MOLECULAR AND CELLULAR BIOLOGY, Nov. 1995, p. 6351–6363 Vol. 15, No. 11
0270-7306/95/$04.00+0

Modulation of $tRNA_i^{Met}$, eIF-2, and eIF-2B Expression Shows that *GCN4* Translation Is Inversely Coupled to the Level of eIF-2 · GTP · Met-$tRNA_i^{Met}$ Ternary Complexes

THOMAS E. DEVER,[1] WEIMIN YANG,[1] STEFAN ÅSTRÖM,[2] ANDERS S. BYSTRÖM,[2] AND ALAN G. HINNEBUSCH[1]*

Section on Molecular Genetics of Lower Eukaryotes, Laboratory of Molecular Genetics, National Institute of Child Health and Human Development, Bethesda, Maryland 20892-2785,[1] and Department of Microbiology, Umeå University, S-901 87 Umeå, Sweden[2]

Received 23 May 1995/Returned for modification 25 July 1995/Accepted 11 August 1995

To understand how phosphorylation of eukaryotic translation initiation factor (eIF)-2α in *Saccharomyces cerevisiae* stimulates *GCN4* mRNA translation while at the same time inhibiting general translation initiation, we examined the effects of altering the gene dosage of initiator $tRNA^{Met}$, eIF-2, and the guanine nucleotide exchange factor for eIF-2, eIF-2B. Overexpression of all three subunits of eIF-2 or all five subunits of eIF-2B suppressed the effects of eIF-2α hyperphosphorylation on both *GCN4*-specific and general translation initiation. Consistent with eIF-2 functioning in translation as part of a ternary complex composed of eIF-2, GTP, and Met-$tRNA_i^{Met}$, reduced gene dosage of initiator $tRNA^{Met}$ mimicked phosphorylation of eIF-2α and stimulated *GCN4* translation. In addition, overexpression of a combination of eIF-2 and $tRNA_i^{Met}$ suppressed the growth-inhibitory effects of eIF-2 hyperphosphorylation more effectively than an increase in the level of either component of the ternary complex alone. These results provide in vivo evidence that phosphorylation of eIF-2α reduces the activities of both eIF-2 and eIF-2B and that the eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complex is the principal component limiting translation in cells when eIF-2α is phosphorylated on serine 51. Analysis of eIF-2α phosphorylation in the eIF-2-overexpressing strain also provides in vivo evidence that phosphorylated eIF-2 acts as a competitive inhibitor of eIF-2B rather than forming an excessively stable inactive complex. Finally, our results demonstrate that the concentration of eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complexes is the cardinal parameter determining the site of reinitiation on *GCN4* mRNA and support the idea that reinitiation at *GCN4* is inversely related to the concentration of ternary complexes in the cell.

The current model for the mechanism of translation initiation in eukaryotic cells derives from biochemical analysis of mammalian cell-free systems and characterization of individual reactions with purified initiation factors. These studies have identified eukaryotic initiation factor (eIF)-2 as the protein responsible for binding the initiator Met-tRNA (Met-$tRNA_i^{Met}$) to the 40S ribosomal subunit in an early step of the initiation pathway (see reviews in references 26 and 31). It is believed that the Met-$tRNA_i^{Met}$ is delivered as part of a ternary complex composed of eIF-2, GTP, and Met-$tRNA_i^{Met}$. After binding of the ternary complex to the 40S ribosomal subunit, the GTP is hydrolyzed to GDP and eIF-2 is released in a binary complex with GDP. Because mammalian eIF-2 has a 100- to 400-fold-higher affinity for GDP than for GTP, a guanine nucleotide exchange factor known as eIF-2B is required to recycle eIF-2 · GDP back to eIF-2 · GTP, allowing eIF-2 to function in a subsequent round of translation initiation (26, 36). Phosphorylation of eIF-2α on serine 51 inhibits the exchange of GTP for GDP on eIF-2. Not only is exchange blocked on the phosphorylated eIF-2 molecule, but phosphorylated eIF-2 also prevents eIF-2B from recycling nonphosphorylated eIF-2 (30, 41, 43). It is generally considered that the inhibition of eIF-2B function is the principal reason why phosphorylation of eIF-2 inhibits translation initiation in vivo (40).

eIF-2 is present in excess of eIF-2B in all cells in which it has been examined (26, 36). Consequently, only limited phosphorylation of eIF-2α produces a sufficient amount of the inhibitor to block translation initiation. The extent of eIF-2α phosphorylation associated with a complete inhibition of protein synthesis varies from as little as 15 to 20% in some cells to as much as 60 to 65% in Ehrlich cells (26). These varied amounts are consistent with the different ratios of eIF-2 to eIF-2B found in the corresponding cells. Thus, the ratio of eIF-2 to eIF-2B is between 7:1 and 4:1 in reticulocyte lysates and just 2:1 in Ehrlich cells (26, 40). In *Saccharomyces cerevisiae*, the ratio of eIF-2 to eIF-2B has been estimated at roughly 10:1; however, approximately 50% phosphorylation of eIF-2α has been observed in slow-growing strains containing a hyperactivated form of the eIF-2α kinase GCN2 and in nutrient-starved wild-type cells (13, 37).

The mechanism by which phosphorylated eIF-2 inhibits eIF-2B has generally been referred to as sequestering, indicating the formation of an extremely stable complex between phosphorylated eIF-2 and eIF-2B (26, 36). Consistent with this model and the relative amounts of eIF-2 and eIF-2B, it was found that addition of eIF-2 · eIF-2B complex stimulates protein synthesis in a catalytic fashion in the inhibited lysates, whereas highly purified eIF-2 appeared to act stoichiometrically in rescuing only one round of translation initiation (26, 27, 30, 43). However, from studies with purified eIF-2 and eIF-2B, Rowlands et al. (41) proposed an alternative model in which phosphorylated eIF-2 [eIF-2(αP)] acts as a competitive inhibitor of eIF-2B. In this competitive inhibition model, sequestration of eIF-2B does not arise from an excessively slow dissociation rate of eIF-2(αP) · eIF-2B complexes but rather from an increased rate of association between eIF-2B and eIF-2(αP) versus eIF-2B and nonphosphorylated eIF-2. In this

* Corresponding author. Phone: (301) 496-4480. Fax: (301) 496-0243. Electronic mail address: ahinnebusch@nih.gov.

TABLE 1. Plasmids used

Yeast gene(s)	Translation factor(s)	Plasmid[a]	Parent vector	Reference or source
SUI2	eIF-2α	p925	YEp24	13
SUI3	eIF-2β	p927	YEp24	This study
GCD11	eIF-2γ	p1781	YEp24	This study
SUI2, SUI3, GCD11	eIF-2αβγ	p1780	YEp24	This study
GCN3	eIF-2Bα	Ep69[b]	YCp50	18
GCD7	eIF-2Bβ	pJB99[b]	pRS316	3
GCD1	eIF-2Bγ	YCp50-Sc4014[b]	YCp50	20
GCD2	eIF-2Bδ			
GCD6	eIF-2Bε			
GCN3, GCD7, GCD2	eIF-2Bαβδ	p1871	pRS426	This study
GCD7, GCD2	eIF-2Bβδ	p1872	pRS426	This study
GCD1, GCD6	eIF-2Bγε	p1873	pRS425	This study
IMT4	$tRNA_i^{Met}$	p1775	pRS425	This study
IMT	$tRNA_i^{Met}$	pIMT100	YEp351	48

[a] All plasmids except those indicated are high copy number in *S. cerevisiae.*
[b] Low-copy-number yeast plasmids.

way, eIF-2B is effectively sequestered in inactive complexes; however, this inhibition can be reversed rapidly by dephosphorylation of free eIF-2(αP).

In *S. cerevisiae*, the translational regulation of *GCN4* expression is mediated in *cis* by four short upstream open reading frames (uORFs) in the *GCN4* mRNA leader. A number of *trans*-acting regulatory factors have also been identified, including the five subunits of eIF-2B (encoded by the *GCN3*, *GCD7*, *GCD1*, *GCD2*, and *GCD6* genes) and the three subunits of eIF-2 (encoded by the *SUI2*, *SUI3*, and *GCD11* genes) (see Table 1). Recessive mutations in the subunits of eIF-2 and eIF-2B that reduce cellular growth, and thus presumably reduce activity of the corresponding factor, lead to increased levels of *GCN4* expression (22, 23). Extensive mutational analysis of the uORFs has led to the following model to explain the translational regulation of *GCN4* expression (1). Following translation of the first (5′-most) uORF, ribosomes resume scanning and must rebind certain initiation factors before they can recognize a downstream AUG codon as a translation start site. When amino acids are plentiful, the critical initiation factors are abundant, and ribosomes can readily reinitiate translation at one of the three remaining uORFs. Translation of uORF 2, 3, or 4 prevents ribosomes from reaching the *GCN4* ORF, and thus *GCN4* expression is repressed. It is postulated that under amino acid starvation conditions, the critical translation initiation factors become limiting. Ribosomes translate uORF 1 and resume scanning; however, ribosomes must scan a greater distance before rebinding the critical factors needed to reinitiate translation. A substantial fraction of the ribosomes bypass the inhibitory uORFs 2, 3, and 4 and become competent to reinitiate translation only while scanning between uORF 4 and the *GCN4* start codon.

It has been shown that the eIF-2α kinase GCN2 is a positive regulator of *GCN4* expression and that phosphorylation of eIF-2α on Ser-51 is required for induction of *GCN4* translation. By analogy with mammalian systems, it was postulated that phosphorylation of eIF-2 would reduce the abundance of the active GTP-bound form of eIF-2 by inhibiting the recycling factor eIF-2B. This, in turn, would lower the concentration of the eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complex. It was proposed that the eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complex is the rate-limiting factor determining the site of reinitiation on *GCN4* mRNA (13). When the levels of this complex are reduced by phosphorylation of eIF-2, this would allow ribosomes to ignore the start codons at uORFs 2 to 4 and reinitiate further downstream at *GCN4* instead. This model is in accord with the fact that mutations in all five subunits of eIF-2B that lead to constitutive derepression of *GCN4* translation have been identified (7, 22, 23). Moreover, mutations in the α (GCN3), β (GCD7), and δ (GCD2) subunits of eIF-2B that impair derepression in the presence of high levels of eIF-2(αP) have been isolated (46).

The structural genes encoding the five subunits of eIF-2B (3, 18, 20, 35) and the three subunits of eIF-2 (11, 14, 17) and the four genes encoding $tRNA_i^{Met}$ (5, 8) have all been cloned from *S. cerevisiae* (Table 1). The availability of these genes and the advantages of the yeast genetic system gave us a novel way of testing in vivo the idea that phosphorylation of eIF-2α decreases the rate of translation initiation primarily by inhibiting eIF-2B function and thereby reducing the formation of eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complexes. In addition, while the current model for *GCN4* regulation is consistent with the phenotypes of various mutations affecting subunits of eIF-2 and eIF-2B, the direct manipulation of ternary-complex levels by altering the dosage of wild-type eIF-2 and eIF-2B offers a straightforward means of testing our model. We have reduced the in vivo concentration of $tRNA_i^{Met}$ by deleting two of its structural genes and increased the levels of $tRNA_i^{Met}$, the three subunits of eIF-2, or all five subunits of eIF-2B and examined the effects of these genetic manipulations on *GCN4* and general translation. The results of this analysis strongly support the idea that the ternary complex is the principal factor which is rate limiting for translation in vivo when high levels of eIF-2α are phosphorylated on serine-51. Our findings also demonstrate more directly than in previous studies that the ternary complex is the cardinal factor determining the site of reinitiation on *GCN4* mRNA and that the frequency of reinitiation at *GCN4* is inversely proportional to the concentration of ternary complexes. The ability to alter the absolute amounts and relative levels of phosphorylated and nonphosphorylated eIF-2 enabled us to obtain in vivo evidence that eIF-2(αP) inhibits eIF-2B in yeast cells by acting as a competitive inhibitor rather than by forming excessively stable inactive complexes with eIF-2B.

