copy-number URA3 plasmid p180 (24) was introduced into all four ascorpore clones from two tetratype tetrads obtained from the cross between H1603 (gcd7-201 GCN3) and H1839 (GCD7::URA3 gcn3-102). To achieve this, 5-fluoro-orotic acid selection (3) was used beforehand to obtain Ura− derivatives of the GCD7::URA3
ascospore clones. Such colonies were readily obtained because URA3 is flanked by tandem repeats of the wild-type GCD7 gene in the GCD7::URA3 allele. The GCN4-lacZ fusion on p180 has the wild-type leader containing all four uORFs and the amino-terminal coding region of GCN4 fused to lacZ. β-Galactosidase assays were conducted as described elsewhere (38).

**GCD6 and GCD7 antibody production.** TrpE-GCD6 and TrpE-GCD7 fusion proteins isolated from *Escherichia coli* were used to raise antibodies directed against GCD6 and GCD7, respectively. The TrpE-GCD6 expression plasmid pJB77 was constructed by inserting the 4-kb BglII-HindIII fragment from pJB6 between the BamHI and HindIII sites of pATH3 (11) (pJB6 contains the 8.6-kb HindIII fragment from pJB1 [4] inserted at the HindIII site of pRS316 [50]). This produces an in-frame fusion of TrpE with amino acids +121 to +712 of GCD6. The TrpE-GCD7 expression plasmid pJB106 was constructed by inserting the 1.1-kb XbaI fragment of pJB99 (4) into the XbaI site of pATH2 (11). This produces an in-frame fusion of TrpE with amino acids +74 to +381 of GCD7. Rabbits were injected with 0.5 to 1.0 mg of the respective fusion proteins and boosted at 4-week and, subsequently, 2-week intervals by Hazleton Laboratories.

**High-copy-number GCD6 and GCD7 plasmids.** pJB115, a high-copy-number LEU2 plasmid containing GCD6, was constructed by inserting the 3-kb SpeI fragment from pJB5 into the SpeI site located in the multiple cloning site of pRS425 (50). In this construct, the BamHI site in the multiple cloning site of the vector is S′ of GCD6. pJB111, a high-copy-number URA3 plasmid containing GCD7, was constructed by inserting the 2.1-kb EagI-SalI fragment of pJB99 between the EagI and SalI sites of YEp24 (45).

**Immunoprecipitation of the GCD-eIF-2 complex.** Subunits of the GCD complex were immunoprecipitated from ribosomal salt wash (RSW) extracts prepared from exponentially growing yeast cells and analyzed essentially as described previously (6). The GCD6- and GCD7-specific antibodies were used at dilutions of 1:500 and 1:250, respectively (6).

**RESULTS**

**gcna alleles impair general translation initiation.** Unlike mutations affecting the GCD1 or GCD2 subunit of the GCD-eIF-2B complex, deletion of GCN3 does not result in an obvious general translation defect, even though GCN3 is associated with the complex (5, 6, 42). We have described numerous mutations in GCN3, called gcna alleles, that result in constitutive derepression of GCN4 translation. In addition to this regulatory phenotype, many gcna alleles reduce the growth rate of cells (17). It seemed likely that the slow-growth phenotype of the gcna mutations results from an inhibitory effect of the mutant GCN3 proteins on the essential function of the GCD-eIF-2B complex in translational initiation. To address this possibility, we introduced gcna alleles that confer slow growth rates (17) into a strain deleted for the chromosomal copy of GCN3 and analyzed the total polysome profiles of the resulting transformants by velocity sedimentation of whole cell extracts on sucrose gradients.

The results shown in Fig. 1A and Table 3 indicate that the gcna alleles with the greatest growth defects (gcna-A26T, gcna-R104K, gcna-E199K, and gcna-V295F) lead to an increase in the ratio of monosomes to polysomes by a factor ranging from 1.3 to 2.0. An increase in the monosome/polysome ratio was reported previously for the gcd1-101 (6) and gcd2-503 (12) mutations and is indicative of a reduction in polysome initiation.}

**FIG. 1.** Effects of gcna alleles on general translation revealed by analysis of polysome profiles. (A) Single-copy plasmids bearing the GCN3 alleles indicated in each panel were introduced into gcna strain H1426. Extracts prepared from the resulting transformants grown to exponential phase at 30°C in SD medium were separated by centrifugation on low-salt 7 to 47% sucrose gradients for 2.5 h at 270,000 × g. Gradients were scanned at 260 nm while fractions were collected, and the absorbance profiles are shown, with the top of each gradient on the left. Positions of 40S, 60S, and 80S ribosomal species are indicated. For each profile, the areas under the 80S (monosome) peak and the polysome peaks were calculated, and the ratio of monosome to polysome areas (M/P) was normalized so that the ratio for the GCN3 transformant is 1.0. OD260 optical density at 260 nm. (B) Distribution of eIF-2 in polysome profiles. Extracts were prepared from GCN3 (H1402), gcnaΔ (H1426), gcna-A26T (H1491), and gcna-R104K (H1489) isogenic yeast strains grown to exponential phase at 30°C in YEPD medium, and polysomes were separated on sucrose gradients as described above. Gradient fractions were collected, and proteins were precipitated, subjected to SDS-PAGE (10% gel), and analyzed by immunoblotting with antibodies against eIF-2B according to the methods described for detection of eIF-2a in polysome gradient fractions (12). The first lane on the left corresponds to the top fraction of each gradient, and the positions of 40S and 60S peaks in the fractions are indicated above. Relevant genotypes are shown on the left. The monosome/polysome ratios (normalized to the ratio for the GCN3 strain) observed for these gradients were as follows: gcnaΔ, 1.0; gcna-A26T, 1.8; and gcna-R104K, 1.4.
in the rate of translation initiation relative to translation elongation. The parental strain lacking GCN3 was indistinguishable from the wild-type GCN3 transformant in growth rate and polysome content. To address the possibility that the decrease in polysome content seen in the gcna3 mutants is an indirect effect of slow growth, we determined the polysome profile of an isogenic petite mutant containing wild-type GCN3 that grows slowly because of respiratory deficiency. This strain had a normal monosome/polysome ratio of 1.0 despite its slow growth rate (data not shown).

