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Unique Classes of Mutations in the *Saccharomyces cerevisiae* G-Protein Translation Elongation Factor 1A Suppress the Requirement for Guanine Nucleotide Exchange

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ABSTRACT

G-proteins play critical roles in many cellular processes and are regulated by accessory proteins that modulate the nucleotide-bound state. Such proteins, including eukaryotic translation elongation factor 1A (eEF1A), are frequently reactivated by guanine nucleotide exchange factors (GEFs). In the yeast *Saccharomyces cerevisiae*, only the catalytic subunit of the GEF complex, eEF1Bα, is essential for viability. The requirement for the *TEF5* gene encoding eEF1Bα can be suppressed by the presence of excess substrate, eEF1A. These cells, however, have defects in growth and translation. Two independent unbiased screens performed to dissect the cause of these phenotypes yielded dominant suppressors that bypass the requirement for extra eEF1A. Surprisingly, all mutations are in the G-protein eEF1A and cluster in its GTP-binding domain. Five mutants were used to construct novel strains expressing only the eEF1A mutant at normal levels. These strains show no growth defects and little to no decreases in total translation, which raises questions as to the evolutionary expression of GEF complexity and other potential functions of this complex. The location of the mutations on the eEF1A-eEF1Bα structure suggests that their mechanism of suppression may depend on effects on the conserved G-protein elements: the P-loop and NKXD nucleotide-binding element.

MANY steps in the process of protein synthesis are regulated or stimulated by energy-requiring ATPases or GTPases. Several critical soluble translation factors are G-proteins, such as the eukaryotic initiation factor 2 (eIF2), the eukaryotic elongation factors 1A and 2 (eEF1A, formerly EF-1α and eEF2) and the release factor 3 (eRF3). Both eIF2 and eEF1A have identified guanine nucleotide exchange factors (GEFs), which help regulate the activity of these proteins to allow the delivery of aminoacyl-tRNAs (Met-tRNA$_i^{met}$ or all other aa-tRNAs, respectively) to the ribosome via the classic "molecular switch" used by many G-proteins (Bourne *et al.* 1991). While the mechanism of regulation is conserved, the structure and the sequence of GEFs themselves vary dramatically. The GEF for eIF2, eIF2B, consists of five subunits (reviewed in Hershey and Merrick 2000). While these include both catalytic and regulatory proteins, the C-terminal amino acids 544–704 of the eIF2Bε subunit define the minimal catalytic region of the GEF *in vitro* and *in vivo* (Gomez *et al.* 2002). The GEF for *Saccharomyces cerevisiae* eEF1A, eEF1B, is composed of two subunits (Anand *et al.* 2001). The eEF1Bα subunit (formerly EF-1β) is catalytic whereas the eEF1Bγ (formerly EF-1γ) subunit appears to regulate the activity of eEF1Bα. A third subunit, eEF1Bβ (formerly EF-1δ), is found only in metazoans and exhibits catalytic GEF activity, although its role in the cell is not well understood (van Damme *et al.* 1990). The minimal catalytic fragments of eIF2Bε and eEF1Bα show no conservation in sequence or structure (Andersen *et al.* 2000; Boesen *et al.* 2004).

The inhibition of the nucleotide exchange reaction on eIF2 is a major regulatory step under cellular conditions of amino acid starvation, heme deficiency, endoplasmic reticulum stress, and viral infection (reviewed in Rabinow *et al.* 1993; Rodnina *et al.* 1995; Chen 2000; Hinnebusch 2000; Kaufman 2000; Ron and Harding 2000). While evidence indicates that eEF1Bα is a target for kinases (Janssen *et al.* 1988; Chang and Traugh 1997) and phosphatases (de Nadal *et al.* 2001), the effects of these modifications on gene expression are not well understood. *In vitro* dephosphorylation of *Artemia salina* eEF1Bα results in increased activity in nucleotide exchange (Janssen *et al.* 1988). In contrast, in the context of the full eEF1 complex *in vivo*, insulin treatment, S6 kinase activity, or *in vitro* protein kinase C treatment stimulates eEF1A, eEF1Bα, and eEF1Bβ (metazoan-specific) phosphorylation, nucleotide exchange, and total translation (Peters *et al.* 1995; Chang and Traugh 1997, 1998). Additionally, loss of eEF1Bγ

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or eEF1Bα in yeast results in resistance to oxidative stress (Olarewaju *et al.* 2004). Thus, much remains to be determined about the regulation of eEF1Bαγ activity and its subsequent effects on the activity of eEF1A.