The overexpression system has proven its utility in several other ways. It has provided new evidence that the GCN3(α)

TABLE 2. Strains used

Strain[a]	Relevant genotype	Reference or source
ASB133-4a[a]	*MAT***a** *trp1-Δ1 ura3-52 IMT1 IMT2 imt3::TRP1 imt4::TRP1 GAL*$^+$	This work
H70	*MAT*α *his1-29 gcn2-101 gcn3-101 ura3-52 gcd1-502 HIS4-lacZ*	19
H1402[b]	*MAT*α *leu2-3 leu2-112 ura3-52 ino1 HIS4-lacZ*	16
H1472[c]	*MAT*α *his1-29 ura3-52 leu2-3 leu2-112 gcn2::LEU2 HIS4-lacZ*	49
H1486[c]	*MAT*α *his1-29 ura3-52 leu2-3 leu2-112 HIS4-lacZ*	49
H1489[b]	*MAT*α *leu2-3 leu2-112 ura3-52 ino1 gcn3*^{c}*-R104K HIS4-lacZ*	16
H1608[b]	*MAT*α *leu2-3 leu2-112 ura3-52 ino1 GCN2*^{c}*-M719V-E1537G HIS4-lacZ*	37
H1794	*MAT*α *leu2-3 leu2-112 ura3-52 gcn2::LEU2 gcd7-201*	3
H2545[a]	*MAT***a** *trp1-Δ1 ura3-52 IMT1 IMT2 imt3::TRP1 imt4::TRP1 leu2::hisG GAL*$^+$	This work
H2546[a]	*MAT***a** *trp1-Δ1 ura3-52 IMT1 IMT2 imt3::TRP1 imt4::TRP1 leu2::hisG gcn2Δ GAL*$^+$	This work
H2547[a]	*MAT***a** *trp1-Δ1 ura3-52 IMT1 IMT2 imt3::TRP1 imt4::TRP1 leu2::hisG gcn3::URA3 GAL*$^+$	This work

[a] Strains with the same superscript are isogenic.

subunit in eIF-2B renders the complex more susceptible to the inhibitory effects of eIF-2(αP), identified a class of eIF-2B mutations likely to cause defects in binding of eIF-2 · GDP substrate, and shown that the requirement for the guanine nucleotide exchange activity of eIF-2B is indispensable even at high cellular concentrations of eIF-2. Finally, it revealed that imbalanced overexpression of the β subunit of eIF-2 has adverse effects on the abundance and function of the trimeric eIF-2 complex, underscoring the importance of coordinately regulating the expression of the eIF-2 components.

MATERIALS AND METHODS

Plasmids. A summary of the plasmids used in these studies is presented in Table 1. A methionine initiator tRNA gene (*IMT4* [5] = group I [8]) on an 850-bp *Hin*dIII fragment isolated from plasmid C50 (a gift of Mark Cigan) was inserted into the *Hin*dIII site of the high-copy-number *LEU2* plasmid pRS425 (6) to create plasmid p1775. YEp351 is a high-copy-number *LEU2* vector (21); the construction of pIMT100 carrying a wild-type chimeric *IMT* gene on YEp351 was described previously (48). The plasmids constructed to overexpress the subunits of eIF-2 are all derivatives of the high-copy-number *URA3* vector YEp24 (39). In all cases, the transcriptional orientation of the inserted gene(s) was the same as that of *URA3*. Plasmid p925 contains a 2.7-kb *Bam*HI fragment containing the *SUI2* (eIF-2α) gene isolated from p597 (13) and inserted in the unique *Bam*HI site of YEp24. Plasmid p596 contains the wild-type *SUI3* (eIF-2β) gene (14) on a 1.8-kb *Hin*dIII fragment inserted at the unique *Hin*dIII site of the low-copy-number *URA3* plasmid YCp50 (39). This same 1.8-kb *Hin*dIII fragment was inserted into the unique *Hin*dIII site of pRS316 (44) to create plasmid p921. The 2.15-kb *Bam*HI-*Pvu*II fragment containing the *SUI3* gene from p921 was used to replace the corresponding 1.7-kb *Bam*HI-*Pvu*II fragment in YEp24 to create p927. The 2.7-kb *SUI2 Bam*HI fragment was inserted into the unique *Bam*HI site of p927 to create plasmid p1778. A 4.3-kb *Bgl*II-*Bam*HI fragment carrying the *GCD11* (eIF-2γ) gene was isolated from Ep264 (17) (a gift of Ernie Hannig, University of Texas at Dallas) and inserted into the unique *Bam*HI sites of plasmids of YEp24 and p927 to create plasmids p1781 and p1779, respectively. The 2.7-kb *SUI2 Bam*HI fragment was then inserted into the unique *Bam*HI sites of plasmids p1781 and p1779 to create plasmids p1782 and p1780, respectively. Thus, a family of high-copy-number YEp24 plasmids carrying various combinations of eIF-2 subunit genes (α, p925; β, p927; γ, p1781; αβ, p1778; αγ, p1782; βγ, p1779; and αβγ, p1780) were constructed.

Plasmid p1871, a high-copy-number *URA3* plasmid containing the intact *GCD2*, *GCD7*, and *GCN3* genes with their own promoters, was constructed by isolating the following three DNA fragments: 2.6-kb *Cla*I-*Eag*I *GCD2* fragment from pMF12 (15), 2.1-kb *Eag*I-*Eco*RI *GCD7* fragment from pJB99 (3), and 4.0-kb *Eco*RI-*Bam*HI *GCN3* fragment from Ep69 (18). These fragments were ligated with *Cla*I- and *Bam*HI-digested pRS426 vector DNA (6). Plasmid p1872, a plasmid similar to p1871 but lacking the *GCN3* gene, was made by ligating the 2.6-kb *GCD2* and 2.1-kb *GCD7* DNA fragments, described above, to *Cla*I- and *Eco*RI-digested pRS426. Plasmid p1873, a high-copy-number *LEU2* plasmid derived from pRS425 (6), containing the intact *GCD1* and *GCD6* genes with their own promoters, was constructed by first isolating a 3.8-kb *Bam*HI DNA fragment containing the *GCD1* gene from p743. Plasmid p743 contains this 3.8-kb *GCD1* fragment from AHp257 inserted at the *Bam*HI site of YEp24. The *Bam*HI ends were end filled by treatment with the Klenow fragment of DNA polymerase I, and *Xho*I linkers were added. The resulting *GCD1* fragment was inserted into *Xho*I-digested pJB115 (4), which contains the *GCD6* gene on pRS425. The $tRNA^{Ile}$ gene located upstream of the *GCD6* gene (3) was removed during this construction.

Genetic methods and construction of yeast strains. Standard methods were used for culturing and transformation of yeast strains (25, 42). The procedures for testing amino acid analog sensitivity (23) have been described previously.

The yeast strains used or constructed are listed in Table 2. ASB133-4a was obtained as an ascospore from a cross of ASB114-18a (*Mat***a** *ura3-52 trp1-Δ1 imt3::TRP1*) and ASB112-1b (*Mat*α *ura3-52 trp1-Δ1 ade2 imt4::TRP1*). The method used to construct the *imt3::TRP1* and *imt4::TRP1* alleles and the procedure used to generate and identify the double-disrupted strain have been described previously (5, 48). H2545 was constructed by transforming ASB133-4a to uracil prototropy with plasmid pNKY85 (2), containing a *leu2::hisG-URA3-hisG* cassette, digested with *Bgl*II (a gift of Nancy Kleckner, Harvard University, Cambridge, Mass.). A Leu^- Ura^+ transformant was isolated and replica plated to 5-fluoroorotic acid medium, yielding H2545 as a spontaneous Leu^- Ura^- segregant. H2546 was constructed by replacing *GCN2* with an unmarked *gcn2Δ* allele by using plasmid p1144, as described previously (13). H2547 is isogenic to ASB133-4a and carries a *gcn3::URA3* disruption allele that was introduced by transforming H2545 with the 3.7-kb *Eco*RI-*Pvu*I fragment isolated from plasmid Ep149, as described previously (18). We verified that the 3-aminotriazole-sensitive ($3\text{-}AT^s$) phenotypes of the *gcn2Δ* and the *gcn3::URA3* alleles in strain H2546 and H2547 could be complemented by the introduction of the corresponding wild-type genes on autonomously replicating low-copy-number plasmids.

Assays of *HIS4-lacZ* and *GCN4-lacZ* expression. Expression assays were conducted as described previously (29, 32) on cell extracts prepared from cultures grown in SD medium containing only the required supplements plus leucine, isoleucine, and valine. The latter supplements were required for the appropriate repression of the fusions in wild-type strains under nonstarvation conditions, especially in strains carrying a high-copy-number *LEU2* plasmid. For repressing conditions, saturated cultures were diluted 1:50 and harvested in mid-logarithmic phase after 6 h of growth. For derepressing conditions, cultures were grown for 2 h under repressing conditions and then for 6 h after the addition of either 3-AT to 10 mM or 8-aza-adenine to 50 μg/ml, as described previously (38). Values shown are the averages of determinations made on two to four independently derived transformants. β-Galactosidase activities are expressed as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per minute per milligram of protein.

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 (11) were carried out as described previously (13) except that antigen-antibody complexes were detected by the enhanced chemiluminescence system (Amersham), following the vendor's instructions.

Gel filtration and immunoblot analysis of eIF-2 overexpression. Two liters of strain H1472 carrying either the empty vector YEp24 or the eIF-2 overexpression plasmid p1780 were grown in SD medium with minimal supplements to an optical density at 600 nm (OD_{600}) of approximately 2, harvested, and disrupted as described previously (10). Extracts were cleared by centrifugation at 22,000 × *g* for 15 min, and ribosomes were pelleted at 200,000 × *g* for 2 h. The ribosome pellets were suspended in lysis buffer (20 mM Tris-HCl [pH 7.5], 100 mM KCl, 30 mM $MgSO_4$, 1 mM dithiothreitol) containing 500 mM KCl and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, pepstatin A [0.7 μg/ml], leupeptin [1 μg/ml], and aprotinin [1 μg/ml]). After suspension, the solutions were mixed for 40 min at 4°C and then centrifuged for 2 h at 200,000 × *g*. The resulting supernatants are referred to as the ribosomal salt wash (RSW). A total of 300 μl of the RSW, containing approximately 1.2 to 1.8 mg of protein, was loaded on a Superose 6 HR 10/30 column (10 by 300 mm; Pharmacia) equilibrated with 20 mM Tris-HCl (pH 7.5)–500 mM KCl–2 mM $MgSO_4$ and the protease inhibitors indicated above. Fractions (400 μl) were collected, and 30-μl aliquots from each

fraction were fractionated by sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis (SDS–10% PAGE). The eIF-2α, β, and γ proteins were identified by immunoblot analysis with antibodies specific for these proteins. Antibodies against eIF-2α and eIF-2β were prepared as described previously (11, 14); antibodies against eIF-2γ (17) were a gift of Ernie Hannig, University of Texas at Dallas. The Superose 6 column was calibrated with thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), and myoglobin (17 kDa) (Bio-Rad Laboratories) as size standards.

Coimmunoprecipitation and immunoblot analysis of eIF-2B overexpression. Three liters of strain H1402 carrying high-copy-number plasmids containing the genes for all five subunits of eIF-2B (p1871 and p1873), plasmids encoding only four of the eIF-2B subunits (lacking *GCN3*; p1872 and p1873), or empty vectors (pRS425 and pRS426) was grown in SD medium with minimal supplements to an OD_{600} of ca. 2. Cells were harvested, and an RSW (final concentration, ca. 3 mg/ml) was prepared as described above for the analysis of eIF-2 overexpression. For coimmunoprecipitation, 0.1 g of protein A-Sepharose CL-4B beads (Pharmacia) was swollen in 100 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 7.5) containing 1 mg of bovine serum albumin per ml. The beads were then washed three times with binding buffer (20 mM Tris [pH 7.5], 50 mM KCl, 1 mM dithiothreitol, 0.1% Triton X-100) and finally resuspended in 2 ml of binding buffer. Ten microliters of GCD6-specific antiserum (4) was incubated with 40 μl of protein A-Sepharose bead suspension in 200 μl of binding buffer for 1 h at room temperature. The beads were pelleted and washed twice with 500 μl of binding buffer. One hundred micrograms of RSW was added to the beads, and the total volume of the sample was brought to 200 μl with binding buffer containing the protease inhibitors described above. The mixtures were incubated at 4°C for 3 h with rocking, and then the beads were pelleted by 5 s of centrifugation and washed three times with binding buffer. Fractions of the pellet or supernatant were subjected to SDS–10% PAGE, immunoblotted, probed with antibodies against the yeast eIF-2B or eIF-2 subunits as described previously (4, 7, 10), and detected with ^{125}I-protein A (Amersham) according to the vendor's instructions.

