Minimum Requirements for the Function of Eukaryotic Translation Initiation Factor 2

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ABSTRACT

Eukaryotic translation initiation factor 2 (eIF2) is a G protein heterotrimer required for GTP-dependent delivery of initiator tRNA to the ribosome. eIF2B, the nucleotide exchange factor for eIF2, is a heteropentamer that, in yeast, is encoded by four essential genes and one nonessential gene. We found that increased levels of wild-type eIF2, in the presence of sufficient levels of initiator tRNA, overcome the requirement for eIF2B *in vivo*. Consistent with bypassing eIF2B, these conditions also suppress the lethal effect of overexpressing the mammalian tumor suppressor PKR, an eIF2α kinase. The effects described are further enhanced in the presence of a mutation in the G protein (γ) subunit of eIF2, *gcd11-K250R*, which mimics the function of eIF2B *in vitro*. Interestingly, the same conditions that bypass eIF2B also overcome the requirement for the normally essential eIF2α structural gene (*SUI2*). Our results suggest that the eIF2βγ complex is capable of carrying out the essential function(s) of eIF2 in the absence of eIF2α and eIF2B and are consistent with the idea that the latter function primarily to regulate the level of eIF2·GTP·Met-tRNA$_i^{Met}$ ternary complexes *in vivo*.

In the current model for eukaryotic translation initiation (Hinnebusch and Liebman 1991; Merrick 1992; Pain 1996), initiator methionyl-tRNA is delivered to the 40S ribosomal subunit in the form of a eukaryotic translation initiation factor 2 (eIF2)·GTP·Met-tRNA$_i^{Met}$ ternary complex. The resulting 43S complex, which also includes eIF3 and eIF1A, binds at or near the 5′ end of capped eukaryotic messenger RNAs in a process that appears to involve interactions between eIF3 and proteins bound at the mRNA 5′ cap (Sachs *et al.* 1997). Once bound, the ribosome traverses the mRNA in a 5′-to-3′ direction and locates the AUG codon representing the translational start site. Recognition of the start site is accompanied by GTP hydrolysis, which releases Met-tRNA$_i^{Met}$ to the ribosomal peptidyl site and converts eIF2 to an eIF2·GDP binary complex. The eIF2·GDP complex must be converted to the GTP form to rebind Met-tRNA$_i^{Met}$ and to participate in another cycle of translation initiation. Mammalian eIF2·GDP is extremely stable *in vitro* in the presence of physiological concentrations of magnesium and requires the guanine nucleotide exchange factor eIF2B to promote rapid conversion of eIF2·GDP to eIF2·GTP. Although the latter complex is less stable, GTP binding is stabilized upon binding Met-tRNA$_i^{Met}$.

In the yeast *Saccharomyces cerevisiae*, subunits of the eIF2 heterotrimer are encoded by the single-copy essential genes *SUI2* (α), *SUI3* (β), and *GCD11* (γ). The primary structures of the eIF2 subunits are conserved between yeast and mammals (Ernst *et al.* 1987; Donahue *et al.* 1988; Pathak *et al.* 1988; Cigan *et al.* 1989; Erickson *et al.* 1997). The γ-subunit of eIF2 is a member of the GTP-binding (G) protein superfamily and is highly similar to eubacterial elongation factor EF1A (formerly EF-Tu; Hannig *et al.* 1993; Gaspar *et al.* 1994). Functional similarity between EF1A and eIF2γ proteins is further suggested by genetic and biochemical data that indicate the γ-subunit plays a significant role in binding nucleotide and tRNA ligands (Erickson and Hannig 1996). The guanine nucleotide exchange factor for eIF2, eIF2B, is a heteropentamer that, in yeast, is encoded by four essential genes (*GCD1*, *GCD2*, *GCD6*, and *GCD7*) and one nonessential gene (*GCN3*) that are also conserved in mammals (Bushman *et al.* 1993a; Cigan *et al.* 1993; Koonin 1995; Price *et al.* 1996a,b). The exchange reaction is regulated indirectly via phosphorylation of the α-subunit of eIF2, which converts eIF2 into a competitive inhibitor of the exchange reaction (Rowlands *et al.* 1988; Dever *et al.* 1995; Kimball *et al.* 1998). *In vivo*, this leads to reduced global rates of translation initiation and, in some cases, gene-specific enhancement of translation (Hershey 1991; Hinnebusch 1993; Rhoads 1993). A mammalian eIF2α kinase, PKR, has been proposed to function as a tumor suppressor and underlies the importance of regulating the exchange reaction for normal homeostasis in higher eukaryotic organisms (Koromilas *et al.* 1992; Meurs *et al.* 1993; Barber *et al.* 1995a,b; Donze *et al.* 1995).

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We previously described a mutation, *gcd11-K250R*, that conferred phenotypes consistent with reduced eIF2 function, *i.e.*, reduced growth rates and increased expression of *GCN4* (Erickson and Hannig 1996). This mutation alters the lysine residue within the NKXD nucleotide-binding motif of eIF2γ that is conserved in G proteins (Dever *et al.* 1987; Bourne *et al.* 1991). Both phenotypes were suppressed by increased dosage of the yeast initiator tRNA gene (*IMT*). *In vitro*, *gcd11-K250R* led to increased dissociation rates for both eIF2·GDP and eIF2·GTP, while the binding of Met-tRNA$_i^{Met}$ to eIF2·GTP complexes stabilized GTP binding for both the wild-type and γ_{K250R} forms of eIF2 (Erickson and Hannig 1996). Together, these data suggested that the γ_{K250R} alteration might also promote more rapid formation of ternary complexes and thereby reduce the requirement for eIF2B *in vivo*. In this article, we confirm these predictions. Furthermore, sufficient levels of wild-type eIF2 also reduce the requirement for eIF2B *in vivo*, though much less efficiently than in the presence of *gcd11-K250R*. This suggests that increasing the rate of dissociation of eIF2·GDP complexes is an essential function provided by eIF2B. Interestingly, the α-subunit of eIF2, which appears to play an important role in eIF2/eIF2B interactions (Vazquez de Aldana and Hinnebusch 1994; Pavitt *et al.* 1997, 1998; Nika *et al.* 2001), is no longer required for viability under conditions that lead to bypass of eIF2B. Our combined data suggest that the eIF2βγ complex is capable of carrying out all essential eIF2 functions, including essential interactions with additional components of the eukaryotic translational machinery. We propose that eIF2B and the α-subunit of eIF2 comprise an elaborate regulatory system for modulating levels of ternary complex that, although not essential *per se* for growth, plays a critical role in maintaining homeostasis and viability in wild-type cells.