Structural and functional studies in the *S. cerevisiae* system have illuminated some aspects of eEF1Bα function. Structural analysis of yeast eEF1A with the catalytic C terminus of eEF1Bα indicates that one face of eEF1Bα interacts with domain II of eEF1A while the other interacts with domain I, which contains the nucleotide-binding region (Andersen *et al.* 2000). This is a significant difference from interactions of the single subunit prokaryotic GEF EF-Ts with domains I and III of EF-Tu (Kawashima *et al.* 1996). Mutations in conserved residues of eEF1Bα that bind domain II of eEF1A cause conditional growth defects, reduce total translation, and enhance fidelity at nonsense codons (Carr-Schmid *et al.* 1999b; Andersen *et al.* 2000). K205 of eEF1Bα is in close proximity to the Mg^{+2}-binding site and is proposed to displace the Mg^{+2} ion, thereby catalyzing nucleotide exchange. In addition, a K205A mutation causes lethality (Andersen *et al.* 2001). While eEF1Bα is normally essential for viability (Hiraga *et al.* 1993), cells can survive without the protein in the presence of excess substrate, eEF1A. Such an eEF1Bα-deficient strain, however, shows significant growth defects such as a 50% increase in doubling time, temperature (Ts^-)- and cold (Cs^-)-sensitive growth, reduced translational fidelity, and increased sensitivity to translation inhibitors (Kinzy and Woolford 1995). When some mutations in the G domain of eEF1A are provided as the extra copy of eEF1A, the Cs^- growth defect is suppressed (Kinzy and Woolford 1995; Carr-Schmid *et al.* 1999a), indicating that it is possible to manipulate the system *in vivo* to more efficiently reduce the requirement for nucleotide exchange.

The result that overexpression of the G-protein substrate can bypass an essential GEF *in vivo* can be used as a genetic system to interpret the effect of GTPase function in the absence of regulation by its GEF. However, eEF1A overexpression also affects actin cytoskeleton organization (Munshi *et al.* 2001). Thus, a system lacking the requirement for eEF1A overexpression focuses the analysis strictly on the loss of GEF function. In the case of eEF1Bα, it can be also used as a model system to determine how the cell responds when the proposed rate-limiting step of translation elongation has been disrupted. We have created an eEF1Bα-deficient strain using suppressors of the requirement for this normally essential protein and normal levels of eEF1A. The screen was performed in strains with and without the [*PSI*+] prion, a form of the release factor eRF3. The screens yielded 4 and 7 mutations, respectively, 10 of which are dominant. While the screen was unbiased, and the strain contained three copies of the gene encoding eEF1A, all 9 unique mutations are located in one of the two genes encoding eEF1A. Interestingly, all mutations are within or in close proximity to the nucleotide-binding domains. One of the mutants, A117V, results in altered mobility of eEF1A in SDS–PAGE. Additionally, all 9 mutants are functional as the only form of eEF1A. A representative subset of mutants was utilized to prepare strains where these forms of eEF1A are the only copy in the cell and the gene encoding eEF1Bα is deleted. Thus, this allows us to determine the function and the necessity of eEF1Bα in the cell. Furthermore, the clustered locations of these mutations in eEF1A provide valuable information on the different roles of the P-loop and the NKXD regions of G-proteins.

MATERIALS AND METHODS

Strains and media: *Escherichia coli* DH5α was used for plasmid preparation. *S. cerevisiae* strains used in these studies are listed in Table 1. Standard yeast genetic methods were employed (Mortimer and Hawthorne 1966; Sherman *et al.* 1986). Yeast cells were grown in either YEPD (1% Bacto yeast extract, 2% peptone, 2% dextrose) or defined synthetic complete media (C or C−) supplemented with 2% dextrose as a carbon source unless noted. Yeast were transformed by the lithium acetate method (Ito *et al.* 1983). Mating-type switching in yeast using the *HO* endonuclease was performed as described (Herskowitz and Jensen 1991). Strains lacking the chromosomal *TEF1*, *TEF2*, and *TEF5* genes were constructed by PCR of the *tef5*::*TRP1* locus from TKY298, transformation into a strain bearing a plasmid-borne eEF1A mutant and *tef1*::*LEU2 tef2*Δ deletions of the eEF1A genes, and selection on C-Trp. All strains were confirmed as lacking eEF1Bα by Western blot analysis.