RESULTS

Reduced *IMT* gene copy number increases *GCN4* expression. *GCN4* expression in yeast cells appears to be sensitive to the level of active eIF-2 (22, 23). Since eIF-2 functions as part of a ternary complex composed of eIF-2, GTP, and Met-$tRNA_i^{Met}$, it was proposed that the levels of this ternary complex dictate the site of translational reinitiation on the *GCN4* mRNA (13). To test this hypothesis, we genetically altered $tRNA_i^{Met}$ levels and examined the effects on *GCN4* expression. Wild-type *S. cerevisiae* has four or five copies of *IMT* genes encoding $tRNA_i^{Met}$ (5). Elimination of the *IMT3* and *IMT4* genes from yeast strains containing four *IMT* genes results in a modest reduction in growth rate (5). It has been shown that such strains contain reduced levels of $tRNA_i^{Met}$; therefore, the slow-growth phenotype probably results from diminished ternary-complex formation (unpublished observation). To determine whether the reduction in $tRNA_i^{Met}$ levels leads to derepression of *GCN4*, an *imt3 imt4* double mutant was transformed with either a high-copy-number vector or the vector carrying an *IMT* gene. The transformants were replica plated to medium containing different amino acid analogs that differentially affect the growth of wild-type yeast strains and mutants defective for *GCN4* translational control. Growth on medium containing 5-fluorotryptophan (5-FT) and 1,2,4-triazolealanine (TRA) requires high constitutive expression of tryptophan and histidine biosynthetic genes, which are regulated by GCN4 (34, 50). Thus, mutants that are constitutively derepressed for *GCN4* can grow on medium containing 5-FT and TRA at concentrations that inhibit wild-type growth. 3-AT is a competitive inhibitor of histidine biosynthesis, and only cells with inducible (wild-type) or constitutively derepressed *GCN4* expression can grow on this medium (24, 50).

Strain H2545, lacking the *IMT3* and *IMT4* genes, grew on first row, both 5-FT plus TRA and 3-AT-containing media (Fig. 1, first row, columns labeled vector). Growth on 3-AT medium was expected, since the strain contains all the *GCN* genes required for induction of *GCN4* expression; however, growth on 5-FT plus TRA medium indicates that the strain is constitutively derepressed for genes subject to *GCN4* control.

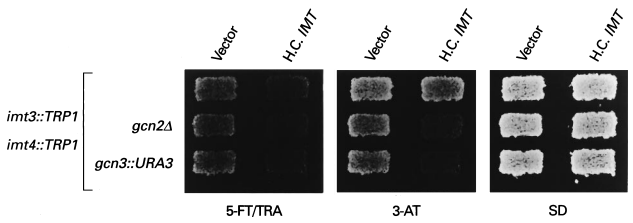


FIG. 1. Modulation of *IMT* gene copy number alters general amino acid control. Isogenic *imt3 imt4* strains H2545 (*GCN2 GCN3*), H2546 (*gcn2*Δ *GCN3*), and H2547 (*GCN2 gcn3*::*URA3*) were transformed with the high-copy-number *IMT* plasmid pIMT100 or with the vector YEp351 alone. Patches of transformants were grown to confluence on SD plates and replica-plated to 5-FT plus TRA plates (0.5 mM 5-FT plus 0.14 mM TRA), 3-AT plates (30 mM), and SD plates. Plates were incubated for 1 to 3 days at 30°C. The first column of strains for each type of medium contains the vector alone; strains in the second column contain the high-copy-number *IMT* plasmid.

Introduction of a high-copy-number plasmid carrying an *IMT* gene prevented growth of the *imt3 imt4* strain on 5-FT plus TRA medium, indicating that the derepressed phenotype of strain H2545 could be reversed by restoring *IMT* expression (Fig. 1, first row, columns labeled H.C. *IMT*). Derepression of *GCN4* expression requires both GCN2, the kinase that phosphorylates eIF-2α, and GCN3, a subunit of eIF-2B thought to mediate the downregulation of eIF-2B by phosphorylated eIF-2 (22, 23). If the elimination of two *IMT* genes in H2545 reduces ternary-complex levels independently of the regulatory mechanism involving phosphorylation of eIF-2, the derepressed phenotype of the strain should be independent of *GCN2* and *GCN3*. As expected, derivatives of H2545 lacking *GCN2* (H2546) or *GCN3* (H2547) and carrying the high-copy-number *IMT* plasmid have a 3-AT-sensitive phenotype, consistent with the requirement for *GCN2* and *GCN3* in derepressing *GCN4* expression under amino acid starvation conditions. In contrast, transformants of strains H2546 and H2547 containing vector alone (i.e., limited for $tRNA_i^{Met}$) were resistant to 3-AT. These results indicate that reduced *IMT* gene dosage mimics the phenotypes of *gcd* mutants in derepressing the general control response in the absence of the positive effectors *GCN2* and *GCN3*. In accord with this interpretation, neither the deletion of *GCN2* nor the disruption of *GCN3* affected the 5-FT/TRA-resistant phenotype associated with the *imt* deletions.

To verify that the reduction in *IMT* copy number led to an increase in *GCN4* expression, we assayed a *GCN4-lacZ* fusion (plasmid p180) in the various *imt3 imt4* strains described above. Because the level of $tRNA_i^{Met}$ overexpression could vary depending on the choice of high-copy-number plasmid, two different sets of high-copy-number vectors and *IMT* plasmids were analyzed. The two sets were found to give similar results for *GCN4-lacZ* expression (Table 3, top four rows versus bottom four rows). As expected, in *GCN2* strains carrying a high-copy-number *IMT* plasmid, we saw relatively low expression of the fusion under the repressing condition of amino acid sufficiency and a four- to sixfold derepression of fusion enzyme in response to amino acid starvation (Table 3). Moreover, this derepression under starvation conditions was largely dependent on *GCN2* in that *GCN4-lacZ* expression increased only 1.4- to 1.8-fold under starvation conditions in the *gcn2*Δ transformants bearing a high-copy-number *IMT* plasmid. Consistent with the results from the growth tests in Fig. 1, *GCN4-lacZ* expression was derepressed two- to threefold under nonstarvation conditions in the *imt3 imt4* transformants carrying the vector alone compared with expression in the corresponding transformants carrying the high-copy-number *IMT* plas-

TABLE 3. Reduced *IMT* gene copy number increases *GCN4* expression at the translational level[a]

GCN2 allele	Plasmid	GCN4-LacZ enzyme activity (U) in transformants containing:			
		p180 (uORFs 1–4)		p226 (uORF 4)	
		R	DR	R	DR
GCN2	pRS425 (vector)	150	230	21	31
	p1775 (H.C. *IMT*)[b]	55	213	20	40
*gcn2*Δ	pRS425	120	150	21	31
	p1775	61	87	23	39
GCN2	YEp351 (vector)	87	210	15	30
	pIMT100 (H.C. *IMT*)	42	250	14	38
*gcn2*Δ	YEp351	79	120	14	26
	pIMT100	25	45	14	32

[a] β-Galactosidase activity was measured in extracts of *imt3*::*TRP1 imt4*::*TRP1* strains H2545 (*GCN2*) and H2546 (*gcn2*Δ) transformed with plasmids harboring *GCN4-lacZ* fusions which contain all four uORFs (p180) or in which uORFs 1 to 3 have been removed by point mutations in their ATG codons while uORF4 remains intact at its normal location (p226). These strains were also transformed with the high-copy-number plasmid p1775 or pIMT100, containing an *IMT* gene, or with a vector (pRS425 or YEp351) alone, as indicated. Assays were done on extracts from cells grown under repressing (R, nonstarvation) and derepressing (DR, histidine starvation imposed by 3-AT) conditions. The results shown are averages of assays conducted on two or three independent transformants, and the individual measurements deviated from the average values shown here by 25% or less.

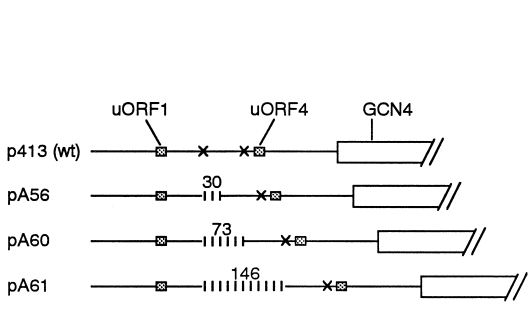
[b] H.C., high copy number.

mid. This increase in *GCN4-lacZ* expression was of the same magnitude in the *gcn2*Δ and *GCN2* strains and therefore occurs independently of GCN2. Under starvation conditions, *GCN4-lacZ* expression was higher in the *GCN2* than the *gcn2*Δ strains, reflecting the GCN2-dependent derepression response to histidine starvation.

The translational regulation of *GCN4* expression is dependent on the uORFs in the *GCN4* mRNA (1). If the reduction in *IMT* gene dosage stimulates *GCN4* expression through the established translational control mechanism, then the increased *GCN4* expression seen in the *imt3 imt4* mutants should require uORF 1. To test this prediction, we assayed a *GCN4-lacZ* construct containing uORF 4 alone, which is defective for derepression under starvation conditions (33). Decreasing the *IMT* gene dosage did not increase *GCN4* expression from this construct (plasmid p226 [Table 3]), indicating that the derepressing effect of reducing the level of $tRNA_i^{Met}$ on *GCN4* expression occurs at the translational level. In previous experiments, we found that the *GCN4-lacZ* constructs on plasmids p180 and p226 were expressed at very similar levels in wild-type strains under nonstarvation conditions (13). In contrast, the strains carrying the high-copy-number *IMT* plasmids showed two- to threefold-higher levels of expression from p180 than from p226 in the nonstarved cultures (Table 3). This may reflect activation of a *GCN2*-independent mechanism to derepress *GCN4* expression in response to uncharged tRNA (28, 47).

To test further the idea that the *imt3 imt4* double mutation mimics *gcd* mutations in derepressing *GCN4* translation, we examined *GCN4-lacZ* alleles with altered spacing between uORFs 1 and 4 in the leader. Previous work has shown that wild-type strains subjected to starvation and *gcd1* mutant strains exhibit high-level translation of *GCN4* that is gradually reduced as the spacing between uORFs 1 and 4 is progressively increased (1) (see Fig. 2, column labeled *gcd1*). This result provided strong evidence that, under derepressing conditions, ribosomes bypass uORF 4 because they have not rebound a critical initiation factor before reaching this site in the leader. When the distance between uORF 1 and uORF 4 is increased, ribosomes are given more time to rebind the factor(s) required for reinitiation, and this prevents them from bypassing uORF 4. Similar to *gcd1* and starved wild-type cells, the *imt3 imt4* transformant bearing the vector alone showed high *GCN4* expression that was eliminated by increasing the spacing between uORFs 1 and 4. For the construct containing an insert of 146 nucleotides between uORFs 1 and 4, there was no significant difference in *GCN4-lacZ* expression between the *imt3 imt4* strains transformed with the *IMT* plasmid and those carrying the vector alone (Fig. 2). These results provide direct support for the idea that ribosomes skip over the start codons at uORFs 2 to 4 under starvation conditions because of reduced levels of the eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complex.

Increasing the gene dosage for eIF-2 or eIF-2B suppresses the slow-growth phenotype of a *GCN2*^{c} mutant. In mammalian cells, phosphorylation of eIF-2α impairs translation by inhibiting the guanine nucleotide exchange factor eIF-2B (30, 41, 43). If the same mechanism operates in yeast cells, then an increase in either the eIF-2 or eIF-2B level would be expected



	GCN4-lacZ activity					
	imt3, imt4					
	+ vector		+ H.C. *IMT*			
	R	DR	R	DR	*gcn2*	*gcd1*
p413 (wt)	132	229	78	199	31	350
pA56	128	194	50	125	30	190
pA60	48	72	31	70	27	89
pA61	34	41	30	56	28	67

FIG. 2. Derepression of *GCN4* elicited by reduced *IMT* gene copy number shows the same dependency as a *gcd1* mutation on the distance between uORFs 1 and 4. The schematics on the left depict constructs containing insertions of 30, 73, or 146 nucleotides between uORFs 1 and 4 in the *GCN4* mRNA leader, which were described previously (1). wt, wild type. X's indicate point mutations in the ATG codons of uORFs 2 and 3. The constructs were introduced into the *imt3 imt4* strain H2545 carrying either the high-copy-number (H.C.) *IMT* plasmid p1775 or the vector pRS425. β-Galactosidase activities were measured in the indicated transformants grown under nonstarvation (R) or histidine starvation (DR) conditions as described in Table 3, footnote *a*. The results obtained from different transformants of the same strain were averaged; the maximum standard error in these experiments was 27% of the mean value. The results for the *gcn2* and *gcd1* mutants represent the averages of the data obtained under both R and DR conditions; these results were taken directly from Fig. 6 in reference 1 and are presented for comparison.

