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Evidence that GCD6 and GCD7, Translational Regulators of *GCN4*, Are Subunits of the Guanine Nucleotide Exchange Factor for eIF-2 in *Saccharomyces cerevisiae*

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Starvation of the yeast *Saccharomyces cerevisiae* for an amino acid signals increased translation of *GCN4*, a transcriptional activator of amino acid biosynthetic genes. We have isolated and characterized the *GCD6* and *GCD7* genes and shown that their products are required to repress *GCN4* translation under nonstarvation conditions. We find that both GCD6 and GCD7 show sequence similarities to components of a high-molecular-weight complex (the GCD complex) that appears to be the yeast equivalent of translation initiation factor 2B (eIF-2B), which catalyzes GDP-GTP exchange on eIF-2. Furthermore, we show that GCD6 is 30% identical to the largest subunit of eIF-2B isolated from rabbit reticulocytes. Deletion of either *GCD6* or *GCD7* is lethal, and nonlethal mutations in these genes increase *GCN4* translation in the same fashion described for defects in known subunits of eIF-2 or the GCD complex; derepression of *GCN4* is dependent on short open reading frames in the *GCN4* mRNA leader and occurs independently of eIF-2α phosphorylation by protein kinase GCN2, which is normally required to stimulate *GCN4* translation. Together, our results provide evidence that GCD6 and GCD7 are subunits of eIF-2B in *S. cerevisiae* and further implicate this GDP-GTP exchange factor in gene-specific translational control.

In response to starvation for an amino acid, the budding yeast *Saccharomyces cerevisiae* increases expression of the GCN4 protein, which in turn activates transcription of more than 30 genes encoding enzymes involved in amino acid biosynthesis (general amino acid control). The increase in *GCN4* expression occurs primarily at the level of translation initiation (for a review, see references 22, 24, and 25) and involves a pathway of positive and negative regulatory factors, some of which have general functions in translation initiation (Fig. 1).

A reduction in the activity of eukaryotic translation initiation factor 2 (eIF-2) appears to be responsible for increasing *GCN4* translation in amino acid-starved cells (25). eIF-2 forms a ternary complex with GTP and initiator tRNA ($tRNA_i^{Met}$) that binds to 40S ribosomes, and it has a role in AUG start codon recognition (12) (for reviews of eukaryotic translation initiation, see references 38 and 44). Dever et al. (8) found that protein kinase GCN2 stimulates *GCN4* translation in amino acid-starved cells by phosphorylating the α subunit of eIF-2 on the serine residue at position 51. By analogy with mammalian systems, this should inhibit exchange of GDP for GTP on eIF-2 catalyzed by initiation factor 2B (eIF-2B) (36, 51, 53). Since only the GTP-bound form of eIF-2 can bind $tRNA_i^{Met}$, phosphorylation of eIF-2α should diminish ternary complex formation as a means of stimulating translation of *GCN4*. Regulation of eIF-2B activity is a widely used mechanism for global translational control in animal cells in response to many different stimuli, including environmental stresses, viral infection, and developmental cues (33, 44).

Exchange factor eIF-2B isolated from rabbit reticulocytes is composed of five subunits and copurifies with a fraction of eIF-2 (29, 36). A complex associated with eIF-2 that contains GCD1, GCD2, and GCN3 was postulated to be the GTP-GDP exchange factor for eIF-2 in *S. cerevisiae* (6, 15). The GCD1 and GCD2 subunits of this "GCD complex" have essential functions in translation initiation in *S. cerevisiae* (6, 15, 56). Nonlethal mutations in these factors lead to increased *GCN4* expression under nonstarvation conditions, as would be expected of mutations in subunits of eIF-2B that reduce guanine nucleotide exchange on eIF-2 and thereby lower eIF-2 activity.

Four upstream open reading frames (uORFs) in the leader of *GCN4* mRNA couple *GCN4* translation to the level of eIF-2 activity (22, 23). Under nonstarvation conditions, it appears that ribosomes scanning from the 5′ end of *GCN4* mRNA translate the first ORF (uORF1) and reinitiate at one of the remaining uORFs, -2, -3, or -4, instead of at *GCN4*. Because of the low level of eIF-2 · GTP · $tRNA_i^{Met}$ ternary complexes present in the cell under starvation conditions, many ribosomes scanning downstream from uORF1 cannot reinitiate at uORF2 to -4 and instead reinitiate further downstream at the *GCN4* start codon (1, 8). Thus, a decrease in the efficiency of reinitiation leads to an increase in *GCN4* translation.

The mutations *gcd6-1* (40) and *gcd7-201* (originally designated *gcd3-201* [39]) have several properties in common with mutations in *GCD1* and *GCD2*, leading us to suspect that the products of *GCD6* and *GCD7* might function in parallel with GCD1, GCD2, and the subunits of eIF-2 in translational repression of *GCN4* and the initiation of general protein synthesis. Our studies show that *GCD6* and *GCD7* encode

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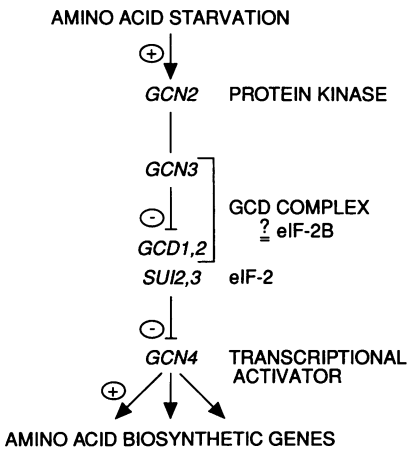


FIG. 1. Regulatory pathway for general amino acid control.

previously unidentified essential proteins that are required for translational control of *GCN4* expression by the uORFs. Cloning and sequence analysis of the *GCD6* and *GCD7* genes reveal sequence similarities between the factors they encode and subunits of the yeast GCD complex, thought to be the eIF-2 guanine nucleotide exchange factor of *S. cerevisiae*. In addition, we report the first amino acid sequence for a known subunit of mammalian eIF-2B and show that this subunit has striking similarity with the sequence of GCD6. These findings strongly suggest that GCD6 and GCD7 are components of eIF-2B in *S. cerevisiae* and that eIF-2B can function in gene-specific translational control as well as global regulation of protein synthesis.

MATERIALS AND METHODS

Construction of strains for the isolation of the *GCD6* and *GCD7* genes. The genotypes of yeast strains used in this study are shown in Table 1. F222 (*MAT***a** *gcd6-1 leu2-2*) and F285 (*MAT***a** *gcd7-201 his4C-207(ts) gcn1-15 ura3-52*) were crossed to H750 (*MAT***a** *gcn2::LEU2 leu2-3,112 ura3-52*) or H751 (*MATα gcn2::LEU2 leu2-3,112 ura3-52*) to create double mutants that combine a deletion of *GCN2*, the *gcn2::LEU2* allele, with *gcd6-1* or *gcd7-201*. Note that *gcd7-201* was originally designated *gcd3-201* (39) and renamed by Hinnebusch (22) to avoid confusion with *gcd3* described by Neiderberger et al. (40). In these crosses, we observed 2+:2− segregation for slow growth on nutrient-rich medium (Slg^-), a phenotype noted for *gcd6-1* (40) but not reported previously for *gcd7-201* (39). As expected, all Slg^- Leu^+ (*gcd gcn2::LEU2*) ascospore clones were 3-aminotriazole resistant ($3\text{-}AT^r$) and all Slg^+ Leu^+ (*GCD gcn2::LEU2*) ascospore clones were 3-aminotriazole sensitive ($3\text{-}AT^s$). Successive backcrosses to H750 or H751 produced the starting strains used for all subsequent experiments, including H1916, H1917, and H2042. The *gcd7-201* strains H1600 and H1603 were Slg^- $3\text{-}AT^r$ ascospore clones from a cross between H2042 and H1511. *gcd6-1* strain H1707 was a Slg^- Leu^- ascospore clone from a cross between H1916 and H1511.