MATERIALS AND METHODS

Plasmids: pSB32 (*LEU2*), YCp50 (*URA3*; Rose and Broach 1991), and pRS316 (*URA3*; Sikorski and Hieter 1989) are yeast-*Escherichia coli* shuttle vectors containing yeast centromere sequences for maintenance in low copy number in yeast. YEp24 (*URA3*), YEp13 (*LEU2*; Rose and Broach 1991), and YEplac112 (*TRP1*; Gietz and Sugino 1988) are yeast-*E. coli* shuttle vectors maintained in high copy in yeast due to the presence of the 2μ origin of replication. Construction of YEp13/*SUI2*/*SUI3*/*GCD11*$_{His8}$ (Ep847), YEp13/*SUI2*/*SUI3*/*gcd11*$_{His8}$*-K50R* (Ep922), and YEplac112/*IMT* (Ep1013) were described previously (Erickson and Hannig 1996). Unless otherwise noted, the *GCD11* alleles used in this study contain a C-terminal octyl-histidine tag. The tag does not appear to alter the function of wild-type eIF2 *in vivo* or affect ligand binding *in vitro* (Erickson and Hannig 1996). The galactose-inducible *PKR* construct (in YCp50) was a generous gift from T. Dever (National Institutes of Health, Bethesda, MD).

Ep1037 is a pSB32/*GCD6*/*GCD7* plasmid. A 3.3-kb *Spe*I fragment from pJB6 (Bushman *et al.* 1993a) containing *GCD6* was converted to a *Hin*dIII fragment following treatment with Klenow enzyme and ligation with *Hin*dIII linkers. This fragment was inserted into *Hin*dIII-cleaved pSB32 to create Ep1033. The *GCD7* fragment was obtained from pJB100 (Bushman *et al.* 1993a) by first converting the *Eco*RI site to an *Eag*I/*Not*I site using oligonucleotide linkers as described above. A 2.1-kb *Not*I fragment containing *GCD7* was removed from the modified pJB100, ligated to *Eag*I-cleaved YEp24 to create Ep673, and then subcloned as a 2.1-kb *Eag*I fragment into *Eag*I-cleaved Ep1033. The resulting plasmid, Ep1037, was cleaved with *Aat*II (in vector sequences), flush-ended, and converted to an *Asc*I site using oligonucleotide linkers to create Ep1066.

The pSB32/*GCD1*/*GCD2*/*GCD6*/*GCD7* plasmid Ep1127 was constructed by inserting an *Asc*I fragment containing *GCD1* and *GCD2* into *Asc*I-cleaved Ep1066. The *GCD1* fragment was obtained as a 2.4-kbp *Bam*HI fragment from YCp50-Sc4014 (Hill and Struhl 1988) that was initially subcloned into *Bam*HI-cleaved pBluescript (Promega, Madison, WI) to create Ep1082. The *GCD2* fragment was obtained from pY26 (Dever *et al.* 1995) as a 2.6-kbp *Eag*I fragment following conversion of the *Cla*I site to an *Eag*I site as above. This fragment was subcloned into *Eag*I-cleaved Ep1082. Vector *Xho*I and *Sac*I sites were sequentially converted to *Asc*I sites by linker tailing to create Ep1120. A 5.0-kb *Asc*I fragment from Ep1120 containing *GCD1* and *GCD2* was then subcloned into *Asc*I-cleaved Ep1066 to create Ep1127.

Additional plasmids containing the four essential eIF2B subunit genes, with or without *GCD11*$_{His8}$, were constructed as follows. Ep1174 (pBluescript with *Xho*I and *Sac*I sites altered to *Asc*I) was cleaved with *Bam*HI and *Hin*dIII and ligated with a 2.1-kb *Bam*HI/*Hin*dIII fragment containing *GCD11*$_{His8}$. The resulting plasmid (Ep1246) was digested with *Bam*HI and ligated with the 2.4-kbp *Bam*HI *GCD1* fragment from YCP50-Sc4014. The resulting plasmid was cleaved with *Eag*I and ligated with the 2.6-kbp *Eag*I *GCD2* fragment to create Ep1247. A 7.1-kb *Asc*I fragment, containing *GCD1*, *GCD2*, and *GCD11*$_{His8}$, was ligated to *Asc*I-cleaved Ep1067 and Ep1066 to create Ep1250 (pRS316/*GCD1*/*GCD2*/*GCD6*/*GCD7*/*GCD11*$_{His8}$) and Ep1262 (pSB32/*GCD1*/*GCD2*/*GCD6*/*GCD7*/*GCD11*$_{His8}$), respectively. The pRS316/*GCD1*/*GCD2*/*GCD6*/*GCD7* plasmid Ep1125 was constructed by first inserting the 2.1-kb *Eag*I *GCD7* fragment from Ep673 into *Eag*I-cleaved pJB5 to create Ep1042. The 5.1-kbp *GCD1*/*GCD2* *Asc*I fragment from Ep1120 was then inserted at the *Sal*I site in Ep1042, which had been modified using *Asc*I oligonucleotide linkers, to create Ep1125.

Plasmids used in the Δ*sui2* suppression experiments contained the 2.1-kbp *Hin*dIII/*Sna*BI *GCD11* fragment (where the *Sna*BI site was altered to a *Bam*HI site by linker tailing; Hannig *et al.* 1993) and/or a 2.5-kbp *Bam*HI fragment from pD14-6 containing *SUI2* (a gift from T. Dever, National Institutes of Health). Construction of YEp13/*GCD11*$_{His8}$ (Ep832) and YEp13/*gcd11-K250R*$_{His8}$ (Ep921) were described previously (Erickson and Hannig 1996). YEp13/*GCD11*$_{His8}$/*SUI3* (Ep1064) and YEp13/*gcd11-K250R*$_{His8}$/*SUI3* (Ep1065) were constructed by removing the 2.5-kbp *Bam*HI *SUI2* fragment from Ep847 and Ep922, respectively.