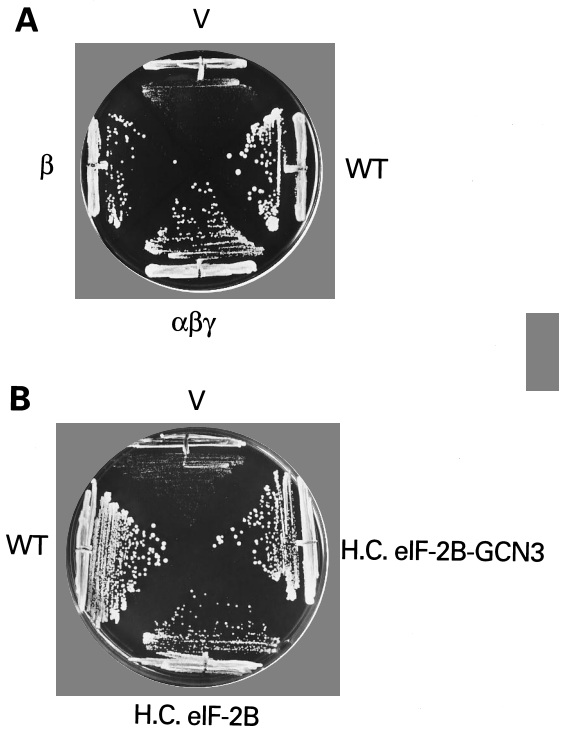


FIG. 3. Overexpression of eIF-2 or eIF-2B alleviates the slow-growth phenotype of strains containing *GCN2^c-M719V-E1537G*. (A) Yeast strain H1608 was transformed with plasmid YEp24 (vector [V]), p927 (β), or p1780 (αβγ). To provide a wild-type (WT) control, the isogenic *GCN2* strain H1402 was transformed with YEp24 (WT). The transformants were streaked on minimal SD medium supplemented only with the required nutrients and incubated for 3 days at 30°C. (B) Yeast strain H1608 was simultaneously transformed with plasmids pRS425 and pRS426 (V), p1871 and p1873 (high-copy-number [H.C.] eIF-2B), or p1872 and p1873 (H.C. eIF-2B-GCN3). The isogenic *GCN2* strain H1402 transformed with pRS425 and pRS426 (WT) is shown as a control. The transformants were streaked on minimal SD medium supplemented only with required nutrients and incubated for 3 days at 30°C.

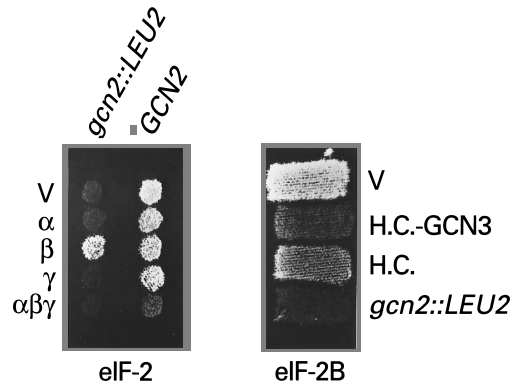


FIG. 4. Overexpression of eIF-2 and eIF-2B alters the general amino acid control response. Isogenic strains H1472 (*gcn2*::*LEU2*) and H1486 (*GCN2*) transformed with different high-copy-number (H.C.) plasmids bearing different genes encoding subunits of eIF-2 or eIF-2B or the vector alone were grown to confluence on SD medium containing minimal supplements, replica plated to SD supplemented with 3-AT (10 mM), and incubated for 3 days at 30°C. (Left) Overexpression of eIF-2. Plasmids YEp24 (vector [V]), p925 (α), p927 (β), p1781 (γ), and p1780 (αβγ) were introduced into the strains with the indicated *GCN2* alleles. (Right) Overexpression of eIF-2B. Plasmids pRS425 and pRS426 (V), p1871 and p1873 (H.C.), and p1872 and p1873 (H.C.-GCN3) were introduced into the *GCN2* strain H1486, and plasmid pRS426 was introduced into strain H1472 (*gcn2*::*LEU2*).

to overcome the effects of eIF-2α phosphorylation on general translation initiation. To test this prediction, we co-overexpressed all three subunits of eIF-2 or either four- or five-subunit forms of eIF-2B in a yeast strain containing a hyperactivated GCN2^c kinase. In addition to causing derepressed *GCN4* expression in the absence of a starvation signal, the kinase allele that we selected (*GCN2^c-M719V-E1537G*) has a severe slow-growth phenotype as a result of the inhibition of general translation initiation (37). Both phenotypes of the *GCN2^c* allele are completely suppressed by the *SUI2-S51A* mutation, which eliminates the phosphorylation site for GCN2 (13, 37).

The genes encoding the α (*SUI2*), β (*SUI3*), and γ (*GCD11*) subunits of eIF-2 were subcloned in a single high-copy-number plasmid, p1780. Introduction of p1780 substantially relieved the slow-growth phenotype of strain H1608 bearing *GCN2^c-M719V-E1537G* (Fig. 3A). This result supports the idea that hyperphosphorylation of eIF-2 is toxic because it reduces the level of active eIF-2 in the cell. High-copy-number plasmids bearing *SUI2* or *GCD11* alone did not suppress (*GCD11*) or only modestly suppressed (*SUI2*) the toxicity of the *GCN2^c* allele (data not shown); however, plasmid p927 bearing *SUI3* alone conferred substantial suppression (Fig. 3A). One interpretation of this latter result is that overexpression of eIF-2β can increase eIF-2 activity and decrease the requirement for eIF-2B. However, as shown in Fig. 7A, overexpression of eIF-2β will not suppress a mutation in the γ (GCD1) subunit of eIF-2B. In addition, the finding that the *GCD1* mutation is suppressed only by co-overexpression of all three subunits of eIF-2 provides genetic evidence that the overexpressed subunits assemble into eIF-2 complexes. The mechanism by which overexpressing the β subunit alone suppresses the *GCN2^c* allele will be discussed below.

Introduction of two high-copy-number plasmids containing the genes encoding all five subunits of yeast eIF-2B (α, *GCN3*; β, *GCD7*; γ, *GCD1*; δ, *GCD2*; and ε, *GCD6*) also partially suppressed the slow-growth phenotype of the *GCN2^c* strain (Fig. 3B), consistent with the notion that phosphorylated eIF-2 inhibits the recycling of eIF-2 by eIF-2B. Interestingly, more complete suppression was conferred by plasmids bearing the genes for only four subunits of eIF-2B, without the α (*GCN3*) subunit (Fig. 3B). This last observation supports our previous conclusion that GCN3 is a regulatory subunit of eIF-2B that mediates the inhibitory effect of eIF-2(αP) on eIF-2B activity (12, 23). Deletion of *GCN3* has been shown to suppress the slow-growth phenotype of *GCN2^c* strains (37). Thus, either increasing the gene dosage for the four-subunit form of eIF-2B lacking GCN3 or deleting *GCN3* from an otherwise wild-type strain suppresses the toxic effects of eIF-2(αP) on translation initiation.

Increasing the gene dosage for eIF-2 or eIF-2B impairs general amino acid control in a wild-type *GCN2* strain. Having found that reducing $tRNA_i^{Met}$ levels derepresses *GCN4* translation, we reasoned that increasing the levels of eIF-2, the protein component of the ternary complex, should have the opposite effect and prevent derepression of *GCN4*. To test this prediction, we introduced the high-copy-number plasmids encoding either individual subunits or all three subunits of eIF-2 into *GCN2* and *gcn2*::*LEU2* strains and replica plated them to medium containing 3-AT. Plasmids carrying single genes for the α, β, or γ subunit of eIF-2 did not diminish the 3-AT-resistant phenotype of the *GCN2* strain, whereas the plasmid encoding all three subunits of eIF-2 substantially reduced 3-AT resistance in this strain (Fig. 4). Curiously, the plasmid encod-

TABLE 4. Overexpression of eIF-2 or eIF-2B lowers *HIS4-lacZ* expression in both wild-type *GCN2* and constitutively derepressed *GCN2*^{c} mutant strains[a]

Plasmid(s)	Overexpressed protein(s)	HIS4-LacZ enzyme activity (U) in transformants of strains:			
		H1486 (*GCN2*)		H1608 (*GCN2*^{c})	
		R	DR	R	DR
YEp24	None	210	970	2,400	2,600
p1780	eIF-2	230	530	1,000	920
pRS425 + pRS426	None	190	830	1,200	1,100
p1871 + p1873	eIF-2B	240	540	600	660
p1872 + p1873	eIF-2B-GCN3[b]	260	370	410	430

[a] β-Galactosidase activities were measured in extracts of nonisogenic yeast strains H1486 (*GCN2*) and H1608 (*GCN2*^{c}*-M719V-E1537G*) bearing the indicated plasmids. The assays on the upper two and lower three sets of strains were conducted independently. Strains were grown under repressing (R, nonstarvation) or derepressing (DR, adenine starvation imposed by 8-azaadenine for H1486 or histidine starvation imposed by 3-AT for H1608) conditions as described in Materials and Methods. The *his1-29* allele in H1486, which makes this strain a sensitive reporter of *GCN4* expression, precludes the use of 3-AT, an inhibitor of histidine biosynthesis, to activate the general control. The results shown are averages of assays conducted on two to four independent transformants, and the individual measurements deviated from the average values shown here by 33% or less.

[b] Co-overexpression of four subunits of eIF-2B (GCD7, GCD1, GCD2, and GCD6), without the GCN3 subunit.

ing the β subunit of eIF-2 alone conferred a 3-AT-resistant phenotype on the *gcn2*::*LEU2* strain, whereas the plasmids encoding eIF-2α, eIF-2γ, or all three subunits of eIF-2 had no effect on the phenotype of this strain (Fig. 4). This last result suggests that overexpression of eIF-2β alone mimics activation of the GCN2 protein kinase in reducing the level of eIF-2 activity in the cell. Introduction of the plasmids encoding all five subunits of eIF-2B into the *GCN2* strain increased 3-AT sensitivity somewhat, whereas the plasmids encoding the four-subunit form of eIF-2B conferred a more substantial 3-AT-sensitive phenotype on this strain (Fig. 4). Thus, it appears that overexpressing intact eIF-2 or the four-subunit form of eIF-2B has comparable effects in preventing derepression of *GCN4* in response to eIF-2α phosphorylation in *GCN2* strains.

A quantitative analysis of the effects of increasing gene dosage for eIF-2 or eIF-2B on *GCN4* expression was achieved by assaying a *HIS4-lacZ* fusion present in the strains used for the replica-plating test. *HIS4* transcription is induced when GCN4 is derepressed in response to starvation for histidine by 3-AT or for purines by 8-aza-adenine (38). Treatment of the *GCN2* strain H1486 with 8-aza-adenine elicited a 4- to 4.5-fold increase in *HIS4-lacZ* expression (Table 4). Introduction of the plasmids encoding all three subunits of eIF-2 or all five subunits of eIF-2B reduced the stimulation of *HIS4-lacZ* expression under starvation conditions to a factor of only 2. As above, the plasmids encoding the four-subunit form of eIF-2B gave more complete reversal of the effects of eIF-2 phosphorylation, reducing the induction of *HIS4-lacZ* to a factor of only 1.4. The *GCN2*^{c} mutation leads to high-level expression of *HIS4* under nonstarvation conditions. Increasing the gene dosage for the eIF-2 complex or for either form of eIF-2B reduced *HIS4-lacZ* expression under both nonstarvation and amino acid starvation conditions in the *GCN2*^{c} strain (Table 4). Thus, introducing additional copies of the genes encoding eIF-2 or eIF-2B reverses the GCN2-mediated derepression of genes subject to GCN4 control. These results provide strong confirmation of the idea that phosphorylation of eIF-2α by GCN2 stimulates