Plasmids derived from the *GCD6* region. See Fig. 3A. pJB1 isolated by complementation of the *gcd6-1* mutation contains a 14.5-kb insert of yeast genomic DNA in the *Bam*HI site of

TABLE 1. Yeast strains

Strain	Genotype	Reference or source
F222	*MAT***a** *gcd6-1 leu2-2*	40
F285	*MAT***a** *gcd7-201 his4C-207(ts) gcn1-15 ura3-52*	39
US21-42C	*MAT***a** *his4 leu2 cdc42 exg1*	F. del Rey (US21-42C)
H750	*MAT***a** *gcn2::LEU2 leu2-3,112 ura3-52*	32
H751	*MATα gcn2::LEU2 leu2-3,112 ura3-52*	32
H1402[a]	*MATα ino1 leu2-3,112 ura3-52 HIS4-lacZ*	18
H1511	*MATα trp1-Δ63 leu2-3,112 ura3-52*	15
H1600	*MATα gcd7-201 trp1-Δ63 gcn2::LEU2 leu2-3,112 ura3-52*	This study
H1603[b]	*MATα gcd7-201 leu2-3,112 ura3-52*	This study
H1707[c]	*MATα gcd6-1 leu2-3,112 ura3-52*	This study
H1725[b]	*MATα gcd7-201 leu2-3,112 ura3-52*	This study
H1728[c]	*MATα gcd6-1 leu2-3,112 ura3-52*	This study
H1727[b]	*MATα GCD7 leu2-3,112 ura3-52*	This study
H1730[c]	*MATα GCD6 leu2-3,112 ura3-52*	This study
H1792[c]	*MATα gcd6-1 gcn2::LEU2 leu2-3,112 ura3-52*	This study
H1793[c]	*MATα GCD6 gcn2::LEU2 leu2-3,112 ura3-52*	This study
H1794[b]	*MATα gcd7-201 gcn2::LEU2 leu2-3,112 ura3-52*	This study
H1795[b]	*MATα GCD7 gcn2::LEU2 leu2-3,112 ura3-52*	This study
H1884	*MAT***a**/*MATα GCD7/gcd7::URA3 gcn2::LEU2/gcn2::LEU2 leu2-3,112/leu2-3,112 ura3-52/ura3-52*	This study
H1885	*MAT***a**/*MATα gcd7-201/gcd7::URA3 gcn2::LEU2/gcn2::LEU2 leu2-3,112/leu2-3,112 ura3-52/ura3-52*	This study
H1905[a]	*MATα gcd6Δ ino1 leu2-3,112 ura3-52 HIS4-lacZ*	This study
H1906[a]	*MATα gcd6Δ ino1 leu2-3,112 ura3-52 HIS4-lacZ*	This study
H1916	*MAT***a** *gcd6-1 gcn2::LEU2 leu2-3,112 ura3-52*	This study
H1917	*MATα gcd6-1 gcn2::LEU2 leu2-3,112 ura3-52*	This study
H2042	*MAT***a** *gcd7-201 gcn2::LEU2 leu2-3,112 ura3-52*	This study

[a] These strains are isogenic to one another; H1905 and H1906 contain episomal plasmid pJB5 (*URA3 GCD6*).
[b] These strains are isogenic to one another.
[c] These strains are isogenic to one another.

YCp50 which destroys this *Bam*HI site. The *Sal*I site in the insert is 1.5 kb from the *Eco*RI site in YCp50. pJB2 was constructed by inserting the 6.8-kb *Bgl*II fragment of pJB1 into the *Bam*HI site of the nonreplicating *URA3* plasmid pRS306 (54). pJB5 was constructed by subcloning the 5.8-kb *Sal*I-*Bam*HI fragment of pJB1 between the *Sal*I and *Bam*HI sites of pRS316, a low-copy-number *URA3* plasmid (54). Unidirectional exonuclease III deletions were generated in pJB5 with the Double-Stranded Nested Deletion Kit from Pharmacia (Piscataway, N.J.). Deletions encroaching from the *Sal*I end of the pJB5 insert, generated from pJB5 digested with *Sal*I and *Kpn*I, gave rise to pJB17, pJB24, and pJB25. pJB30 was obtained by making deletions from the *Bam*HI end of the insert, beginning with pJB5 digested with *Bam*HI and *Sac*I. pJB71 was constructed by deletion of the 1.5-kb *Xho*I fragment from pJB5 (one of the *Xho*I sites in pJB5 occurs in the multiple cloning site [MCS] of the vector sequence). pJB85 was constructed by inserting the 2.8-kb *Sca*I fragment of pJB5 at the *Sma*I site in the MCS of pRS316 such that the *Hin*dIII site in the MCS was adjacent to the $tRNA^{Ile}$.

GCD6-integrating plasmid pJB98, used for the construction of isogenic *gcd6-1* and wild-type strains, contains the 5.8-kb *Sal*I-*Bam*HI insert from pJB5 inserted between the *Sal*I and *Bam*HI sites of pRS306. The low-copy-number *GCD6 LEU2* vector pJB102 was constructed by inserting the 2.4-kb *Xho*I-*Bam*HI fragment from pJB85 between the *Xho*I and *Bam*HI sites of pRS315 (54).

pJB96, an integrating *URA3* vector containing only the first 92 amino acid residues of the of the *GCD6* ORF, was constructed in two steps. (i) The 1.9-kb *Sca*I-*Xba*I fragment of pJB5 (region 3′ to the *GCD6* ORF beginning at the *Sca*I site at position +2297) was inserted between the *Xba*I and *Sma*I sites of pRS306 to make pJB95. (ii) The 750-bp *Eco*RI fragment of pJB5 (region 5′ to the *GCD6* ORF beginning at position +276) was inserted in the correct orientation at the *Eco*RI site of pJB95 to create pJB96.

Plasmids derived from the *GCD7* region. See Fig. 3B. pJB13 and pJB14, isolated by complementation of the *gcd7-201* mutation, contain overlapping inserts of 10.5 and 12 kb, respectively, in the *Bam*HI site of YCp50. In both plasmids, the *Bam*HI site adjacent to the *Sal*I site of YCp50 is destroyed but the *Bam*HI site adjacent to the *Eco*RI site of YCp50 is preserved. Both inserts are oriented such that the 5′ end of the *GCD7* ORF is proximal to the *Sal*I site of YCp50. pJB87 was constructed by inserting the 5.2-kb *Eco*RI fragment of pJB13 into the *Eco*RI site of pRS306. pJB57 was produced by deletion of the 6-kb *Bam*HI fragment from pJB14. pJB99 and pJB100 were constructed by isolating the 2.1-kb *Sna*BI-*Bst*EII fragment containing the *GCD7* gene, filling in the *Bst*EII end with the Klenow fragment of DNA polymerase I, and inserting this fragment at the *Sma*I site in the MCS of pRS316; in the process, the *Sna*BI, *Bst*EII, and *Sma*I sites were destroyed. In pJB99, the insert is oriented such that the *Bst*EII end of the insert is adjacent to the *Eco*RI site in the MCS of pRS316; pJB100 has the opposite orientation. pJB101 was constructed by deletion of the 1-kb *Xba*I fragment from pJB100 (one of the *Xba*I sites in pJB100 occurs in the MCS of the vector).

pJB110, an integrating *URA3* vector in which 66% of the *GCD7* ORF is deleted, was constructed in three steps. (i) The 2.2-kb *Eco*RI-*Xba*I fragment (region 5′ of *GCD7* up to position +220 in the *GCD7* ORF) was inserted between the *Sma*I and *Eco*RI sites of the vector pUC19 after filling in the *Xba*I overhang as described above, thereby creating pJB108. (ii) The 1.5-kb *Bam*HI-*Sph*I fragment (3′ coding region of *GCD7* beginning at position +975) was inserted between the *Bam*HI and *Sph*I sites of pJB108 to create pJB109. (iii) The 3.8-kb *Bam*HI-*Bgl*II fragment from pNKY51 (3) (*URA3* gene flanked by *hisG* direct repeats) was inserted into the *Bam*HI site of pJB109 to create pJB110.

Genetic demonstration that *GCD6* and *GCD7* were cloned. *GCD6*-derived plasmid pJB2 digested with *Bam*HI and *GCD7*-derived plasmid pJB87 digested with *Bgl*II were used to transform strain H750 (*MAT***a** *gcn2::LEU2 leu2-3,112 ura3-52*) to Ura^+. In all 17 tetrads analyzed from the cross between the pJB2 transformant and H1917 (*MATα gcd6-1 gcn2::LEU2 leu2-3,112 ura3-52*), the Ura^-, Slg^-, and $3\text{-}AT^r$ phenotypes cosegregated 2+:2−. In all but one of the 46 tetrads analyzed from the cross between the pJB87 transformant and H1600 (*MATα gcd7-201 gcn2::LEU2 leu2-3,112 ura3-52 trp1Δ63*), the Ura^-, Slg^-, and $3\text{-}AT^r$ phenotypes cosegregated 2+:2−. These results indicate that sequences in pJB2 and pJB87 directed plasmid integration to sites closely linked to *gcd6-1* and *gcd7-201*, respectively.

Construction of *GCD* strains isogenic to *gcd6-1* and *gcd7-201* mutants. *GCD* strains were constructed from *gcd6-1* and *gcd7-201* mutants by integrating a *URA3* plasmid containing the corresponding cloned *GCD* gene at the respective *gcd* chromosomal locus, producing a nontandem duplication consisting of the *gcd* and *GCD* alleles separated by plasmid sequences and *URA3*. Selecting for loss of *URA3* by growing the transformants on medium containing 0.1% 5-fluoroorotic acid (5-FOA) (4) yielded isogenic *gcd* and *GCD* strains as Slg^- and Slg^+ derivatives, respectively. For *GCD6*, strain H1707 (*MATα gcd6-1 leu2-3,112 ura3-52*) was transformed with *Mlu*I-digested pJB98 (see Fig. 3A). A single Ura^+ transformant gave rise to the $5\text{-}FOA^r$ derivatives H1728 (*MATα gcd6-1 leu2-3,112 ura3-52*) and H1730 (*MATα GCD6 leu2-3,112 ura3-52*). For *GCD7*, strain H1603 (*MATα gcd7-201 leu2-3,112 ura3-52*) was transformed with *Bgl*II-digested pJB87 (see Fig. 3B). A single Ura^+ transformant gave rise to the $5\text{-}FOA^r$ derivatives H1725 (*MATα gcd7-201 leu2-3,112 ura3-52*) and H1727 (*MATα GCD7 leu2-3,112 ura3-52*). In these isogenic *gcd* mutant and wild-type strains, the *GCN2* gene was replaced by the *gcn2::LEU2* allele as described previously (43), producing strains H1792, H1793, H1794, and H1795.