Strain construction: The parent strain for EY878 (*MAT*α *leu2-3, 112 trp1-Δ63 ura3-52 gcd1::hisG gcd2::hisG gcd6Δ gcd7::hisG gcn3::hisG* <Ep1125>) is EY809 (*MAT*α *leu2-3, 112 trp1-Δ63 ura3-52 gcd6Δ gcd7::hisG* <Ep1042 (pRS316[*URA3*]/*GCD6*/*GCD7*>)). The *gcd7::hisG* allele (from pJB110; Bushman *et al.* 1993a) removes 66% (residues 75–325) of the 381-amino-acid *GCD7* open reading frame (ORF). The *gcd6Δ* allele was from pJB96 (Bushman *et al.* 1993a), and removes 87% (residues 93–713) of the 713-amino-acid *GCD6* ORF. The remainder of the eIF2B subunit genes were deleted in EY809 after replacing the pRS316-based plasmid with the low-copy

LEU2 plasmid Ep1127 that contains *GCD1*, *GCD2*, *GCD6*, and *GCD7*. The *gcd1*Δ*::hisG-URA3-hisG* allele (Ep1191) was derived from Ep175 (a URA3 disruption version of Ep174; Hannig and Hinnebusch 1988) by replacing the disrupting *URA3* fragment with the *hisG-URA3-hisG* cassette from pNKY51 (Alani *et al.* 1987). This removes 52% (residues 1–299) of the 578-amino-acid *GCD1* ORF. The *gcd2*Δ*::hisG-URA3-hisG* allele (Ep1145) removes 93% (residues 26–632 on a *Pvu*II/*Eco*RI restriction fragment) of the 651-amino-acid *GCD2* ORF (Paddon *et al.* 1989). The *gcn3*Δ*::hisG-URA3-hisG* allele was derived from Ep308 (Hannig *et al.* 1990) by replacing the disrupting *LEU2* fragment with *hisG-URA3-hisG* as above to create Ep545. This construct removes the entire 305-amino-acid *GCN3* ORF. DNA fragments used for gene disruptions were obtained following digestion of the corresponding plasmids with *Bam*HI (*GCD1*), *Not*I (*GCD2*), or *Bgl*II/*Pvu*II (*GCN3*) and purification by agarose gel electrophoresis. Gene disruptions were confirmed by Southern blot analysis of Ura^+ transformants, using appropriate probes to distinguish chromosomal and plasmid-borne (in Ep1127) alleles (data not shown). Chromosomal disruptants were plated on 5-fluoroorotic acid (5-FOA) medium to select for recombination between the direct *hisG* repeats, an event that evicts the *URA3* gene, leaving behind a single copy of the *hisG* repeat. *GCD2*, *GCD1*, and *GCN3* were disrupted sequentially in the EY809 background. The *URA3* plasmid Ep1125 was then used to replace Ep1127 to create EY878. The absence of each essential eIF2B subunit gene in EY878 was also demonstrated genetically by the inability of plasmids containing only three of the four essential eIF2B subunit genes, in all possible combinations, to complement in the absence of Ep1125.

Disruption of *GCD11* in EY878 utilized a derivative of EY878 in which Ep1262(*LEU2*) replaced Ep1125(*URA3*). *GCD11* was then disrupted using the *gcd11::hisG-URA3-hisG* allele from Ep523 as described (Dorris *et al.* 1995), followed by growth on 5-FOA. The *URA3* plasmid Ep1250 was used to replace the *LEU2* plasmid Ep1262 to create EY923.

EY740 (*MAT***a** *leu2-3, -112 ura3-52 trp1-*Δ*63 gcd11::hisG GAL2*$^+$ <Ep293; YCp50/*GCD11*>) was obtained as a meiotic segregant from a cross between a *GAL2*$^+$ derivative of H1515 (Marton *et al.* 1993) and EY585 (*MAT*α *leu2-3, -112 ura3-52 gcd11::hisG GAL2*$^+$ <Ep293>). The *gcd11::hisG* allele lacks the entire *GCD11* open reading frame (Hannig *et al.* 1993). EY835 (*MAT*α *leu2-3, -112 ura3-52 trp1-*Δ*63 sui2*Δ *gcd11::hisG GAL2*$^+$ <Ep1130; Ycp50/*GCD11*/*SUI2*>) was obtained from a cross between EY740 and EY779 (*MAT*α *leu2-3, -112 ura3-52 trp1-*Δ*63 sui2*Δ <pSB32/*SUI2*>). The *sui2*Δ allele removes the N-terminal two-thirds of the *SUI2* ORF (Dever *et al.* 1992). EY779 is a derivative of H1816 (Vazquez de Aldana and Hinnebusch 1994).

Growth rate determination: Doubling times at 30° were determined for log-phase cultures grown in minimal (SD) media supplemented as necessary (Sherman *et al.* 1986).

eIF2B assay: Reactions (30 μl) contained 20 mm Tris-HCl (pH 7.5), 100 mm KCl, 5 mm $MgCl_2$, 0.1 mm Na_2EDTA, 1 mm dithiothreitol, 5% glycerol, 1 mg/ml creatine kinase (as carrier), 10 μm GDP, 100 μm GTP, and 300 nm [^{3}H]Met-$tRNA_i^{Met}$ (65 kcpm/pmol)[3]. eIF2 preparations (3 μg, >80% pure; Erickson and Hannig 1996) were prebound to GDP for 10 min at 23° in the absence or presence of 1 μg yeast eIF2B (Nika *et al.* 2001) and then transferred to a 10° water bath for an additional 5 min. Under these conditions, both wild-type eIF2 and $eIF2_{\gamma K250R}$ are maximally bound to GDP (Erickson and Hannig 1996). GTP and [^{3}H]Met-$tRNA_i^{Met}$ were then added, and 5-μl aliquots were withdrawn and filtered through nitrocellulose as described previously (Erickson and Hannig 1996).