The gcda1 and gcd2 mutants mentioned above that exhibited a reduction of polysome content were found previously to contain increased amounts of eIF-2 migrating with small ribosomal subunits, suggesting an accumulation of 43S or 48S translational initiation intermediates (6, 12). A similar result was observed here (Fig. 1B) for the gcna3-A267 and gcna3-R104K mutants. Cell extracts were fractionated on sucrose gradients, and antibodies specific for the β subunit of eIF-2 were used for immunoblot analysis to determine the eIF-2 content of each gradient fraction. Whereas the parental gcna3 strain and the GCN3 transformant had no detectable eIF-2β in the 40S–60S region of the gradient, the gcna3 mutants exhibited significant amounts of eIF-2β distributed in these fractions, particularly for the slowest-growing gcna3-A267 strain. Taken together, the results shown in Fig. 1 suggest that gcna3 mutants have defects in general translation initiation similar to those present in gcd1-101 and gcd2-503 strains, consistent with the idea that GCN3 is an integral component of the essential GCD-eIF-2β complex of S. cerevisiae (5, 6).

Sequence of three GCD2 mutations showing different genetic interactions with GCN3. Harashima et al. (20) reported that the gcd2-502 and gcd2-503 mutations are conditionally lethal in the presence of gcn3-101 or a gcn3 deletion but not in strains containing wild-type GCN3. In contrast, the slow-growth and temperature-sensitive phenotypes of the gcd2-1 mutation were found to be relatively unaffected by the presence or absence of GCN3 (43) (see Table 5). Given the fact that GCD2 and GCN3 exhibit regions of significant sequence similarity, one hypothesis to explain the ability of GCN3 to suppress certain gcd2 mutations but not others was that the gcd2-502 and gcd2-503 mutations alter amino acids in regions of sequence similarity between GCD2 and GCN3 such that GCN3 can functionally substitute for these mutant gcd2 proteins. In contrast, the gcd2-1 mutation would affect a region unrelated to GCN3 in both sequence and function, preventing complementation of gcd2-1 by GCN3 (42). To test this hypothesis, we determined the nature of all three gcd2 mutations. Our interest in the sequence of these mutant alleles was further stimulated by our recent finding that GCD7 exhibits strong sequence similarity with GCN3 and to a lesser extent with GCD2 (4).

We mapped the three GCD2 mutations by marker rescue of their temperature-sensitive phenotypes. Deletion derivatives of GCD2 were generated by using suitable restriction sites and subcloned into the high-copy-number yeast plasmid YEp24 (Table 1). As expected, none of these gcd2 deletion alleles complemented the Tsm− phenotype of the three gcd2 mutations. If the position of the chromosomal gcd2 mutation is contained in the wild-type GCD2 sequences remaining in the plasmid, then homologous recombination events between the chromosome and the plasmid can generate the wild-type allele of GCD2. We tested for such events in transformants of various gcd2 mutants containing the plasmid-borne gcd2 deletion alleles by observing the frequency of temperature-resistant (wild-type GCD2) papillae that arose spontaneously in each transformant (Table 4). The results of this analysis localized the gcd2-502 and gcd2-503 mutations to the 343-bp interval between the BamHI and NdeI sites near the middle of GCD2, whereas the gcd2-1 mutation was localized to the C-terminal segment 3′ of the NdeI site in GCD2.

We then used PCR to amplify and clone the appropriate regions containing the gcd2 mutant alleles from genomic DNA and determined the nature of each mutation by DNA sequence analysis (see Materials and Methods). The gcd2-502 and gcd2-503 mutations were found to contain single-point mutations resulting in glycine and serine substitutions, respectively, at the same amino acid, arginine 334. At odds with the complementation model described above, this residue is located in a region of GCD2 that shows no significant sequence similarity with GCN3 or GCD7 (Fig. 2). Moreover, the gcd2-1 allele contains a nonsense mutation that eliminates the last 21 amino acids from the C terminus of GCD2, thus affecting the region of highest similarity among these three proteins. The location of these gcd2 mutations suggests that the ability of GCN3 to completely suppress the lethal effects of gcd2-502 and gcd2-503, but not that of gcd2-1, cannot be explained simply by proposing that the regions of sequence similarity between GCD2 and GCN3 represent different copies of a functionally redundant domain that is impaired by gcd2-502 and gcd2-503 but unaffected by gcd2-1. Rather, it appears that these mutations

![Table 3: Monosome/polysome ratios in gcna3 mutant extracts](image)

![Table 4: Marker rescue mapping of gcd2 mutations](image)
affect the function of the GCD–eIF-2B complex in ways that are
differentially influenced by the presence of GCN3.

