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Mutations in the GCD7 Subunit of Yeast Guanine Nucleotide Exchange Factor eIF-2B Overcome the Inhibitory Effects of Phosphorylated eIF-2 on Translation Initiation

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Phosphorylation of the α subunit of eukaryotic translation initiation factor 2 (eIF-2α) impairs translation initiation by inhibiting the guanine nucleotide exchange factor for eIF-2, known as eIF-2B. In *Saccharomyces cerevisiae*, phosphorylation of eIF-2α by the protein kinase GCN2 specifically stimulates translation of *GCN4* mRNA in addition to reducing general protein synthesis. We isolated mutations in several unlinked genes that suppress the growth-inhibitory effect of eIF-2α phosphorylation catalyzed by mutationally activated forms of GCN2. These suppressor mutations, affecting eIF-2α and the essential subunits of eIF-2B encoded by *GCD7* and *GCD2*, do not reduce the level of eIF-2α phosphorylation in cells expressing the activated GCN2^c kinase. Four *GCD7* suppressors were shown to reduce the derepression of *GCN4* translation in cells containing wild-type *GCN2* under starvation conditions or in *GCN2*^{c} strains. A fifth *GCD7* allele, constructed in vitro by combining two of the *GCD7* suppressors mutations, completely impaired the derepression of *GCN4* translation, a phenotype characteristic of deletions in *GCN1*, *GCN2*, or *GCN3*. This double *GCD7* mutation also completely suppressed the lethal effect of expressing the mammalian eIF-2α kinase dsRNA-PK in yeast cells, showing that the translational machinery had been rendered completely insensitive to phosphorylated eIF-2. None of the *GCD7* mutations had any detrimental effect on cell growth under nonstarvation conditions, suggesting that recycling of eIF-2 occurs efficiently in the suppressor strains. We propose that GCD7 and GCD2 play important roles in the regulatory interaction between eIF-2 and eIF-2B and that the suppressor mutations we isolated in these genes decrease the susceptibility of eIF-2B to the inhibitory effects of phosphorylated eIF-2 without impairing the essential catalytic function of eIF-2B in translation initiation.

The best-characterized mechanism for the regulation of general protein synthesis in mammalian cells involves phosphorylation of the α subunit of translation initiation factor 2 (eIF-2) in response to various kinds of stress, including hemin or amino acid limitation and virus infection (reviewed in references 18 and 26). Composed of three different subunits (α, β, and γ), eIF-2 delivers the initiator Met-tRNA$_i^{Met}$ to the small ribosomal subunit in a ternary complex with GTP (eIF-2–GTP–tRNA$_i^{Met}$). In the course of this reaction, the GTP on eIF-2 is hydrolyzed to GDP, and the guanine nucleotide exchange factor known as eIF-2B is required to regenerate eIF-2–GTP from eIF-2–GDP (reviewed in reference 30). eIF-2 that is phosphorylated on the serine residue at position 51 of the α subunit inhibits the recycling activity of eIF-2B, decreasing the level of eIF-2–GTP–tRNA$_i^{Met}$ ternary complexes in the cell and thereby inhibiting general translation initiation (29, 43; reviewed in references 18 and 26).

Phosphorylation of eIF-2α in the yeast *Saccharomyces cerevisiae* mediates gene-specific translational control in addition to a general inhibition of protein synthesis. *S. cerevisiae* cells respond to amino acid limitation by increasing the transcription of more than 30 genes encoding amino acid biosynthetic enzymes. This global response to starvation (general amino acid control) occurs by an increase in the levels of GCN4 protein, which binds upstream of the coregulated genes and coordinately activates their transcription (reviewed in reference 21). GCN4 levels are regulated by a unique translational control mechanism that couples amino acid availability with the level of active eIF-2 (1, 10). When amino acids are abundant, four short open reading frames (uORFs) in the leader of *GCN4* mRNA restrict the flow of scanning ribosomes from the 5′ cap to the *GCN4* AUG codon. According to our model, ribosomes translate the 5′-proximal uORF (uORF1) and resume scanning downstream. Under nonstarvation conditions, levels of the eIF-2–GTP–tRNA$_i^{Met}$ ternary complex are high and ribosomes are rapidly recharged with the ternary complex after translation of uORF1. This allows them to reinitiate translation at one of the remaining uORFs (uORF2 to uORF4), after which they dissociate from the mRNA and fail to reach the *GCN4* start codon. Under conditions of amino acid or purine limitation, the protein kinase GCN2 is activated and phosphorylates the α subunit of eIF-2 on Ser-51 (10, 36). By analogy with mammalian systems, phosphorylation of eIF-2α in yeasts is thought to inhibit the activity of eIF-2B, reducing the rate of GDP-GTP exchange on eIF-2 following the completion of each initiation cycle. We proposed that the resulting decrease in the levels of eIF-2–GTP–tRNA$_i^{Met}$ ternary complexes allows many ribosomes to scan past uORF2 to uORF4 without rebinding initiator tRNA$_i^{Met}$, suppressing recognition of these start sites and allowing reinitiation to occur further downstream at *GCN4* (10).

In support of this model, recent studies have shown that several of the *trans*-acting factors that regulate *GCN4* translation are subunits of a high-molecular-weight complex that is structurally similar to mammalian eIF-2B (7) and possesses guanine nucleotide exchange activity for eIF-2 (6). The yeast equivalent of eIF-2B contains four subunits encoded by the essential genes *GCD1*, *GCD2*, *GCD6*, and *GCD7* (3, 4, 6). The

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fifth subunit, a nonessential protein encoded by *GCN3*, is believed to function primarily in mediating the inhibitory effect of phosphorylated eIF-2 on the nucleotide exchange activity of eIF-2B (9). Thus, deletion of *GCN3* has no effect on cell growth under nonstarvation conditions but prevents derepression of *GCN4* translation in response to eIF-2α phosphorylation by GCN2 in amino acid-starved cells (15). Mutant forms of the GCN3 protein, encoded by *gcn3*^{c} alleles, mimic the effects of eIF-2α phosphorylation in reducing rates of general translation initiation and in derepressing *GCN4* expression (4, 13).

Previously, we reported the isolation of *GCN2* mutations which increase the ability of GCN2 to phosphorylate eIF-2α in the absence of an imposed amino acid starvation, causing constitutive derepression of *GCN4* translation (35, 47). The more potent of these *GCN2*^{c} mutations reduce the cellular growth rate by inhibiting general translation at the initiation step. In all cases, the toxic effects of high-level eIF-2α phosphorylation catalyzed by such GCN2^c kinases were completely reversed by substituting serine 51 in eIF-2α with a nonphosphorylatable alanine residue (9, 10, 35). Deletion of *GCN3* also reduced the growth inhibition associated with eIF-2α hyperphosphorylation by GCN2^c proteins, in accord with the postulated role of GCN3 as a regulatory subunit of yeast eIF-2B (9). The GCN1 protein is needed in addition to GCN2 and GCN3 for increased translation of *GCN4* mRNA in amino acid-starved yeast cells, and it was shown recently that GCN1 is required in vivo for eIF-2α phosphorylation by wild-type GCN2 under starvation conditions (28). The GCN2^c kinases also require GCN1 for efficient eIF-2α phosphorylation in vivo. In contrast, phosphorylation of eIF-2α in yeast cells catalyzed by two different mammalian eIF-2α kinases was found to be completely independent of GCN1 (28). Thus, it appears that GCN1 is specifically required for activation of GCN2; however, the way in which it regulates GCN2 kinase function remains to be determined.

In an effort to identify other proteins involved in eIF-2α phosphorylation or in mediating the inhibitory effects of phosphorylated eIF-2 on translation initiation in yeasts, we have isolated and characterized mutations that suppress the slow-growth phenotype conferred by one of the *GCN2*^{c} alleles. We recently reported a detailed molecular characterization of one group of these suppressor mutations which affect the α subunit of eIF-2 (46). In this report, we describe the isolation of these and other suppressor mutations mapping in six different genes, all of which restore wild-type growth in cells expressing the *GCN2*^{c}*-E532K,E1522K*-activated kinase. We found that suppressor mutations in the positive regulator GCN1 have the expected effect of reducing the level of eIF-2α phosphorylation (data not shown). In contrast, mutations affecting the GCD2 and GCD7 subunits of the eIF-2B complex actually lead to increased levels of eIF-2α phosphorylation and thus resemble the suppressors we isolated in eIF-2α (46). The same phenotype was reported recently for a deletion of the nonessential GCN3 subunit of yeast eIF-2B (9). The *GCD2* and *GCD7* mutations do not appear to affect the catalytic function of eIF-2B in cells incapable of eIF-2α phosphorylation. On the basis of these and other findings presented below, we propose that the *GCD7* and *GCD2* suppressor mutations render the GDP-GTP exchange factor eIF-2B insensitive to the inhibitory effects of eIF-2α phosphorylation and that GCD7 and GCD2 participate with GCN3 in the regulation of eIF-2B function by phosphorylated eIF-2.

MATERIALS AND METHODS

Plasmids. The plasmids we employed carrying wild-type copies of general control regulatory genes on the low-copy-number *URA3* vector YCp50 (34) were as follows: p256 containing the *GCD1* gene (19); p585 containing *GCN2* (47); p597 containing *SUI2* (10); p655 containing *GCN1* (28); Ep69 and p1182 containing *GCN3* and *GCD2*, respectively (13); and pJB5 and pJB99 containing *GCD6* and *GCD7*, respectively (3). Ep293 contains the *GCD11* gene (14) on a 2-kb *Hin*dIII-*Sna*BI fragment (E. Hannig, The University of Texas at Dallas), and p596 contains *SUI3* on a 1.8-kb *Hin*dIII fragment (11).

Plasmids derived from the low-copy-number *URA3* vector pRS316 (45) containing *GCN2*^{c}*-E532K, E1522K* (plasmid p1056), *GCN2*^{c}*-R699W, D918G, E1537K* (plasmid p1053), *GCN2*^{c}*-M719V, E1537G* (plasmid p1052), and *GCN2*^{c}*-E532K, E1537G* (plasmid p1054) were described previously (35), as were the low-copy-number plasmids containing *gcn3*^{c}*-R104K* (plasmid Ep305), *gcn3*^{c}*-V295F* (plasmid Ep306), *gcn3*^{c}*-AA25, 26VV* (plasmid Ep313), *gcn3*^{c}*-A26T* (plasmid Ep314), *gcn3*^{c}*-Δ303-305* (plasmid Ep319), *gcn3*^{c}*-D71N* (plasmid Ep324), and *gcn3*^{c}*-E199K* (plasmid Ep325) (13). Plasmids p1097, p1098, and p1350 carry the *SUI2*, *SUI2-S51A*, and *SUI2-L84F* alleles (10, 46), respectively, on the single-copy-number *LEU2* vector pSB32 (37). Plasmids p1420 and p1421 contain cDNAs encoding wild-type dsRNA-PK and the catalytically inactive mutant dsRNA-PK-K296R, respectively, under the control of a galactose-inducible yeast promoter (9).

Plasmid p1353, a derivative of the single-copy-number *URA3* vector YCp50, was constructed by inserting the 7-kb *Sal*I-*Xba*I fragment obtained from p1056 containing the *GCN2*^{c}*-E532K, E1522K* allele between the *Bam*HI and *Sal*I sites of the vector. p1558 was constructed by isolating the 2.1-kb *Eco*RI-*Spe*I fragment containing *GCD7* from plasmid pJB99, generating blunt ends with T4 DNA polymerase and inserting this fragment between the *Sal*I and *Sac*I sites of the *LEU2* vector pRS315 (45) after making these sites blunt ended with the same polymerase. In the process, the four sites were destroyed and the insert is oriented such that its *Eco*RI site end is adjacent to the modified *Sac*I site in the vector.