Deletion of chromosomal *GCD6*. *Mlu*I-digested pJB96 (see Fig. 3A) was used to transform strain H1402 to Ura^+, resulting in a nontandem duplication at the *GCD6* locus containing the *gcd6Δ* allele and *GCD6* separated by vector sequences and *URA3*. This strain was transformed with *GCD6 LEU2* plasmid pJB102. Ura^- derivatives of the resulting transformant were isolated by growth on 5-FOA medium (4) and screened for the inability to lose pJB102. These strains were shown to contain only the *gcd6Δ* allele and the pJB102-borne copy of *GCD6* by DNA blot hybridization analysis of total yeast DNA digested with *Bam*HI or *Eco*RI (55) by using the 0.7-kb *Eco*RI fragment from the *GCD6* ORF radiolabeled by the random primer technique (13) as a probe. To confirm that *gcd6Δ* is lethal, plasmid pJB5 (containing *URA3* and *GCD6*) or pRS316 (*URA3* alone) were introduced into *gcd6Δ* Leu^+ transformants containing *GCD6* on pJB102. The transformants containing pJB5, but not those containing pRS316, were able to lose pJB102 and become Leu^-. Thus, we concluded that *GCD6* is essential for vegetative growth on nutrient-rich medium.

Deletion of chromosomal *GCD7*. The *Eco*RI-*Sph*I fragment of pJB110, containing the *gcd7::URA3* deletion/disruption allele (see Fig. 3B), was used to transform the diploid strain JBX42 (*GCD7/gcd7-201 gcn2::LEU2/gcn2::LEU2 leu2-3/*

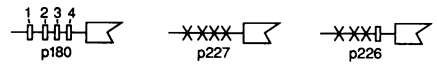
leu2-3 leu2-112/leu2-112 ura3-52/ura3-52) to Ura$^+$. A Gcd$^+$ transformant (H1884) was sporulated, and all 20 tetrads analyzed contained only two viable spores that were Ura$^-$ Slg$^+$ 3-AT^s, as expected if the *gcd7-201* allele was replaced by *gcd7::URA3* and if *gcd7::URA3* was lethal, as all of the viable progeny of the transformed diploid have a Gcd$^+$ phenotype. Similarly, a Gcd$^-$ Ura$^+$ transformant of JBX42 (H1885) was sporulated, and 16 tetrads were found to contain only two viable spores that were all Ura$^-$ Slg$^-$ 3-AT^r, as expected if the *GCD7* allele was replaced by *gcd7::URA3* since all of the viable progeny exhibit the phenotypes of *gcd7-201*. These interpretations were confirmed by DNA blot hybridization analysis of *Hin*dIII-digested DNA from two of the viable ascospore clones from each cross, the Gcd$^+$ Ura$^+$ and Gcd$^-$ Ura$^+$ parental diploid strains, and JBX42; the *GCD7* probe employed was the 1.1-kb *Xba*I fragment from pJB99 (see Fig. 3B), radiolabeled as described above.

DNA sequence analysis. The nucleotide sequences of both strands of the *GCD6* region were determined with the Sequenase kit from U.S. Biochemical Corp. (Cleveland, Ohio). Both strands of the *GCD7* region were determined by Lofstrand Labs Limited (Gaithersburg, Md.) with pJB99 DNA. Sequences were assembled and analyzed by using the Genetics Computer Group sequence analysis software package (9). Dotplots were generated using a window size of 30 and a stringency of 15. The symbol comparison table of Gribskov and Burgess (16) was used in all sequence comparisons reported here and is based on the Dayhoff PAM-250 matrix. Parameters used for sequence alignments with "bestfit" were gap weight = 3.0 and gap length weight = 0.1. We report percent identities for protein pairs based on best fit alignments. The three-way alignment reported for GCD7-GCN3-GCD2 was generated by using the "pileup" program. We searched the GCD6 and GCD7 sequences for protein motifs represented in the PROSITE Dictionary of Protein Sites and Patterns compiled by Amos Bairoch of the University of Geneva (9). We also searched for similarities to sequences in the GenBank, EMBL, and SWISSPROT data bases.

Nucleotide sequence accession numbers. The nucleotide sequences of the *GCD6* and *GCD7* regions have been deposited into GenBank under accession numbers L07115 and L07116, respectively.

A.

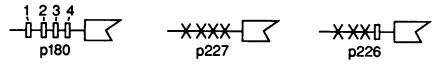
β-Galactosidase Activity (U) from *GCN4-lacZ*



Relevant Genotype	p180 R	p180 DR	p227 R	p227 DR	p226 R	p226 DR
GCD6	24	82	450	480	11	26
gcd6-1	170	220	750	610	39	37
GCD6 gcn2Δ	10	17				
gcd6-1 gcn2Δ	160	160				

B.

β-Galactosidase Activity (U) from *GCN4-lacZ*



Relevant Genotype	p180 R	p180 DR	p227 R	p227 DR	p226 R	p226 DR
GCD7	13	56	1000	740	12	20
gcd7-201	160	130	1700	1200	19	16
GCD7 gcn2Δ	6	5				
gcd7-201 gcn2Δ	130	120				

FIG. 2. The *gcd6-1* and *gcd7-201* mutations lead to constitutive derepression of *GCN4-lacZ* translation independent of the positive regulator *GCN2*. β-Galactosidase activity was measured in yeast strains grown to mid-logarithmic phase under nonstarvation, repressing (R) conditions, or derepressing (DR) conditions of histidine starvation induced by 3-AT, as described previously (34). *GCN4-lacZ* fusions were introduced into the strains on low-copy-number plasmids. The fusion on p180 has the wild-type leader containing all four uORFs (shown as open boxes numbered 1 to 4). p227 is identical to p180 except that point mutations remove the AUG codons of all four uORFs (shown as X's). p226 is identical to p227 except that uORF4 remains intact. The relevant genotypes of the strains are shown on the left. The *gcd6-1* and *GCD6* strains shown in panel A (H1728 and H1730, respectively) are isogenic, as are the *gcd7-201* and *GCD7* strains in panel B (H1725 and H1727, respectively); however, strains in panel A are not isogenic to those in panel B. β-Galactosidase was assayed as described previously (37). Enzyme activities are expressed as units (nanomoles of *o*-nitrophenyl-β-D-galactopyranoside cleaved per minute per milligram of total protein). Results shown are the mean values of measurements made with two to five independent transformants for each strain; individual measurements differed from the mean by less than 30%.

RESULTS

The *gcd6-1* and *gcd7-201* mutations elevate *GCN4* expression independent of protein kinase GCN2. It was shown previously that *GCN4* function is required for the derepression of amino acid-biosynthetic enzymes that occurs in *gcd6-1* and *gcd7-201* mutants, suggesting that GCD6 and GCD7 negatively regulate *GCN4* function (39, 40). To test this possibility, we examined the effects of the *gcd6-1* and *gcd7-201* mutations on the levels of β-galactosidase activity produced from a *GCN4-lacZ* fusion introduced on plasmid p180 into pairs of isogenic *gcd* mutant and wild-type strains (constructed as described in Materials and Methods). As expected, in the wild-type strains *GCN4-lacZ* expression was low under nonstarvation conditions and increased in response to histidine starvation elicited by 3-AT, an inhibitor of the *HIS3* product (22). In contrast, expression of the fusion was constitutively elevated in the *gcd6-1* and *gcd7-201* mutant strains (Fig. 2, columns labeled p180). These results indicate that GCD6 and GCD7 are required for repression of *GCN4* under nonstarvation conditions.

Deletion of the positive regulator *GCN2* in the wild-type strains prevented derepression of *GCN4-lacZ* expression in response to histidine starvation. However, deletion of *GCN2* in the *gcd6-1* and *gcd7-201* mutants had virtually no effect on *GCN4-lacZ* expression (Fig. 2, compare *GCN2* with *gcn2Δ* strains). Thus, the *gcd6-1* and *gcd7-201* mutations completely overcome the requirement for protein kinase GCN2 to achieve high-level expression of *GCN4*. The same observation has been made for mutations in other *GCD* genes (20) and is consistent with the notion that GCN2 stimulates *GCN4* expression by antagonism of GCD factors (Fig. 1).

Evidence that GCD6 and GCD7 repress *GCN4* expression at the translational level. GCN2 stimulates *GCN4* translation by overcoming the inhibitory effects of uORFs in the *GCN4* mRNA leader. It has been shown that uORF4 efficiently represses *GCN4* and that uORF1 is required to overcome

this repression in response to amino acid starvation (22). Thus, a *GCN4-lacZ* fusion containing uORF4 alone (p226) is translated constitutively at low levels, whereas the fusion containing no uORFs (p227) is translated constitutively at very high levels (Fig. 2). If the *gcd6-1* and *gcd7-201* mutations increase *GCN4* expression at the translational level, their effects should be greatly diminished by removal of the uORFs from the *GCN4* mRNA leader. The results in Fig. 2 show that these mutations have less than a 2-fold effect on expression from the p227 construct lacking all four uORFs, in contrast to the 7-fold (170 from 24 units) and 12-fold (160 from 13 units) increases associated with the *gcd6-1* and *gcd7-201* mutations, respectively, for the wild-type fusion on p180. Thus, our results indicate that these *gcd* mutations increase *GCN4* expression primarily by overcoming the inhibitory effects of the uORFs on *GCN4* translation rather than by increasing *GCN4* mRNA levels or protein stability.