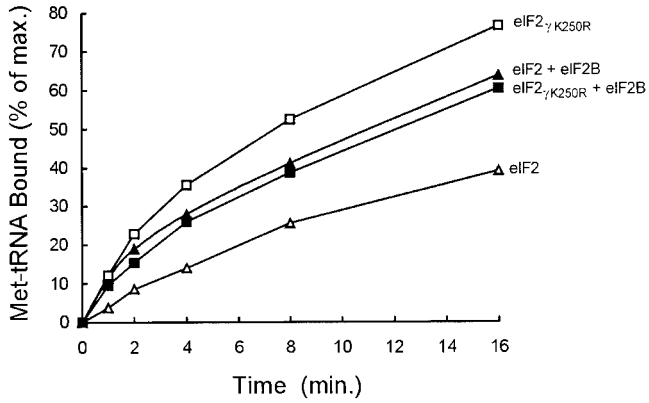

Figure 1.—Effect of the γK250R alteration on formation of ternary complexes by eIF2. The amount of [^{3}H]Met-$tRNA_i^{Met}$ bound by eIF-2 was determined by nitrocellulose filter binding (Erickson and Hannig 1996) and reported as a percentage of maximal Met-$tRNA_i^{Met}$ bound. △, wild-type eIF2; □, $eIF2_{\gamma K250R}$; ▲, wild-type eIF2 and eIF2B; ■, $eIF2_{\gamma K250R}$ and eIF2B. The data shown are the average of two experiments wherein data points differ by <15% at each time point. GTP-independent [^{3}H]Met-$tRNA_i^{Met}$ binding was minimal (<0.1 pmol in all cases) and was not subtracted. Reactions were repeated in the absence of added GDP to determine maximal [^{3}H]Met-$tRNA_i^{Met}$ binding. The figure 100% corresponds to the following amount of [^{3}H]Met-$tRNA_i^{Met}$ bound in each 5-μl aliquot: eIF2, 1.2 pmol; $eIF2_{\gamma K250R}$, 1.0 pmol; eIF2 + eIF2B, 1.2 pmol; $eIF2_{\gamma K250R}$ + eIF2B, 1.1 pmol.

RESULTS

Yeast eIF2 exhibits an intrinsic nucleotide exchange activity: We measured nucleotide exchange activity by testing the ability of GDP-bound yeast eIF2 to form eIF2·GTP·Met-$tRNA_i^{Met}$ ternary complexes *in vitro* (Figure 1). In mammalian systems, this reaction requires eIF2B to dissociate the eIF2·GDP complex (reviewed in Merrick 1992). In contrast to the mammalian system, wild-type yeast eIF2·GDP readily formed ternary complexes in the absence of eIF2B, with 40% of maximal complex formation seen after 16 min. We attribute this effect to an appreciable intrinsic GDP off-rate for yeast eIF2 that is not seen for the mammalian factor (Ahmad *et al.* 1985; Erickson and Hannig 1996). Addition of catalytic amounts of yeast eIF2B further increased ternary complex formation by wild-type eIF2 to ~64% of maximum over the same time period. In the absence of eIF2B, $eIF2_{\gamma K250R}$ displayed an apparent rate of ternary complex formation (at early time points) that was greater than that for wild-type eIF2 alone and similar to the eIF2B-promoted reaction. The possibility that the $eIF2_{\gamma K250R}$ preparation was contaminated with eIF2B is unlikely, since the addition of eIF2B to the $eIF2_{\gamma K250R}$ reaction did not further enhance ternary complex formation (*i.e.*, reaction rates at early time points are similar). The ability of the γ_{K250R} alteration to mimic eIF2B activity *in vitro* suggested that *gcd11-K250R* strains might demonstrate a reduced requirement for eIF2B function *in vivo*.

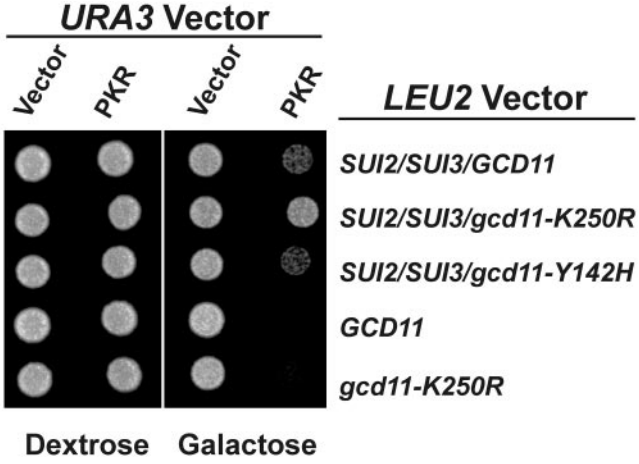

Figure 2.—Suppression of lethality associated with PKR expression in yeast. Three plasmids were introduced into yeast strain EY740 (*MAT***a** *ura3-52 trp1-Δ63 leu2-3 leu2-112 gcd11::hisG GAL2*$^+$): (i) a high-copy *LEU2* plasmid (YEp13) containing genes encoding the α(*SUI2*), β(*SUI3*), and γ(*GCD11*) subunits of eIF-2, or a low-copy *LEU2* plasmid (pSB32) encoding the γ-subunit only; (ii) a high-copy *TRP1* plasmid (YEplac112; Gietz and Sugino 1988) containing the yeast *IMT* gene; and (iii) a low-copy *URA3* plasmid (YCp50; Rose and Broach 1991) with (PKR) or without (vector) the mammalian PKR gene expressed from the inducible yeast *GAL1* promoter. Strains representing independent isolates were grown to saturation in liquid minimal media containing 2% dextrose (Sherman *et al.* 1986), diluted, and ~10^4 cells were spotted onto minimal media plates containing either 2% dextrose or 2% galactose and were then incubated for 2 days at 30°.