DNA manipulations: Recombinant DNA techniques were performed as described (Sambrook *et al.* 1989). Restriction endonucleases and DNA-modifying enzymes were obtained from Roche Biochemicals (Indianapolis). The *TEF1* and *TEF2* genes were recovered from the genome of *SBD* mutants by PCR and the coding region was directly sequenced. Plasmids expressing the identified mutant forms of eEF1A were prepared by either *in vivo* recombination of a pRS316 (*URA3 CEN*)-based plasmid or use of the Quikchange mutagenesis kit (Stratagene, La Jolla, CA) using pTKB754 as the template. The resulting plasmids were transformed into TKY767 and grown on C-Ura to confirm that the eEF1Bα-deficiency phenotype was suppressed. Each cloned gene containing an *SBD* mutant was transformed into MC214, loss of the wild-type eEF1A plasmid was monitored by growth on 5-fluoroanthranilic acid (5-FAA) (Toyn *et al.* 2000), and the recovered strain was analyzed for growth defects.

EMS mutagenesis and isolation of suppressors of the eEF1Bα-deficiency: TKY298 (2×10^8 cells) or TKY604 cells (2×10^8) were mutagenized by a modification of standard procedures (Lawrence 1991). Cells were washed and resuspended in 2 ml of 50 mm potassium phosphate buffer. Resuspended cells (100 μl) were added to a 10% sodium thiosulfate solution and kept as a zero time point. Ethyl methanesulfonate (EMS; 40 μl) was added to the remaining cells and shaken at 30°. Aliquots of 600 μl of cells were transferred at 30, 60, and 90 min to a 10% sodium thiosulfate solution to neutralize the EMS, washed twice in sterile water, resuspended in 5 ml of 15% glycerol, and stored at −80°. The percentage of surviving cells was determined by plating a dilution of cells from each time point and counting the colonies after 2–3 days of incubation. For TKY298 [*PSI*+], ~30,000 colonies from the 30-min time point (53% killing) and 70,000 colonies from the 60-min time point (61% killing) were screened by plating on YEPD at the

TABLE 1

***S. cerevisiae* strains**

Strain	Genotype	Source
MC213	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF2 TRP1*	Sandbaken and Culbertson (1988)
MC 214	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF2* URA3	Sandbaken and Culbertson (1988)
JWY4231	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 pTEF5 URA3*	Carr-Schmid *et al.* (1999b)
TKY235	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 pTEF5 LEU2*	Carr-Schmid *et al.* (1999b)
TKY298	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 pTEF2 URA3*	This work
TKY299	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 pTEF2 LEU2*	This work
TKY352	*MATa ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 pTEF1 LEU2*	This work
TKY372	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 TEF1 SBD1* (*TEF2-20* R164K)	This work
TKY373	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-his4-713 tef5::TRP1 TEF2 sbd2* (*TEF1-21* D156E)	This work
TKY374	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 TEF1 SBD3* (*TEF2-17* D156N)	This work
TKY375	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 TEF1 SBD4* (*TEF2-22* T22S)	This work
TKY603	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 pTEF5 LEU2*[*psi*−]	This work
TKY604	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 pTEF2 URA3*[*psi*−]	This work
TKY 631	*MATα ura3-52 lys2-81 ade2-101trp1Δ63 his3Δ200leu2Δ1 tef5::KanMX pTEF5 URA3*	This work
TKY646	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 SBD1 pTEF2 URA3*	This work
TKY647	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2- l his4-713 tef5::TRP1 sbd2 pTEF2 URA3*	This work
TKY648	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 SBD3 pTEF2 URA3*	This work
TKY649	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 SBD4 pTEF2 URA3*	This work
TKY 726	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 pTEF1 LEU2*	This work
TKY767	*MATα ura3-52 lys2-81 ade2-101trp1Δ63 his3Δ200 leu2Δ1 tef5::KanMX pTEF1 TRP1*	This work
TKY781	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 TEF2 SBD5* (*TEF1-4* E122K) [*psi*−]	This work
TKY782	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 TEF1 SBD6* (*TEF2-17* D156N) [*psi*−]	This work
TKY783	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1his4-713 tef5::TRP1 TEF2 SBD7* (*TEF1-20* R164K) [*psi*−]	This work
TKY784	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 TEF1 SBD8* (*TEF2-23* A112T) [*psi*−]	This work
TKY785	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 TEF2 SBD9* (*TEF1-24* A117T) [*psi*−]	This work
TKY786	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 TEF2 SBD10* (*TEF1-25* A117V) [*psi*−]	This work
TKY787	*MATα ura3-52 trp1Δ101 lys2-801 leu2Δ1 met2-1 his4-713 tef5::TRP1 TEF2 SBD11* (*TEF1-26* A117V T172A) [*psi*−]	This work
TKY 789	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF1 URA3* (R164K)	This work
TKY 791	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF1 URA3* (A117V T172A)	This work
TKY 846	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF1 URA3* (D156E)	This work
TKY 847	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF1 URA3* (D156N)	This work
TKY 848	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF1 URA3* (T22S)	This work
TKY 849	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF1 URA3* (A112T)	This work
TKY 850	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF1 URA3* (E122K)	This work
TKY 851	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF1 URA3* (A117T)	This work
TKY 852	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ pTEF1 URA3* (A117V)	This work
TKY 961	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3* (R164K)	This work
TKY 962	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3* (D156N)	This work
TKY 963	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3* (T22S)	This work
TKY 964	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3* (A112T)	This work
TKY 965	*MATα ura3-52 leu2-3,112 trp1Δ1 lys2-20 met2-1 his4-713 tef1::LEU2 tef2Δ tef5::TRP1 pTEF1 URA3* (A117V)	This work