Allele-specific interactions between gc3 mutations and
different alleles of GCD2. An alternative explanation for the
sequence relatedness between GCN3 and GCD2 is that the
regions of similarity mediate interactions between these two
proteins in the GCD–eIF-2B complex. If this hypothesis is
correct, it should be possible to detect extensive allele
specificity in the interactions between mutants in GCD2 and
GCN3. We reasoned that the gc3 alleles isolated
previously (17) provide a group of altered-function GCN3
mutations ideal for probing interactions between GCN3 and
GCD2. As mentioned above, gc3 alleles resemble gcd2
mutations in causing constitutive derepression of GCN4
expression and slow growth under nonstarvation conditions;
however, they do not confer temperature-sensitive growth.
The gc3 mutations are located throughout the GCN3
sequence (Fig. 2), and their recessive nature suggests that
they encode proteins which assemble into the GCD–eIF-2B
complex less efficiently than does wild-type GCN3. Thus, if
GCD2 and GCN3 are in physical contact, certain gc3
mutations might exhibit markedly different interactions with
the gcd2-I allele that truncates the protein at the C terminus
versus the gcd2-502 and gcd2-503 mutations that alter arginine
334. To test this possibility, we constructed the full
matrix of double mutants that combine gcd2-502, gcd2-503,
or gcd2-I with each of seven different gc3 alleles and
characterized the double mutants for the ability to form
colonies at temperatures permissive (23°C) or restrictive
(36°C) for gcd2 single mutants (Table 5).

If GCN3 and GCD2 both contribute to the essential
function of eIF-2B, we would expect that most gc3 gcd2
double mutants would be more impaired for growth than is
either single mutant. This type of additive interaction was
observed for many of the double mutants, which displayed a
growth defect at 23°C equal to or greater than that of the
corresponding gc3 single mutant and showed poorer
growth at 36°C than at 23°C, characteristic of the tempera-
ture-sensitive gcd2 mutations. This additivity was
particularly striking in the case of gcd2-503, which was
inviable in combination with four of the gc3 alleles, gc3-A26T,
gc3-A425,26,26, V, gc3-E199K, and gc3-V295F. Not
surprisingly, this lethality involved the four gc3 alleles
that conferred the slowest growth rate in the wild-type
GCD2 strain. Three of these alleles (gc3-A26T, gc3-
A425,26, V, and gc3-E199K) also produced the most
growth-impaired gc3 gcd2-502 double mutants, one of which
was inviable.

In contrast with the additive interactions just described,
the slow growth rate at 23°C associated with several of the
gc3 alleles was suppressed by certain mutations in gcd2,
and the temperature sensitivity of gcd2 mutants was
suppressed by certain gc3 alleles, in some cases even when
the gc3 was very deleterious in the GCD2 strain. The slow
growth at 23°C conferred by gc3-V295F, gc3-R104K,
and gc3-S65F was significantly suppressed by gcd2-502 and
gc3-S65F was also suppressed by gcd2-503. Another
noteworthy feature of the gc3-R104K and gc3-S65F
alleles is that they conferred better growth at 36°C in the
GCD2-502 and gcd2-503 strains than did gc3-D71N, even
though the latter allele produced a lesser growth defect at
23°C in the GCD2 strain. These suppressive interactions
suggest that the mutations involved cause offsetting bio-
chemical defects, or that the GCN3 and GCD2 proteins are
in close contact in eIF-2B and these mutations lead to
offsetting structural changes in the complex.

There were many similarities in the interactions between
the various gc3 mutations and the gcd2-502 and gcd2-503
alleles
TABLE 5. Genetic interactions among gcn3<sup>F</sup> and gcd2 alleles

<table>
<thead>
<tr>
<th>Plasmid-borne GCN3 allele</th>
<th>Colony formation in transformants of strain&lt;sup&gt;a&lt;/sup&gt;:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>GCD2 gen3&lt;sup&gt;A&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>23°C</td>
</tr>
<tr>
<td>None (vector)</td>
<td>++++</td>
</tr>
<tr>
<td>GCN3</td>
<td>++++</td>
</tr>
<tr>
<td>gen3&lt;sup&gt;F&lt;/sup&gt;-A26T</td>
<td>+</td>
</tr>
<tr>
<td>gen3&lt;sup&gt;F&lt;/sup&gt;-AA25,26VV</td>
<td>+/−</td>
</tr>
<tr>
<td>gen3&lt;sup&gt;F&lt;/sup&gt;-S65F</td>
<td>+/−</td>
</tr>
<tr>
<td>gen3&lt;sup&gt;F&lt;/sup&gt;-D71N</td>
<td>++++</td>
</tr>
<tr>
<td>gen3&lt;sup&gt;F&lt;/sup&gt;-R104K</td>
<td>+</td>
</tr>
<tr>
<td>gen3&lt;sup&gt;F&lt;/sup&gt;-E199K</td>
<td>+</td>
</tr>
<tr>
<td>gen3&lt;sup&gt;F&lt;/sup&gt;-V295F</td>
<td>+</td>
</tr>
</tbody>
</table>

<sup>a</sup> Alleles of GCN3 borne on the low-copy-number plasmid YCp50 (17) were introduced into gen3<sup>A</sup> yeast strains H1426 (GCD2), EY462 (gcd2-502), H652 (gcd2-503), and H954 (gcd2-1). Growth was scored for five independent transformants by streaking for single colonies on minimal medium and incubating at 23 or 36°C. Relative colony sizes were scored 3 to 6 days later. A minus sign indicates that no visible colonies were formed. A superscript plus indicates less growth than that signified by a normal plus.

alleles, which may reflect the fact that these two mutations alter the same amino acid in GCD2. In general, combinations with gcd2-503 had phenotypes similar to those seen with gcd2-502, but more severe. Notable exceptions involved gcn3<sup>F</sup>-R104K and gcn3<sup>F</sup>-V295F, which were suppressed by gcd2-502 at 23°C but exacerbated by gcd2-503. Some differences in the interactions among gcd2-502 and gcd2-503 and gcn3<sup>F</sup> alleles were not unexpected, since these two gcd2 mutations have quite different effects on polysome profiles in vivo (12).