PKR-mediated growth inhibition is suppressed by increased eIF2 gene dosage: We reasoned that yeast cells less dependent upon eIF2B might show decreased sensitivity to overexpression of the mammalian eIF2α kinase PKR, which confers a severe slow-growth phenotype in wild-type yeast (Dever *et al.* 1993). This phenotype appears to result from an increase in phosphorylated eIF2α (at residue serine 51), which converts eIF2 to a competitive inhibitor of the exchange reaction (Rowlands *et al.* 1988; Chong *et al.* 1992; Dever *et al.* 1995). Because eIF2B is typically present at reduced levels relative to eIF2 (Price and Proud 1994), the resulting functional sequestration of eIF2B leads to decreased levels of ternary complex and thus reduced growth rates. We constructed yeast strains harboring a high-copy plasmid containing *GCD11* or *gcd11-K250R* in combination with *SUI2* and *SUI3*, or a low-copy plasmid containing the *GCD11* allele alone. Co-overexpression of the three eIF2 subunit genes has been shown to increase the level of this initiation factor complex at least fivefold *in vivo* (Hannig *et al.* 1993; Dever *et al.* 1995; Erickson and Hannig 1996). The chromosomal *GCD11* allele was deleted in these strains, which, in addition, harbored a plasmid containing a galactose-inducible PKR construct, as well as a high-copy *IMT* plasmid (or the empty vector). All strains grew well on dextrose media (Figure 2). In the absence of the high-copy *IMT* plasmid, all strains failed to grow upon induction of PKR expression on galactose media (data not shown). However, in the presence of increased *IMT* gene dosage, *gcd11-K250R* suppressed the PKR-mediated growth defect in the presence of elevated levels of eIF2α and eIF2β (Figure 2), suggesting that suppression is mediated by the eIF2 complex. Under these same conditions wild-type eIF2 was a much weaker suppressor, as would be predicted on the basis of its slower intrinsic off-rate for GDP. eIF2$_{\gamma Y142H}$, which demonstrates a wild-type GDP off-rate *in vitro* (Erickson and Hannig 1996), suppresses at a level similar to wild-type eIF2. The effects of overexpression of eIF2 are likely not due to inhibition of phosphorylation of the α-subunit by PKR, as these conditions have been shown to increase the level of phosphorylated eIF2 in the cell (Dever *et al.* 1995). Our results suggest that elevated levels of eIF2 and initiator tRNA reduce the requirement for eIF2B at a level proportional to the intrinsic nucleotide off-rate for yeast eIF2.

Bypass of the essential function of eIF2B: If the only essential function provided by eIF2B is to promote the rapid dissociation of eIF2·GDP complexes, we reasoned that *gcd11-K250R* might suppress a deletion of some or all of the four essential eIF2B subunit genes. To test this hypothesis, we constructed a yeast strain lacking the chromosomal *GCD1*, *-2*, *-6*, and *-7* genes that encode the essential eIF2B subunits (Cigan *et al.* 1991, 1993), as well as the nonessential eIF2B subunit gene *GCN3* (Hannig and Hinnebusch 1988). The resulting strain (EY878) is viable because it contains a low-copy *URA3* plasmid harboring the four essential eIF2B subunit genes. To test the requirement for eIF2B in *gcd11-K250R* strains, we constructed *LEU2* plasmids containing either *gcd11-K250R* or *GCD11* in combination with *SUI2* and *SUI3*. These plasmids were introduced into EY878 that also harbored either a high-copy *IMT* plasmid or the empty vector. Transformants were then plated on 5-FOA medium to examine the ability of the eIF2 plasmids to suppress the complete loss of essential eIF2B genes. 5-FOA selects for Ura$^-$ cells that have lost the *URA3* plasmid (Boeke *et al.* 1987), which, in the case of EY878, contains the only copies of the essential eIF2B subunit genes. As shown in Figure 3, the *gcd11-K250R/SUI2/SUI3* combination was an effective suppressor in the eIF2B quintuple-deletion strain. Suppression was dependent upon increased *IMT* gene dosage and, in addition, required high-copy expression of all three eIF2 subunit genes (Figure 3; data not shown). Western blot analysis confirmed the absence of detectable eIF2B subunits in the suppressed strain (data not shown). Overexpression of wild-type eIF2 also suppressed the complete absence of eIF2B in an *IMT*-dependent manner, albeit at a reduced level, consistent with the intrinsic nucleotide off-rate seen for wild-type eIF2·GDP complexes. Enhanced dissociation of eIF2·GDP resulting from the *gcd11-K250R* mutation (Figure 1) is consistent with

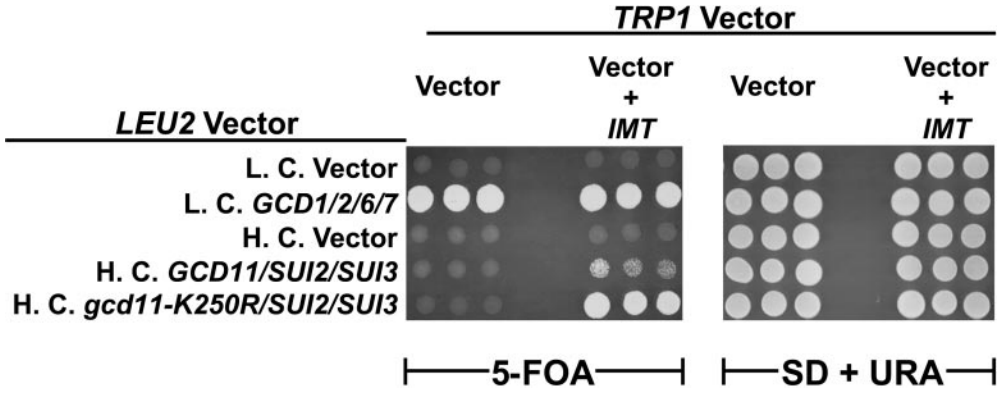

FIGURE 3.—Suppression of the lethal effect associated with deletion of the five eIF2B subunit genes. Low-copy (L.C.; pSB32) or high-copy (H.C.; YEp13) *LEU2* plasmids indicated to the left of the figure were introduced into the eIF2B quintuple deletion strain EY878, which also harbored either YEplac112 [*TRP1*] (Vector) or YEplac112/*IMT* (Vector + *IMT*). Plasmid transformants were selected, grown in liquid media containing uracil, and 5 μl of a 1:10 dilution was spotted onto minimal media containing uracil + 5-FOA (5-FOA) or uracil alone (SD + URA). Each spot corresponds to an independent isolate. This photograph was taken following incubation at 30° for 5 days. Low-copy *LEU2* plasmids harboring any combination of three of the four essential eIF2B subunit genes failed to complement in EY878 (data not shown).

more efficient bypass of the nucleotide exchange function of eIF2B. Our results lend strong support to the notion that the rapid dissociation of eIF2·GDP complexes is an essential eIF2B function.