nonpermissive temperature of 37°. Colonies that grew well at 37° were patched onto fresh YEPD plates, grown at 30°, and then confirmed for growth at 37°. For TKY604 [*psi*−], ~7500 colonies from the 30-min time point (40% killing) were plated on YEPD at the permissive temperature of 30° to allow recovery and then replica plated to 5-FOA at 37° and 24°. Colonies that grew better on 5-FOA at 37° than at 24° were patched onto YEPD plates, grown at 30°, and then confirmed for growth at 37° by streaking and spotting of cells on YEPD.

Drug sensitivity and growth assay: Two-milliliter cultures of each strain were grown at 30° in the appropriate media to midlog phase and independent colonies of each strain were assayed as previously described (Carr-Schmid *et al.* 1999b). The concentrations of drugs used were 2 mm cycloheximide, 25 mm hygromycin B, and 1.3 m (800 mg/ml) paromomycin. Sensitivity to each drug was measured by the radius of inhibition of growth around each disc in millimeters. Relative growth of wild-type, eEF1Bα-deficient, and suppressor strains was assayed by streaking or serial dilution spotting as previously described (Carr-Schmid *et al.* 1999b).

Western blot analysis: Proteins were extracted from suppressor, wild-type, and eEF1Bα-deficient strains at an OD_{600} of 0.3–0.8. Cells were spun down for 5 min at 5000 rpm, resuspended in 0.4 ml of cold lysis buffer (100 mm Tris–HCl, pH 8, 20% glycerol, 1 mm DTT, and 1 mm PMSF), and lysed by vortexing with glass beads. Concentration of protein in the extract was determined using Bradford's reagent (Bio-Rad, Hercules, CA) and protein levels were analyzed by Western blot analysis using polyclonal antibodies to Rpa1p (provided by Steven Brill, Rutgers University) and yeast eEF1A (Carr-Schmid *et al.* 1999a).

Nonsense and missense suppression and total translation assays: Nonsense suppression assays were performed on strains containing *URA3*-based *CEN* plasmids containing *lacZ* expressed from the *PGK1* promoter with the *PGK1* transcriptional terminator with either the wild-type sequence (pUKC815tail) or an in-frame UAA (pUKC817tail), UAG (pUKC818tail), or UGA (pUKC819tail) codon (Carr-Schmid *et al.* 1999b). The strains containing each plasmid were grown overnight at 30° in C-Ura to midlog phase. At least four samples for each strain were analyzed in duplicate using the ortho-nitrophenyl-β-galactoside assay as previously described (Dinman and Kinzy 1997) and standard deviations were calculated. Dual luciferase assays were performed with reporter systems to measure nonsense and missense suppression. Nonsense suppression assays were performed on strains containing *URA3*-based *CEN* plasmids containing renilla and firefly luciferase reporters expressed from the *ADH1* promoter with the *CYC1* terminator and either the wild-type sequence (AAA) or a stop codon (UAA) (Harger and Dinman 2003). Missense suppression assays were performed on strains containing *URA3*-based *CEN* plasmids containing a CAC (His)-to-CGC (Arg) mutation in firefly luciferase at codon 245 (Salas-Marco and Bedwell 2005). Strains containing each plasmid were grown overnight at 30° in C-Ura to midlog phase. Cells were harvested by centrifugation and washed twice with 0.5–1 ml of cold lysis buffer (1× PBS, pH 7.4, 1 mm PMSF). Cell suspensions were lysed with glass beads and resuspended in cold lysis buffer. At least three independent colonies of each strain were analyzed in triplicate using the Dual-Luciferase system (Promega, Madison, WI) and a microtiter plate luminometer (MTX Lab Systems) and standard deviations were calculated. *In vivo* [^{35}S]methionine incorporation was performed as described (Carr-Schmid *et al.* 1999b).