The gcd2-1 mutation, which deletes the C terminus of the protein, displayed a distinctly different pattern of interactions with the gcn3<sup>F</sup> alleles. In several cases, gcn3<sup>F</sup> mutations that were exacerbated by combination with gcd2-502 or gcd2-503 were suppressed by combination with gcd2-1. Specifically, the gcn3<sup>F</sup>-AA25,26VV and gcn3<sup>F</sup>-E199K mutations, which were very deleterious in the GCD2 strain and lethal or very severe in combination with gcd2-502 and gcd2-503, were suppressed for their growth defects at 23°C by gcd2-1. Finally, it is interesting that the gcn3<sup>F</sup>-A26T gcd2-1 and gcn3<sup>F</sup>-V295F gcd2-1 double mutants exhibit no temperature sensitivity and grow at rates very similar to those of the corresponding gcn3<sup>F</sup> single mutants, suggesting that the gcd2-1 mutation is completely suppressed by these two gcn3<sup>F</sup> alleles. The numerous instances of nonadditivity and mutual suppression seen in the interactions between the gcd2 mutations and gcn3<sup>F</sup> alleles, plus the fact that gcd2-1 shows a distinct and specific set of interactions versus gcd2-502 and gcd2-503, suggest to us that the GCD2 and GCN3 proteins are in close physical contact within the GCD complex.

Genetic evidence that GCD6 and GCD7 are subunits of the GCD-eIF-2B complex. In view of our recent findings that gdc6 and gdc7 mutations affect GCN4 translational control, that GCD6 and GCD1, and GCD7, GCD2, and GCN3, are related in sequence, and that GCD6 is highly similar to the largest subunit of rabbit eIF-2B (4), we decided to determine whether GCD6 and GCD7 are additional subunits of the GCD-eIF-2B complex. We reasoned that if this hypothesis was correct, it might be possible to observe genetic interactions between GCD6 and GCD7 and GCN3 similar to those just described for GCD2. To test our prediction, we constructed double mutants containing gdc6-1 or gdc7-201 and one of four different GCN3 alleles: wild-type GCN3; a gcn3 deletion, gcn3<sup>F</sup>-R104K; or gcn3-102.

As observed for a mutation in the GCD1 subunit of the GCD-eIF-2B complex (19, 26), deletion of GCN3 was lethal in the presence of gdc6-1 and gdc7-201 (Table 6). This result is striking because in an otherwise wild-type strain, deletion of GCN3 does not reduce cell viability and its effect on GCN4 expression is the opposite of that associated with gdc6-1 and gdc7-201 mutations (see below). We also observed synthetic lethality between the gdc6-1 and gdc7-201 mutations and the gcn3<sup>F</sup>-R104K allele. These interactions are consistent with the idea that GCD6 and GCD7 are subunits of the GCD-eIF-2B complex and that gdc6-1 and gdc7-201 impair these subunits in a way that is exacerbated either by eliminating GCN3 or by altering it in a way that reduces the biochemical function of the complex.

The gcn3-102 allele is defective for promoting derepression of GCN4 expression; however, it resembles wild-type GCN3 in its ability to suppress the temperature-sensitive growth exhibited by certain gdc1 and gdc2 mutations (20). Similar to its effect on gdc1 and gdc2 mutations, the gcn3-102 allele did not exacerbate the growth defect of gdc6-1 and completely suppressed the slow-growth phenotype of gdc7-
TABLE 7. Effects of gcn3-102 and gcd7-201 mutations on GCN4 expression

<table>
<thead>
<tr>
<th>Strain</th>
<th>Ascospor clone</th>
<th>Relevant genotype</th>
<th>GCN4-lacZ expression&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Nonstarvation</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>H1875</td>
<td>1C</td>
<td>GCN3 GCD7</td>
<td>10</td>
</tr>
<tr>
<td>H1882</td>
<td>2D</td>
<td>GCN3 GCD7</td>
<td>17</td>
</tr>
<tr>
<td>H1876</td>
<td>1D</td>
<td>GCN3 gcd7-201</td>
<td>190</td>
</tr>
<tr>
<td>H1880</td>
<td>2C</td>
<td>GCN3 gcd7-201</td>
<td>170</td>
</tr>
<tr>
<td>H1872</td>
<td>1A</td>
<td>gcn3-102 GCD7</td>
<td>7</td>
</tr>
<tr>
<td>H1879</td>
<td>2B</td>
<td>gcd7-201 GCD7</td>
<td>11</td>
</tr>
<tr>
<td>H1873</td>
<td>1B</td>
<td>gcn3-102 gcd7-201</td>
<td>23</td>
</tr>
<tr>
<td>H1877</td>
<td>2A</td>
<td>gcd7-201 gcd7-201</td>
<td>23</td>
</tr>
</tbody>
</table>

<sup>a</sup> The low-copy-number plasmid p180 bearing GCN4-lacZ was introduced into ascospor clones 1A to 1D and 2A to 2D from two tetratype tetrads (1 and 2) from the cross between H1603 (gcd7-201 Gcn3) and H1839 (gcd7-201 gcn3-102); see Materials and Methods for details.