Bypass of the essential function of *SUI2* (eIF2α): In cells that no longer require eIF2B, it is possible that certain eIF2 subunit(s) with which eIF2B interacts are not required. We chose the α-subunit of eIF2 to test this idea, on the basis of previous genetic evidence that suggested a direct interaction between the α-subunit of eIF2 and eIF2B (VAZQUEZ DE ALDANA *et al.* 1993; PAVITT *et al.* 1997, 1998). We constructed a Δ*gcd11* Δ*sui2* strain (EY835) that harbored a *URA3/GCD11/SUI2* plasmid and used the plasmid shuffle technique described above to examine the ability of *GCD11* and *gcd11-K250R* constructs (*LEU2*) to suppress the Δ*sui2* mutation by conferring viability in the absence of the resident *URA3* plasmid. To demonstrate the absence of chromosomal *SUI2* and *GCD11* in this strain, a low-copy *LEU2* plasmid containing both *SUI2* and *GCD11*, but not plasmids harboring either gene alone, supported the viability of EY835 in the absence of the *URA3/GCD11/SUI2* plasmid (Figure 4). High-copy plasmids containing *gcd11-K250R*, either alone or in combination with *SUI3* (eIF2β), suppressed the Δ*sui2* mutation, increasing doubling times 1.5- to 2.2-fold compared with controls (Figure 5, bottom panel). Suppression by *gcd11-K250R* was independent of the presence of a multi-copy *IMT* plasmid, although suppression was more efficient with the *IMT* plasmid (20–40% decrease in doubling times). This result suggests that the two-subunit form (βγ) of eIF2 is functional in this strain, but does not rule out the additional possibility that the γ-subunit alone is functional. A low-copy plasmid containing *gcd11-K250R* also suppressed Δ*sui2*, albeit less efficiently (data not shown). Overexpression of wild-type *GCD11* weakly suppressed Δ*sui2* (5-fold increase in doubling time) and suppression required co-overexpression of *SUI3* and *IMT*. Our results suggest that the contribution of the α-subunit to eIF2 function is not essential for ligand binding or the interaction of eIF2 with additional components of the translational apparatus.

Comparative requirements for bypass of essential eIF2B and eIF2α functions: Examination of results presented in Figures 3 and 4 reveals a difference in the requirements for suppression in the eIF2B deletion

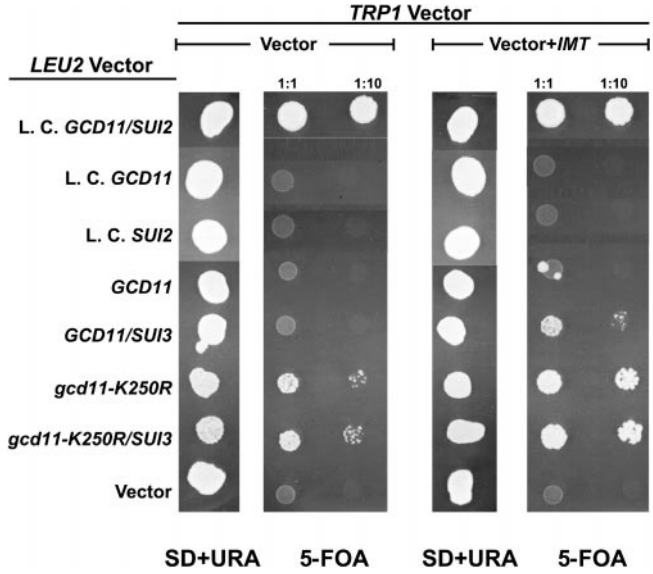

FIGURE 4.—The α-subunit of eIF2 is dispensable in *gcd11-K250R* strains. *LEU2* plasmids harboring alleles listed to the left were introduced into yeast strain EY835 (*MATα leu2-3, -112 trp1-Δ63 ura3-52 sui2Δ gcd11::hisG* <YCp50/*SUI2/GCD11*>) containing either YEplac112 (Vector) or YEplac112/*IMT* (Vector + *IMT*). Transformants were grown in minimal media containing uracil for 2 days at 30°, plated as described in Figure 2 (either undiluted or at a 1:10 dilution) on minimal media (SD) containing uracil and 5-FOA (5-FOA) or uracil alone (SD + URA), and grown for 4 days at 30°. Results are representative of analysis of a larger number of independent transformants (data not shown). L.C., plasmids based on the low-copy vector pSB32; the remainder of the plasmids listed to the left are based upon the high-copy vector YEp13.

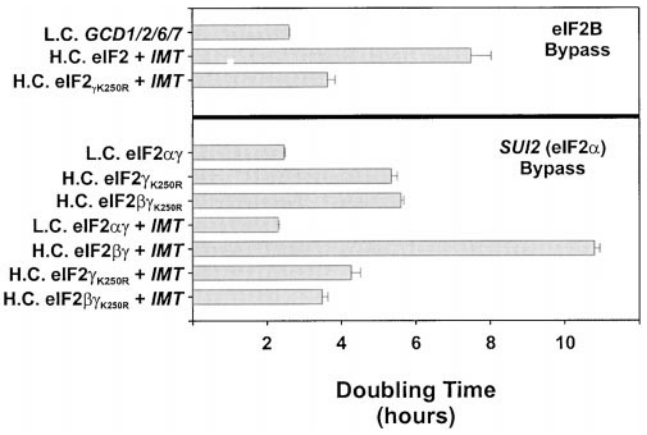

FIGURE 5.—Growth rates of suppressed strains lacking eIF2B or eIF2α. Growth rates were determined in EY923 (eIF2B bypass; see Figure 6) or EY835 (eIF2α bypass; see Figure 4) following growth on 5-FOA as described in RESULTS. Low-copy (L.C.) or high-copy (H.C.) *LEU2* plasmids present in each strain containing eIF2 or eIF2B subunit genes are indicated to the left. All strains contained a high-copy *TRP1* vector or the same vector containing *IMT* as indicated. Doubling times were determined in triplicate as described in MATERIALS AND METHODS.