The *gcd7-201* mutation has little effect on expression from the p226 construct containing uORF4 alone, as expected if the derepressing effect of this mutation is mediated by the positively acting uORF1. The *gcd6-1* mutation also appears to be strongly dependent on uORF1 for stimulating *GCN4* translation; however, at least under nonstarvation conditions, this allele leads to a small increase in *GCN4-lacZ* expression (from 11 to 39 units) in the presence of uORF4 alone. The latter finding may indicate that the *gcd6-1* mutation causes a small number of ribosomes scanning from the 5′ end of the mRNA to ignore the uORF4 start codon even when it occurs as the sole upstream AUG in the leader (leaky scanning [30]). According to our model for the regulation of *GCN4* expression (1), prior translation of uORF1 exacerbates leaky scanning at uORF4 because reinitiating ribosomes are more likely to ignore the uORF4 start site than are ribosomes engaged in primary initiation events (30). Taken together, the results in Fig. 2 lead us to conclude that GCD6 and GCD7 repress *GCN4* expression at the translational level by ensuring that ribosomes which have translated uORF 1 will reinitiate at uORF2, -3, or -4 and thus fail to reach the *GCN4* start site.

Isolation and characterization of the *GCD6* and *GCD7* genes. We exploited the ability of the *gcd6-1* and *gcd7-201* mutations to restore high-level *GCN4* expression in *gcn2* mutants to clone the *GCD6* and *GCD7* genes. In otherwise wild-type strains, deletion of *GCN2* prevents induction of histidine-biosynthetic enzymes via *GCN4* and thus causes increased sensitivity to 3-AT. By elevating *GCN4* expression, the *gcd* mutations confer resistance to 3-AT in the absence of *GCN2* function. The *gcd6-1* and *gcd7-201* mutations also cause slow growth on rich media (Slg^-). We used complementation of the Slg^- and $3\text{-}AT^r$ phenotypes of the *gcd6-1 gcn2::LEU2* and *gcd7-201 gcn2::LEU2* mutants H1916 and H2042 to isolate the *GCD6* and *GCD7* genes, respectively, from a yeast genomic library constructed in a low-copy-number plasmid (49). Restriction maps of the genomic DNA inserts present in the plasmids we isolated (pJB1, pJB13, and pJB14) are shown in Fig. 3. Fragments from the putative *GCD6* and *GCD7* genomic inserts were used to direct integration of a nonreplicating *URA3* plasmid into the yeast genome at sites homologous to the inserted yeast DNA sequences. Genetic analysis revealed that the integrated *URA3* marker was tightly linked to *GCD6* or *GCD7*, confirming that our plasmids contain the authentic *GCD6* and *GCD7* genes (see Materials and Methods for details).

To define the boundaries of the *GCD6* and *GCD7* genes, subclones of the genomic inserts present in pJB1 and pJB13/pJB14 were constructed in low-copy-number plasmids and tested for complementation of the *gcd6-1* and *gcd7-201* mutations, respectively (Fig. 3). The nucleotide sequence of a region encompassing each complementation unit was determined and found to contain a single long ORF of 2,139 bp for *GCD6* and 1,143 bp for *GCD7* (Fig. 4 and 5). For each gene, we found that deletions which remove sequences from the 5′ or 3′ end of the long ORF abolished complementing activity (Fig. 3). Using RNA blot hybridization analysis, we detected *GCD6* and *GCD7* transcripts of 2.3 and 1.3 kb, respectively, large enough to encode the predicted ORFs (data not shown). We deduced that the $tRNA^{Ile}$ gene located 3′ of the *GCD6* ORF is not required for *GCD6* function since pJB71 lacks this $tRNA^{Ile}$ gene but fully complements the *gcd6-1* mutation (Fig. 3).

In view of the Slg^- phenotype of *gcd6-1* and *gcd7-201*, it seemed likely that GCD6 and GCD7 have essential functions in addition to their role in *GCN4* translational control. To test this possibility, we created chromosomal deletions of *GCD6* and *GCD7* in strains that were functionally diploid for these genes (see Materials and Methods for details). We found that yeast strains containing the deletion allele as the only copy of *GCD6* and *GCD7* were inviable, indicating that both *GCD6* and *GCD7* are essential genes.

GCD6 and *GCD7* are located on chromosomes IV and XII, respectively. Three hundred fifty base pairs of sequence located 3′ to the *GCD6* ORF are identical to the 5′ noncoding region of the *TCP1* gene (Fig. 3). *TCP1* has been mapped 13.9 centimorgans (cM) distal to *pet14* on the right arm of chromosome IV (57). *GCD7* was localized to chromosome XII between *cdc42* and *cdc25* by hybridization of a *GCD7* probe to an ordered lambda library containing mapped yeast genomic DNA (data not shown) (48). Subsequently, we used tetrad analysis to map *GCD7* approximately 13 cM from *exg1* and 38 cM from *cdc42* (Table 2).

Sequence similarity between GCD6 and GCD7 and other translational regulators of *GCN4* in the GCD complex. We found 27% amino acid sequence identity between GCD7 and the GCN3 protein (19), which is a nonessential component of the high-molecular-weight GCD complex (6) and a positive regulator of *GCN4* translation (22). The statistical significance of the sequence relatedness was tested by determining the quality scores (a value incorporating similarities and gaps) between these sequences and 100 randomized sequences of the same composition and length. The quality score for GCD7 aligned with GCN3 was 13.8 standard deviations above the mean. The dot plot sequence comparison shown in Fig. 6A indicates that the greatest similarity occurs within the carboxyl-terminal two-thirds of these proteins. It was reported earlier (42) that GCN3 is related in sequence to GCD2, an essential component of the GCD complex that is required for translational repression of *GCN4* (22). We found that the amino acid sequences of GCD7 and GCD2 are 23% identical, with a quality score that is 4.4 standard deviations above the mean. A three-way alignment among GCD7, GCD2, and GCN3 shown in Fig. 6B confirms that GCD7 and GCN3 show the greatest similarity, followed by the GCN3-GCD2 pair. The GCD7-GCD2 pair shows the least similarity, except in the carboxyl-terminal 40 amino acids. A 21-amino-acid stretch in this region is 38% identical in all three proteins. We conclude that GCD7, GCN3, and GCD2 constitute a group of similar proteins with related functions in *GCN4* translational control.

Numerous mutations in *GCN3* that lead to constitutive derepression of *GCN4* expression and slow growth under nonstarvation conditions have been described. These *gcn3*^{c}