Genetic methods and construction of yeast strains. Standard techniques for growth, genetic analysis, and plasmid transformation of yeast strains were performed as described (23, 42). Resistance to 3-amino-1,2,4-triazole (3-AT) was determined by replica plating to SD medium (42) containing 30 mM 3-AT as previously described (22).

The yeast strains we employed or constructed are listed in Table 1. H1175 and H1176 were constructed by tetrad analysis of several genetic crosses between *GCN* strains in our collection and a *gcn1-1* mutant kindly provided by Peter Niederberger and Ralf Hütter, scoring the *gcn1-1* mutation (41) by sensitivity to 3-AT. H1627 and H1641 are Leu$^-$ 3-AT-resistant (3-AT^R) ascospores derived from a cross between H750 and H1613. H1691 was obtained by transforming H1641 to Leu$^+$ with the 2-kb *Sal*I-*Xho*I fragment that contains the *LEU2* gene isolated from vector YEp13 (2). H1836 is a Ura$^+$ 3-AT^R segregant from a cross between H1627 and H1834 that contains the *GCN2*^{c}*-E532K,E1522K* allele in combination with the *GCD7::URA3* marked allele. ED190 carries *GCN2*^{c}*-E532K, E1522K* along with the *GCD2::URA3* marked allele and was constructed by transforming H1627 with the integrating plasmid p790 digested with *Bgl*II to direct its integration to the *GCD2* locus. H1857 and H1858 were constructed by transforming, respectively, strains H1627 and H1691 to Ura$^+$ 3-AT-sensitive (3-AT^S) phenotypes with the 3-kb *Bst*EII-*Sna*BI fragment from plasmid p781 containing the *gcn2::URA3*

TABLE 1. Strains used

Strain	Genotype	Reference or source
H601	*MATa lys1 gcn2-101*	17
H602	*MATα lys2 gcn2-101*	17
H741	*MATα lys2 leu2-3 leu2-112 gcn3::LEU2*	32
H742	*MATa lys2 leu2-3 leu2-112 gcn3::LEU2*	32
H750	*MATa leu2-3 leu2-112 ura3-52 gcn2::LEU2*	25
H1145	*MATα ura3-52 gcn1-1 HIS4-lacZ*	This study
H1146	*MATa leu2-3 leu2-112 ura3-52 gcn1-1 HIS4-lacZ*	This study
H1402	*MATα leu2-3 leu2-112 ura3-52 ino1 HIS4-lacZ*	13
H1613	*MATα leu2-3 leu2-112 ura3-52 ino1 GCN2^c-E532K,E1522K HIS4-lacZ*	35
H1627	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K HIS4-lacZ*	This study
H1641	*MATa leu2-3 leu2-112 ino1 ura3-52 GCN2^c-E532K,E1522K HIS4-lacZ*	This study
H1642	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63*	10
H1691	*MATa ura3-52 ino1 GCN2^c-E532K,E1522K HIS4-lacZ*	This study
H1822	*MATa ura3-52 ino1 GCN2^c-E532K,E1522K SUI2-V89I HIS4-lacZ*	This study
H1823	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K SUI2-L84F HIS4-lacZ*	This study
H1824	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K SUI2-R88C HIS4-lacZ*	This study
H1825	*MATa ura3-52 ino1 GCN2^c-E532K,E1522K SUI2-I58M HIS4-lacZ*	This study
H1826	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K GCD7-I348V HIS4-lacZ*	This study
H1827	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K GCD7-I118T HIS4-lacZ*	This study
H1828	*MATa ura3-52 ino1 GCN2^c-E532K,E1522K GCD7-D178Y HIS4-lacZ*	This study
H1834	*MATa leu2-3 leu2-112 ura3-52 gcn2::LEU2 GCD7::URA3*	4
H1836	*MATa leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K GCD7::URA3*	This study
H1857	*MATα leu2-3 leu2-112 ura3-52 gcn2::URA3 HIS4-lacZ*	This study
H1858	*MATa ura3-52 ino1 gcn2::URA3 HIS4-lacZ*	This study
H1895	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcn2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63*	T. E. Dever
H1930	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K GCD2-510 HIS4-lacZ*	This study
H1931	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K gcn1-501 HIS4-lacZ*	This study
H1932	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K GCD7-K329E HIS4-lacZ*	This study
H1933	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K gcn20-1 HIS4-lacZ*	This study
H2116	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcn2Δ gcn3Δ sui2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p919[*SUI2 LEU2*]	46
H2215	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG* p1108 [*GCN4-lacZ TRP1*] at *trp1-Δ63* p1558[*GCD7 LEU2*]	This study
H2216	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ* p1108 [*GCN4-lacZ TRP1*] at *trp1-Δ63* pJB99 [*GCD7 URA3*]	This study
H2218	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* pJB99[*GCD7 URA3*]	This study
H2221	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1558[*GCD7 LEU2*]	This study
H2222	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* pJB99[*GCD7 URA3*]	This study
H2223	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1558 [*GCD7 LEU2*]	This study
H2224	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1559[*GCD7-I118T LEU2*]	This study
H2225	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1560[*GCD7-D178Y LEU2*]	This study
H2226	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1561[*GCD7-K329E LEU2*]	This study
H2227	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1562[*GCD7-I348V LEU2*]	This study
H2228	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1563[*GCD7-I118T,D178Y LEU2*]	This study
H2422	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1558[*GCD7 LEU2*]	This study
H2423	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1559[*GCD7-I118T LEU2*]	This study
H2424	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1560[*GCD7-D178Y LEU2*]	This study
H2425	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1561[*GCD7-K329E LEU2*]	This study
H2426	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1562[*GCD7-I348V LEU2*]	This study
H2427	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 gcd7::hisG gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1563[*GCD7-I118T,D178Y LEU2*]	This study
H2428	*MATa leu2-3 leu2-112 ura3-52 trp1-Δ63 sui2Δ gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1097[*SUI2 LEU2*]	46

Continued on following page

TABLE 1—*Continued*

Strain	Genotype	Reference or source
H2429	*MAT***a** *leu2-3 leu2-112 ura3-52 trp1-Δ63 sui2Δ gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1098[*SUI2-S51A LEU2*]	46
H2430	*MAT***a** *leu2-3 leu2-112 ura3-52 trp1-Δ63 sui2Δ gcn2Δ gcn3Δ* p1108[*GCN4-lacZ TRP1*] at *trp1-Δ63* p1350[*SUI2-L84F LEU2*]	46
H2442	*MAT***a** *ura3-52 ino1 GCN2^c-E532K,E1522K gcn1-502 HIS4-lacZ*	This study
H2443	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K gcn1-503 HIS4-lacZ*	This study
H2444	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K gcn1-504 HIS4-lacZ*	This study
H2445	*MAT***a** *ura3-52 ino1 GCN2^c-E532K,E1522K gcn3-501 HIS4-lacZ*	This study
H2446	*MAT***a** *ura3-52 ino1 GCN2^c-E532K,E1522K gcn1 HIS4-lacZ*	This study
H2447	*MAT***a** *ura3-52 ino1 GCN2^c-E532K,E1522K gcn1 HIS4-lacZ*	This study
H2448	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K gcn3 HIS4-lacZ*	This study
H2449	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K gcn3 HIS4-lacZ*	This study
ED190	*MATα leu2-3 leu2-112 ura3-52 GCN2^c-E532K,E1522K GCD2::URA3 HIS4-lacZ*	This study
TD14-2	*MAT***a** *leu2-3 leu2-112 ura3-52 ino1 HIS4-lacZ SUI2::URA3*	T. E. Dever
TD46-1	*MATα leu2-3 leu2-112 ura3-52 trp1-Δ63 (GCN4-lacZ TRP1) SUI2::URA3*	T. E. Dever

allele. Replacement of *GCN2^c* with the *gcn2::URA3* allele in each strain was confirmed by complementation analysis with *gcn2-1* tester strains.

The *gcd7::hisG* deletion allele was introduced into transformants of strains H1642 and H1895 bearing the *GCD7 LEU2* plasmid p1558 by using the *Eco*RI-*Sph*I fragment from plasmid pJB110 (3) that carries the *gcd7::URA3* allele (the *URA3* gene is flanked by *hisG* direct repeats). After selection of Ura$^+$ transformants, segregants resistant to 5-fluoroorotic acid were isolated, creating strains H2215 and H2216. H2216 was transformed with the *URA3 GCD7* plasmid pJB99 and grown on medium containing leucine to permit loss of p1558, yielding the Leu$^-$ Ura$^+$ strain H2218. H2222 was constructed by introducing unmarked *gcn2* and *gcn3* deletion alleles into H2215 by using plasmids p1144 (10) and p1143 (9), respectively, to create strain H2221, and then replacing plasmid p1558 with pJB99, as described above. Strains H2428 (wild-type *SUI2*), H2429 (*SUI2-S51A*), and H2430 (*SUI2-L84F*) are isogenic to H2116 and contain the *SUI2* alleles on the *LEU2* plasmids p1097, p1098, and p1350, respectively, as the only copy of *SUI2*.

Isolation and characterization of suppressors of the slow-growth phenotype of *GCN2^c-E532K,E1522K*. Strains H1627 and H1691 were streaked for individual colonies on YEPD medium (42), and a large number of colonies from each strain were restreaked on the same medium to identify independent clones in which the slow-growth phenotype (Slg$^-$) of *GCN2^c-E532K,E1522K* had reverted to wild-type growth (Slg$^+$). The revertants were identified as large, fast-growing colonies in a background of smaller, more slowly growing cells. Each revertant was streaked for single colonies on YEPD two consecutive times to purify it and to compare its growth rate with that of wild-type *GCN2* strain H1402. Revertants that grew more slowly than H1402 were discarded.

Revertants with a 3-AT^S phenotype were subjected to genetic complementation analysis to test for allelism between the suppressor mutations in the different revertants and the *gcn1*, *gcn2*, or *gcn3* mutations in the tester strains H1145, H1146, H601, H602, H741, and H742. Diploids formed between the revertants or their wild-type parental strains and each of the tester strains were analyzed for growth on SD plates containing 3-AT.

Revertants with a 3-AT-resistant (3-AT^R) or leaky 3-AT-sensitive (3-AT^L) phenotype were analyzed for complementation of their Slg$^+$ phenotype by transformation with low-copy-number *URA3* plasmids containing wild-type alleles of different general control regulatory genes (Table 2). For each revertant, three independent Ura$^+$ transformants for each plasmid were streaked for single colonies on minimal SD plates lacking uracil and the sizes of the resulting colonies were compared with those of transformants of the same revertants and of the parental *GCN2^c-E532K,E1522K* strain bearing vector alone. Reappearance of the slow-growth phenotype characteristic of *GCN2^c-E532K,E1522K* in all three transformants containing a given plasmid-borne gene was taken as an indication that the revertant carried a recessive chromosomal allele of that gene. When appropriate, complementation of 3-AT^L or 3-AT^S phenotypes was tested in the same transformants.