strain compared with the Δ*sui2* strain. In the former instance, suppression in all cases required increased *IMT* gene dosage, whereas suppression of Δ*sui2* by *gcd11-K250*R is independent of, though enhanced by, the presence of additional copies of *IMT*. A trivial explanation for this difference may be related to the presence of the chromosomal *GCD11* allele in EY878 used in the eIF2B bypass experiments (Figure 3). In this case, the presence of wild-type eIF2 complexes may compete with $eIF2_{\gamma K250R}$ and thereby reduce the efficiency of suppression in these strains. To test this idea, we created a Δ*gcd11* strain (EY923) isogenic with EY878 and repeated the eIF2B bypass experiments. The results, shown in Figure 6, are essentially identical to those shown in Figure 3; *i.e.*, bypass of the essential function of eIF2B requires overexpression of both eIF2 and initiator tRNA and is independent of the presence of a chromosomal *GCD11* allele in the host strain. Again, $eIF2_{\gamma K250R}$ is a more efficient suppressor, resulting in a 1.4-fold increase in doubling time (*vs.* the control) compared with a 3-fold increase for wild-type eIF2 (Figure 5, top).

DISCUSSION

Previous biochemical studies using mammalian factors indicated that eIF2 and eIF2B play critical roles in the initiation of eukaryotic protein synthesis (reviewed in MERRICK 1992; PRICE and PROUD 1994; PAIN 1996). Genetic analyses in yeast provided evidence that both factors are required for growth and viability (HINNEBUSCH 1997). In yeast, each of the three conserved eIF2 subunits and four of the five conserved eIF2B subunits are encoded by essential genes. We previously described a mutation in the gene encoding the γ-subunit of eIF2, *gcd11-K250R*, that increased the intrinsic rate of dissociation of guanine nucleotides from binary complexes *in vitro* (ERICKSON and HANNIG 1996). Although $eIF2_{\gamma K250R}$ showed increased dissociation for both GDP and GTP *in vitro*, GTP binding by both $eIF2_{\gamma K250R}$ and wild-type eIF2 could be stabilized by forming ternary complexes with charged initiator tRNA. These results were consistent with *in vivo* experiments that demonstrated increased *IMT* gene dosage suppressed both the slow growth and increased expression of *GCN4* (*i.e.*, the Gcd^- phenotype) in *gcd11-K250R* strains. The latter are indicative of at least partial restoration of eIF2 function (HIN-

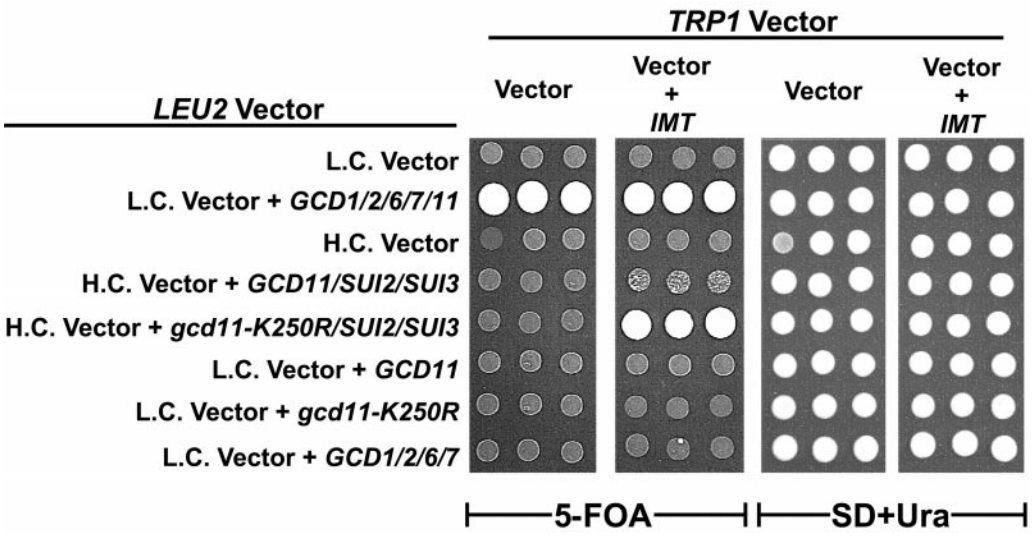

FIGURE 6.—Increased *IMT* gene dosage is required for bypass of eIF2B. Low-copy (L.C.; pSB32) or high-copy (H.C.; YEp13) *LEU2* plasmids indicated to the left of the figure were introduced into EY923, which also harbored either YEplac112 [*TRP1*] (Vector) or Ep1013 (Vector + *IMT*). EY923 lacks all five chromosomal eIF2B subunit genes as well as *GCD11*, but harbors a *URA3* plasmid (Ep1250) containing *GCD11* and the essential eIF2B subunit genes. Independent transformants were grown, diluted, and plated on media as described in the legend to Figure 3. This photograph was taken following growth at 30° for 5 days.

nebusch 1994). These combined results suggested to us that *gcd11-K250R* strains might exhibit a reduced dependence upon eIF2B, resulting in partial or complete bypass of the requirement for eIF2B dependent upon (or enhanced by) increased *IMT* gene dosage. The data presented here demonstrate that overexpression of either the wild-type or γ_{K250R} form of eIF2 suppresses deletion of the four essential eIF2B subunit genes and that bypass of essential eIF2B function(s) requires co-overexpression of initiator tRNA. Consistent with a reduced requirement for eIF2B function, strains grown under bypass conditions but containing all eIF2B subunit genes show a reduced sensitivity to the eIF2α kinases PKR (Figure 2) and Gcn2p (Dever *et al.* 1995). The efficiency of suppression of both the PKR-induced growth phenotype and the absence of eIF2B correlated directly with the rate of dissociation of guanine nucleotides determined previously with purified eIF2 preparations; *i.e.*, *gcd11-K250R* strains were more efficient than strains harboring the wild-type *GCD11* allele. Our data imply that enhancing the rate of nucleotide dissociation from eIF2 is an essential function of eIF2B.