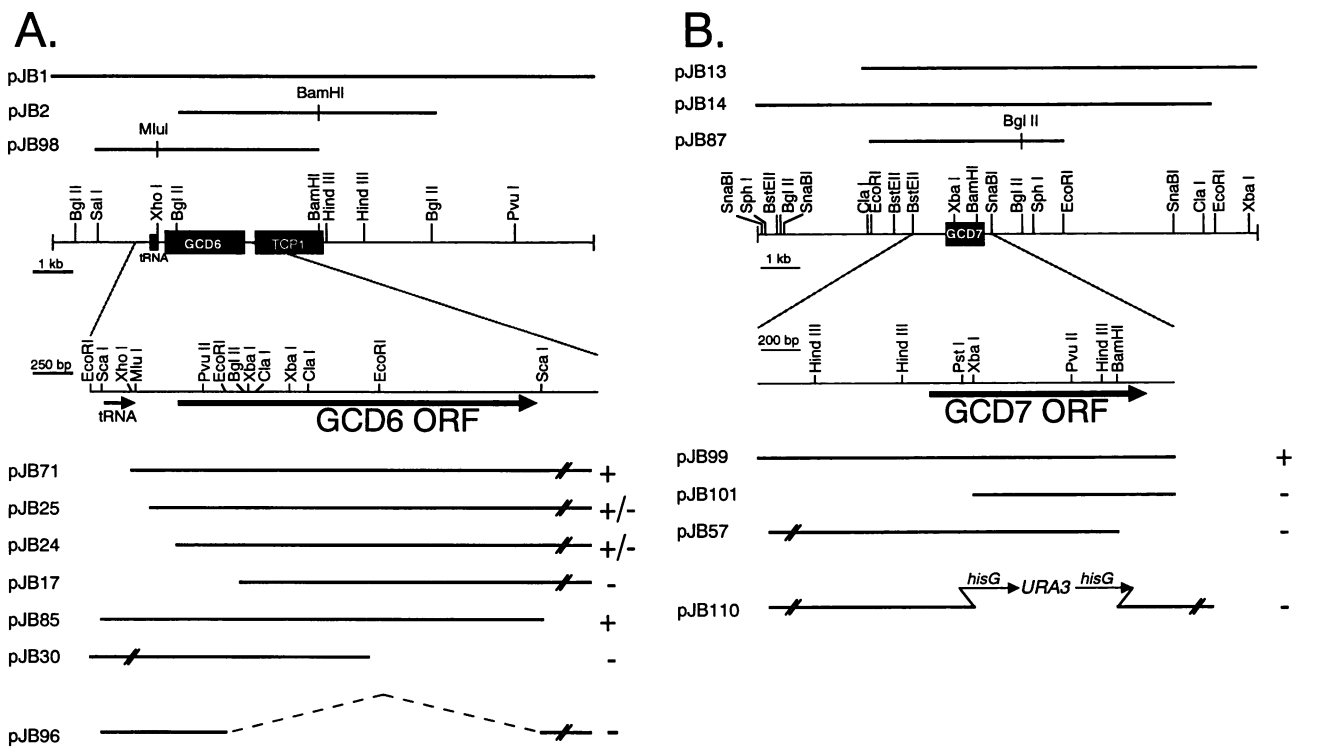


FIG. 3. Physical and functional maps of the *GCD6* and *GCD7* chromosomal regions. (A) Restriction map of the *GCD6* region, giving the positions of the *GCD6* ORF (box labeled GCD6), the upstream $tRNA^{Ile}$ (41), and the downstream *TCP1* ORF (57) (boxes labeled tRNA and TCP1, respectively). The extent of DNA present in pJB1, pJB2, and pJB98 is shown above the map, including restriction sites used to linearize pJB2 and pJB98 to direct integration. Below the map is an enlarged view of the *GCD6* region for which the nucleotide sequence was determined in this study, with the positions of the $tRNA^{Ile}$ and *GCD6* ORF indicated by arrows. Solid lines represent the DNA present in subclones (pJB71 to pJB30), and the results of complementation tests are shown to the right. A double slash indicates where the insert in that plasmid continues beyond the region shown in the enlargement. The extent of the deletion in the *gcd6Δ* allele constructed in pJB96 is shown by the dashed line. (B) Restriction map of the *GCD7* region along with the location of the *GCD7* ORF indicated by the box in the complete map and by the arrow in the enlarged map. The structure and complementing activity of subclones are summarized as in panel A. The extent of the deletion in the *gcd7::URA3* allele constructed in pJB110 (bottom construct) is shown by the portion replaced with the *URA3* gene flanked by *hisG* repeats.

alleles have the same phenotype as mutations in *GCD* genes and are thought to impair the essential function of the GCD complex in translation initiation. The amino acid substitutions produced by the $gcn3^c$ mutations (18) are indicated in the sequence alignment shown in Fig. 6B. Many of these mutations affect amino acids conserved among GCN3, GCD7, and GCD2, and these mutations generally have greater effects on *GCN4* expression and cell growth than those affecting nonconserved positions. This observation suggests that the most deleterious $gcn3^c$ mutations alter a structure or function of GCN3 that is shared with GCD7 and GCD2.

We also discovered that GCD6 is related in sequence to GCD1, another essential component of the high-molecular-weight GCD complex (6). The sequence of GCD1 used in this comparison differs from that reported previously (21) and incorporates sequence corrections determined by A. M. Cigan and A. G. Hinnebusch (6a). GCD6 is 22.5% identical to GCD1, and the overall similarity between the two sequences has a quality score of 4.7 standard deviations above the mean; moreover, two stretches of more significant similarity were observed. The quality score for amino acids 40 to 78 of GCD6 aligned with amino acids 57 to 95 of GCD1 is 12.1 standard deviations above the mean, and the quality score for amino acids 225 to 311 of GCD6 aligned with amino acids 422 to 508 of GCD1 is 12.7 standard deviations above the mean.

Sequence similarity between GCD6 and the largest subunit of mammalian eIF-2B. We found that GCD6 is very similar in sequence to the largest subunit of the rabbit eIF-2 guanine nucleotide exchange factor, eIF-2B. A cDNA clone encoding the rabbit eIF-2Bε protein was obtained recently by screening a λZAP cDNA library prepared from rabbit reticulocyte lysate (kindly provided by J.-J. Chen [5]) with a chicken antiserum raised against rabbit eIF-2B. The following evidence indicates that the cDNA clone thus obtained encodes eIF-2Bε: (i) The cDNA codes for an isopropylthiogalactoside-inducible 86-kDa fusion protein that reacts with the anti-eIF-2B antiserum on immunoblots; (ii) the cDNA contains a single long ORF whose predicted amino acid sequence encompasses the sequence of a peptide isolated after partial hydrolysis of the purified eIF-2Bε subunit; (iii) translation in reticulocyte lysate of mRNA transcribed in vitro from the cDNA yields a ^{35}S-labeled protein that comigrates with endogenous eIF-2Bε (3a). The observed molecular weight of rabbit eIF-2Bε is 82,000, similar to the 81,160 deduced from the DNA sequence of *GCD6* (Fig. 4). A dot plot comparison of GCD6 and rabbit eIF-2Bε (Fig. 7A) shows that these proteins are similar over their entire lengths, with no large deletions or insertions in one sequence

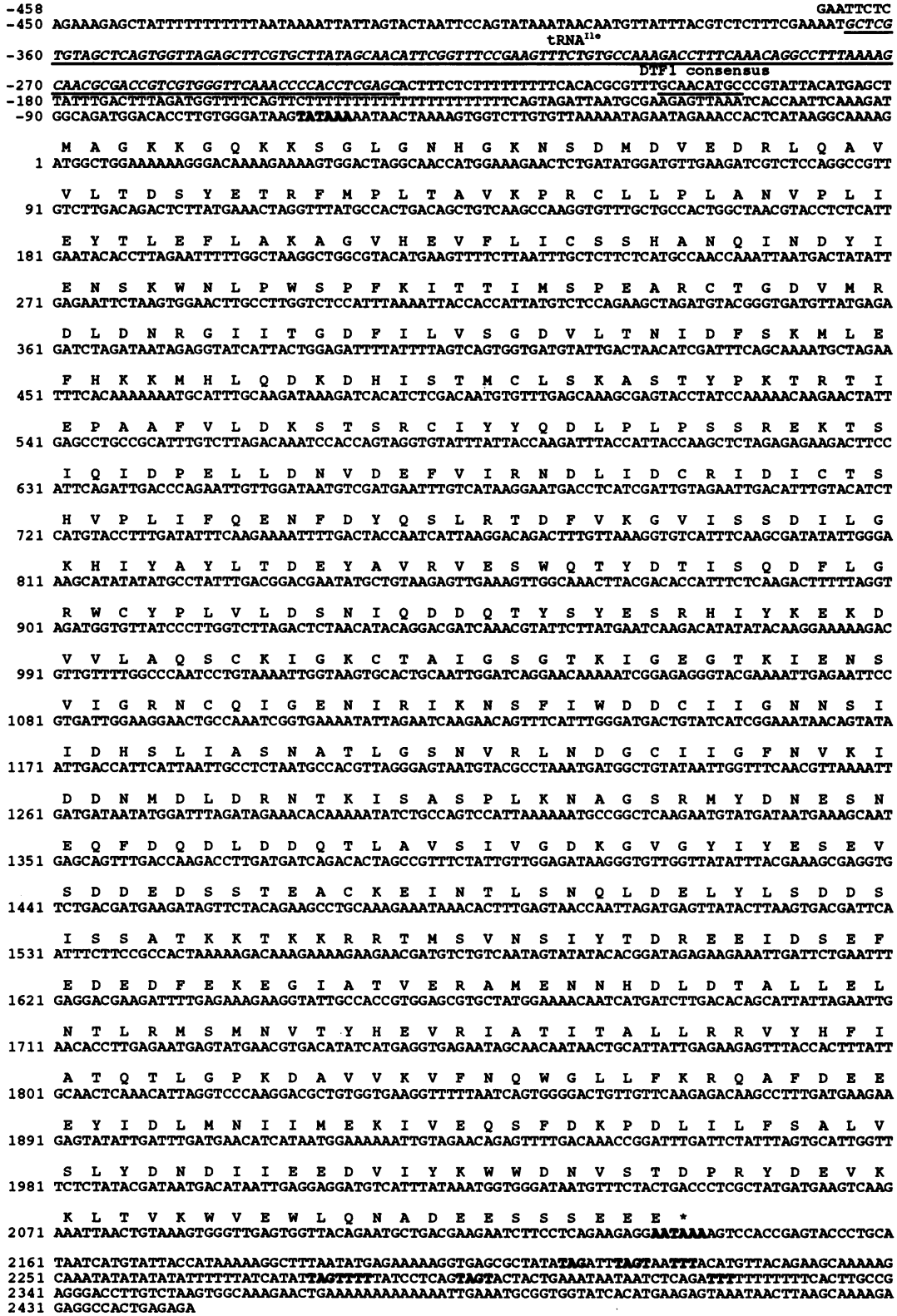


FIG. 4. Nucleotide sequence of the *GCD6* region. The nucleotide sequence is numbered relative to the putative translation initiation codon (+1) of the *GCD6* ORF. The deduced amino acid sequence of GCD6 is given above the coding region in single-letter code. The $tRNA^{Ile}$ sequence present upstream of *GCD6* is italicized and underlined. The region from positions −239 to −149 is necessary for full complementation of the *gcd6-1* mutation; compare pJB71 with pJB25 in Fig. 3A. Underlined within this region is the consensus sequence for the binding site of *Drosophila* transcription factor 1 (46); although we are aware of no corresponding yeast factor, it is interesting that this 9-bp sequence is also present upstream from the *GCD7* ORF. A potential TATA box sequence (11) is present beginning at nucleotide −64.