Genetic analysis of mutations in *GCD2*, *GCD7*, and *SUI2*. Revertants believed to contain mutations in *GCD2* (H1930), *GCD7* (H1826, H1827, H1828, and H1932), or *SUI2* (H1822, H1823, H1824, and H1825) were crossed with the parental strains H1627 or H1691, and 10 to 12 tetrads from each cross were analyzed. In all cases, we observed 2+:2− segregation

TABLE 2. Genetic identification of suppressors of the Slg$^-$ phenotype of *GCN2^c-E532K,E1522K* by complementation analysis

Locus of suppressor mutation	No. of Slg$^+$ revertants identified[a]			
	AT^S	AT^L	AT^R	Total
GCN1	33			33
GCN2	105			105
GCN3	10			10
GCD1				0
GCD2			H1930	1
GCD6				0
GCD7		H1826, H1827	H1828, H1932	4
GCD11				0
SUI2	H1822, H1823	H1825	H1824	4
SUI3				0
Unidentified		9	21	30

[a] The identities of *GCN1*, *GCN2*, and *GCN3* suppressor mutations in the 3-AT^S revertants were established by genetic complementation analysis with *gcn1*, *gcn2*, and *gcn3* tester strains. The identities of the suppressor mutations in the remaining revertants listed by name were established by complementation analysis with plasmids carrying wild-type copies of the indicated genes and by linkage analysis with suitably marked strains (see text for details). Plasmid complementation experiments were also carried out to confirm the assignment of the *GCN1*, *GCN2*, and *GCN3* suppressors in the 3-AT^S revertants.

for the Slg phenotype, indicative of a single nuclear suppressor present in each revertant. Revertant H1930 (*GCN2^c-E532K, E1522K GCD2-510*) was crossed to strain ED190 (*GCN2^c-E532K,E1522K GCD2::URA3*), and allelism between the suppressor and the *GCD2* gene was confirmed by observing 2+:2− segregation for both Slg and Ura phenotypes and the absence of Slg$^+$ Ura$^+$ and Slg$^-$ Ura$^-$ recombinant spores in 10 tetrads. For the *GCD7* suppressors, revertants H1826 (*GCN2^c-E532K,E1522K GCD7-I348V*), H1827 (*GCN2^c-E532K,E1522K GCD7-I118*) and H1932 (*GCN2^c-E532K, E1522K GCD7-K329E*) were crossed to strain H1836 (*GCN2^c-E532K,E1522K GCD7::URA3*), and all 10 tetrads analyzed from each cross were parental ditypes (two Slg$^+$ Ura$^-$ and two Slg$^-$ Ura$^+$ spores). The suppressor in revertant H1828 (*GCN2^c-E532K,E1522K GCD7-D178Y*) was shown to be allelic to *GCD7* by crossing H1828 to H1826 and H1827, proven to contain suppressors mapping at *GCD7*, and observing 4+:0− segregation for the Slg phenotype in a total of 23 tetrads analyzed from the two crosses. Finally, for the *SUI2* revertants, H1822 (*GCN2^c-E532K,E1522K SUI2-V89I*) and H1825 (*GCN2^c-E532K,E1522K SUI2-I58M*) were crossed to TD14-2 (*GCN2 SUI2::URA3*), and revertants H1823 (*GCN2^c-E532K, E1522K SUI2-L84F*) and H1824 (*GCN2^c-E532K,E1522K SUI2-R88C*) were crossed to TD46-1 (*GCN2 SUI2::URA3*). As expected for a suppressor at *SUI2*, we observed 4+:0−, 3+:1−, and 2+:2− segregation for the Slg phenotype and 2+:2− segregation of the Ura phenotype, with all the Slg$^+$ spores being Ura$^-$.

Cloning and sequence analysis of *GCD7* suppressor alleles. The four *GCD7* suppressor mutations were cloned by PCR with primers that hybridized 186 nucleotides upstream of the ATG (5′-GCCAGATCTGGCTTTGAACTATACAGCTTG AGT-3′, containing a *Bgl*II restriction site) and 115 nucleotides downstream of the stop codon (5′-GCCCTCAGACTAGTG GATCCCCCGTAAATATTCTG-3′, containing a *Xho*I restriction site) of the *GCD7* gene. The 1.5-kb amplified fragments were digested with *Bgl*II and *Xho*I and inserted between the *Bam*HI and *Xho*I sites of the vector pRS315. Plasmids Jp162, Jp164, Jp166, and Jp168 contain the PCR-amplified *Bam*HI-*Xho*I fragments isolated from revertants H1826, H1827, H1828, and H1932, respectively. The complete coding region of *GCD7* in these four plasmids was sequenced (40) with specific oligonucleotide primers. The authenticity of the observed *GCD7* mutations was confirmed by sequencing several independent plasmids derived from two different PCRs for each suppressor allele analyzed.

DNA fragments containing each of the cloned *GCD7* suppressor mutations were subcloned into a plasmid-borne copy of wild-type *GCD7*, as follows. The 205-bp *Xba*I-*Sac*I fragment from Jp164, the 187-bp *Sac*I-*Nru*I fragment from Jp166, and the *Ava*I-*Xho*I (present in the multiple cloning region of the vector) fragments from Jp162 and Jp168 were used to replace the corresponding fragments in the wild type *GCD7* gene carried on the low-copy-number *LEU2* plasmid p1558 to create, respectively, plasmids p1559 (*GCD7-I118T*), p1560 (*GCD7-D178Y*), p1561 (*GCD7-K329E*), and p1562 (*GCD7-I348V*). Plasmid p1563 was constructed by combining the 205-bp *Xba*I-*Sac*I from Jp164 and the 187-bp *Sac*I-NruI fragment from Jp166 in the same gene, generating the *GCD7-I118T,D178Y* allele.

Yeast strains H2218 and H2222 were transformed to Leu$^+$ with these plasmids and the transformants were transferred to medium containing 5-fluoroorotic acid to evict the preexisting *URA3* plasmid carrying wild-type *GCD7*. This resulted in a set of isogenic strains containing the appropriate *GCD7* suppressor allele on the *LEU2* vector as the only copy of *GCD7* in the cell, in combination with either *gcn2*Δ (strains H2223, H2224, H2225, H2226, H2227, and H2228) or *gcn2*Δ *gcn3*Δ chromosomal mutations (strains H2422, H2423, H2424, H2425, H2426, and H2427).

Analysis of *GCN4-lacZ* expression. β-Galactosidase activity in whole-cell extracts was assayed as described previously (27) after strains were grown in SD medium. For repressing conditions, saturated cultures were diluted 1:50 and grown for 6 h to mid-logarithmic phase. For derepressing conditions, cultures were grown as just indicated for 2 h and then for 6 h after addition of 3-AT to 10 mM.

Isoelectric-focusing gel electrophoresis. Growth of yeast strains, preparation of samples, vertical slab gel isoelectric focusing, and detection of eIF-2α by immunoblot analysis with antiserum prepared against a trpE–eIF-2α fusion protein (8) and ^{125}I-labelled protein A were carried out as described by Dever et al. (10).

RESULTS

Isolation of suppressors of the slow-growth phenotype of a constitutively activated *GCN2^c* allele. Yeast strains containing the *GCN2^c-E532K,E1522K* allele exhibit a slow-growth phenotype on rich medium because of hyperphosphorylation of eIF-2α and concomitant inhibition of general translation initiation (35). In an effort to identify genes whose products are involved in translational control by eIF-2 phosphorylation, we isolated fast-growing revertants of strains H1627 and H1691 harboring the *GCN2^c-E532K,E1522K* allele integrated into the chromosome. A total of 187 independent revertants that grew on rich medium (YEPD) at the same rate as the wild-type *GCN2* strain H1402 were isolated; this indicated that they contained mutations that completely suppressed the growth defect associated with *GCN2^c-E532K,E1522K*.

To determine whether any of the revertants contained mutations that impaired translational derepression of *GCN4* in response to amino acid starvation, we analyzed their growth on minimal medium (SD) containing the inhibitor of histidine biosynthesis, 3-AT. Derepression of *GCN4* and histidine biosynthetic genes under its control is required for growth in the presence of 3-AT. We found that most of the revertants (150 of 187) exhibited a 3-AT^S phenotype that is characteristic of *gcn* mutants. An additional 12 revertants showed leaky sensitivity to 3-AT (3-AT^L), and the remaining 25 mutants were resistant to 3-AT (3-AT^R) (Table 2).

The large number of revertants we obtained with a 3-AT^S or 3-AT^L phenotype was expected because the known *GCN* genes are dispensable for cell viability and are required both for derepression of *GCN4* (20) and for the slow-growth phenotype associated with *GCN2^c* alleles (35). Thus, it was already shown that deletion of *GCN1* or *GCN3* suppresses the slow-growth phenotype of the *GCN2^c-E532K,E1522K* allele (28, 35). Moreover, loss-of-function mutations in *GCN2* itself would be expected to produce 3-AT^S revertants that grow like the wild type on rich medium. To test whether the revertants contained suppressor mutations in any of these three *GCN* genes, we tested each revertant for the ability to complement the 3-AT^S phenotype of known *gcn1*, *gcn2*, and *gcn3* mutants (see Materials and Methods). The results of this complementation analysis suggested that 70% of the 150 3-AT^S revertants contained intragenic suppressor mutations at *GCN2*, 22% contained *gcn1* mutations, and 7% contained *gcn3* mutations (Table 2). Given that *gcn1* and *gcn3* deletion mutants have the same phenotype as the 43 revertants assigned to the *GCN1* and *GCN3* complementation groups, we presumed that these revertants carry loss-of-function *gcn1* or *gcn3* alleles and chose

not to study them further. Nor did we characterize the large number of revertants placed in the *GCN2* complementation group. Interestingly, two of the 3-AT^S revertants, H1822 and H1823, complemented all three of the *gcn* tester strains we used, suggesting that they carry suppressor mutations in some other gene that is required for derepression of *GCN4* in response to phosphorylation of eIF-2. We verified that the suppressor mutations in H1822 and H1823 were recessive for their 3-AT^S phenotypes by crossing each mutant with a *GCN2^c-E532K,E1522K* strain of opposite mating type and showing that the resulting diploids were 3-AT^R. The suppressor mutations contained in H1822 and H1823 are discussed further below.

Analysis of suppressor strains that do not abolish derepression of *GCN4* expression in the presence of *GCN2^c-E532K, E1522K*. For the 3-AT^R and 3-AT^L revertants, we used a different complementation test to identify suppressor mutations mapping in genes already known to affect the general control response. The 37 3-AT^R and 3-AT^L revertants, along with the 3-AT^S revertants H1822 and H1823 described above, were transformed with low-copy-number plasmids carrying different *GCN*, *GCD*, or *SUI* genes. (*SUI2* and *SUI3* encode the α and β subunits of eIF-2, respectively.) The resulting transformants were tested for complementation of the suppressor mutations, as indicated by restoration of the Slg$^-$ phenotype characteristic of the *GCN2^c-E532K,E1522K* allele. We found that revertant H1930 recovered the Slg$^-$ phenotype of the *GCN2^c* allele when transformed with the plasmid bearing *GCD2* but retained the Slg$^+$ suppressor phenotype when transformed with each of the other plasmids. Similarly, the Slg$^+$ suppressor phenotype in the four revertants H1826, H1827, H1828, and H1932 was complemented specifically by the plasmid bearing *GCD7*, and the suppressor mutations in the four revertants H1822, H1823, H1824, and H1825 were complemented by the plasmid bearing *SUI2* (Table 2). Note that the last group contains the 3-AT^S revertants H1822 and H1823 described above that were not assigned to the *GCN1*, *GCN2*, or *GCN3* complementation groups. These results suggested that we had isolated mutations in the subunits of eIF-2B encoded by *GCD2* and *GCD7* and the α subunit of eIF-2 that overcome the inhibitory effects of the *GCN2^c-E532K,E1522K* allele on general translation initiation.