RESULTS

An eEF1Bα-deficient strain shows altered translation phenotypes: Prior work indicates a strain lacking eEF1Bα where viability is restored by excess eEF1A, is sensitive to translation inhibitors such as paromomycin, and shows suppression of the *lys2-801* (UAG) nonsense mutation *in vivo* (Kinzy and Woolford 1995). The alteration in nonsense suppression was quantitated using a *lacZ* reporter construct. Wild-type (TKY235) and eEF1Bα-deficient (TKY299) strains were assayed for the production of β-galactosidase indicative of suppression of an in-frame UAA, UAG, or UGA codon. The eEF1Bα-deficient strain showed a 5- to 10-fold increase in nonsense suppression at all three stop codons (Figure 1A), indicating omnipotent nonsense suppression. Total translation was also monitored, and an eEF1Bα-deficient strain showed an ~50% reduction in total protein synthesis as measured by ^{35}Met incorporation (Figure 1B). Neither effect is due to the extra copy of the *TEF2* gene encoding eEF1A, since a strain with the normal copies of eEF1A and eEF1Bα genes on the chromosome as well as a *TEF2* plasmid shows wild-type nonsense suppression and total translation (Munshi

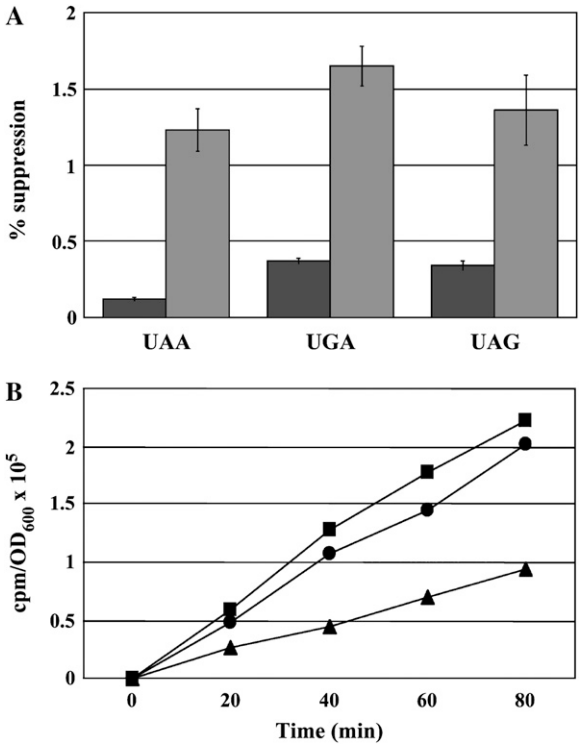

Figure 1.—(A) A wild-type eEF1Bα (TKY235, p*TEF5 LEU2*, solid bars) and an eEF1Bα-deficient (TKY299, *TEF2 LEU2*, shaded bars) strain were assayed for the ability to readthrough the three stop codons using a *lacZ*-based assay. Results show the percentage of readthrough of the indicated stop codon and represent the average of a minimum of four samples. The error bars represent the propagation of error calculated as the standard deviation of a minimum of three samples. (B) Strains, as in A, containing pRS316 (TKY235, squares and TKY299, triangles) or with a *URA3 TEF2* plasmid (TKY235, circles) were grown to midlog phase in C-Ura-Met, diluted, and grown for varying times in C-Ura-Met with [^{35}S]methionine and total protein synthesis measured by TCA precipitation. Data are represented as counts per minute per A_{600} unit.