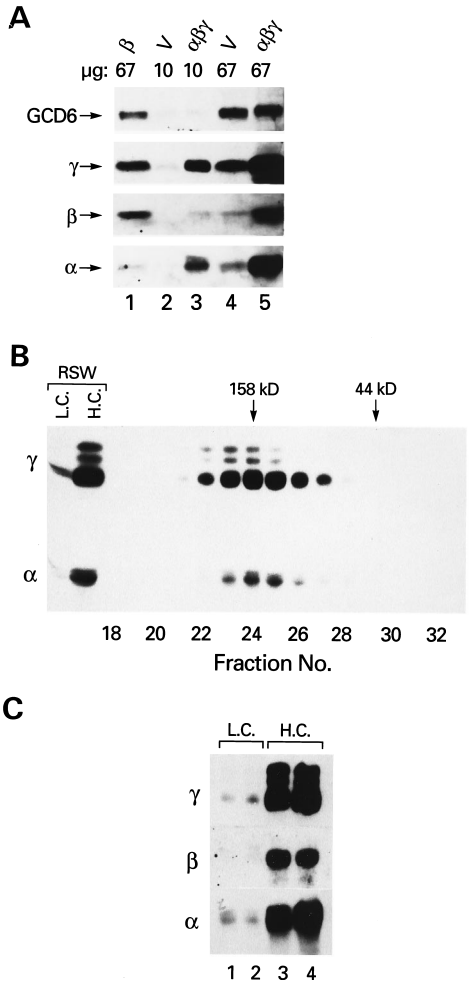


FIG. 5. Biochemical analysis of eIF-2 overexpression by immunoblot analysis of whole-cell extracts and the RSW fractionated by Superose 6 gel filtration chromatography. (A) Samples (10 or 67 μg, as indicated) of whole-cell extracts from strain H1472 transformed with YEp24 (vector [V]; lanes 2 and 4), p927 (β; lane 1), or p1780 (αβγ; lanes 3 and 5) were fractionated by electrophoresis on SDS–10% polyacrylamide gels. Proteins were transferred to nitrocellulose filters and probed with antibodies specific for eIF-2α (1:1,500 dilution), eIF-2γ (1:3,000 dilution), and GCD6 (the δ subunit of eIF-2B; 1:1,500 dilution). The blot was then stripped and probed with antibodies specific for eIF-2β (1:500 dilution). Immune complexes were detected by using the enhanced chemiluminescence system of Amersham, according to the vendor's instructions. The immunoreactive polypeptides are indicated to the left of the appropriate portions of the immunoblot. (B) Immunoblot analysis of the RSW fractionated by Superose 6 gel filtration chromatography. The RSW was prepared as described in Materials and Methods from strain H1472 carrying the high-copy-number eIF-2 plasmid p1780. The RSW was fractionated by molecular size on a Superose 6 gel filtration column, and 0.4-ml fractions were collected. A 30-μl sample of each fraction was subjected to SDS–10% PAGE and analyzed by immunoblotting with antibodies to eIF-2α and eIF-2γ at the dilutions described above. The elution positions of molecular size standards are indicated above the panel, and the positions of the eIF-2α and eIF-2γ polypeptides are indicated on the left. In the two lanes labeled RSW, 90 μg of unfractionated RSW from strain H1472 transformed with the vector (L.C.) or p1780 (H.C.) was analyzed in parallel. (C) Immunoblot analysis of peak eIF-2 fractions from Superose 6 column chromatography of the RSW from the high-copy (H.C.) eIF-2 strain (H1472 transformed with p1780). Fractions 23 and 24 from the same column analyzed in panel B were loaded in lanes 3 and 4, respectively (30 μl each). Equivalent aliquots from the corresponding Superose 6 fractions from the low-copy (L.C.) eIF-2 strain (H1472 transformed with the vector) were loaded in lanes 1 and 2. The panel represents composites of identical gels that were blotted and probed separately with different antibodies. The immunoreactive polypeptides are indicated at the left. The proteins had the expected mobilities except that the predominant eIF-2γ polypeptide was truncated. As shown in the lanes at the far left of panel B, this partial degradation of eIF-2γ occurred in the RSW preparations from both the low-copy-number and high-copy-number eIF-2 strains.

GCN4 translation by decreasing eIF-2B activity and lowering the concentration of eIF-2 · GTP · Met-tRNA$_i^{Met}$ ternary complexes.

Overexpression of eIF-2 and eIF-2B subunits leads to increased amounts of the initiation factor complexes. It was important to demonstrate that the overexpressed subunits of eIF-2 and eIF-2B combine to increase the amounts of the intact heteromeric complexes rather than functioning as independent subunits. To establish this point, we used gel filtration chromatography and coimmunoprecipitation experiments to evaluate the amounts and integrity of the eIF-2 and eIF-2B complexes produced in the overexpressing strains. We first measured the levels of the individual eIF-2 subunits by immunoblot analyses of crude extracts prepared from the appropriate strains. We found that the amounts of eIF-2 α, β, and γ proteins present in 10 μg of crude extract from the strain overexpressing eIF-2 were similar to those present in 67 μg of extract from the strain bearing the vector alone (Fig. 5A, lanes 3 and 4). In contrast, GCD6 protein was found in proportion to the amount of total protein loaded on the gel. In subsequent titration experiments comparing the amounts of the three subunits in wild-type versus overexpressing strains, all three subunits of eIF-2 appeared to be overexpressed between five- and eightfold (data not shown). The apparent decrease in the steady-state level of eIF-2α in strains overexpressing eIF-2β is reproducible and will be discussed further below. Similar experiments conducted on strains overexpressing either four or five subunits of eIF-2B revealed that the eIF-2B subunits were overexpressed between four- and sixfold in the strains bearing the high-copy-number plasmids (Fig. 6, lanes 1 to 3, and data not shown). The levels of overexpression of the different eIF-2B subunits were similar in the transformants overexpressing the four- and five-subunit forms of eIF-2B (except, of course, for GCN3).

To address whether the overexpressed proteins were being incorporated into the appropriate complexes, RSW was prepared from strains overexpressing eIF-2 or eIF-2B or containing the vectors alone. For the strain overexpressing eIF-2, the RSW was fractionated by Superose 6 gel filtration chromatography under high-salt conditions. We found that the α, β, and γ subunits of eIF-2 from the overexpressing strain coeluted from the column in fractions 23 to 25 (Fig. 5B and data not shown). A similar elution profile was obtained for the eIF-2 subunits from the control strain bearing the vector alone (data not shown). In each case, the elution position of the eIF-2 subunits corresponded to a molecular mass of approximately 150 kDa, consistent with the known size of the eIF-2 complex. The GCD6 protein, the ε subunit of eIF-2B, eluted earlier from the column at a position consistent with the larger size of eIF-2B (data not shown). To estimate how much eIF-2 complex was present in the overexpressing strain, we compared equivalent amounts of the two peak eIF-2 fractions from the wild-type and high-copy-number eIF-2 strains. As shown in Fig. 5C, the amounts of all three subunits of eIF-2 were substantially increased in the peak fractions from the overexpressing strain (compare lanes 3 and 4 with 1 and 2). From titration experiments, we estimated that eIF-2 complexes were present in the RSW at ca. 10-fold-higher levels in the overexpressing than in the control strain (data not shown). In accord with these observations, it was shown previously that extracts from strains overexpressing all three subunits of eIF-2, using a related set of plasmids, led to a four- to fivefold increase in ternary-complex-forming activity (17).

The formation of eIF-2B complexes was assessed by coimmunoprecipitation analysis. By using antibodies specific for GCD6, eIF-2B was immunoprecipitated under nondenaturing conditions from RSW prepared from strains overexpressing eIF-2B or bearing vectors alone. The immunoprecipitates were subjected to immunoblot analysis with antibodies against all five subunits of eIF-2B and the α subunit of eIF-2. As shown in Fig. 6, antibodies against GCD6 coimmunoprecipitated the majority of the overexpressed GCD6, GCD1, GCD7, and GCN3 (lanes 6 to 9). The amounts of these four proteins were increased in the immunoprecipitates from the high-copy eIF-2B strain (compare pellets in Fig. 6, lanes 7 and 11 versus 6 and 10) roughly in proportion to their amounts in the RSW (compare lanes 7 and 11 with lane 5). Only a fraction of the overexpressed GCD2 was coimmunoprecipitated with the other four subunits of eIF-2B; in addition, it appears that the GCD2 subunit was truncated by proteolysis. Both of these unexpected results regarding GCD2 were also observed in the wild-type strain (Fig. 6 and data not shown) and thus are not attributable to overexpression of the protein. From a comparison of the immunoblots of GCD2 in whole-cell extracts (Fig. 6, lanes 2 and 3) versus the RSW and immune complexes (Fig. 6, lanes 5, 7, and 11), it seems likely that proteolysis of GCD2

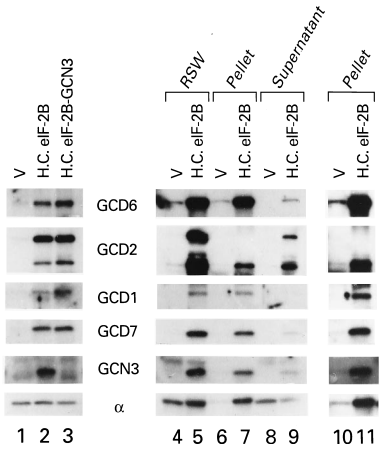


FIG. 6. Biochemical analysis of eIF-2B overexpression by immunoblot analysis of whole-cell extracts and by coimmunoprecipitation from RSW. Protein samples from strain H1402 transformed with plasmids pRS425 and pRS426 (vector [V]; lanes 1, 4, 6, 8, and 10), p1871 and p1873 (high-copy-number [H.C.] eIF-2B; lanes 2, 5, 7, 9, and 11), or p1872 and p1873 (H.C. eIF-2B-GCN3; lane 3) were subjected to SDS–10% PAGE and analyzed by immunoblotting with antiserum against eIF-2B subunits (GCD6, 1:500 dilution; GCD2, 1:500 dilution; GCD1, 1:300 dilution; GCD7, 1:250 dilution; and GCN3, 1:25 dilution) or eIF-2α (α, 1:500 dilution), as indicated between the left two panels. Lanes 1 to 3, whole-cell extracts (25 μg) from the indicated yeast transformants analyzed by immunoblotting; lanes 4 to 9, coimmunoprecipitation of eIF-2B and eIF-2 with antibodies against GCD6, the largest subunit of yeast eIF-2B; lanes 4 and 5, RSW (30 μg) prior to immunoprecipitation; lanes 6 and 7, pellet fractions from RSW (30 μg) following incubation with GCD6 antibody bound to protein A-Sepharose CL-4B beads, as described in Materials and Methods; lanes 8 and 9, supernatant fractions from the immunoprecipitations prepared as described in Materials and Methods (the amount loaded corresponds to 15 μg of RSW); lanes 10 and 11, longer exposure of lanes 6 and 7. For the immunoblot analysis, three identical blots (one blot for detecting GCD6, GCD1, and GCN3; the second blot for detecting GCD7 and the α subunit of eIF-2; and the third blot for detecting GCD2) were prepared for each set of samples. The blots were then divided across the lanes into strips containing fractions of the appropriate molecular weights. The GCD2 protein was partially cleaved during preparation of the extracts and immunoprecipitation reactions (lane 2 versus lanes 5 and 7). The upper bands migrated at the position corresponding to the full-length GCD2 protein. The lower band, approximately 55 kDa, is a truncated form of GCD2. As can be seen in the longer exposure (lane 10), the truncated GCD2 proteins were also present in the samples from yeast cells transformed with empty vectors, indicating that the cleavage is not caused by overexpression. The apparent increase in the level of GCD1 protein in lane 3 versus lane 2 was not reproducible. Additional analyses revealed little or no difference in the levels of overexpression of the four largest eIF-2B subunits whether the α (GCN3) subunit was overexpressed or not.

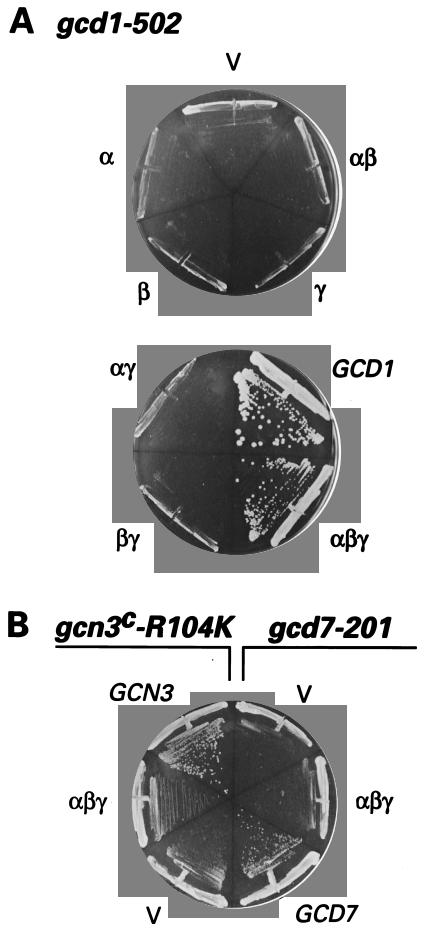


FIG. 7. Overexpression of eIF-2 alleviates the slow-growth phenotype of a *gcd1-502* strain but not of a *gcn3^c-R104K* or *gcd7-201* strain. (A) Yeast strain H70 was transformed with high-copy-number plasmid YEp24 (V), YEp24 carrying the indicated yeast eIF-2 subunit genes (α and/or β and/or γ), or the wild-type *GCD1* gene on low-copy-number plasmid YCp50-Sc4014 (*GCD1* [20]). The indicated strains were streaked on SD plates supplemented with required nutrients, and the plates were incubated at 37°C for 5 days. (B) Yeast strains H1489 (*gcn3^c-R104K*) and H1794 (*gcd7-201*) were transformed with high-copy-number eIF-2 plasmid p1780 (αβγ), with vector YEp24 alone (V), or with the wild-type genes on low-copy-number plasmid Ep69 (*GCN3* [18]) or pJB99 (*GCD7* [3]), as indicated on the panel. The indicated transformants were streaked on SD medium supplemented with required nutrients and incubated at 37°C for 3 days.