To confirm that the nine revertants whose Slg$^+$ phenotypes were complemented by plasmid-borne copies of *GCD2*, *GCD7*, or *SUI2* all contain single nuclear suppressor mutations, we crossed each mutant to the *GCN2^c-E532K,E1522K* parental strain of the opposite mating type and analyzed segregation of the suppressor phenotype in the meiotic products. In all cases, the Slg$^+$ phenotype segregated 2+:2− in a minimum of 10 tetrads tested. For those crosses involving revertants with a 3-AT^S or 3-AT^L phenotype, these phenotypes cosegregated with the Slg$^+$ suppressor phenotype. To prove that the suppressor mutations map in the particular genes implicated by plasmid complementation tests, we crossed each of the nine revertants with a strain containing a *URA3*-marked allele of the gene under consideration (e.g., *GCD7::URA3* in the case of the *GCD7* suppressors) and analyzed segregation of the Ura$^+$ and Slg$^+$ phenotypes in the meiotic products. In each case, we observed no recombination between the Slg$^+$ suppressor phenotype and the Ura$^+$ phenotype among the ascospore segregants, confirming that the revertant strains contained mutations in *GCD2*, *GCD7*, or *SUI2*, in accord with the results of the plasmid complementation tests (see Materials and Methods for details).

To determine whether the mutations in *GCD2*, *GCD7*, and *SUI2* were fully recessive for their suppressor phenotypes, we compared the rate of colony formation on rich medium for each mutant strain transformed by its complementing wild-type gene with that of isogenic *GCN2* and *GCN2^c-E532K, E1522K* strains transformed with vector alone. The resulting transformants of strains H1823 and H1825 bearing the *SUI2* plasmid, strains H1828 and H1932 bearing the *GCD7* plasmid, and strain H1930 containing plasmid-borne *GCD2* formed colonies at the same rate as the parental *GCN2^c-E532K, E1522K* strain, indicating that these five revertants contain fully recessive suppressor mutations. In contrast, the appropriate transformants of strains H1822, H1824, H1826, and H1827 formed colonies at a rate intermediate between that observed for the wild-type and *GCN2^c-E532K,E1522K* control strains, indicating that the mutations in these strains are semidominant for their suppressor phenotype.

TABLE 3. Genetic identification of 3-AT^R and 3-AT^L suppressors of the Slg$^-$ phenotype of *GCN2^c-E532K,E1522K* by meiotic analysis[a]

Recombination group	Locus of gene	No. (identity) of revertants
I	*GCN2*	24
II	*GCN1*	4 (H1931, H2442, H2443, H2444)
III	*GCN3*	1 (H2445)
IV	*GCN20*	1 (H1933)

[a] 3-AT^R and 3-AT^L revertants of opposite mating types containing suppressor mutations that were not complemented by plasmid-borne wild-type genes were crossed together, and the resulting diploids were sporulated. Segregation of the Slg phenotype in the meiotic progeny was analyzed in 10 to 12 tetrads from each cross to determine whether or not the two revertants from each cross contained linked suppressor mutations (see text for details).

We next turned our attention to the 30 3-AT^R and 3-AT^L revertants whose Slg$^+$ phenotype was not complemented by any of the plasmid-borne genes. To determine the number of suppressor genes that were altered in this set of revertants, we crossed revertants in one mating type with those of the opposite mating type and analyzed 10 tetrads from each cross to determine the frequency of recombination between the two suppressor mutations in each cross. The occurrence of approximately 25% Slg$^-$ recombinant ascospores among the meiotic progeny was taken to indicate that the two suppressor mutations in the cross mapped to unlinked genes, whereas crosses involving suppressors mapping in the same gene yielded only Slg$^+$ ascospores. The results of this analysis indicated that the 30 remaining suppressor mutations mapped to four unlinked loci (recombination groups I to IV [Table 3]). Subsequently, we confirmed that nine of the group I revertants and all of the revertants from groups II to IV contained single suppressor mutations by crossing them to the *GCN2^c-E532K,E1522K* parental strains of opposite mating type and observing 2+:2− segregation for the Slg phenotype in all tetrads analyzed from each cross.

One explanation for revertants with 3-AT^R Slg$^+$ phenotypes which were not complemented by any of the cloned genes would be an alteration in the *GCN2^c-E532K,E1522K* allele that lowers its kinase activity enough to restore a normal growth rate on rich medium but not enough to prevent derepression of *GCN4* to levels which confer resistance to 3-AT. In fact, this phenotype has been described for *GCN2^c* alleles containing a single mutation in any of three regions of the GCN2 protein, including the kinase domain, the His-RS-related domain, and the C-terminal segment of GCN2 (35). To test this possibility, we crossed revertants from groups I to IV to *gcn2Δ* strains H1857 or H1858 and analyzed the resulting meiotic progeny from each cross for segregation of the Slg and 3-AT pheno-

types. For all nine crosses involving revertants in group I, we obtained no Slg$^-$ progeny and 2+:2− segregation for the 3-AT phenotype in every tetrad examined (83 analyzed in total). These were the results expected for intragenic suppressor mutations at *GCN2*, and accordingly, the revertants in recombination group I were not studied further. For the corresponding crosses involving revertants in the other three recombination groups, we observed 4+:0−, 3+:1−, and 2+:2− segregation of the Slg phenotype in different asci, indicative of frequent recombination between the suppressor mutation and the *GCN2^c-E532K,E1522K* allele. The latter results indicated that the mutations in recombination groups II to IV were unlinked suppressors of the *GCN2^c* allele (Table 3).

To test the possibility that the suppressors in groups II to IV were dominant mutations in *GCN1* or *GCN3* that cannot be complemented by the cloned wild-type alleles, we crossed the four revertants in group II to the *GCN2^c-E532K,E1522K gcn1* strains H2446 or H2447 (which are Slg$^+$ and 3-AT^S) and analyzed the meiotic progeny. We observed no Slg$^-$ ascospores and 2+:2− segregation for the 3-AT phenotype in 27 tetrads obtained from the four different crosses. On the basis of these results, we concluded that the four revertants in group II contain 3-AT^R alleles of *GCN1* which are dominant to wild-type *GCN1* for suppression of the Slg$^-$ phenotype of *GCN2^c-E532K,E1522K*. By a similar analysis with the *GCN2^c-E532K,E1522K gcn3* strains H2448 and H2449, we concluded that the single revertant in group III contains a 3-AT^R allele of *GCN3* that is dominant for its ability to suppress the Slg$^-$ phenotype of the *GCN2^c* mutation. Finally, the mutation contained in the single group IV revertant H1933 was judged to be recessive for its suppressor phenotype in a diploid constructed by crossing it with a *GCN2^c-E532K,E1522K* strain. On the basis of the fact that the Slg$^+$ suppressor in H1933 is recessive but cannot be complemented by any of the plasmid-borne genes we tested, we concluded that it harbors a suppressor mutation in an unknown gene, henceforth designated *GCN20*. We recently cloned *GCN20* and verified that it is not identical to any previously described yeast gene (data not shown).

In summary, our genetic analysis of suppressor mutations that overcome the inhibitory effects on cell growth conferred by genetic activation of the eIF-2α kinase GCN2 has identified mutations in the essential subunits *GCD2* and *GCD7* and the regulatory subunit *GCN3* of the guanine nucleotide exchange factor eIF-2B. Four suppressors affect the α subunit of eIF-2, the substrate of GCN2 kinase, and extensive genetic and molecular characterization of these mutations was presented recently (46). We also isolated dominant and recessive suppressor mutations in the positive regulator GCN1 and a single mutation in a novel gene we named *GCN20*. The molecular basis for suppression of *GCN2^c-E532K,E1522K* by the single-mutation mapping in *GCN20* will be the subject of a future report. Below, we present a detailed analysis of the suppressor mutations mapping in *GCD7*.

Suppressor mutations in the GCD2 and GCD7 subunits of the eIF-2B complex do not reduce eIF-2α phosphorylation. We considered it unlikely that the suppressor mutations mapping in the GCD2 and GCD7 subunits of eIF-2B would overcome the toxicity of the *GCN2^c-E532K,E1522K* kinase by reducing the level of eIF-2α phosphorylation. To test this presumption, we used isoelectric-focusing gel electrophoresis to resolve eIF-2α isoforms that differ by phosphorylation on Ser-51 and then immunoblot analysis with antibodies against eIF-2α to visualize the two isoforms. In accord with previous results (35), the *GCN2^c-E532K,E1522K* allele in the parental strain led to a relatively high level of eIF-2α phosphorylation that was largely

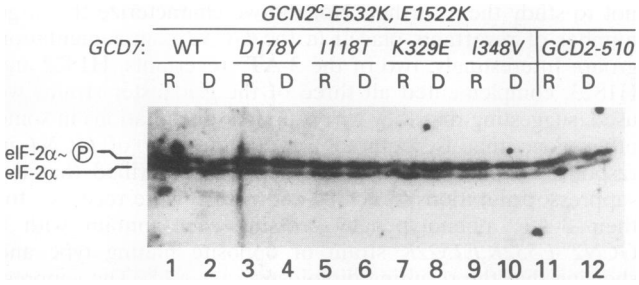

FIG. 1. Isoelectric focusing gel electrophoresis of eIF-2α from strains containing *GCN2^c-E532K,E1522K* and different *GCD7* or *GCD2* suppressor alleles. Strains H1927, H1828, H1827, H1932, H1826, and H1930 containing *GCN2^c-E532K,E1522K* and the indicated *GCD7* or *GCD2* alleles all present at their normal chromosomal loci were grown under nonstarvation conditions (repressing, R) or under conditions of histidine starvation (derepressing, D) for a total of 6 h. For derepressing conditions, cultures were supplemented with 3-AT at 10 mM for 1 h prior to harvesting. Samples of total cellular proteins were separated by isoelectric focusing on a vertical slab gel as previously described (10) and subjected to immunoblot analysis with polyclonal antiserum specific for yeast eIF-2α and ^{125}I-labeled protein A to visualize immune complexes, all as described previously (8). The ~Ⓟ symbol denotes the position of eIF-2α phosphorylated on Ser-51.

independent of amino acid availability, with the species phosphorylated on Ser-51 occurring at roughly the same level as the basally phosphorylated form of the protein (Fig. 1, lanes 1 and 2). The suppressor mutations in *GCD7* and *GCD2* clearly did not reduce the level of eIF-2α phosphorylation in the presence of *GCN2^c-E532K,E1522K*; in fact, the proportion of eIF-2α that was phosphorylated appeared to be increased in the suppressor mutants compared with that seen in the corresponding *GCD2 GCD7* strain (Fig. 1, compare lanes 1 and 2 with lanes 3 through 12). A similar increase in the level of eIF-2α phosphorylation was also conferred by a deletion of the GCN3 subunit of eIF-2B (9) and by three of the four mutations in eIF-2α described above that were isolated as suppressors of *GCN2^c-E532K,E1522K* (46). These results imply that the *GCD2* and *GCD7* suppressor mutations overcome the toxic effects of the hyperactivated *GCN2^c-E532K,E1522K* kinase by rendering eIF-2B insensitive to the inhibitory effects of phosphorylated eIF-2.

Cloning and sequence analysis of the *GCD7* alleles that suppress the slow-growth phenotype of *GCN2^c-E532K,E1522K*. To learn more about the four mutations in *GCD7* that overcome the toxic effects of eIF-2α hyperphosphorylation, the chromosomal alleles were cloned by PCR and inserted into low-copy-number plasmids. DNA sequence analysis revealed that each suppressor contained a different missense mutation in the *GCD7* coding region (Fig. 2A). Two of the suppressor alleles (*GCD7-I118T* and *GCD7-D178Y*) have alterations in the central part of the protein, 60 amino acids apart (Fig. 2B). The other two suppressor alleles (*GCD7-K329E* and *GCD7-I348V*) contain substitutions in the C-terminal domain of GCD7, a region which exhibits significant sequence similarity with the C-terminal domains of GCN3 and GCD2; however, neither *GCD7* mutation alters a residue that is conserved among these three subunits of eIF-2B (Fig. 2C).