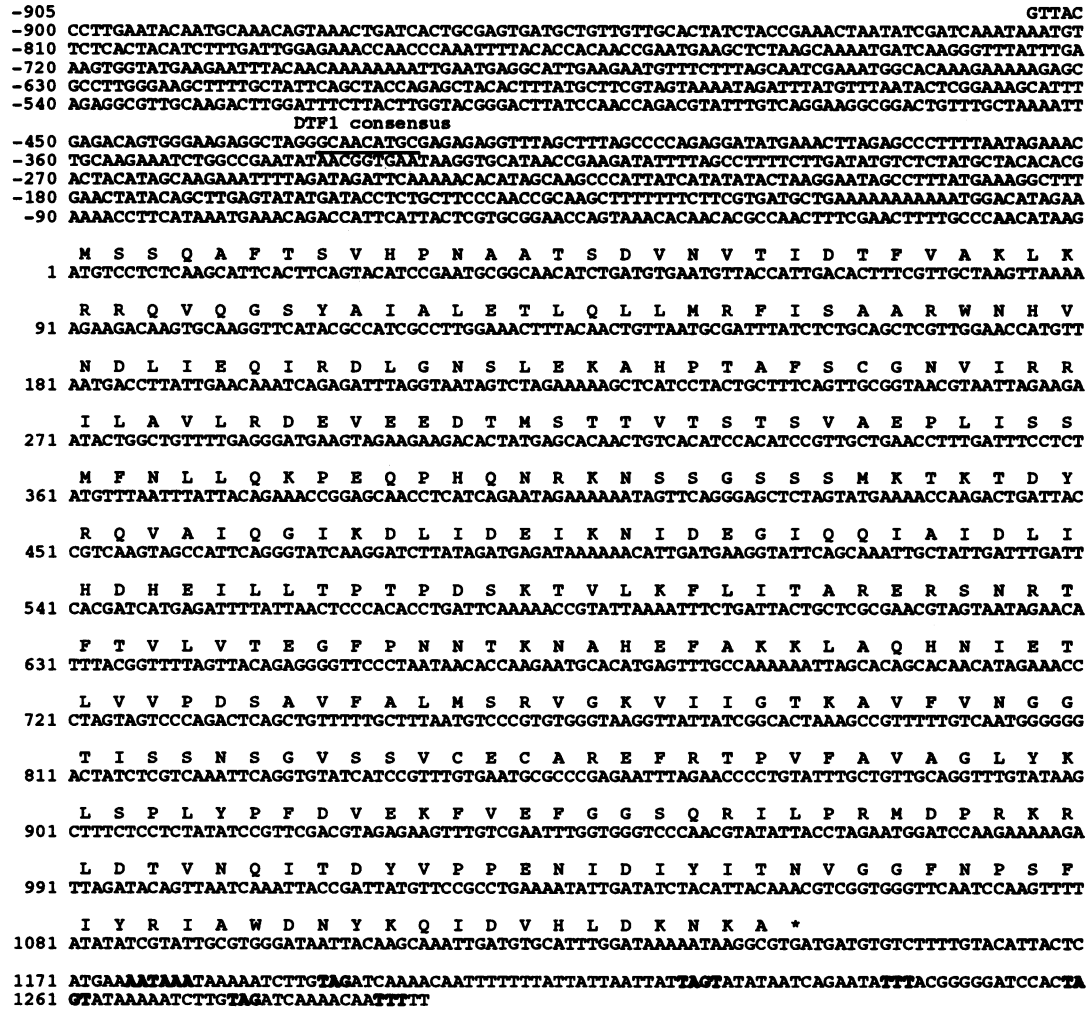


FIG. 5. Nucleotide sequence of the *GCD7* region. The nucleotide sequence is numbered relative to the putative translation initiation codon (+1) of the *GCD7* ORF. The deduced amino acid sequence of *GCD7* is shown above the coding region in single-letter code. The DTF-1 consensus binding site also present upstream of GCD6 begins at nucleotide −428 (underlined). There are several AT-rich stretches but no clear TATAAA consensus sequence in the putative promoter region. The first ATG in frame with the GCD7 ORF was designated +1 and is presumed to be the translational start site. The *GCD7* ORF extends for 381 amino acids, and the encoded polypeptide is predicted to be 43 kDa and to have an isoelectric point of 8.0. A putative polyadenylation site, AATAAA (boldface), begins at +1176, 30 nucleotides downstream of the TGA stop codon (indicated by an asterisk). Sequence elements corresponding to the *S. cerevisiae* tripartite transcription termination signal TAG...TAGT...TTT are also shown in boldface (47, 58).

relative to the other. A best fit alignment of the amino acid sequences of GCD6 and eIF-2Bε shown in Fig. 7B reveals 30% identity and 55% similarity between the two proteins, with the greatest similarity occurring at their amino termini. The quality score for GCD6 aligned with eIF-2Bε was 36 standard deviations above the mean score for the randomized sequences, indicating a very high level of statistical significance for the sequence similarity between GCD6 and eIF-2Bε.

Overall, the sequences of rabbit eIF-2Bε and GCD1 are only 18% identical; however, amino acids that are conserved between GCD6 and GCD1 are also conserved in eIF-2Bε more frequently than would be expected from the 30% identity between GCD6 and eIF-2Bε (data not shown). Perhaps these conserved residues contribute to a structural feature or biochemical function which all three proteins have in common. Furthermore, the carboxy termini of GCD6, GCD1, and eIF-2Bε are composed of extremely acidic and serine-rich sequences which are potential phosphorylation sites for casein kinases I and II (14, 31, 35). Interestingly, it was reported that casein kinase II phosphorylation of eIF-2Bε increases eIF-2B activity in vitro (10).

The 5′-most ATG in frame with the *GCD6* ORF was designated +1 and is presumed to be the translational start site. The *GCD6* ORF extends for 712 amino acids, and the encoded polypeptide is predicted to be 81 kDa and to have an isoelectric point of 4.5. The extremely low pI reflects the high content of acidic residues in GCD6 (7.4% Glu and 9.7% Asp). The termination codon TAA (indicated by an asterisk) lies within a putative polyadenylation site AATAAA (boldface) (47) beginning at bp 2135. Sequence elements corresponding to the *S. cerevisiae* tripartitetranscription termination signal TAG...TAGT...TTT are also shown in boldface (58).

TABLE 2. Linkage analysis of *GCD7*

Cross	Marker pair	No. of tetrads[a]			Map distance[b] (cM)
		PD	T	NPD	
F431 × H1603[c]	*gcd7 exg1*	98	10	3	13
	gcd7 cdc42	41	67	3	38
	exg1 cdc42	42	64	5	42

[a] For definitions of abbreviations, see footnote *b*.

[b] Calculated by using Perkins' formula (45), Xp = 50(T + 6N)/(P + T + N), where P, N, and D are the numbers of parental ditype (PD), nonparental ditype (NPD), and tetratype (T) tetrads, respectively, and Xp is the map distance in centimorgans.

[c] F431 (*MATa his4 leu2 cdc42 exg1*) was crossed with H1603 (*MATα gcd7-201 leu2-3,112 ura3-52*). The *gcd7-201* allele was identified in ascospore clones by its phenotypes of slow growth and resistance to 0.5 mM 5-fluoro-DL-tryptophan (40), and the *exg1* and *cdc42* alleles were identified as described previously (7, 28).

DISCUSSION

GCD6 and GCD7 function downstream of protein kinase GCN2 in the translational control of *GCN4* expression by amino acid availability. The *gcd6-1* and *gcd7-201* mutations were isolated on the basis of causing increased expression of amino acid-biosynthetic genes subject to general amino acid control. To account for the dependence on *GCN4* function for this phenotype, it was proposed that GCD6 and GCD7 are negative regulators of *GCN4* (39, 40). We provided direct evidence for this hypothesis by demonstrating high-level expression of a *GCN4-lacZ* fusion in *gcd6-1* and *gcd7-201* mutants in the absence of amino acid limitation. In addition, we showed that GCN2 is not required for elevated *GCN4* expression in these *gcd* mutants. Thus, GCD6 and GCD7 appear to act downstream of GCN2 at the same step in the regulatory pathway identified previously for components of the GCD complex and eIF-2 (Fig. 1).

Although it was previously suggested that GCD7 regulates *GCN4* at the level of transcription (39), our measurements of the expression of a *GCN4-lacZ* fusion lacking uORFs indicate that *gcd6-1* and *gcd7-201* affect *GCN4* expression primarily at the translational level. Our experiments do not rule out the possibility that the *gcd6-1* and *gcd7-201* mutations affect the steady-state level of *GCN4* mRNA; however, the fact that they cause little change in *GCN4-lacZ* expression when uORF4 alone is in the leader indicates that their predominant effect is to increase the ability of ribosomes which have translated uORF1 to scan past uORF2 to -4 and reinitiate at *GCN4* instead. Thus, these *gcd* mutations alter ribosomal recognition of the *GCN4* uORFs in the same manner observed with amino acid-starved wild-type cells.