<sup>b</sup> β-Galactosidase activity in yeast strains was measured in a mid-logarithmic phase under repressing (nonstarvation) conditions or under derepressing conditions of histidine starvation induced by 3-AT as described previously (55). Enzyme activities are expressed as units (nanomoles of o-nitrophenyl-β-D-galactosidase [ONPG] cleaved per minute per milligram of total protein). Results are mean values of measurements made on two independent transformants for each strain; individual measurements differed from the mean by less than 20%.

201 (Table 6). It is noteworthy that gcn3-102 was isolated as a suppressor of the temperature-sensitive phenotype of gcd1-101 in a GCN3 strain (26). These results can be explained by proposing that the gcd3-102 product is competent for complex formation and that it compensates for defects in the structure or function of the GCD-eIF-2B complex associated with particular mutations in GCD1 or GCD7.

The inability of gcd3-102 to promote derepression of GCN4 expression is suppressed by the gcd6-1 mutation, as indicated by the wild-type resistance to 3-AT exhibited by the double mutant (Table 6). 3-AT inhibits histidine biosynthesis, and wild-type resistance requires efficient derepression of genes in the histidine pathway subject to transcriptional regulation by GCN4. In contrast to what was seen with gcd6-1, the 3-AT<sup>a</sup> phenotype of gcd3-102 was not fully suppressed by gcd7-201 (Table 6). To quantitate the effects on GCN4 expression associated with combining these last two mutations, we assayed the expression of a GCN4-lacZ fusion in strains containing different combinations of the gcd7-201 and gcn3-102 mutant alleles (Table 7).

The wild-type GCN3 GCD7 strain showed the expected increase in GCN4-lacZ expression under conditions of histidine starvation. The gcn3-102 single mutant was impaired for this derepression, consistent with the idea that GCN3 is the regulatory subunit of eIF-2B that couples increased translation of GCN4 to reduced amino acid availability. The gcd7-201 single mutant showed constitutive derepression of GCN4-lacZ expression, as would be expected for a reduction in eIF-2B function irrespective of amino acid availability. The double mutant gave GCN4-lacZ expression intermediate between that seen in the single mutants and similar to what we observed in the wild-type strain. Thus, the gcd7-201 and gcn3-102 mutations appear to be compensatory in nature.

Coimmunoprecipitation of GCD6 and GCD7 with other subunits of the GCD-eIF-2B · eIF-2 complex. The genetic interactions between mutations in GCN3 and the gcd6-1 and gcd7-201 alleles described above lent strong support to the idea that GCD6 and GCD7 are present with GCN3 in the GCD·eIF-2B complex. In an effort to obtain physical evidence for this interaction, we used antibodies against GCD6 and GCD7 to investigate whether they coimmunoprecipitate and copurify with other subunits of the GCD·eIF-2B · eIF-2 complex. The antibodies were raised against TrpE-GCD6 and TrpE-GCD7 fusion proteins produced in E. coli (see Materials and Methods), and their specificity was established by immunoblot analysis of total protein extracts of wild-type strains and transformants containing high-copy-number plasmids bearing GCD6 or GCD7. As shown in Fig. 3, each antiserum reacted with a single polypeptide having an electrophoretic mobility consistent with the predicted molecular weight of the corresponding GCD protein (81,000 for GCD6 and 43,000 for GCD7 [4]) that was present at higher levels in the transformants containing the appropriate high-copy-number plasmids.

The GCD6-specific antiserum was used to immunoprecipitate proteins under nondenaturing conditions from a high-salt extract of yeast ribosomes (RSW) found previously to be enriched for the GCD1, GCD2, and GCN3 proteins (6). The immunoprecipitated proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by immunoblotting with antibodies against GCD6, GCD7, GCD1, GCD2, GCN3, and the α and β subunits of eIF-2 (4). We found that GCD6, GCD7, GCD2, GCN3, and GCD1 proteins present in the RSW were recovered in the immune complexes (Fig. 4A, lanes 2 and 7) and substantially depleted from the supernatants (Fig. 4B, lanes 2 and 7). In addition, a small fraction of the eIF-2α and eIF-2B subunits were coimmunoprecipitated with GCD6. In control immunoprecipitations done with preimmune serum, none of these proteins was isolated in the immune complexes or depleted from the supernatants (Fig. 4A and B, lanes 3 and 8). The depletion of proteins from the supernatants by immunoprecipitations using GCD6-specific antibodies indicates that the majority of the GCD7, GCD2, GCN3, and GCD1 proteins in the RSW are complexed with GCD6.

These results suggest that GCD6 and GCD7 are present in the same complexes that were previously found to contain GCD1, GCD2, GCN3, and a fraction of eIF-2 (6). If this is