To demonstrate that the cloned *GCD7* alleles were necessary and sufficient to confer the suppressor phenotype, we introduced each plasmid-borne suppressor or the wild-type allele as the only copy of *GCD7* into a strain lacking chromosomal copies of both *GCD7* and *GCN2*. We also introduced a fifth suppressor allele constructed in vitro, *GCD7-*

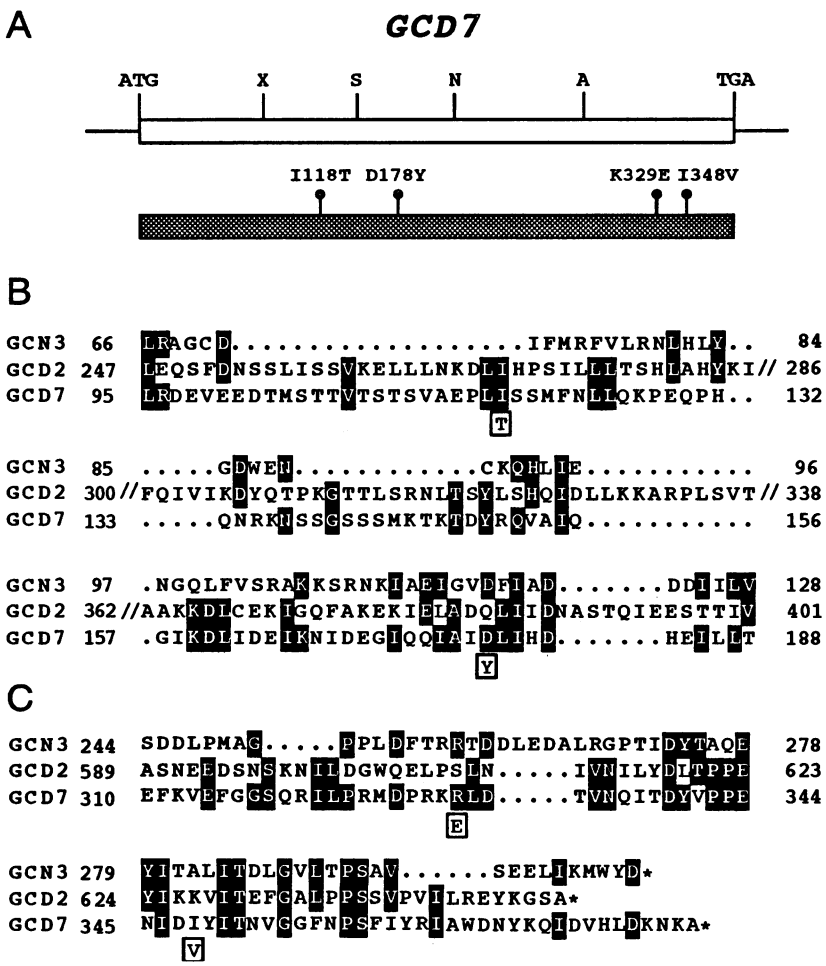

FIG. 2. Suppressor mutations in the GCD7 protein that overcome the slow-growth phenotype of the *GCN2^c-E532K,E1522K* protein kinase. (A) Location of the suppressor mutations in GCD7 protein are shown as closed circles at the appropriate positions below the restriction map of the *GCD7* gene. The mutations are designated by the wild-type amino acid (in single-letter code), its position relative to the N terminus, and the replacing amino acid, in that order. A, *Ava*I; N, *Nru*I; S, *Sac*I; X, *Xba*I. (B) Alignment of a portion of the GCD7 protein between amino acid positions 95 and 188 with segments of the GCN3 and GCD2 proteins, as reported by Bushman et al. (3). Identities are shown in black, gaps in the alignment are indicated by dots, and // denotes an interruption in the GCD2 sequence in a region of no obvious similarity to GCD7 or GCN3. Amino acid changes in GCD7 that result in the suppressor phenotype are shown boxed below the GCD7 sequence. (C) Partial alignment of the C-terminal regions of GCD7, GCD2, and GCN3 where two of the GCD7 suppressor mutations map. The alignment in these regions differs slightly from that previously reported (3).

I118T,D178Y, that contains both mutations mapping in the central domain of the GCD7 protein. When a single-copy-number plasmid containing *GCN2^c-E532K,E1522K* (p1353) was introduced into these six transformants, we found that all five *GCD7* suppressor alleles overcame the slow-growth phenotype conferred by the activated GCN2^c kinase (Fig. 3B). These results indicate that the single point mutations we identified in *GCD7* are sufficient to suppress the growth defect observed in *GCN2^c-E532K,E1522K* strains. When the same *GCN2^c-E532K,E1522K* allele was introduced on plasmid p1056, which appears to be present in several copies per cell (47), the Slg$^-$ phenotype in the wild-type *GCD7* transformant was more pronounced than that given by the single-copy-number plasmid p1353 (Fig. 3C). In the strains containing p1056, the *GCD7-I118T*, *GCD7-I348V*, and *GCD7-I118T, D178Y* alleles gave complete suppression, whereas the *GCD7-D178Y* and *GCD7-K329E* alleles incompletely suppressed the Slg$^-$ phenotype of *GCN2^c-E532K,E1522K* (Fig. 3C). It is noteworthy that none of the *GCD7* suppressor alleles (including the double mutant) had any effect on the rate of colony formation in the *gcn2*Δ strain (Fig. 3A), where eIF-2α phosphorylation does not occur (10). This last result strongly suggests that the *GCD7* suppressors do not reduce the essential function of eIF-2B in recycling eIF-2.

The *GCD7* suppressor mutations impair derepression of *GCN4* to different extents. The transformants bearing the cloned suppressor genes were analyzed to determine the effects of the *GCD7* mutations on derepression of *GCN4* and its target genes in the histidine biosynthetic pathway. In the first experiment, we analyzed the level of *HIS3* gene expression by determining resistance to 3-AT (Fig. 4). Strains containing the two weakest suppressors, *GCD7-D178Y* and *GCD7-K329E*, were 3-AT^R in the presence of either *GCN2* or *GCN2^c-E532K,E1522K*. The *GCD7-I118T* and *GCD7-I348V* alleles conferred partial sensitivity to 3-AT in the transformant containing *GCN2* but not in the *GCN2^c-E532K,E1522K* transformant, whereas the *GCD7-I118T,D178Y* double mutation conferred complete sensitivity to 3-AT in both *GCN2* and *GCN2^c-E532K,E1522K* strains.

To determine more directly the effects of the *GCD7* sup-

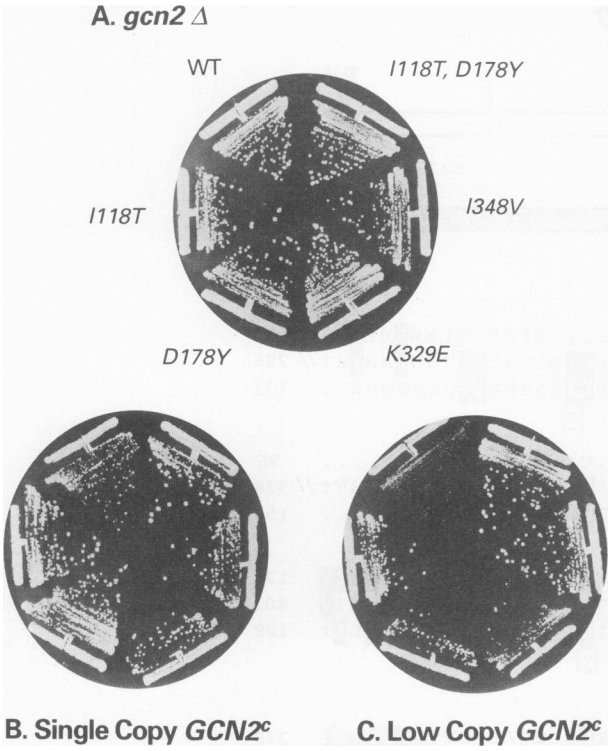

FIG. 3. Suppressor mutations in *GCD7* abolish the growth defect conferred by the *GCN2c-E532K,E1522K*-encoded protein kinase. The indicated *GCD7* alleles were introduced on low-copy-number plasmids into strain H2218 that contains deletions of the chromosomal copies of both *GCD7* and *GCN2*. The resulting strains H2224 (*GCD7-I118T*), H2225 (*GCD7-D178Y*), H2226 (*GCD7-K329E*), H2227 (*GCD7-I348V*), and H2228 (*GCD7-I118T,D178Y*) and the isogenic wild-type *GCD7* control strain H2223 (WT) were transformed with the single-copy-number plasmid p1353 (B) or with the low-copy-number plasmid p1056 (C), both containing the *GCN2c-E532K,E1522K* allele, or with vector alone (A). Transformants were streaked on SD plates and incubated for 2 days at 30°C.

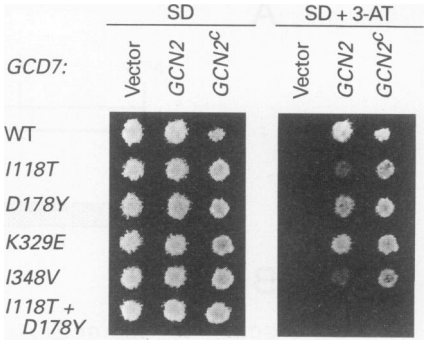

FIG. 4. Effects of the *GCD7* suppressor alleles on derepression of the general control system. The same strains described in the legend to Fig. 3 containing the indicated *GCD7* alleles and plasmid p1353 containing *GCN2c-E532K,E1522K*, p585 containing *GCN2*, or vector alone were tested for the ability to derepress *HIS* genes subject to *GCN4* control. Patches of transformants were grown to confluence on SD plates, replica plated to SD plates or to SD plates containing 10 mM 3-AT (SD + 3-AT), and incubated for 3 days at 30°C.

pressor mutations on *GCN4* expression, we assayed β-galactosidase activity produced from a *GCN4-lacZ* fusion present in the same strains analyzed for 3-AT sensitivity. In accord with previous results, the wild-type *GCD7* strain bearing wild-type *GCN2* showed a sixfold derepression of *GCN4-lacZ* expression when starved for histidine by addition of 3-AT, whereas the corresponding *GCN2c* strain was constitutively derepressed (Table 4). The double mutation *GCD7-I118T,D178Y* significantly reduced *GCN4-lacZ* expression in both *GCN2* and *GCN2c* transformants, whereas *GCD7-I348V* and *GCD7-I118T* had lesser effects. The *GCD7-D178Y* and *GCD7-K329E* alleles were very similar to wild-type *GCD7* when the *GCN2* allele was present but showed a significant reduction in *GCN4-lacZ* expression in combination with *GCN2c-E532K,E1522K*, particularly under conditions of amino acid sufficiency. We believe that the *GCD7* mutations lead to more pronounced reductions in *GCN4-lacZ* expression under nonstarvation conditions in the *GCN2c* background versus starvation conditions in the *GCN2* background, because this strain exhibits a significant *GCN2*-independent derepression response to histidine starvation (10). The results on *GCN4-lacZ* expression shown in Table 4 are in complete agreement with the different levels of 3-AT sensitivity observed for the *GCD7* suppressor strains shown in Fig. 4. We conclude that the *GCD7* suppressor alleles impair derepression of *GCN4* and the general control response to different extents, with the *GCD7-I118T,D178Y* allele conferring the greatest resistance to the effects of eIF-2α phosphorylation and *GCD7-D178Y* and *GCD7-K329E* being the least altered in this response.