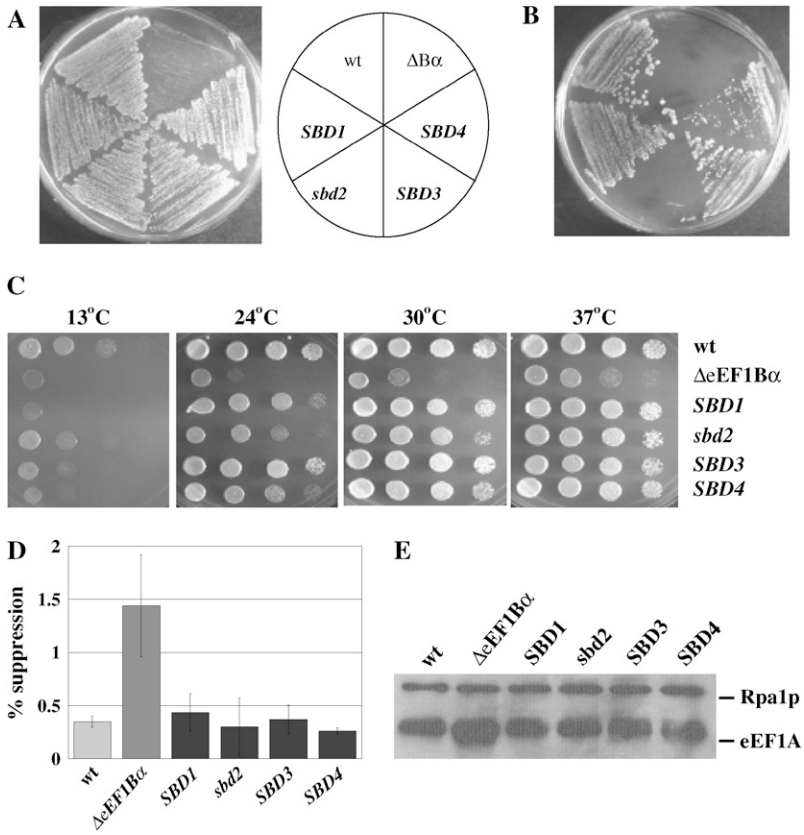

FIGURE 2.—(A). Wild-type eEF1Bα (TKY235), the parental eEF1Bα-deficient (TKY298), and the *SBD1–4* (TKY646-649) strains were grown on 5-FOA for 3 days at 30°. (B) Strains, as in A, were mated to the eEF1Bα-deficient strain TKY352, diploids were selected, streaked onto 5-FOA, and grown for 3 days at 30°. (C) Wild type (JWY4231, p*TEF5 URA3*), the parental eEF1Bα-deficient (TKY298), and *SBD1–4* lacking the *TEF2 URA3* plasmid (TKY372-375) strains were grown in YEPD and diluted to an A_{600} of 1.0. Ten-fold serial dilutions were spotted and grown at 13,° 24,° 30,° and 37° for 2–8 days. (D) Wild-type (TKY235), eEF1Bα-deficient (TKY299), or *SBD1–4* strains (TKY372-375) were assayed for the ability to readthrough a UAA stop codon using a *lacZ*-based assay. Results show the percentage of readthrough of the stop codon and represent the average of a minimum of four samples. The error bars represent the propagation of error calculated as the standard deviation for a minimum of four samples. (E) Equal amounts of total yeast proteins from wild-type (TKY235), eEF1Bα-deficient (TKY298), and *SBD1–4* (TKY372-375) strains, as determined by Bio-Rad assay, were analyzed by SDS–PAGE and Western blot analysis with polyclonal antibodies to yeast eEF1A and yeast Rpa1p (as a loading control).

et al. 2001; Figure 1B). Thus, while an eEF1Bα-deficient strain is viable, there are consequences to the lack of catalyzed nucleotide exchange and/or the presence of eEF1Bα, which could be illuminated by the analysis of suppressors of the defects of eEF1Bα-deficient strains.

Isolation of mutations that suppress the conditional growth defect of a cell lacking eEF1Bα: To identify mutations in genes that suppress the defects of a cell lacking eEF1Bα, yeast strain TKY298 bearing two chromosomal and one plasmid-borne gene encoding eEF1A as well as a chromosomal deletion of the *TEF5* gene encoding eEF1Bα were mutagenized with EMS to 53–61% lethality. Approximately 100,000 colonies were screened for growth at the restrictive temperature of 37°, yielding four independent colonies. To determine if the putative suppressor mutations were a result of a mutation in the plasmid-borne *TEF2* gene, all four strains were transformed with a *TEF1 LEU2* plasmid (pTKB168) and loss of the *TEF2 URA3* plasmid was monitored by growth on 5-FOA (BOEKE *et al.* 1987). The resulting strains still demonstrated wild-type growth at 37° (data not shown), indicating that the four suppressor mutations reside in the chromosomal DNA. Surprisingly, all four strains also grew on 5-FOA when an empty *LEU2* plasmid was present, exhibiting suppression of not only the Ts^- defect of the eEF1Bα-deficient strain, but also the requirement for excess eEF1A (Figure 2A). The suppressor strains were termed *SBD* for *s*uppressor of an eEF1*B*α-*d*eficiency. To determine if the mutations were dominant or recessive, each of the *SBD* mutant strains with the *TEF2 URA3* plasmid (TKY646-649) was mated to the eEF1Bα-deficient strain TKY352 and diploids were selected. The *TEF1 LEU2* plasmid was lost by nonselective growth and the diploids were streaked on 5-FOA. Loss of the suppressor phenotype, 5-FOA resistance, was seen for TKY647 (*sbd2*), indicating a recessive mutation (Figure 2B). However, diploids containing the suppressor mutation from TKY646, TKY648, and TKY649 (*SBD1*, *-3*, and *-4*) allowed the strains to survive without excess eEF1A and are thus dominant (Figure 2B).