Evidence that GCD6 and GCD7 are subunits of the eIF-2B of *S. cerevisiae*. The GCD complex contains GCD1 and GCD2, two factors that function in translation initiation in *S. cerevisiae* (6, 15, 56), and is physically associated with a fraction of the eIF-2 present in cells. These findings led to the proposal that the GCD complex represents yeast eIF-2B, the GDP-GTP exchange factor for eIF-2 (6). The results of more recent biochemical studies of the GCD complex support this hypothesis (5a). Although direct biochemical experiments will be required to demonstrate an association of GCD6 and GCD7 with other GCD factors, the following observations strongly suggest that GCD6 and GCD7 are additional subunits of the GCD complex.

(i) The *GCD6* and *GCD7* genes were found to be essential. The slow growth phenotype of the *gcd6-1* and *gcd7-201* mutants probably reflects a disruption of the essential functions of these gene products in translation initiation. The GCD1 and GCD2 subunits of the GCD complex are also essential, and nonlethal mutations in these factors which derepress *GCN4* translation cause slow growth (21, 43).

(ii) GCD6 and GCD7 are similar in sequence to other subunits of the GCD complex. GCD7 is most closely related to the regulatory subunit GCN3 but also shares significant similarity with GCD2. GCD6 contains regions of significant similarity with GCD1.

(iii) GCD6 is 30% identical in sequence to the largest subunit of rabbit eIF-2B, the first mammalian subunit of eIF-2B for which a complete amino acid sequence has been obtained. This result lends strong support to our hypothesis that GCD6 is a subunit of a complex that functions as the guanine nucleotide exchange factor for eIF-2 in *S. cerevisiae*. The regions of sequence similarity observed between GCD1 and GCD6 are also conserved between the rabbit subunit of eIF-2B and GCD1, suggesting an important role for the conserved residues in catalysis or regulation of eIF-2B activity.

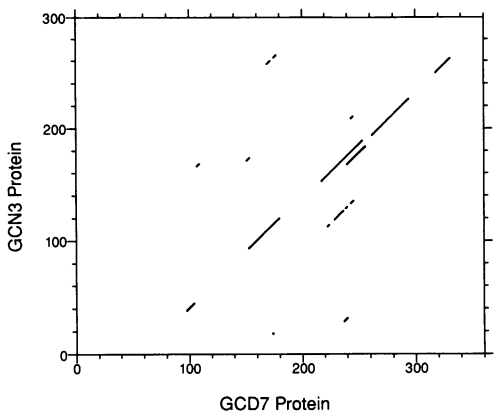
(iv) If GCD6 and GCD7 are included as subunits in the GCD complex, then the number and molecular masses of the subunits (GCN3, 34 kDa; GCD7, 43 kDa; GCD1, 58 kDa; GCD2, 71 kDa; and GCD6, 81 kDa) would correspond closely with the subunit composition of mammalian eIF-2B (α, 34 kDa; β, 40 kDa; γ, 57 kDa; δ, 65 kDa; and ε, 82 kDa [36]).

(v) Mutations in *GCD6*, *GCD7*, *GCD1*, *GCD2*, and genes encoding subunits of eIF-2 (*SUI2* and *SUI3*) all increase *GCN4* translation by the same mechanism involving uORFs that operates in amino acid-starved wild-type cells when eIF-2α is phosphorylated by GCN2. This can be explained by proposing that the GCD factors are subunits of the eIF-2B complex and that the *gcd6-1* and *gcd7-201* mutations lead to constitutive derepression of *GCN4* translation by impairing the ability of eIF-2B to catalyze GDP-GTP exchange on eIF-2. Thus, the common feature of these mutations would be to diminish the level of eIF-2 activity. The resulting decrease in eIF-2 activity would enable ribosomes scanning downstream from uORF1 to avoid reinitiation at uORF2 to -4 and reinitiate at the *GCN4* start site instead (8).

GCN3, GCD7, and GCD2 may mediate contacts between eIF-2B and the α subunit of eIF-2. The sequence similarity among GCN3, GCD7, and GCD2 (Fig. 6B) suggests that these proteins share a structural feature or biochemical function. The only effect of deleting GCN3 from otherwise wild-type cells is to prevent derepression of *GCN4* translation mediated by protein kinase GCN2 (18, 19). This led to the idea that GCN3 would mediate the inhibitory effect of phosphorylated eIF-2 on the catalytic activity of eIF-2B (6, 8). Studies of mammalian systems have demonstrated that phosphorylated eIF-2 forms a stable complex with eIF-2B that is inactive for GDP-GTP exchange on eIF-2 (36, 51, 53). If this mechanism operates in *S. cerevisiae*, then GCN3 might make direct contact with the α subunit of eIF-2 to stabilize the physical interaction between eIF-2B and phosphorylated eIF-2. Because GCN3 is dispensable, additional components of the GCD complex may also interact with the α subunit of eIF-2. Perhaps regions of sequence similarity among GCN3, GCD7, and GCD2 contribute to a recognition surface on eIF-2B for the α subunit of eIF-2.

Regulatory functions of eIF-2B. In translation factor eIF-2B, the cell appears to dedicate five proteins to GDP-GTP exchange on eIF-2, whereas other GTP exchange factors such as the guanine nucleotide releasing factors for Ras proteins (52), the translation elongation factor Ts of *Esch-*

A.



B.

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GCD2   1                                MSESEA KSRSATPPSK AKQATPTTTA AANGEKKLTN KELKELKKQE KAAKRAAMKQ

GCD2  57   ANGISIEQQQ QQAQMKKEKK QLQREQQQKR EQKQKNANKK KQNERNVKKS TLFGHLETTE ERRATILALT SAVSSPKTSR

                                                    V-V
                                          I         T  V
GCN3   1                      MSEFNI T.ETYLRFLE EDTEMTMPIA AIEALVTLLR IKTP...... .....ETAAE MINTIKSSTE
GCD7   1   MSSQAFTSVH PNAATSDVNV TIDTFVAKLK RRQVQGSYAI ALETLQLLMR FISAARW... .....NHVND LIEQIRDLGN
GCD2 137   ITAAGLMVPV VASALSGSNV LTASSLMPVG PNASSTVSAS APASTTTTLP ASSAALSAGT SSASTNTPTA IQQEIASSNA
consensus  .......... ...a.S..Nv t..t.l..l. .....t..a. A.e.l.tllr ..saa..... .....nt... .i..I.ss..

               F      F                        M                              Y
GCN3   55  ELIKSIPNSV S......... .......... LRAGCD.... .......... .....IFMRF VLRNLHLY.. ..........
GCD7   73  SLEKAHPTAF S........C GNVIRRILAV LRDEVEEDTM STTVTSTSVA EPLISSMFNL LQKPEQPH.. ..........
GCD2 217   SDVAKTLASI SLEAGEFNVI PGISSVIPTV LEQSFDNSSL ISSVKELLLN KDLIHPSILL LTSHLAHYKI VGSIPRCIAM
consensus  sl.k..p.s. S......... ......i..v Lr...d.... ...v...... ..li.....l l...l..y.. ..........

                                                                                          K
GCN3   85  ........GD WEN..... .....CKQHL IE........ .......... .......... ......NGQL FVSRAKKSRN
GCD7  133  ........QN RKNSSGSSSM KTKTDYRQVA IQ........ .......... .......... ......GIKD LIDEIKNIDE
GCD2  297  LEVFQIVIKD YQTPKGTTLS RNLTSYLSHQ IDLLKKARPL SVTMGNAIRW LKQEISLIDP STPDKAAKKD LCEKIGQFAK
consensus  .........d ..n..g.... ...t.y.qh. I......... .......... .......... ........kd l...ik....

GCN3  111  KIAEIGVDFI AD.......D DIILVH.GYS RAVFSLLNHA ANKFIR.FRC VVTESRP..S KQGNQLYTLL EQKGIPVTLI
GCD7  171  GIQQIAIDLI HD.......H EILLTPTPDS KTVLKFLITA RERSNRTFTV LVTEGFPNNT KNAHEFAKKL AQHNIETLVV
GCD2  377  EKIELADQLI IDNASTQIEE STTIVTYGSS KVLTELLLHN AISLKKNIKV IVVDSRPLF. .EGRKMAETL RNAGVNVMYA
consensus  .i.eia.dlI .D........ .i.lv..g.S k.v..lL.ha a....r.f.v .VtesrP... k.g...a..L .q.gi.v...