The *GCD7* mutations that most strongly affect *GCN4* expression suppress the slow-growth phenotype conferred by other *GCN2c* alleles and by a human eIF-2α kinase expressed in yeast cells. To test whether the most efficient *GCD7* suppressor alleles were able to overcome the toxicity associated with higher levels of eIF-2α phosphorylation than are produced by the *GCN2c-E532K,E1522K* product, we analyzed the *GCD7* mutations for suppression of the slow-growth phenotype conferred by the *GCN2c-R699W,D918G,E1537G*, *GCN2c-M719V,E1537G*, and *GCN2c-E532K,E1537G* alleles. The products of these three genes produce higher levels of

TABLE 4. *GCN4-lacZ* expression in *GCN2c* or *GCN2* strains containing *GCD7* suppressor alleles[a]

Strain (allele)	Growth on 3-AT		*GCN4-lacZ* expression (U)			
			GCN2		*GCN2c*	
	GCN2	*GCN2c*	R	DR	R	DR
H2223 (*GCD7*)	+	+	16	96	160	210
H2224 (*GCD7-I118T*)	±	+	16	62	36	81
H2225 (*GCD7-D178Y*)	+	+	14	81	52	99
H2226 (*GCD7-K329E*)	+	+	15	97	78	130
H2227 (*GCD7-I348V*)	±	+	16	52	32	65
H2228 (*GCD7-I118T,D178Y*)	−	−	13	40	16	45

[a] Isogenic yeast strains H2223, H2224, H2225, H2226, H2227, and H2228 carrying the indicated *GCD7* alleles on low-copy-number plasmids were transformed with the single-copy-number plasmid p585 containing wild-type *GCN2* or p1353 containing the *GCN2c-E532K,E1522K* allele and were grown for 8 h under nonstarvation conditions (SD minimal medium) in which the general control system is repressed (R) or for 6 h under conditions of histidine starvation (SD plus 10 mM 3-AT) in which the system is derepressed (DR). Expression of β-galactosidase from a *GCN4-lacZ* fusion integrated in the chromosome at *trp1-Δ63* was measured in cell extracts prepared from the different strains. Each value is the average obtained from two different transformants; the individual measurements varied from the mean values by 10% or less. Units of enzyme activity are given as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside cleaved per minute per milligram of protein.

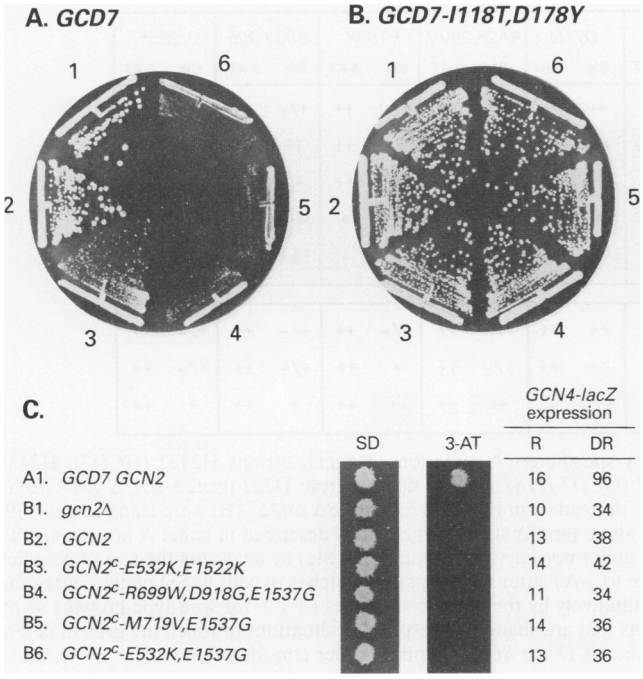

FIG. 5. Effects of the *GCD7-I118T,D178Y* suppressor allele on growth rate under nonstarvation conditions and on *GCN4* expression in transformants containing different *GCN2*^{c} alleles. Strains H2223 (*GCD7*) and H2228 (*GCD7-I118T,D178Y*) were transformed with plasmids containing *GCN2* (p585, number 2), *GCN2*^{c}*-E532K,E1522K* (p1056, number 3), *GCN2*^{c}*-R699W,D918G, E1537G* (p1053, number 4), *GCN2*^{c}*-M719V,E1537G* (p1052, number 5), *GCN2*^{c}*-E532K, E1537G* (p1054, number 6), or vector alone (number 1). Strains carrying *GCD7* (A) or *GCD7-I118T,D178Y* (B) and the *GCN2* alleles indicated above were streaked on SD plates and incubated for 2 days at 30°C. (C) Patches of transformants were grown to confluence on SD and replica plated to SD or SD containing 10 mM 3-AT (3-AT) and incubated for 3 days at 30°C. Expression of β-galactosidase from a *GCN4-lacZ* fusion integrated at *trp1-Δ63* was measured in cell extracts prepared from different strains following the procedures given in Table 4. Each value is the average of two independent transformants; the individual measurements varied from the mean by 15% or less. The results shown on line A1 were obtained with strain 1 shown in panel A; the results on lines B1 to B6 were obtained with strains 1 to 6 shown in panel B.

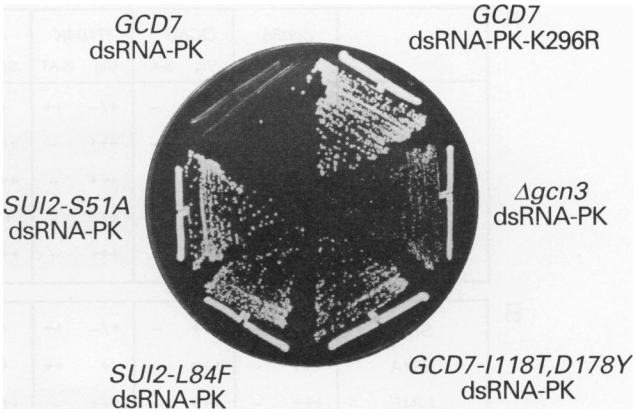

FIG. 6. The *GCD7-I118T,D178Y* allele alleviates the growth-inhibitory effects of expressing human dsRNA-PK. Yeast strains H2223 (*GCD7*), H2228 (*GCD7-I118T,D178Y*), and H2422 (*GCD7 gcn3*Δ) and the isogenic control strains H1817 (*SUI2-S51A*) and H1927 (*SUI2-L84F*) were transformed with plasmid p1420 (9) containing the coding sequences for wild-type dsRNA-PK under the control of a galactose-inducible promoter. Strain H2223 (*GCD7*) was also transformed with plasmid p1421, containing coding sequences for the catalytically inactive kinase dsRNA-PK-K296R. Strains containing the indicated *GCD7* alleles and the human eIF-2α kinase constructs were streaked on synthetic medium containing 10% galactose (to induce expression of the dsRNA-PK constructs) and incubated for 10 days at 30°C.

eIF-2α phosphorylation and a more severe growth defect in otherwise wild-type strains than the *GCN2*^{c}*-E532K,E1522K* kinase does (35). As expected, strains carrying these more highly activated *GCN2*^{c} alleles and the weaker suppressors *GCD7-D178Y* or *GCD7-K329E* had a slow-growth phenotype, whereas the stronger suppressors *GCD7-I118T*, *GCD7-I348V*, and *GCD7-I118T,D178Y* restored wild-type growth in strains containing any of the *GCN2*^{c} alleles (Fig. 5B, and data not shown). Interestingly, only the *GCD7-I118T,D178Y* allele impaired the general control response in the presence of the three more highly activated kinases, conferring a 3-AT^S phenotype regardless of the *GCN2*^{c} allele present in the strain (Fig. 5C).

We quantitated the effects of the *GCD7-I118T,D178Y* suppressor on *GCN4* expression under these conditions by measuring the β-galactosidase activity produced in transformants containing the different *GCN2*^{c} alleles. The *GCD7-I118T, D178Y* allele greatly reduced *GCN4-lacZ* expression when combined with any of the four *GCN2*^{c} alleles (strains B3 to B6 in Fig. 5C). The fact that the strain deleted for *GCN2* (strain B1) showed virtually the same *GCN4-lacZ* expression as that seen in the *GCN2*^{c} mutants in the presence of *GCD7-I118T,D178Y* (strains B3 to B6) indicates that this *GCD7* double mutation completely abolishes the effects of eIF-2 phosphorylation on *GCN4* expression. (The residual increase in *GCN4-lacZ* expression seen in the *gcn2*Δ strain under starvation conditions is a manifestation of the GCN2-independent derepression response mentioned above.)

Finally, we tested the ability of the *GCD7-I118T,D178Y* allele to suppress the lethal effect of expressing the human eIF-2α kinase dsRNA-PK (31) (also known as p68, DAI, or dsI) in yeast cells under the control of a galactose-inducible promoter (5, 9). As shown previously, the growth inhibitory effect of expressing dsRNA-PK in yeast cells is completely abolished by a mutation that changes the phosphorylation site at position 51 of eIF-2α from serine to alanine (*SUI2-S51A*) (Fig. 6) (9). Remarkably, the *GCD7-I118T,D178Y* mutation mimics the *SUI2-S51A* mutation and restores wild-type growth in cells expressing dsRNA-PK. Note that the *GCD7-I118T,D178Y* allele and one of our suppressors of *GCN2*^{c}*-E532K,E1522K* which alter eIF-2α (*SUI2-L84F*) (46) are both more effective than a deletion of *GCN3* in suppressing the lethal effect of expressing dsRNA-PK in yeast cells (Fig. 6). As expected, the *GCD7-I118T* and *GCD7-K329E* alleles were less effective suppressors of the mammalian eIF-2α kinase, conferring a growth rate similar to that seen in the *gcn3*Δ strain (data not shown). We conclude that the *GCD7-I118T,D178Y* double mutation renders eIF-2B completely insensitive to the inhibitory effects of phosphorylated eIF-2 without affecting the essential function of the GCD7 protein in the eIF-2B complex.