Growth was assayed for the four mutants without an extra eEF1A encoding plasmid. At 37°, growth was essentially as for wild type for the suppressor strains; at 24°, *SBD1* and *SBD3* grew slightly better than *sbd2* and *SBD4* strains, while, at 13°, all the suppressor strains resemble the eEF1Bα-deficient strain (Figure 2C). The *SBD* strains recovered the nonsense suppression phenotype of the eEF1Bα-deficient strain as monitored by a reduction in the level of β-galactosidase activity back to the wild-type levels (Figure 2D). To confirm that the strain had lost the extra copy of eEF1A, Western blot analysis was performed. All four suppressor strains show wild-type levels of eEF1A equivalent to two chromosomal genes encoding eEF1A, and not the excess protein seen in the eEF1Bα-deficient strain (Figure 2E). These are the first strains shown to be able to bypass the need for the nucleotide exchange factor eEF1Bα without the presence of excess eEF1A.

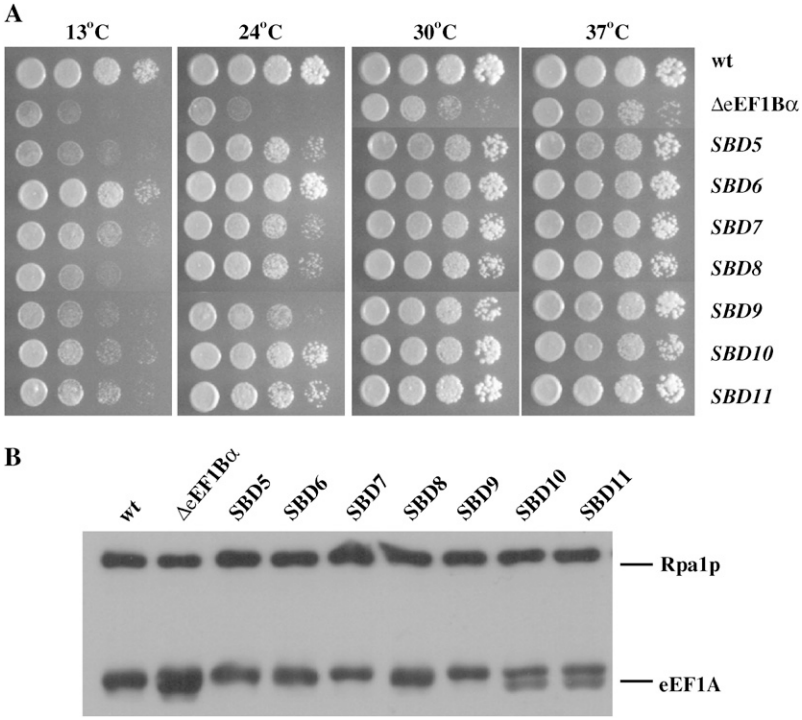

FIGURE 3.—(A) The [*psi*−] wild-type (TKY603), parental eEF1Bα-deficient (TKY604), or *SBD5-11* (TKY781-787) strains were grown in YEPD and diluted to an A_{600} of 1.0. Ten-fold serial dilutions were spotted and grown at 13°, 24°, 30,° and 37° for 2–8 days. (B) Equal amounts of total yeast proteins from strains, as in A, as determined by Bio-Rad assay, were analyzed by SDS–PAGE and Western blot analysis with polyclonal antibodies to yeast eEF1A and yeast Rpa1p (as a loading control).

Isolation of mutations that bypass eEF1Bα in a [*psi*−] background: In the course of analysis of the mutants, it was determined that the parent of the suppressor strains also contains the yeast prion [*PSI*$^+$]. The presence of [*PSI*$^+$], a form of the eukaryotic release factor 3 (eRF3), could alter the translational status of the cell and bias the screen results. Thus, a similar screen was performed specifically for bypass suppressors of the eEF1Bα deficiency using the [*psi*−] derivative of TKY298, TKY604. Since all the mutants in the first screen could lose the extra eEF1A-encoding *URA3* plasmid, we identified strains that, following mutagenesis, could grow on 5-FOA at 37° better than at 25°. From 7500 colonies, 13 mutants were identified. Growth assays of the cells on YEPD indicated that the suppressors grew faster than the parent eEF1Bα-deficient strain and in many cases grew similarly to a wild-type strain (Figure 3A; data not shown). Diploids were prepared by mating each mutant back to an eEF1Bα-deficient parent strain of the opposite mating type (TKY726). All 13 diploids grew on 5-FOA as for the first screen and were thus dominant. Western analysis of equalized protein extracts from all suppressor, wild-type, and eEF1Bα-deficient strains showed that eEF1A protein levels for all *SBD* suppressors approximated wild-type levels and were qualitatively much less than the eEF1Bα-deficient strain (Figure 3B; data not shown). Four suppressor strains show heterogeneity as a doublet in the eEF1A band by Western analysis (Figure 3B; data not shown). This is not observed in the wild-type strain, because the protein sequences of the *TEF1* and *TEF2* open reading frames are 100% identical. This doublet suggests that, in these mutants, either *TEF1* or *TEF2* is changed in some way that causes a shift in mobility on an SDS–PAGE gel.