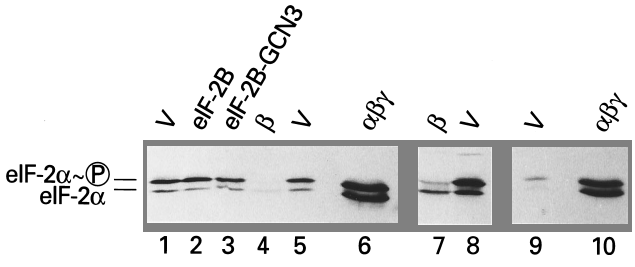


FIG. 8. Isoelectric focusing gel electrophoresis of eIF-2α from strains carrying *GCN2^c-M719V-E1537G* and different high-copy-number plasmids. Yeast strain H1608 transformed with the indicated high-copy-number plasmids was grown under nonstarvation conditions (repressing) for 6 to 7 h. Samples of total cellular protein were separated by isoelectric focusing on a vertical slab gel (13), followed by immunoblot analysis as described previously (11). The anti-yeast eIF-2α antibody was raised against a carboxyl-terminal fusion protein that lacks the serine 51 site of phosphorylation, and thus reactivity should be independent of the phosphorylation state of the protein. The more acidic hyperphosphorylated form of eIF-2α focuses above the less phosphorylated species. The high-copy-number plasmids used were pRS425 and pRS426 (V, lane 1), YEp24 (V, lanes 5, 8, and 9), p927 (β, lanes 4 and 7), p1780 (αβγ, lanes 6 and 10), p1871 and p1873 (eIF-2B, lane 2), and p1872 and p1873 (eIF-2B-GCN3, lane 3). Lanes 7 and 8 are a longer exposure of lanes 4 and 5, respectively. Lanes 9 and 10 are a shorter exposure of lanes 5 and 6, respectively.

occurred during preparation of the RSW and the immunoprecipitations and that this truncated form of GCD2 has reduced affinity for the complex.

It is noteworthy that increased amounts of eIF-2 were coimmunoprecipitated with GCD6 in the strain overexpressing eIF-2B. Previously, we demonstrated that eIF-2 exists both alone and stably associated with eIF-2B in the RSW (7, 10). This explains why the eIF-2 subunits are present in both the pellet and the supernatant fractions after eIF-2B is immunoprecipitated with anti-GCD6 antibodies. For the strain bearing the vector alone, most of the eIF-2α remained in the supernatant, reflecting the larger size of the free pool than of the eIF-2B-bound pool of eIF-2 (Fig. 6, lanes 8 versus 6). In contrast, the majority of eIF-2α was coimmunoprecipitated with GCD6 from extracts of the strain overexpressing eIF-2B (Fig. 6, lanes 7 and 9). The fact that most of the eIF-2 is stably associated with eIF-2B when the latter is being overexpressed is consistent with the idea that all of the eIF-2B in the cell is complexed with eIF-2. In addition, these results provide strong additional support that the overexpressed eIF-2B subunits assemble into functional eIF-2B complexes.

Overexpression of eIF-2 does not bypass the essential requirement for eIF-2B. One possible mechanism by which overexpression of eIF-2 suppresses the effects on translation associated with phosphorylation of eIF-2α is by completely eliminating the requirement for eIF-2B. eIF-2B catalyzes the exchange of GTP for GDP on eIF-2, restoring eIF-2 to its active GTP-bound state (27). At high levels of eIF-2, spontaneous uncatalyzed nucleotide exchange on eIF-2 might be sufficient to support growth, so that overexpression of eIF-2 would bypass the requirement for eIF-2B. To test this possibility, we investigated whether overexpressing eIF-2 could suppress the lethal effect of deleting *GCD1*. A *gcd1*::*LEU2* strain carrying wild-type *GCD1* on a *TRP1* plasmid was transformed with a single-copy *URA3* plasmid, encoding wild-type *GCD1*, or the high-copy-number *URA3* plasmid p1780, encoding all three subunits of eIF-2. The resulting strains were grown on medium containing tryptophan and uracil to permit loss of both plasmids. In the strains carrying two different *GCD1* plasmids, either the *TRP1* or the *URA3* plasmid was frequently lost, with the strain acquiring a Ura^+ Trp^- or Ura^- Trp^+ phenotype, respectively. In contrast, no Trp^- segregants were obtained from the strain overexpressing eIF-2 from the *URA3* plasmid. Thus, overexpression of eIF-2 did not compensate for a complete inactivation of the γ subunit of eIF-2B.

To examine whether overexpression of eIF-2 can compensate for a reduction rather than lack of eIF-2B function, we tested whether overexpressing eIF-2 could suppress the phenotypes of different point mutations in subunits of eIF-2B. Overexpression of eIF-2 did not suppress the growth defect conferred by a *gcn3^c-R104K* or *gcd7-201* mutation in the α and β subunits of eIF-2B, respectively (Fig. 7B). These results indicate that overexpression of eIF-2 does not even dampen the need for eIF-2B function. In addition, they show that the *gcd7-201* and *gcn3^c-R104K* mutations decrease the recycling of eIF-2 by eIF-2B in a way that cannot be overcome simply by producing more substrate eIF-2.

In an additional set of experiments, a set of high-copy-number plasmids encoding all possible combinations of eIF-2 subunits were introduced into the *gcd1-502* strain H70, which is

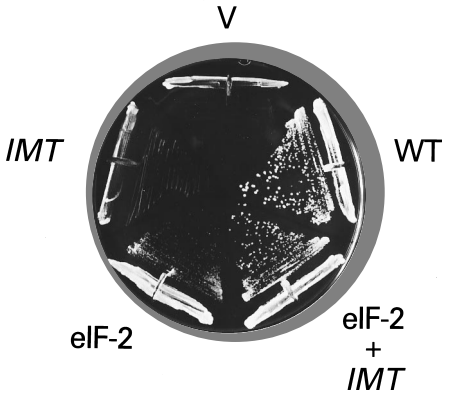


FIG. 9. Overexpressed eIF-2 and $tRNA_i^{Met}$ cooperate to alleviate the slow-growth phenotype of strains containing *GCN2^c-M719V-E1537G*. Yeast strain H1608 (*GCN2^c-M719V-E1537G*) was transformed with high-copy-number plasmids YEp24 and YEp351 (V); YEp24 and pIMT100 carrying a wild-type *IMT* gene (*IMT*); YEp351 and p1780 carrying the eIF-2 subunit genes (eIF-2); or p1780 and pIMT100 (eIF-2 plus *IMT*). As a control, the isogenic wild-type yeast strain H1402 was transformed with YEp24 and YEp351 (WT). The transformants were streaked on SD medium supplemented with the required nutrients and incubated for 2 days at 30°C. Note that the poorer suppression of the *GCN2^c* mutation by the high-copy-number eIF-2 plasmid observed here versus that in Fig. 3A may reflect the fact that the strains in this analysis carry two high-copy-number plasmids instead of one, which may reduce the plasmid copy number relative to the situation in Fig. 3A.

thermosensitive for growth on rich medium at 37°C (19) because of an alteration in the γ subunit of eIF-2B. The high-copy-number plasmid encoding all three subunits of eIF-2 almost completely suppressed this temperature sensitivity, whereas plasmids encoding the individual subunits or combinations of only two subunits of eIF-2 did not (Fig. 7A). Immunoblot analysis showed that the appropriate eIF-2 subunits were being overexpressed in strains bearing these latter plasmids (data not shown). Identical results were obtained for the *gcd1-101* mutation (data not shown). The observation that the temperature sensitivity of these *gcd1* mutants could be suppressed only by co-overexpression of all three eIF-2 subunits supports our biochemical data (Fig. 5) that the overexpressed subunits assemble into active eIF-2 complexes. One possible interpretation of these results is that the *gcd1* mutations impair the ability of eIF-2B to bind its substrate eIF-2, and thus increasing the substrate eIF-2 concentrations can suppress this defect.

Overexpression of eIF-2 affects the level of eIF-2α phosphorylation. One way in which overexpression of eIF-2 or eIF-2B could suppress the slow-growth phenotype of a *GCN2^c* mutation would be to decrease eIF-2α phosphorylation. To test this possibility, we measured eIF-2α phosphorylation on Ser-51 by isoelectric focusing gel electrophoresis of whole-cell extracts followed by immunoblotting with eIF-2α-specific antibodies. Consistent with our previous findings (13, 37), the majority of eIF-2α was phosphorylated on Ser-51 in the *GCN2^c* strain (Fig. 8, lanes 1, 5, 8, and 9). Overexpression of intact eIF-2B or the eIF-2B–GCN3 complex did not reduce the proportion of eIF-2α that was phosphorylated (Fig. 8, lanes 1 to 3), ruling out a mechanism involving a decrease in eIF-2α phosphorylation when eIF-2B is overexpressed. Unexpectedly, eIF-2α phosphorylation as well as the steady-state level of eIF-2α was dramatically lowered in the *GCN2^c* strain overexpressing only eIF-2β (Fig. 8, lanes 7 and 8), providing an explanation for why overexpressing eIF-2β suppressed the *GCN2^c* mutation, as shown in Fig. 3 (see Discussion). This reduction in the steady-state level of eIF-2α in strains overexpressing eIF-2β alone was also observed in Fig. 5A. Co-overexpression of all three subunits of eIF-2 had two marked effects on phosphorylation of eIF-2α on Ser-51. First, the absolute amount of the phosphorylated (inhibitory) form was dramatically increased (Fig. 8, lanes 5 and 6 and 9 and 10). Second, the ratio of phosphorylated to nonphosphorylated eIF-2α was reduced. Shorter exposures of the immunoblot shown in Fig. 8 revealed a ca. 1:1 ratio of phosphorylated to nonphosphorylated eIF-2α when the entire eIF-2 complex was overexpressed (data not shown). As discussed below, the fact that overexpression of eIF-2 can suppress the growth defect of a *GCN2^c* allele even though it greatly increases the absolute amount of eIF-2(αP) has important implications for the mechanism by which eIF-2B is inhibited by phosphorylated eIF-2.

Enhanced suppression of the slow-growth phenotype of a *GCN2^c* strain by co-overexpression of $tRNA_i^{Met}$ and eIF-2. Because reducing *IMT* gene dosage and overexpressing eIF-2 had opposite effects on *GCN4* expression, we wondered if increasing *IMT* gene dosage would augment the effects of overexpressing eIF-2. To examine this possibility, the high-copy-number *IMT* plasmid pIMT100 was introduced into the *GCN2^c* strain alone or in combination with the high-copy-number eIF-2 plasmid p1780. Overexpression of *IMT* alone had no effect on the growth rate of the *GCN2^c* strain (Fig. 9). In contrast, co-overexpression of $tRNA_i^{Met}$ and eIF-2 resulted in more complete suppression of the slow-growth phenotype of the *GCN2^c* allele than occurred with overexpression of eIF-2 alone (Fig. 9). The synergistic effect of overexpressing $tRNA_i^{Met}$ along with eIF-2 suggests that $tRNA_i^{Met}$ can become limiting in cells overexpressing eIF-2. This is consistent with the idea that limitations in the levels of the eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complex, and not simply eIF-2, are responsible for the slow-growth phenotype of *GCN2^c* strains.

DISCUSSION

Evidence that phosphorylation of eIF-2 in yeast cells regulates translation initiation by reducing the formation of eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complexes. According to our model for *GCN4* translational control, ribosomes translate uORF 1, resume scanning, and reinitiate at one of the four AUG start codons located downstream in the *GCN4* mRNA. Under nonstarvation conditions, reinitiation occurs at uORFs 2 to 4, precluding recognition of the *GCN4* start site. In amino acid-deprived cells, a significant fraction of the ribosomes bypass the start sites at uORFs 2 to 4 and reinitiate at *GCN4* instead. We proposed that the relative utilization of these downstream start sites is dictated by the rate at which ribosomes scanning downstream of uORF 1 are reloaded with charged initiator $tRNA^{Met}$ (1, 13). Because Met-$tRNA_i^{Met}$ is thought to be delivered to the ribosome in a ternary complex with eIF-2 and GTP, the frequency of reinitiation at *GCN4* should be coupled to the concentration of this ternary complex.