***GCD7* suppressors overcome certain *gcn3*^{c} mutations that mimic the inhibitory effect of eIF-2α phosphorylation on translation initiation.** We recently reported that the four suppressors of *GCN2*^{c}*-E532K,E1522K* affecting eIF-2α do not reduce the level of eIF-2α phosphorylation, suggesting that they render phosphorylated eIF-2 incapable of inhibiting eIF-2B function (46). Thus, these mutations overcome the

A

	gcn3Δ		*GCN3*		*R104K*		*A26T*		*D71N*		*AA25,26VV*		*E199K*		*Δ303-305*		*V295F*	
	Slg	3-AT	Slg	3-AT	Slg	3-AT	Slg	3-AT	Slg	3-AT	Slg	3-AT	Slg	3-AT	Slg	3-AT	Slg	3-AT
GCD7	+++	-	+++	-	+/-	++	+	++	++	++	+/-	++	+/-	++	+/-	++	+/-	++
K329E	+++	-	+++	-	+++	-	+++	+/-	+++	-	++	+	++	++	++	++	+	++
D178Y	+++	-	+++	-	+++	-	+++	-	+++	-	++	+/-	++	++	++	++	+	++
I118T	+++	-	+++	-	+++	-	++	-	+++	-	++	+/-	+++	+	++	++	+	++
I118T,D178Y	+++	-	+++	-	+++	-	+++	-	+++	-	+++	+/-	+++	-	+++	-	++	++

B

SUI2	+++	-	+++	-	+/-	++	+	++	++	++	+/-	++	+/-	++	+/-	++	+/-	++
S51A	+++	-	+++	-	+	++	+	++	++	++	+/-	++	+	++	+/-	++	+/-	++
L84F	+++	-	+++	-	+++	-	+++	-	+++	-	++	++	++	++	+	++	+	++

FIG. 7. *GCD7* suppressor alleles alleviate the growth defects caused by specific *gcn3*^{c} mutations. Isogenic strains H2422 (*GCD7*), H2423 (*GCD7-I118T*), H2424 (*GCD7-D178Y*), H2425 (*GCD7-K329E*), and H2427 (*GCD7-I118T,D178Y*) derived from H222 (*gcn2Δ gcn3Δ gcd7::hisG*) (A) and strains H2428 (*SUI2*), H2429 (*SUI2-S51A*), and H2430 (*SUI2-L84F*) derived from H2116 (*gcn2Δ gcn3Δ sui2Δ*) (B) were transformed with plasmids containing the *gcn3*^{c} alleles indicated across the top or with vector alone (*gcn3Δ* strain). The strains described in panel A are isogenic to those shown in panel B. The resulting transformants were tested for growth under nonstarvation conditions (Slg) by analyzing the size of colonies formed from single cells after incubation for 2 days at 30°C and for resistance to 3-AT after replica plating patches of cells to SD plates containing 3-AT at 10 mM (3-AT). Degree of growth in each medium is indicated qualitatively by the number of pluses (+++ for wild-type growth) or by a minus sign (essentially no growth after 7 days of incubation at 30°C). Results that are shaded correspond to situations in which the growth defect or 3-AT^R phenotype of the *gcn3*^{c} allele is wholly or largely suppressed by the *GCD7* or *SUI2* mutation under consideration.

inhibitory effect of phosphorylated eIF-2 by altering eIF-2 rather than eIF-2B. Interestingly, we found that all four of these eIF-2α mutations also suppressed a certain mutation in *GCN3*, called *gcn3*^{c}*-R104K*, that leads to constitutively derepressed *GCN4* expression (3-AT resistance) and slow growth under nonstarvation conditions in the absence of *GCN2*. The phenotype of *gcn3*^{c}*-R104K* indicates that this mutation reduces eIF-2B function independently of eIF-2α phosphorylation by GCN2 (13). The fact that *gcn3*^{c}*-R104K* is suppressed by the same mutations in eIF-2α that overcome the deleterious effects of eIF-2α phosphorylation suggests that *gcn3*^{c}*-R104K* reduces eIF-2B activity in a way that mimics the inhibitory effect of phosphorylated eIF-2. By contrast, the *gcn3*^{c}*-Δ303-305* allele was not suppressed by any of the four suppressors in eIF-2α, indicating that this mutation reduces eIF-2B function by a different mechanism, e.g., destabilizing the eIF-2B complex (46). To classify additional *gcn3*^{c} alleles according to whether or not they mimic the inhibitory effect of phosphorylated eIF-2, we introduced the eIF-2α suppressor allele *SUI2-L84F* into a strain lacking chromosomal copies of *SUI2*, *GCN2*, and *GCN3* and transformed the resulting strain with plasmids carrying each of seven different *gcn3*^{c} alleles, all of which lead to 3-AT resistance and slow growth in strains lacking *GCN2* (Fig. 7B, row *SUI2*). Analysis of the resulting transformants revealed that the *SUI2-L84F* allele suppressed the Slg$^-$ and 3-AT^R phenotypes of a particular subset of *gcn3*^{c} mutations, including the R104K, A26T, and D71N *gcn3*^{c} alleles, conferring growth on minimal medium at wild-type levels and uncovering the 3-AT sensitivity associated with the *gcn2Δ* allele present in these strains. The *SUI2-L84F* allele partially suppressed the AA25,26VV and E199K *gcn3*^{c} alleles but showed no suppression of the Δ303-305 and V295F alleles (Fig. 7B). As expected, the *SUI2-S51A* allele, which suppresses the Slg$^-$ phenotype of *GCN2*^{c} alleles by preventing eIF-2α phosphorylation, is incapable of suppressing any of the *gcn3*^{c} mutations.

Our ability to isolate *GCD7* mutations that render eIF-2B less sensitive to eIF-2α phosphorylation suggested to us that GCD7 acts in conjunction with GCN3 to mediate inhibition of eIF-2B activity by phosphorylated eIF-2. We reasoned that if this explanation was correct, the *GCD7* mutations might suppress the same *gcn3*^{c} alleles that are suppressible by *SUI2-L84F* and which seem to impair eIF-2B function in a way that mimics phosphorylated eIF-2. To test this possibility, we constructed a set of six isogenic strains deleted for *GCN2* and *GCN3*, each carrying a different *GCD7* suppressor allele or wild-type *GCD7*, and introduced into these strains the plasmid-borne *gcn3*^{c} alleles. The results shown in Fig. 7A indicate that the *GCD7-K329E*, *GCD7-D178Y*, and *GCD7-I118T* alleles closely resemble *SUI2-L84F* in completely suppressing the R104K, A26T, and D71N alleles and in partially suppressing the AA25,26VV *gcn3*^{c} allele. These three *GCD7* mutations also partially suppressed the E199K and Δ303-305 *gcn3*^{c} alleles but gave no suppression of *gcn3*^{c}*-V295F*. The *GCD7-I118T,D178Y* double mutation was superior to the *GCD7* single mutations, strongly suppressing the phenotypes of all *gcn3*^{c} alleles tested, except for the 3-AT^R phenotype of *gcn3*^{c}*-V295F*. It is noteworthy that the *GCD7-I118T,D178Y* allele is the strongest suppressor of both the *GCN2*^{c} mutations and those *gcn3*^{c} mutations that are suppressible by *GCD7* mutations. These results imply that the suppressible class of *gcn3*^{c} alleles reduce eIF-2B function in a way that mimics the inhibitory effect of eIF-2α phosphorylation and that *SUI2-L84F* and the *GCD7* suppressors listed in Fig. 7A overcome the deleterious effects of phosphorylated eIF-2 by closely related mechanisms.

The *GCD7-I384V* allele interacted with the *gcn3*^{c} alleles differently than did the other four *GCD7* suppressors. Deletion of *GCN3* impaired the growth of strains bearing *GCD7-I348V*; in addition, the *GCD7-I348V* mutation suppressed the 3-AT^S phenotype associated with the *gcn2Δ* chromosomal mutation when *GCN3* was also deleted (data not shown). These results indicate that the *GCD7-I348V* allele acquires a Gcd$^-$ phenotype in the absence of *GCN3* even though it displays a Gcn$^-$ phenotype in strains containing *GCN3* (Fig. 4 and Table 4). This interaction resembles that seen for other reduced-function mutations in subunits of eIF-2B whose Gcd$^-$ and Slg$^-$ phenotypes are exacerbated by inactivation of *GCN3*. In fact, the *gcd1-101*, *gcd6-1*, and *gcd7-201* mutations are lethal in

combination with *gcn3Δ* (4, 15). These similarities suggest that the *GCD7-I348V* mutation differs from the other *GCD7* suppressors in causing a reduction in eIF-2B catalytic function when the GCN3 subunit is missing from the complex.

DISCUSSION

We have used genetic reversion analysis to identify proteins involved in the regulation of translation initiation by phosphorylation of eIF-2 in *S. cerevisiae*, a mechanism employed in mammalian cells to reduce total protein synthesis under stress conditions (18, 24, 29, 43). In yeasts, this mechanism is employed to elicit specific derepression of *GCN4* translation in response to starvation for amino acids or purines (21, 36). The protein kinase GCN2 becomes activated under these starvation conditions and phosphorylates the α subunit of eIF-2. By analogy with mammalian systems, phosphorylated eIF-2 is expected to reduce the activity of eIF-2B, the guanine nucleotide exchange factor for eIF-2, and this should stimulate ribosomal reinitiation at *GCN4* (10). In accord with this prediction, a protein complex containing five translational regulators of *GCN4* was shown to be the yeast equivalent of eIF-2B (3, 6). In addition, it was demonstrated that deletion of the nonessential GCN3 subunit of yeast eIF-2B impairs derepression of *GCN4* and substantially reduces the growth inhibition associated with hyperphosphorylation of eIF-2 by GCN2^c proteins or by the mammalian eIF-2α kinases dsRNA-PK and HCR when expressed in yeasts (9, 35). These latter findings implicated GCN3 as a regulatory subunit that mediates the inhibitory effects of phosphorylated eIF-2 on the essential function of eIF-2B in translation initiation.

By isolating revertants with a wild-type growth rate in strains expressing the *GCN2^c-E532K,E1522K* kinase, we have identified mutations in several genes whose products are involved either in regulating GCN2 kinase function or in mediating the inhibitory effects of phosphorylated eIF-2 on translation initiation (Fig. 8). The isolation of mutations in *GCN1* was expected because inactivation of this gene suppresses the phenotypes of numerous *GCN2^c* mutations (35) and because a recent work indicates that GCN1 is required in vivo for high-level eIF-2α phosphorylation by both wild-type GCN2 and a GCN2^c kinase (28). We have verified that the suppressor mutations mapping in *GCN1* decrease the level of eIF-2α phosphorylation in vivo (data not shown), and we presume that the suppressors mapping in *GCN2* itself have the same effect. Some of the *GCN1* mutations we isolated (those in group II [Table 3]) are of particular interest because they exhibit a dominant-negative phenotype and thus probably encode functionally defective proteins that compete with wild-type GCN1 either in generating the starvation signal or in transducing the signal to GCN2.

Instead of reducing GCN2 kinase function, suppressors in the second group mapping in *SUI2*, *GCN3*, *GCD7*, and *GCD2* appear to make the translational machinery less sensitive to phosphorylated eIF-2. The suppressor mutations in eIF-2α alter residues located between positions 58 and 89 that are perfectly conserved in eIF-2α from different sources (8, 12, 46). Since eIF-2α is expected to physically contact eIF-2B in the course of inhibiting its recycling activity (18, 26, 33), it is reasonable to have found that other amino acids in eIF-2α besides the phosphorylation site at Ser-51 contribute to this regulatory interaction. The isolation of mutations in *GCN3* was anticipated because deletion of this gene suppresses the phenotypes associated with numerous *GCN2^c* alleles and alleviates the toxic effect of expressing mammalian eIF-2α kinases in yeast cells (9, 35). Interestingly, a *GCN3* deletion does not