                        K   TK
GCN3  180  VDSAVGAVID K.VDKVFVGA EGV.AESGGI INLVGTYSVG VLAHNARKPF YVVTESHKFV RMFPLSSDDL PMAG......
GCD7  244  PDSAVFALMS R.VGKVIIGT KAVFVNGGTI SSNSGVSSVC ECAREFRTPV FAVAGLYKLS PLYPFDVEKF VEFGGSQRIL
GCD2  455  LITSLDTIFN MDVDYVFLGA HSILSN.GFL YSRAGTAMLA MSAKRRNIPV LVCCESLKFS QRVQLDSVTF NELADPNDLV
consensus  .dsav.a... ..VdkVf.Ga ..v..n.G.i .s..Gt.sv. ..A...r.Pv .vv.es.Kfs ...plds..f .e.g......

GCN3  252  .......... .......... .......... .PPLDFTRRT DDLED..... .......... .......... .......ALR
GCD7  323  .......... .......... .......... .PRMDPRKRL DTVNQ..... .......... .......... ..........
GCD2  534  NIDYENPVER RGNKGALLNQ FIKERKFEKK KLAMENKPKG NKIGGKKGSE GESKDASNEE DSNSKNILDG WQELPSLNIV
consensus  .......... .......... .......... .p.md...r. d......... .......... .......... ..........

                              F                  del
GCN3  269  GPTIDYTAQE YITALITDLG VLTPSAV... ...SEELIKM WYD*
GCD7  337  ..ITDYVPPE NIDIYITNVG GFNPSFIYRI AWDNYKQIDV HLDKNKA*
GCD2  614  NILYDLTPPE YIKKVITEFG ALPPSSVPVI LREYKGSA*
consensus  ....DytppE yI...IT..G .l.PS.v..i .......i.. ..d....
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FIG. 6. Sequence similarity among GCD7, GCN3, and GCD2. (A) A dot plot comparison of the GCD7 and GCN3 amino acid sequences reveals sequence similarity between these two proteins. (B) A three-way alignment of the complete sequences of GCN3, GCD7, and GCD2 was generated by using progressive pairwise alignments. Numbers refer to the positions of the amino acids in the protein sequences relative to the amino termini, periods represent gaps inserted in the sequence, and asterisks occur at the C terminus of each sequence. A consensus sequence is shown below the GCD2 sequence; uppercase letters indicate that three sequences are identical at that position, and lowercase letters indicate that two of the three sequences are identical. Positions of identity among all three sequences are in boldface. For the GCD7 sequence, the GCN3-GCD2 alignment differs from the alignment proposed by Paddon et al. (42) in the amino-terminal region preceding amino acid 129 of GCN3 and amino acid 402 of GCD2. In our alignment, there are more identities in this region, 24 compared with 18 in the previous alignment; however, we have allowed more gaps, 7 compared with only 4. The exact placement of the large GCD2 insert region (GCD2 residues 528 to 615 in our alignment) is also slightly different, but this placement is ambiguous. Amino acid changes in *gcn3*^{c} alleles are shown above the GCN3 sequence, with boldface type indicating alleles that result in the greatest derepression of *GCN4* expression (18). All of the *gcn3*^{c} alleles alter single amino acids except for two: *gcn3*^{c}*-AA25,26VV*, in which the alanines at positions 25 to 26 are replaced by valines, and *gcn3*^{c}*Δ303-305*, in which the last three amino acids of GCN3 are missing. The sequence information for GCD7 and the revised alignment between GCD2 and GCN3 presented here affect the interpretation of certain *gcn3*^{c} alleles mapping in the amino-terminal region from that discussed previously (18).

erichia coli (26), and the eukaryotic translation elongation factor 1β (27) seem to be single polypeptides. None of these simple exchange factors shows obvious similarity to any of the yeast factors we propose to be components of eIF-2B, leaving open the question of which subunit(s) of eIF-2B catalyzes the exchange reaction. One possible reason for the existence of multiple subunits in eIF-2B is to perform regulatory functions. We already suggested that the principal

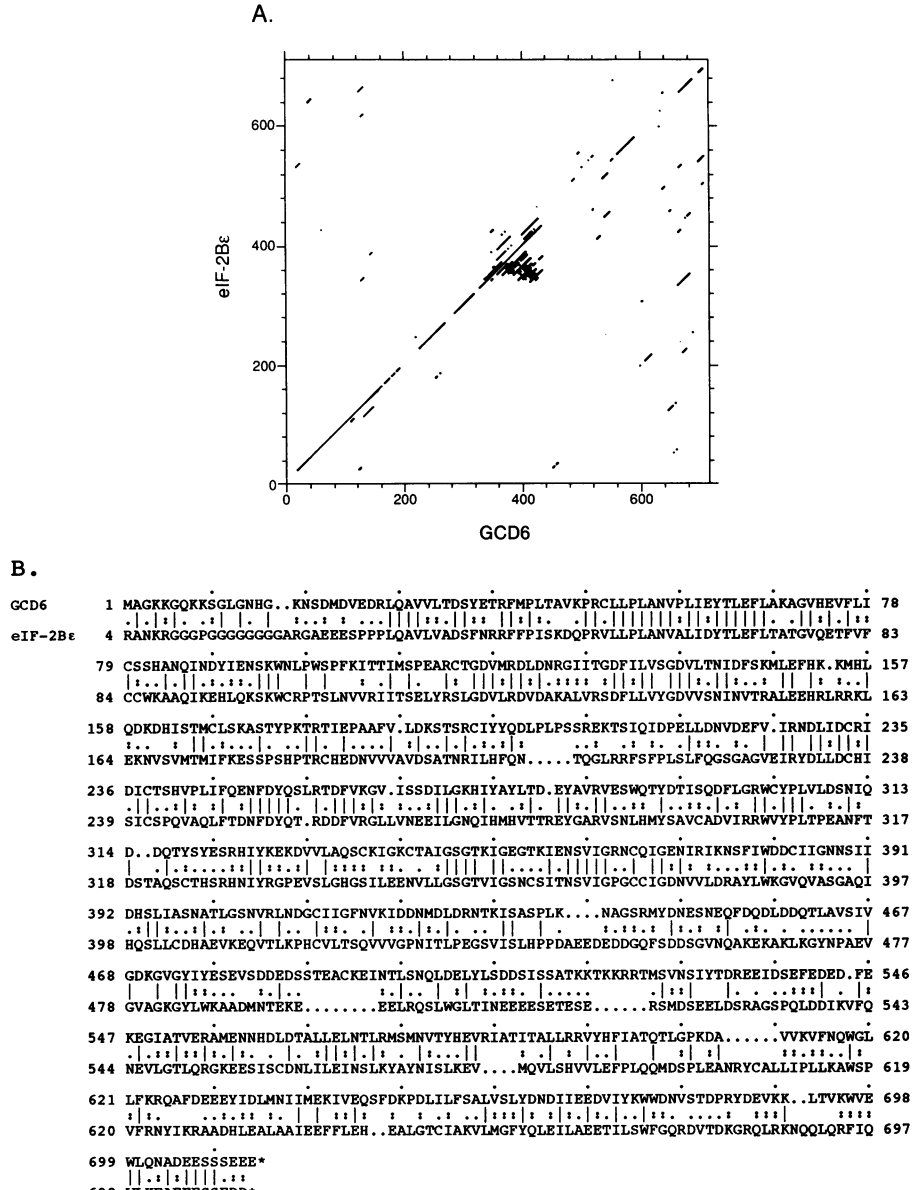

FIG. 7. Amino acid sequence alignment between GCD6 and rabbit eIF-2Bε. (A) A dot plot comparison of the GCD6 and eIF-2Bε amino acid sequences shows extensive similarity between these two proteins. A small central region consists of several diagonal lines rather than the single diagonal expected from colinear sequence similarity. Inspection of the eIF-2Bε and GCD6 sequences revealed that this cluster of lines derives from alternative alignments of overlapping internal repeats that are shared by the two proteins. The GCD1 protein also contains nested internal repeats in this region, which corresponds to one of the stretches of most significant similarity between GCD6 and GCD1 (data not shown). (B) An alignment of the predicted amino acid sequences of the GCD6 and eIF-2Bε proteins was generated by using the best fit program. Numbers refer to the positions of the amino acids in the sequences relative to the amino termini, and the carboxy termini are represented by asterisks. Identities between the two sequences are indicated by lines, conservative replacements with a similarity value higher than 1.0 are indicated by colons, and replacements with a similarity value between 0.5 and 1.0 are indicated by periods. The similarity values used for sequence comparisons are based on evolutionary distance such that amino acids with different physical properties may be considered to be conservative replacements.

role of GCN3 is to mediate the inhibitory effect of phosphorylated eIF-2 on the function of eIF-2B. This inhibitory interaction has been shown to reduce translation in mammalian cells under various stress conditions, including viral infection, heat shock, and hemin deprivation in reticulocytes (33, 50). Studies with metazoans have shown that eIF-2B function can also be regulated independently of eIF-2α phosphorylation. The increase in eIF-2B activity involved in the postfertilization activation of protein synthesis in the sea urchin *Strongylocentrotus purpuratus* may be elicited by changes in redox potential (2); and in mammalian cells, regulation of eIF-2B activity has been postulated to occur in response to glutamine starvation (50) and glucose-6-phosphate levels (17). Perhaps one of the essential subunits of the eIF-2B complex is involved in these regulatory responses. It will be interesting to determine whether *S. cerevisiae* em-

ploys related mechanisms to control eIF-2B activity independent of eIF-2α phosphorylation.

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