Previously, we showed that the protein kinase GCN2 stimulates *GCN4* translation by phosphorylating the α subunit of eIF-2 (13). By analogy with mammalian systems, we hypothesized that phosphorylated eIF-2 inhibits guanine nucleotide exchange on eIF-2 catalyzed by the yeast equivalent of eIF-2B, thereby decreasing the concentration of ternary complexes in the cell. This hypothesis is in accord with the isolation of mutations in subunits of yeast eIF-2 or eIF-2B that either mimic eIF-2 phosphorylation and constitutively derepress *GCN4* in strains lacking GCN2 (22, 23) or prevent *GCN4* derepression in the presence of high-level eIF-2α phosphorylation (45, 46). The ability to modulate the levels of wild-type eIF-2, eIF-2B, and $tRNA_i^{Met}$ has provided us with a more

straightforward means of testing our model for *GCN4* translational control. In addition, it has allowed us to obtain in vivo evidence that the eIF-2 · GTP · Met-$tRNA_i^{Met}$ ternary complex is the critical factor limiting translation in cells where high levels of eIF-2α are phosphorylated on serine 51 and to probe the molecular mechanism of inhibition of eIF-2B by phosphorylated eIF-2 as it occurs in *S. cerevisiae.*

Previously, Cigan et al. (9) demonstrated that a mutation in the anticodon loop of $tRNA_i^{Met}$ from 3′-UAC-5′ to 3′-UCC-5′ allowed translation to initiate from AGG codons. In addition, mutations that permit recognition of non-AUG codons by wild-type $tRNA_i^{Met}$ have been isolated in the α, β, and γ subunits of eIF-2 (11, 13a, 14). These findings demonstrate important roles for both $tRNA_i^{Met}$ and eIF-2 in AUG codon recognition. Since $tRNA_i^{Met}$ and eIF-2 are both components of the ternary complex, it is reasonable to suppose that the ternary complex functions directly in AUG codon recognition. We have shown that reducing $tRNA_i^{Met}$ gene dosage increases the frequency with which ribosomes scanning downstream from uORF 1 will bypass the start sites at uORFs 2 to 4 and reinitiate further downstream at *GCN4* (Fig. 1 and 2 and Table 3). Overexpressing eIF-2 under conditions in which the level of eIF-2 activity was being diminished by phosphorylation had the opposite effect, decreasing reinitiation at *GCN4* (Fig. 4 and Table 4). These results support the idea that eIF-2 and Met-$tRNA_i^{Met}$ function together as components of the ternary complex to dictate the selection of AUG codons during the process of reinitiation on the *GCN4* mRNA.

Overexpression of eIF-2B had the same effect as overexpressing eIF-2, i.e., decreasing the probability of reinitiation at *GCN4* under conditions in which eIF-2 is being phosphorylated by GCN2 (Fig. 4 and Table 4). Overexpressing either eIF-2 or eIF-2B also reversed the general inhibition of translation initiation that occurs when eIF-2 is phosphorylated at high levels by a genetically activated $GCN2^c$ kinase (Fig. 3). These findings provide in vivo evidence that phosphorylation of eIF-2 in yeast cells impairs translation initiation by inhibiting the recycling of eIF-2 by eIF-2B. The fact that overexpressing a combination of eIF-2 and $tRNA_i^{Met}$ suppressed the slow-growth phenotype of a $GCN2^c$ kinase mutant more completely than did overexpressing either component alone (Fig. 9) strongly supports the idea that the ternary complex is the critical component that limits translation initiation when eIF-2α is phosphorylated on serine 51 in yeast cells.

The more complete suppression of the slow-growth phenotype of a *GCN2^c^* mutation observed in strains overexpressing the four-subunit form of eIF-2B lacking GCN3 than in strains overexpressing all five subunits supports the idea that GCN3 is a regulatory subunit of eIF-2B. Previously, we proposed that GCN3 mediates the inhibitory effect of eIF-2(αP) on eIF-2B function (12, 23), so that deletion of *GCN3* alters the interaction between eIF-2B and phosphorylated eIF-2 and allows eIF-2B to remain more active in the presence of eIF-2(αP). According to this model, overexpression of all five subunits of eIF-2B would suppress the slow-growth phenotype of *GCN2^c^* alleles by increasing the ratio of eIF-2B to its inhibitor eIF-2(αP). The four-subunit form of eIF-2B lacking GCN3 would be a more effective suppressor because, in addition to increasing the eIF-2B/eIF-2(αP) ratio, the overexpressed form of eIF-2B is less susceptible to inhibition by phosphorylated eIF-2.

The only instance in which overexpressing a single subunit of eIF-2 affected translation initiation involved the eIF-2β subunit (encoded by *SUI3*). Overexpression of this protein alone alleviated the growth-inhibitory effect of a *GCN2^c^* mutation to the same extent as did overexpression of intact eIF-2 (Fig. 3A). In contrast, overexpression of eIF-2β alone did not complement any of the mutations affecting subunits of eIF-2B (Fig. 7 and data not shown). Moreover, it appeared to elicit partial derepression of *GCN4* in a *gcn2* mutant (Gcd^- phenotype [Fig. 4 and data not shown]), whereas overexpression of the intact eIF-2 complex impaired derepression of *GCN4* (Gcn^- phenotype [Fig. 4 and Table 4]). The results in Fig. 8 show that suppression of the *GCN2^c^* mutation by excess eIF-2β can be attributed to a reduction in the level of eIF-2 phosphorylation, although it is not clear why this occurs. One possibility is that the β subunit can directly inhibit GCN2 kinase function. An alternative model is that overexpressed eIF-2β stimulates partial dissociation of eIF-2 into αβ and βγ dimers that are not phosphorylated by GCN2 and might even inhibit phosphorylation of intact eIF-2. This partial dissociation of eIF-2 could also account for the Gcd^- phenotype associated with overexpressing eIF-2β (Fig. 4). In addition, we observed a decrease in the steady-state level of eIF-2α when the β subunit alone was overexpressed (Fig. 5A and 8), perhaps indicating that αβ dimers are degraded more rapidly than is the intact eIF-2 complex.

The partial suppression of the *gcn2* mutation by overexpression of only eIF-2β implies a reduction in eIF-2 function that mimics eIF-2α phosphorylation. This seems to be at odds with the fact that overexpressing eIF-2β suppressed the toxicity of eIF-2α hyperphosphorylation by a $GCN2^c$ kinase. To resolve this apparent discrepancy, we propose that the slow-growth phenotype in the *GCN2^c^* mutant results from extensive inhibition of eIF-2B and that relieving this inhibitory effect by lowering eIF-2 phosphorylation restores normal growth even though it involves a reduction in the absolute amount of functional eIF-2 complex.

In addition to demonstrating the importance of ternary-complex levels in translational control by phosphorylated eIF-2, our results revealed two different classes of mutations in eIF-2B. It was originally supposed that mutations in the GCD1, GCD2, GCD6, and GCD7 subunits of eIF-2B all impair cell growth and derepress *GCN4* expression because they reduce the steady-state level or catalytic activity of eIF-2B. Our finding that the slow-growth phenotypes of *gcd1-101* and *gcd1-502* mutants can be suppressed by overexpressing eIF-2 suggests that these mutations reduce the catalytic activity of eIF-2B by decreasing its affinity for the substrate eIF-2 · GDP and thus can be overcome through mass action by increasing the concentration of eIF-2. The fact that *gcd7-201* and *gcn3^c^-R104K* are not suppressed by overexpressing eIF-2 (Fig. 7) implies that these mutations diminish eIF-2B function by altering some other aspect of the exchange reaction distinct from the binding of eIF-2 · GDP substrate. In addition, our results show that even a 10-fold increase in eIF-2 levels does not obviate the requirement for eIF-2B in recycling eIF-2 · GDP to eIF-2 · GTP after each round of initiation.

Evidence that eIF-2(αP) is a competitive inhibitor of eIF-2B. Overexpression of eIF-2 substantially suppressed the growth-inhibitory effects of a $GCN2^c$ kinase (Fig. 3). Direct measurements of eIF-2α phosphorylation indicated that ca. 80% of the eIF-2 was phosphorylated in the *GCN2^c^* strain and that this proportion decreased to ca. 50% in transformants that were overexpressing eIF-2. Because the level of eIF-2 was 5- to 10-fold higher in these transformants, however, the absolute amount of phosphorylated eIF-2 increased by a factor of 3 to 6 when eIF-2 was overexpressed (Fig. 8). Thus, suppression of the slow-growth phenotype of the *GCN2^c^* mutant clearly did not result from a decrease in the cellular concentration of phosphorylated eIF-2.

One widely accepted model for the inhibition of eIF-2B by

phosphorylated eIF-2 proposes that eIF-2(αP) sequesters eIF-2B in a nondissociable complex (26, 36). Because eIF-2 is more abundant than eIF-2B, this model can explain why phosphorylation of only a fraction of eIF-2 is sufficient to completely inhibit eIF-2B. Indeed, the percentage of eIF-2 phosphorylation associated with translational inhibition is higher in Ehrlich ascites cells than in rabbit reticulocyte lysates, consistent with the higher molar ratio of eIF-2B to eIF-2 in Ehrlich cells than in reticulocytes (26, 40). More recently, however, Rowlands et al. (41) proposed that phosphorylated eIF-2 acts as a competitive inhibitor rather than an irreversible inhibitor of eIF-2B. This was based on the fact that complexes between eIF-2B and eIF-2 dissociated too rapidly to be measured, whether or not eIF-2 was phosphorylated. To account for the difference in dissociation constants for the eIF-2 · eIF-2B and eIF-2(αP) · eIF-2B complexes, they inferred that the rate of binding to eIF-2B was much greater for eIF-2(αP) than for eIF-2. In addition, they proposed that the difference in the affinities of eIF-2(αP) and eIF-2 for eIF-2B was so great that the competition between the two forms for binding eIF-2B would effectively mimic sequestering by irreversible inhibition. This difference in affinities would also account for the fact that limited phosphorylation of eIF-2α can completely inhibit translation. An important feature of the competitive-inhibition model is that the inhibition can be rapidly reversed: if the eIF-2(αP) · eIF-2B complexes dissociate rapidly, then dephosphorylation of eIF-2(αP) will immediately abolish inhibition. The irreversible-inhibition model, in contrast, requires dephosphorylation of eIF-2(αP) while it is still complexed to eIF-2B. In view of genetic evidence that Ser-51 of eIF-2α interacts directly with eIF-2B (45), it seems unlikely that this region of eIF-2α would be accessible to a phosphatase when eIF-2 is bound to eIF-2B.

These two mechanisms predict different outcomes for the large increase in the concentration of eIF-2(αP) that occurs in the *GCN2*^{c} strains overexpressing eIF-2 (Fig. 8). The mechanism of irreversible inhibition should be sensitive to the absolute amount of the inhibitor eIF-2(αP), whereas competitive inhibition should be less dependent on the absolute amount of the inhibitor and more sensitive to the ratio of inhibitor eIF-2(αP) to substrate eIF-2. In yeast cells, there is a ca. 10-fold-higher molar amount of eIF-2 than of eIF-2B (6a, 7, 10). Thus, in our *GCN2*^{c} mutant, in which 80% of the eIF-2 is phosphorylated, there should be about an eightfold molar excess of eIF-2(αP) to eIF-2B. In the transformants of this strain that are overexpressing eIF-2, the amount of eIF-2(αP) should be in 25- to 50-fold molar excess over eIF-2B; yet translation initiation was stimulated relative to that in the parental *GCN2*^{c} strain. These observations are difficult to reconcile with the mechanism of irreversible inhibition. Although overexpressing eIF-2 in the *GCN2*^{c} strain greatly increased the molar amount of eIF-2(αP), it led to a decrease in the eIF-2(αP)/eIF-2 ratio from ca. 80% to ca. 50%. The fact that stimulation of translation initiation in the *GCN2*^{c} strain was accompanied by a large increase in the absolute amount of eIF-2(αP) but a decrease in the eIF-2(αP)/eIF-2 ratio is much more consistent with the competitive-inhibition model.