Kinzy and Woolford (1995) demonstrated previously that additional copies of the *TEF2* gene, encoding elongation factor eEF1A (previously eEF1α), were sufficient to bypass the requirement for its exchange factor, eEF1B, when provided on a low-copy-number plasmid. The requirement for elevated levels of both wild-type eIF2 and initiator tRNA in bypassing eIF2B function suggests that eIF2 and/or initiator tRNA are normally maintained at limiting levels such that eIF2B is essential for promoting levels of ternary complex required in rapidly growing cells. Such a mechanism would also allow for rapid and effective changes in the level of ternary complexes by modulating eIF2B activity in response to various stimuli and, as such, may play an important role in regulating cell growth. Our results make the prediction that cells harboring mutations analogous to *gcd11-K250R* may be less sensitive to growth regulation mediated through protein kinases that phosphorylate the α-subunit of eIF2 (Samuel 1993; Wek 1994; Shi *et al.* 1998; Sood *et al.* 2000a,b). Additional strategies developed to circumvent this regulatory mechanism, such as specific alterations in eIF2α that render it refractory to phosphorylation (Donze *et al.* 1995) or dominant negative mutations in PKR (Koromilas *et al.* 1992; Meurs *et al.* 1993; Barber *et al.* 1995a), have been shown to promote tumor formation in mammals, implying that PKR may function as a tumor suppressor (Lengyel 1993).

Conditions required to suppress a Δ*sui2* mutation differed somewhat from those required to suppress the deletion of essential eIF2B subunit genes. In the latter case, increased *IMT* gene dosage was absolutely required, whereas suppression of Δ*sui2* in *gcd11-K250R* strains was more efficient in the presence of, but did not require, additional copies of *IMT*. The difference in gene dosage requirements for *IMT* raises the possibility that eIF2B provides a function, in addition to nucleotide exchange, that is substituted (in the eIF2B bypass experiments) by elevated levels of initiator tRNA. It is possible that catalyzed nucleotide exchange proceeds through an eIF2·GTP·eIF2B intermediate that facilitates the interaction of eIF2 with initiator tRNA, perhaps by increasing the on-rate for tRNA relative to eIF2·GTP binary complexes. Manchester and Stasikowski (1990) proposed a similar model based upon theoretical considerations of association and dissociation rate constants under physiological conditions and the reaction rates for protein synthesis initiation. If this is indeed the preferred pathway for ternary complex formation *in vivo*, increasing the level of initiator tRNA may overcome the requirement for this eIF2B function via mass action. However, this model does not fully explain the requirement for increased *IMT* gene dosage in *gcd11-K250R* strains in the absence of eIF2B. In the presence of eIF2B, the viability of *gcd11-K250R* Δ*sui2* strains does not require additional copies of *IMT*, despite the fact that these conditions would be expected to bypass the eIF2B nucleotide exchange function. In fact, co-overexpression of *gcd11-K250R*, *SUI3*, and *IMT* is sufficient to bypass eIF2B *in vivo* (J. Nika and E. M. Hannig, unpublished observations). Furthermore, we demonstrated recently that eIF2α is required to promote efficient interaction between eIF2 and eIF2B *in vitro* (Nika *et al.* 2001). These combined observations suggest that eIF2B may contribute to the formation of $eIF2_{\gamma K250R}$ ternary complexes in a manner that does not appear to require catalyzed nucleotide exchange and that is independent of (or less dependent upon) direct eIF2/eIF2B interaction. An alternative means through which eIF2B may facilitate ternary complex formation is by increasing local concentrations of tRNA, perhaps through a channeling type of mechanism. Such a mechanism may be direct or indirect, would not require direct interaction between eIF2 and eIF2B, and may be facilitated by a ribosomal localization of at least a portion of the eIF2B pool (Matts *et al.* 1988; Cigan *et al.* 1991; Chakrabarti and Maitra 1992; Ramaiah *et al.* 1992; Bushman *et al.* 1993b; Mueller *et al.* 1998). However, we cannot rule out completely the involvement of at least some form of an eIF2·eIF2B intermediate. In this respect, it is interesting to note recent data demonstrating genetic as well as physical interaction between *GCD11* and *LOS1* (Hellmuth *et al.* 1998; Grosshans *et al.* 2000). *LOS1* encodes a member of the β-importin family that plays a nonessential role in transport of tRNA across the yeast nuclear membrane. However, it is unclear whether this interaction is important in the specific transport and/or localization of initiator tRNA. On the other hand, eEF1A (the functional homolog of prokaryotic EF1A) does appear to be required for efficient nuclear export of certain noninitiator tRNAs and has been suggested to func-

tion in coordinating translation and tRNA export in yeast (Grosshans *et al.* 2000).

Our combined data predict that eIF2βγ carries out all eIF2 functions required for translation initiation, including interactions with ribosomes and other translational factors, start site recognition, nucleotide exchange (in the presence or absence of eIF2B), and formation of ternary complexes. This suggests a model in which eIF2βγ comprises the eIF2 functional core, whereas the α-subunit of eIF2 and the eIF2B heteropentamer form a regulatory core that modulates the level of eIF2 function by regulating nucleotide exchange and formation of ternary complexes *in vivo*. The availability of yeast strains lacking normally essential subunits of eIF2 and eIF2B should provide valuable tools for dissecting the functions of individual polypeptides in these multisubunit complexes. Such functions could include roles in catalysis, as well as regulatory functions involved in cellular responses to stress or other environmental stimuli (Welsh and Proud 1992; Engelberg *et al.* 1994; Kimball and Jefferson 1994; Brostrom *et al.* 1996; Gallie *et al.* 1997; Qu *et al.* 1997; Scheper *et al.* 1997). In this regard, results of previous studies have indicated a role for Gcd6p in catalysis, whereas Gcd2p, Gcd7p, and Gcn3p appear to form a regulatory subcomplex (Yang and Hinnebusch 1996; Fabian *et al.* 1997; Pavitt *et al.* 1997, 1998; Gomez and Pavitt 2000). Biochemical analysis of individual eIF2B polypeptides and subcomplexes that are devoid of contaminating subunits, purified from strains using the genetic backgrounds developed in this article, in addition to further genetic analyses of these strains, will allow these questions to be addressed more directly.

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