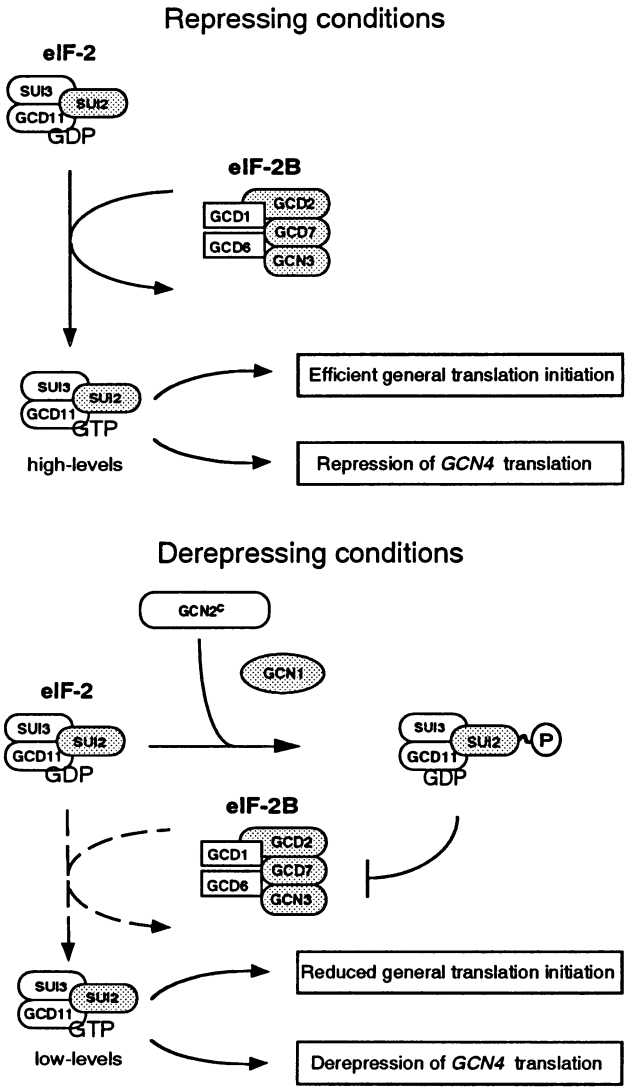

FIG. 8. The eIF-2α protein, three different subunits of eIF-2B, and GCN1 mediate the effects of a constitutively activated form of the eIF-2α kinase GCN2 on general translation initiation and *GCN4* expression. *SUI2*, *SUI3* and *GCD11* encode, respectively, the α, β, and γ subunits of eIF-2, and *GCD1*, *GCD6*, *GCD2*, *GCD7*, and *GCN3* encode the five subunits of eIF-2B, of which GCD2, GCD7, and GCN3 are interrelated in sequence. Only the GTP-bound form of eIF-2 is active in translation initiation, and eIF-2B is required to exchange GDP for GTP on eIF-2 following each round of translation initiation. Amino acid starvation or the presence of genetically activated GCN2^c protein kinases lead to phosphorylation of the α subunit of eIF-2 on Ser-51, dependent on the GCN1 protein. The phosphorylated form of eIF-2α inhibits the recycling factor eIF-2B, decreasing the concentration of the GTP-bound form of eIF-2. This reduces the rate of general translation initiation, thereby decreasing cellular growth, but specifically derepresses *GCN4* translation. Dashed lines indicate processes or functions which are diminished in response to eIF-2α phosphorylation. Gene products that can be mutated to suppress the phenotypic effects of a GCN2^c kinase are shown shaded. Loss-of-function mutations in GCN1 reduce or eliminate eIF-2α phosphorylation. Altered-function mutations in eIF-2α, GCN3, GCD7, and GCD2 render eIF-2B insensitive to eIF-2α phosphorylation.

completely restore wild-type growth when the mammalian kinases are expressed at very high levels from the strong galactose-inducible promoter. This result suggests that additional proteins are involved in negatively regulating the GDP-GTP exchange activity of eIF-2B by phosphorylated eIF-2α.

The isolation of mutations in *GCD7* and *GCD2* that overcome the toxic effects of eIF-2 hyperphosphorylation suggests that these two essential subunits of eIF-2B also have important roles in mediating the negative regulatory interaction between phosphorylated the form of eIF-2 and eIF-2B. The *GCD7* mutations resemble both the *SUI2* suppressors and a *gcn3* deletion in overcoming the growth inhibition conferred by various *GCN2*^{c} alleles and by expression of human dsRNA-PK (9, 46) in the presence of even higher levels of eIF-2α phosphorylation than are seen in isogenic strains containing wild-type *GCD7*. In fact, the doubly mutant allele *GCD7-I118T,D178Y* impairs the derepression of *GCN4* translation and overcomes the toxic effect of expressing dsRNA-PK to the same extent as mutating the phosphorylation site on eIF-2α does and more completely than occurs when *GCN3* is deleted. Thus, it appears that eIF-2B complexes containing the GCD7-I118T,D178Y protein have completely lost the ability to be negatively regulated by phosphorylated eIF-2. At present, we cannot eliminate the possibility that the *GCD7* and *GCD2* suppressors stimulate the catalytic activity of eIF-2B rather than making it insensitive to phosphorylated eIF-2. We consider this an unlikely possibility, however, because the level of eIF-2α phosphorylation is so high in these mutants (Fig. 1) that all of the eIF-2B should be sequestered by phosphorylated eIF-2, given the large molar excess of eIF-2 versus eIF-2B (7). That deletion of *GCN3* and the *SUI2*, *GCD7*, and *GCD2* suppressor mutations all lead to elevated levels of eIF-2α phosphorylation suggests that phosphorylation of eIF-2 is negatively autoregulated and that this feedback mechanism is disrupted when phosphorylated eIF-2 does not inhibit eIF-2B function in translation initiation.

It is noteworthy that none of the *GCD7* suppressors has any detectable effect on growth in the absence of eIF-2α phosphorylation and only one, *GCD7-I348V*, decreases the growth rate in strains deleted for *GCN3*. Reduced-function *gcd* mutations frequently have more severe phenotypes in the absence of *GCN3*, presumably because removal of GCN3 decreases the stability or activity of the eIF-2B complex when one of the other essential GCD subunits is also mutated (4, 15, 16). Thus, four of the five *GCD7* suppressors isolated in this study appear to have little or no effect on the essential catalytic function of the complex in translation, and only *GCD7-I348V* reduces the stability or activity of eIF-2B in strains lacking *GCN3*. On the basis of this finding, we propose that the regions of GCD7 protein affected by the suppressor mutations are dedicated primarily to the regulation of eIF-2B function by phosphorylated eIF-2.

The role of GCD7 in the regulation of eIF-2B activity by phosphorylated eIF-2. Studies on mammalian systems have led to the idea that phosphorylated eIF-2 present in a binary complex with GDP inhibits eIF-2B function either by forming an excessively stable complex from which the GDP on eIF-2 cannot be released (reviewed in reference 33) or by acting as a competitive inhibitor of eIF-2B catalytic activity (38, 39). In the context of these models, the amino acids which are altered by the *SUI2* suppressors described here and elsewhere (46) could identify residues in the amino-terminal half of the eIF-2α protein which participate directly in the stable interaction between eIF-2B and eIF-2(αP)–GDP that sequesters eIF-2B in an inactive form. Because GCN3 is required for the inhibitory effect of phosphorylated eIF-2 on translation initiation, we suggested that eIF-2α might interact directly with a particular segment of GCN3 in forming the stable contacts between eIF-2(αP)–GDP and eIF-2B that are responsible for inhibiting eIF-2B function (46). One way to explain the ability of the *GCD7* mutations to overcome the effects of phosphorylated eIF-2 would be to propose that a region in GCD7 also directly interacts with a portion of eIF-2α and is required to stabilize the catalytically inactive eIF-2(αP)–GDP–eIF-2B complex. Perhaps the sequence similarity among GCD7, GCD2, and GCN3 (3) reflects a common structural motif that is responsible for independent contacts between eIF-2α and GCN3, GCD7, and GCD2, each of which is required to stabilize the interaction between eIF-2B and phosphorylated eIF-2(αP)–GDP.

The idea that GCD7 and GCN3 make independent contacts with eIF-2α can explain the ability of the *GCD7* suppressors to overcome the growth inhibition conferred by the class of *gcn3*^{c} mutations which appear to mimic the inhibitory effects of phosphorylated eIF-2. That the growth inhibition conferred by the *gcn3*^{c}*-A26T*, *gcn3*^{c}*-R104K*, and *gcn3*^{c}*-D71N* mutations is completely abolished by the same mutations in *GCD7* and *SUI2* which overcome the inhibitory effects of eIF-2α phosphorylation suggests that these *gcn3*^{c} mutations decrease eIF-2B activity in a way that closely resembles the inhibitory effect of phosphorylated eIF-2α. This could occur, for example, if the *gcn3*^{c} mutations lead to an excessively stable interaction between eIF-2 and eIF-2B, impeding the dissociation of the two complexes following nucleotide exchange. If this model is correct, then *GCD7* suppressor mutations that weaken independent contacts between eIF-2α and GCD7 could counteract the more stable interactions between eIF-2α and GCN3 imposed by the suppressible *gcn3*^{c} mutations. In contrast, the phenotypes associated with *gcn3*^{c}*-E199K*, *gcn3*^{c}*-Δ303-305*, and *gcn3*^{c}*-V295F* would derive partly (or almost completely in the case of the V295F substitution) from a reduction in the stability or catalytic activity of eIF-2B that is unrelated to the effect of phosphorylation on the affinity of eIF-2α for the subunits of eIF-2B. Consequently, these *gcn3*^{c} mutations are only partially suppressed by the *GCD7* mutations and are totally insensitive to the *SUI2-L84F* allele.

An alternative model could be proposed to account for the suppressor activity of the *GCD7* mutations in which only the GCN3 subunit directly contacts eIF-2α and the role of GCD7 is to present GCN3 in a way that permits stable interaction between GCN3 and phosphorylated eIF-2. An extreme version of this second class of models would be that GCD7 has no direct role in the regulation of eIF-2B function by phosphorylated eIF-2 and is required only to anchor GCN3 to the rest of the complex. In this view, the *GCD7* mutations would suppress the effects of both eIF-2α hyperphosphorylation and the *gcn3*^{c} mutations simply by causing the nonessential GCN3 subunit to dissociate from eIF-2B. One problem with this model is its failure to explain why the *GCD7* mutations preferentially suppress the same *gcn3*^{c} mutations which are suppressed by *SUI2-L84F*. To account for this allele specificity would require that those *gcn3*^{c} alleles that do not mimic the effect of eIF-2α phosphorylation (and thus are not suppressed by *SUI2-L84F*) would also be less dependent on GCD7 for stable incorporation of GCN3 into the eIF-2B complex. At present, it is not obvious why this relationship would exist. A second argument is that the *GCD7-I348V* allele leads to a slow-growth phenotype in a *gcn3Δ* strain but not in a *GCN3* strain. If the effect of the *GCD7-I348V* mutation was to exclude *GCN3* from the eIF-2B complex, we might not expect a deletion of *GCN3* to produce a synthetic Slg$^-$ phenotype in the *GCD7-I348V* strain. Perhaps the strongest argument

against this alternative model is the fact that the *GCD7-I118T,D178Y* double mutation suppresses the effects of very high levels of eIF-2α phosphorylation catalyzed by dsRNA-PK significantly better than a deletion of *GCN3* does. This latter finding indicates that eIF-2B activity can still be inhibited by high levels of phosphorylated eIF-2 in the absence of GCN3 and suggests that GCD7 is directly involved in stabilizing the interaction between eIF-2(αP)–GDP and eIF-2B.

Taking all of the results together, we prefer a model in which GCD7 makes one or more direct contacts with eIF-2α that contribute to the stability of the eIF-2(αP)–GDP–eIF-2B complex. Weakening these contacts would decrease the amount of eIF-2B that is sequestered and inactivated by eIF-2 in response to either phosphorylation or certain *gcn3*^{c} mutations, restoring a normal growth rate and, in some cases, complete repression of *GCN4* expression. In mammalian cells, phosphorylation of eIF-2α not only increases the affinity of eIF-2–GDP for eIF-2B but also impairs the nucleotide exchange activity of eIF-2B, i.e., the GDP cannot be released from an eIF-2(αP)–GDP–eIF-2B complex (29, 44; reviewed in reference 26). Thus, it is possible that GCD7 also transmits the effects of binding phosphorylated eIF-2α to the active site of eIF-2B, perhaps by triggering an allosteric transition in the eIF-2B complex. Such a dual role for GCD7 in the regulatory mechanism might account for the presence of *GCD7* suppressor mutations in two noncontiguous sections of the GCD7 protein.

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