The results presented in this work provide valuable information about the mechanism and regulation of translation initiation in eukaryotic cells. They demonstrate that the ternary complex performs a critical function in AUG start codon recognition during the process of reinitiation. In addition, they provide in vivo evidence that phosphorylation of eIF-2α on Ser-51 impairs initiation by inhibiting the recycling of eIF-2 by eIF-2B and thereby decreases the concentration of ternary complexes. Our results strongly suggest that eIF-2(αP) inhibits eIF-2B in yeast cells by competitive inhibition, in agreement with the in vitro studies of Rowlands et al. (41) on the corresponding mammalian factors. They also raise the interesting possibility that increasing expression of eIF-2β alone or decreasing expression of the α subunit of eIF-2B (GCN3 in yeast cells) could be used as regulatory mechanisms to dampen or counteract the effects of eIF-2α kinases on translation initiation. Finally, our demonstration that the overexpressed subunits of eIF-2 and eIF-2B assemble into the appropriate complexes suggests that the techniques developed in this study will facilitate biochemical analysis of the many interesting regulatory mutations in eIF-2 and eIF-2B that are available for *S. cerevisiae.*

ACKNOWLEDGMENTS

We thank our colleagues for helpful discussions and Graham Pavitt, Ronda Rolfes, and Carlos Vazquez de Aldana for comments on the manuscript.

Work in the laboratory of A.S.B. is supported by grants from the Swedish Natural Science Research Council (project B-BU-4856-305) and the Swedish Cancer Society (project 3516-B94-01XAB).

REFERENCES

1. **Abastado, J. P., P. F. Miller, B. M. Jackson, and A. G. Hinnebusch.** 1991. Suppression of ribosomal reinitiation at upstream open reading frames in amino acid-starved cells forms the basis for *GCN4* translational control. Mol. Cell. Biol. **11:**486–496.
2. **Alani, E., L. Cao, and N. Kleckner.** 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. Genetics **116:**541–545.
3. **Bushman, J. L., A. I. Asuru, R. L. Matts, and A. G. Hinnebusch.** 1993. Evidence that GCD6 and GCD7, translational regulators of *GCN4*, are subunits of the guanine nucleotide exchange factor for eIF-2 in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**1920–1932.
4. **Bushman, J. L., M. Foiani, A. M. Cigan, C. J. Paddon, and A. G. Hinnebusch.** 1993. Guanine nucleotide exchange factor 2 in *Saccharomyces cerevisiae*: interactions between the essential subunits GCD2, GCD6, and GCD7 and the regulatory subunit GCN3. Mol. Cell. Biol. **13:**4618–4631.
5. **Bystrom, A. S., and G. R. Fink.** 1989. A functional analysis of the repeated methionine initiator tRNA genes (*IMT*) in yeast. Mol. Gen. Genet. **216:**276–286.
6. **Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter.** 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene **110:** 119–122.

6a. **Cigan, A. M.** Unpublished data.

7. **Cigan, A. M., J. L. Bushman, T. R. Boal, and A. G. Hinnebusch.** 1993. A protein complex of translational regulators of *GCN4* is the guanine nucleotide exchange factor for eIF-2 in yeast. Proc. Natl. Acad. Sci. USA **90:**5350–5354.
8. **Cigan, A. M., and T. F. Donahue.** 1986. The methionine initiator tRNA genes of yeast. Gene **41:**343–348.
9. **Cigan, A. M., L. Feng, and T. F. Donahue.** 1988. $tRNA_i^{Met}$ functions in directing the scanning ribosome to the start site of translation. Science **242:**93–97.
10. **Cigan, A. M., M. Foiani, E. M. Hannig, and A. G. Hinnebusch.** 1991. Complex formation by positive and negative translational regulators of *GCN4*. Mol. Cell. Biol. **11:**3217–3228.
11. **Cigan, A. M., E. K. Pabich, L. Feng, and T. F. Donahue.** 1989. Yeast translation initiation suppressor *sui2* encodes the alpha subunit of eukaryotic initiation factor 2 and shares identity with the human alpha subunit. Proc. Natl. Acad. Sci. USA **86:**2784–2788.
12. **Dever, T. E., J.-J. Chen, G. N. Barber, A. M. Cigan, L. Feng, T. F. Donahue, I. M. London, M. G. Katze, and A. G. Hinnebusch.** 1993. Mammalian eukaryotic initiation factor 2α kinases functionally substitute for GCN2 in the *GCN4* translational control mechanism of yeast. Proc. Natl. Acad. Sci. USA **90:**4616–4620.
13. **Dever, T. E., L. Feng, R. C. Wek, A. M. Cigan, T. D. Donahue, and A. G. Hinnebusch.** 1992. Phosphorylation of initiation factor 2α by protein kinase GCN2 mediates gene-specific translational control of *GCN4* in yeast. Cell **68:**585–596.

13a. **Donahue, T. F.** Personal communication.

14. **Donahue, T. F., A. M. Cigan, E. K. Pabich, and B. Castilho-Valavicius.** 1988. Mutations at a Zn(II) finger motif in the yeast eIF-2β gene alter ribosomal start-site selection during the scanning process. Cell **54:**621–632.
15. **Foiani, M., A. M. Cigan, C. J. Paddon, S. Harashima, and A. G. Hinnebusch.** 1991. GCD2, a translational repressor of the *GCN4* gene, has a general function in the initiation of protein synthesis in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**3203–3216.

16. **Hannig, E. H., N. P. Williams, R. C. Wek, and A. G. Hinnebusch.** 1990. The translational activator GCN3 functions downstream from GCN1 and GCN2 in the regulatory pathway that couples *GCN4* expression to amino acid availability in *Saccharomyces cerevisiae*. Genetics **126:**549–562.
17. **Hannig, E. M., A. M. Cigan, B. A. Freeman, and T. G. Kinzy.** 1992. *GCD11*, a negative regulator of *GCN4* expression, encodes the gamma subunit of eIF-2 in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**506–520.
18. **Hannig, E. M., and A. G. Hinnebusch.** 1988. Molecular analysis of *GCN3*, a translational activator of *GCN4*: evidence for posttranslational control of *GCN3* regulatory function. Mol. Cell. Biol. **8:**4808–4820.
19. **Harashima, S., and A. G. Hinnebusch.** 1986. Multiple *GCD* genes required for repression of *GCN4*, a transcriptional activator of amino acid biosynthetic genes in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **6:**3990–3998.
20. **Hill, D. E., and K. Struhl.** 1988. Molecular characterization of *GCD1*, a yeast gene required for general control of amino acid biosynthesis and cell-cycle initiation. Nucleic Acids. Res. **16:**9253–9265.
21. **Hill, J. E., A. M. Myers, K. J. Koerner, and A. Tzagaloff.** 1986. Yeast/*E. coli* shuttle vectors with multiple unique restriction sites. Yeast **2:**163–167.
22. **Hinnebusch, A. G.** 1992. General and pathway-specific regulatory mechanisms controlling the synthesis of amino acid biosynthetic enzymes in *Saccharomyces cerevisiae*, p. 319–414. *In* E. W. Jones, J. R. Pringle, and J. R. Broach (ed.), The molecular and cellular biology of the yeast *Saccharomyces*: gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Hinnebusch, A. G.** 1993. Gene-specific translational control of the yeast GCN4 gene by phosphorylation of eukaryotic initiation factor 2. Mol. Microbiol. **10:**215–223.
24. **Hinnebusch, A. G., and G. R. Fink.** 1983. Positive regulation in the general amino acid control of *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. USA **80:**5374–5378.
25. **Ito, H., Y. Fukada, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:**163–168.
26. **Jackson, R. J.** 1991. Binding of met-tRNA, p. 193–229. *In* H. Trachsel (ed.), Translation in eukaryotes. CRC Press, Boca Raton, Fla.
27. **Konieczny, A., and B. Safer.** 1983. Purification of the eukaryotic initiation factor 2-eukaryotic initiation factor 2B complex and characterization of its guanine nucleotide exchange activity during protein synthesis initiation. J. Biol. Chem. **258:**3402–3408.
28. **Krupitza, G., and G. Thireos.** 1990. Translational activation of GCN4 mRNA in a cell-free system is triggered by uncharged tRNAs. Mol. Cell. Biol. **10:**4375–4378.
29. **Lucchini, G., A. G. Hinnebusch, C. Chen, and G. R. Fink.** 1984. Positive regulatory interactions of the *HIS4* gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **4:**1326–1333.
30. **Matts, R., D. Levin, and I. London.** 1983. Effect of phosphorylation of the alpha-subunit of eukaryotic initiation factor 2 on the function of reversing factor in the initiation of protein synthesis. Proc. Natl. Acad. Sci. USA **80:**2559–2563.
31. **Merrick, W. C.** 1992. Mechanism and regulation of eukaryotic protein synthesis. Microbiol. Rev. **56:**291–315.
32. **Moehle, C. M., and A. G. Hinnebusch.** 1991. Association of RAP1 binding sites with stringent control of ribosomal protein gene transcription in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**2723–2735.
33. **Mueller, P. P., and A. G. Hinnebusch.** 1986. Multiple upstream AUG codons mediate translational control of *GCN4*. Cell **45:**201–207.
34. **Niederberger, P., M. Aebi, and R. Huetter.** 1986. Identification and characterization of four new *GCD* genes in *Saccharomyces cerevisiae*. Curr. Genet. **10:**657–664.
35. **Paddon, C. J., E. M. Hannig, and A. G. Hinnebusch.** 1989. Amino acid sequence similarity between GCN3 and GCD2, positive and negative translational regulators of *GCN4*: evidence for antagonism by competition. Genetics **122:**551–559.
36. **Pain, V. M.** 1986. Initiation of protein synthesis in mammalian cells. Biochem. J. **235:**625–637.
37. **Ramirez, M., R. C. Wek, C. R. Vazquez de Aldana, B. M. Jackson, B. Freeman, and A. G. Hinnebusch.** 1992. Mutations activating the yeast eIF-2α kinase GCN2: isolation of alleles altering the domain related to histidyl-tRNA synthetases. Mol. Cell. Biol. **12:**5801–5815.
38. **Rolfes, R. J., and A. G. Hinnebusch.** 1993. Translation of the yeast transcriptional activator *GCN4* is stimulated by purine limitation: implications for activation of the protein kinase GCN2. Mol. Cell. Biol. **13:**5099–5111.
39. **Rose, M. D., and J. R. Broach.** 1991. Cloning genes by complementation in yeast. Methods Enzymol. **194:**195–230.
40. **Rowlands, A. G., K. S. Montine, E. C. Henshaw, and R. Panniers.** 1988. Physiological stresses inhibit guanine-nucleotide-exchange factor in Ehrlich cells. Eur. J. Biochem. **175:**93–99.
41. **Rowlands, A. G., R. Panniers, and E. C. Henshaw.** 1988. The catalytic mechanism of guanine nucleotide exchange factor action and competitive inhibition by phosphorylated eukaryotic initiation factor 2. J. Biol. Chem. **263:**5526–5533.
42. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1974. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
43. **Siekierka, J., V. Manne, and S. Ochoa.** 1984. Mechanism of translational control by partial phosphorylation of the alpha subunit of eukaryotic initiation factor 2. Proc. Natl. Acad. Sci. USA **81:**352–356.
44. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
45. **Vazquez de Aldana, C. R., T. E. Dever, and A. G. Hinnebusch.** 1993. Mutations in the α subunit of eukaryotic translation initiation factor 2 (eIF-2α) that overcome the inhibitory effects of eIF-2α phosphorylation on translation initiation. Proc. Natl. Acad. Sci. USA **90:**7215–7219.
46. **Vazquez de Aldana, C. R., and A. G. Hinnebusch.** 1994. Mutations in the GCD7 subunit of yeast guanine nucleotide exchange factor eIF-2B overcome the inhibitory effects of phosphorylated eIF-2 on translation initiation. Mol. Cell. Biol. **14:**3208–3222.
47. **Vazquez de Aldana, C. R., R. C. Wek, P. San Segundo, A. G. Truesdell, and A. G. Hinnebusch.** 1994. Multicopy tRNA genes functionally suppress mutations in yeast eIF-2α kinase GCN2: evidence for separate pathways coupling *GCN4* expression to uncharged tRNA. Mol. Cell. Biol. **14:**7920–7932.
48. **von Pawel-Rammingen, U., S. Åstrom, and A. S. Bystrom.** 1992. Mutational analysis of conserved positions potentially important for initiator tRNA function in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:**1432–1442.
49. **Wek, R. C., J. F. Cannon, T. E. Dever, and A. G. Hinnebusch.** 1992. Truncated protein phosphatase GLC7 restores translational activation of *GCN4* expression in yeast mutants defective for the eIF-2α kinase GCN2. Mol. Cell. Biol. **12:**5700–5710.
50. **Wolfner, M., D. Yep, F. Messenguy, and G. R. Fink.** 1975. Integration of amino acid biosynthesis into the cell cycle of *Saccharomyces cerevisiae*. J. Mol. Biol. **96:**273–290.