FIG. 3. Characterization of GCD6-specific and GCD7-specific antibodies by immunoblot analysis of unfraccionated yeast extracts. For GCD6, 50-μg samples of extracts prepared from wild-type strain H1730 bearing vector pRS425 (SC) or high-copy-number GCD6 plasmid pJB115 (HC) were fractionated by SDS-PAGE (10% polyacrylamide gel) and subjected to immunoblot analysis using a 1:500 dilution of GCD6-specific antiserum. The immunoreactive species visible only in the HC extract is presumed to be GCD6 and is labeled as such. For GCD7, 50-μg samples of extracts prepared from wild-type strain H1727 bearing vector alone (SC) or high-copy-number GCD7 plasmid pJB111 (HC) were analyzed as for GCD6, using a 1:250 dilution of GCD7-specific antiserum. The immunoreactive species visible only in the HC extract is presumed to be GCD7 and is labeled as such. Migration of Bio-Rad prestained low-molecular-weight markers is indicated on the left of each panel, with the molecular weights of the markers given in kilodaltons.
true, we would expect to find that GCD6 and GCD7 can be quantitatively immunoprecipitated by an antiserum against one of the other subunits of the complex. In accord with this expectation, we obtained results essentially identical to those just described for GCD6-specific antiserum when we used antibodies against GCD1 instead (compare lanes 2 and 4 in Fig. 4A and B). As a final control, we showed that GCD6 and GCD7 could be immunoprecipitated by a monoclonal antibody that recognizes an epitope-tagged version of GCD1. An allele of GCD1 in which nine amino acids corresponding to an epitope of the hemagglutinin (HA) protein of influenza virus were added to the C terminus of GCD1 has been constructed (5). This epitope-tagged version of GCD1 complements a gcd1 deletion like the wild-type allele, and the HA-tagged GCD1 protein can be immunoprecipitated by a monoclonal antibody against the HA epitope (5). As shown in Fig. 4, this monoclonal antibody coimmunoprecipitated a significant portion of the GCD6, GCD7, and GCD2 proteins and a small fraction of eIF-2α and eIF-2β along with the majority of the GCD1 and GCN3 present in the RSW prepared from yeast cells expressing the epitope-tagged version of GCD1. In contrast, none of these proteins was immunoprecipitated by the monoclonal antibody when the RSW was prepared from a strain expressing wild-type GCD1 (compare lanes 5 and 6 in Fig. 4A and B). The GCD6 and GCD2 proteins were selectively depleted from these immunoprecipitates compared with those obtained by using antibodies against GCD1 or GCD6. We presume that either addition of the HA epitope or binding of the HA monoclonal antibody to GCD1 partially disrupted the GCD–eIF-2B complex and decreased the affinity of GCD6 and GCD2 for the remainder of the complex.

By comparing immunoprecipitates from wild-type and gcn2Δ strains, we tested whether eIF-2α phosphorylation was required for the association of a fraction of eIF-2 with the GCD complex described above. In the case of factors purified from Ehrlich ascites tumor cells, eIF-2 phosphorylated on the α subunit was deduced to have an affinity 150-fold higher than that of the nonphosphorylated species for eIF-2B in vitro. This difference in affinity is believed to be important for the inhibition of eIF-2B function by phosphorylated eIF-2 (47). However, we found that the same fraction of eIF-2 subunits was coimmunoprecipitated by antibodies against GCD6 from an RSW prepared from a wild-type GCN2 strain or a gcn2 deletion strain (compare lanes 2 and 7 in Fig. 4A and B). As no eIF-2α phosphorylation has been detected in a gcn2 deletion strain (10), we conclude that phosphorylation of eIF-2α is not required for the interaction between the GCD–eIF-2B complex and eIF-2 detected under our immunoprecipitation conditions.

GCD6 and GCD7 copurify with other subunits of the GCD–eIF-2B complex. To provide independent biochemical evidence that GCD6 and GCD7 are components of the GCD–eIF-2B complex, we used immunoblot analysis to determine whether GCD6 and GCD7 copurify with other subunits of the complex. Cigan et al. have recently described a purification scheme for the GCD–eIF-2B complex and shown that the most highly purified preparations of this complex contain the guanine nucleotide exchange activity for eIF-2 that is characteristic of mammalian eIF-2B (5). Typical results from one of the final steps in this purification scheme, velocity sedimentation on a glycerol gradient, are shown in Fig. 5. Immunoblot analysis of the gradient fractions revealed that GCD1, GCD2, and GCN3 were distributed between fractions 12 and 13 with most of the eIF-2α and eIF-2β that were present in the starting sample. In similar
In this study, we extend our characterization of the subunit composition of the GCD complex of *S. cerevisiae* and use genetic analysis to explore interactions among its various polypeptide components. This complex is the yeast equivalent of mammalian eIF-2B (5), the guanine nucleotide exchange factor for eIF-2, which is essential for protein synthesis and plays a central role in the gene-specific translational control of *GCN4*. Consistent with the view that the regulatory subunit GCD6 is an integral component of the GCD-eIF-2B complex, our observations suggest that *gcn3* mutations lead to defects in general translation initiation. Our genetic data showing extensive allele-specific interactions between *GCN3* and *GCD2* suggest that GCD3 and GCD2 interact directly within the GCD-eIF-2B complex. In addition, we obtained strong genetic and biochemical evidence that GCD6 and GCD7 are additional components of this complex (5). Mammalian eIF-2B contains five subunits (44) with molecular weights very similar to those deduced from the cloned genes encoding GCD1, GCD2, GCD6, GCD7, and GCN5, leading us to suggest that all of the subunits of eIF-2B in *S. cerevisiae* have now been identified (4, 5).

Figure 6 summarizes many of our conclusions about the structure and function of the GCD-eIF-2B complex. Its principal activity is guanine nucleotide exchange on eIF-2. Under normal growth conditions in wild-type cells, eIF-2-GTP is rapidly regenerated from eIF-2-GDP and GTP, leading to high levels of translation initiation and repression of *GCN4*. In response to amino acid starvation, the α subunit of eIF-2 is phosphorylated by the protein kinase GCN2. By analogy with mammalian systems, phosphorylated eIF-2 is thought to inhibit the GCD-eIF-2B complex. The resulting decrease in the levels of eIF-2-GTP reduces the efficiency of general translation initiation and thereby stimulates the expression of *GCN4*. Mutations in one of the subunits of eIF-2B or eIF-2 itself can mimic the effect of eIF-2 phosphorylation in reducing the level of eIF-2 activity, with the
attendant decrease in growth rate and derepression of GCN4.

Genetic properties of GCN3 suggest a key regulatory role for this subunit of the GCD-eIF-2B complex. Deletion of GCN3 prevents translational derepression of GCN4 and overcomes the slow-growth phenotype associated with constitutive activation of GCN2 but does not affect cell growth or general translation under nonstarvation conditions (19, 25). Furthermore, recent results (9) indicate that deletion of GCN3 does not diminish the phosphorylation of eIF-2α by GCN2. These findings have led to the idea that GCN3 directly interacts with the α subunit of eIF-2 and mediates the inhibition of eIF-2B function exerted by the phosphorylated form of eIF-2 (5, 9).

Evidence that GCD6 and GCD7 are subunits of the GCD complex. Allele-specific interactions between GCD6 and GCD7 and alleles of GCN3 provided strong genetic evidence that GCD6 and GCD7 are components of the eIF-2B - eIF-2 complex of S. cerevisiae. The synthetic lethality reported here between mutations in GCD6 or GCD7 and a deletion of GCN3 is in accord with earlier observations that certain mutations affecting GCD1 and GCD2 and the α and γ subunits of eIF-2 (encoded by SUI2 [7] and GCD11 [18], respectively) were more severe in strains lacking GCN3 than in otherwise wild-type strains (19, 20, 53). By showing that GCD6 and GCD7 coimmunoprecipitate and copurify with the GCD1, GCD2, and GCN3 subunits of the GCD-eIF-2B complex and with a fraction of the α and β subunits of eIF-2, we provided two independent lines of biochemical evidence establishing that GCD6 and GCD7 are subunits of eIF-2B. The six of the eight different polypeptides contained in the GCD-eIF-2B - eIF-2 complex of S. cerevisiae, GCD1, GCD2, GCD6, GCD7, GCN3 (subunits of eIF-2B), and GCD11 (the γ subunit of eIF-2), were first identified genetically as translational regulators of GCN4 expression. This finding provides strong support for the idea that translational derepression of GCN4 expression is mediated by a reduction in the recycling of eIF-2 catalyzed by eIF-2B (Fig. 6).

Involvement of GCN3 in the essential function of the GCD-eIF-2B complex in translation initiation. Although GCN3 is a dispensable subunit of yeast eIF-2B and is thought to be required primarily for down-regulation of eIF-2B activity by phosphorylated eIF-2α (29), it can be mutated to have strong inhibitory effects on cell growth in the absence of starvation. We showed that gcen3 mutations with this phenotype lead to reduced polysome content and increased amounts of 80S monosomes relative to wild-type yeast strains. This polypeptide defect is characteristic of a reduction in the initiation stage of protein synthesis with little or no accompanying defect in elongation or termination. Thus, with initiation occurring slowly but elongation and termination proceeding at normal rates, the number of translating 80S ribosomes on polysomes at any given time is diminished from the wild-type state. Under the conditions of our sucrose gradient analysis, the excess 40S and 60S subunits generated by polysome runoff accumulate as nonfunctional 80S couples (12).

A second indication that general translation initiation is impaired in gcen3 mutants came from our finding that two of the most severely affected gcen3 alleles produce an accumulation of eIF-2B in the region of the polysome gradient containing small ribosomal subunits. This could indicate an accumulation of 48S preinitiation complexes containing eIF-2 · GTP · Met-tRNA1Met, mRNA, and other initiation factors bound to 40S subunits in gcen3 strains. The same phenomenon has been observed in gcd1-101 (6) and gcd2-503 (12) mutants and in mammalian cell lysates in which eIF-2B function was inhibited (8, 13, 14, 52) and may be an indication of eIF-2B function late in the initiation pathway. This hypothesis would explain the occurrence of halfmer polysomes in gcd2-502 mutants (12) as an accumulation of 48S preinitiation complexes on mRNAs containing translating 80S ribosomes. It is interesting that the gcd2-502 and gcd2-503 mutations have markedly different effects on polysome profiles despite the fact that both mutations alter the same amino acid in GCD2. Perhaps these substitutions have differential effects on two distinct functions of eIF-2B in the initiation pathway.

Interactions between GCN3 and other subunits of the GCD-eIF-2B complex. A common feature of most gcd mutations affecting the essential subunits of eIF-2B is that deletion of GCN3 exacerbates the slow-growth phenotype associated with the mutation. One way to account for this interaction has been to propose that the presence of GCN3 in the GCD complex lessens the destabilizing effect of the gcd mutation on the structure or function of the complex (42). The postulated reduction in eIF-2B activity resulting from inactivation of GCN3 would not be severe enough to reduce the rate of initiation in otherwise wild-type cells but would have noticeable consequences when combined with defects in other eIF-2B subunits, including gcd6-1 and gcd7-201 mutations analyzed here. This additivity of separate biochemical defects can also explain how mutations in the α and γ subunits of eIF-2 can be exacerbated by deletion of GCN3 (20, 53), as GCN3 is a subunit of the eIF-2-recycling factor. Insertion of a gcen3 deletion in preventing derepression of GCN4 under starvation conditions (28), suggesting that it renders the GCD-eIF-2B complex insensitive to phosphorylated eIF-2. It resembles wild-type GCN3, however, in not exacerbating the effects of gcd mutations, suggesting that the gcen3-102 protein is stably incorporated into the GCD-eIF-2B complex and does not appreciably decrease the efficiency of guanine nucleotide exchange on nonphosphorylated eIF-2. The gcen3-102 product is superior to wild-type GCN3 for suppressing the gcdl-101 and gcd7-201 mutations but not for suppressing other gcen alleles, such as gcd6-1. This observation implies that the gcdl-101 and gcd7-201 mutations are lethal when eIF-2B activity is reduced by a combination of factors that differs from that involved with mutations like gcd6-1 which are not suppressed by gcen3-102. We suspect that the gcen3-102 product increases the activity of the GCD-eIF-2B complex in a way that offsets the particular biochemical defect(s) associated with the gcdl-101 and gcd7-201 mutations.

A panel of seven gcen3 alleles was found to have substantially different patterns of interaction with the gcd2-502, gcd2-503, and gcd2-1 mutations, and we observed several striking cases of mutual suppression by particular gcen3 and gcd2 alleles. First, we consider the suppression of gcd2 mutations by gcen3 alleles. Because wild-type GCN3 either partially or completely suppresses the growth defects of the gcd2 mutations, one component of the suppression conferred by the gcen3 alleles probably involves the residual wild-type function of the gcen3 product. However, the gcen3 alleles displayed distinctly different profiles of suppression of the three gcd2 mutations at 36°C. For example, gcen3- E199K and gcen3- V295F gave no suppression of gcd2-502, exacerbated gcd2-503, and suppressed gcd2-1 nearly as well as did wild-type GCN3. In addition, gcen3- D71N and gcen3- S63F gave no suppression of gcd2-1 but were two of the best suppressors of gcd2-502 and gcd2-503. These results imply that different gcen3 alleles and the three gcd2 mutations have
distinguishable effects on the structure and function of the eIF-2B complex. A particular gcna product is able to compensate for one gcd2 lesion but not another, and it may contribute an additional defect that further impairs the structure or function of the complex.

Of greater interest were the cases in which a gcd2 mutant allele was superior to wild-type GCDC in suppressing the growth defect of certain gcna mutations at 23°C. These interactions indicate that a mutant gcd2 protein can compensate for a defect caused by a particular gcna mutation that exhibits a similar phenotype. The most striking examples of this phenomenon were the suppression of gcna-426T and gcna-E199K by gcd2-1 and the suppression of gcna-R104K and gcna-V295F by gcd2-502. One way to account for this type of interaction would be to suggest that the two mutant proteins have offsetting biochemical defects. For example, a gcna mutation might lead to an excessively stable interaction between eIF-2 and eIF-2B, mimicking the inhibitory effect of eIF-2α phosphorylation on the recycling function of eIF-2B. A gcd2 mutation that reduces eIF-2B-recycling function by weakening the interaction between eIF-2B and its substrate eIF-2 could suppress this particular gcna allele.

Although we cannot rule out the possibility that all of the cases of mutual suppression that we observed are the result of compensatory biochemical defects, we believe that at least some of these interactions are indicative of direct contact between GCDC and GCN3 in the eIF-2B complex. This view is motivated by our observation of regions of sequence similarity between these two proteins, which could form a conserved structural motif that mediates protein-protein interactions. This notion, combined with the fact that regions of sequence similarity between GCN3 and GCDC are also conserved in GCD7 (Fig. 2), leads to the idea that these three proteins form a heterotrimeric unit within the GCD-eIF-2B complex that is stabilized by interactions between conserved regions of each protein (Fig. 6). Given that GCN3 is a nonessential subunit of the complex that seems to be required only for the regulation of eIF-2B function by phosphorylated eIF-2, perhaps GCD7 and GCDC also have important regulatory roles, whereas the GCD1 and GCD6 proteins might be concerned primarily with catalysis.

Allele-specific interactions characteristic of multigene complexes. The observation of both synthetic lethality and mutual suppression between alleles of two genes is a strong indication that the factors involved form a multigene complex, as shown here for eIF-2B · eIF-2 of S. cerevisiae. The interactions that we observed among the trans-acting regulators of GCN4 are remarkably similar to allele-specific interactions seen among other groups of genes, including: (i) interactions between a translational activator of mitochondrial genes and mitochondrial ribosomal proteins (15, 16, 37), (ii) cdc genes implicated in initiation of DNA replication in S. cerevisiae (22), (iii) certain transcription factors postulated to function as intermediaries between transcriptional activators and the basic transcription machinery (46, 51), and (iv) actin and SAC genes presumed to encode other cytoskeletal components (2, 41). The high frequency with which we observed synthetic lethality among mutations in GCN3 and genes encoding subunits of eIF-2B or eIF-2 suggests that screens to identify synthetic lethal mutations may be extremely productive in the identification of interacting components. In addition, they provide a convenient way of cloning the genes once identified. While allele-specific interactions often have been interpreted as evidence for complex formation among a set of factors, in this study we attempted to use such genetic data to provide information about physical relationships among the components of the complex. This kind of detailed genetic approach to subunit interactions can provide a focus for molecular and biochemical studies. Thus, experiments are now under way to test the idea that cases of mutual suppression observed between different alleles of GCN3, GCDC, and GCDC arise from direct protein-protein interactions between these two subunits of the GCD-eIF-2B complex. In addition, it will be interesting to determine whether gcd2-1 and gcna-E199K have offsetting effects on the eIF-2-recycling function of eIF-2B.

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REFERENCES


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