All SBD mutants are in a chromosomal eEF1A-encoding gene: On the basis of prior results of some eEF1A mutations permitting enhanced viability in the absence of eEF1Bα, the dominant phenotype of the 16 mutants from the two screens, and the heterogeneity of the eEF1A band in Western blot analysis, the genes encoding eEF1A were analyzed from the mutant strains. Initially, the *TEF1* and *TEF2* genes were cloned from the *SBD7* and *SDB11* mutant strains by *in vivo* recombination into a pRS316 plasmid (*URA3 CEN*). The resulting plasmids were transformed into the eEF1Bα-deficient strain TKY767, plated on 5-FAA to monitor the loss of the *TEF1 TRP1* plasmid, recovered on YEPD, and spotted at various temperatures. The plasmids encoding *TEF1*, but not *TEF2*, from either suppressor allowed enhanced growth at 24°, 30°, and 37°, indicating the *SBD* phenotype (Figure 4A). The genes were sequenced and mutations of R164K (*SBD7*) and A117V T172A (*SBD11*) were identified in the *TEF1* gene; however, the *TEF2* sequences were wild type. On the basis of these results *TEF1* and *TEF2* were amplified from the genomic DNA from the remaining 11 mutants identified in the [*psi*−] screen. A total of seven different mutations were identified, three of them appearing more than once. Mutants that maintained a single eEF1A band by Western-contained mutations of E112K (*SBD5*, three isolates), D156N (*SBD6*), R164K (*SBD7*, three isolates), A112T (*SBD8*), and A117T (*SBD9*). Mutants with the doublet by Western analysis contained mutations of A117V (*SBD10*, three isolates) and A117V T172A

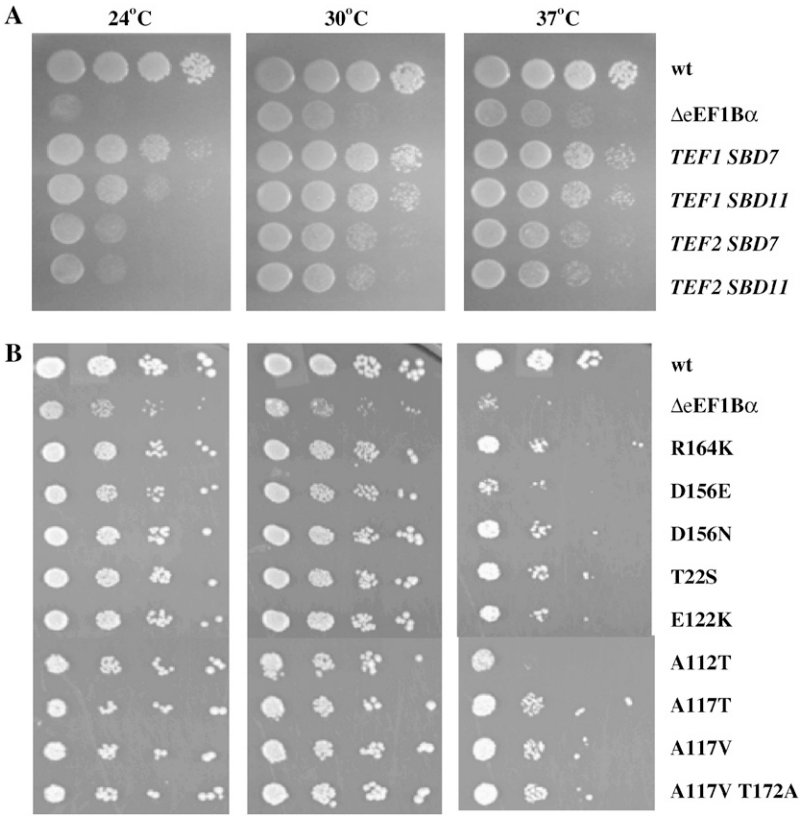

FIGURE 4.—(A) Wild type (JWY235), eEF1Bα-deficient (JWY767, *pTEF1 TRP1*) with pRS316, and JWY767 transformed with the plasmids containing the *TEF1* or *TEF2* gene cloned from TKY789 (*SBD7*) and TKY791 (*SBD11*) strains were grown in C-Ura and diluted to an A_{600} of 1.0. Tenfold serial dilutions were spotted onto C-Ura medium and grown at 24°, 30°, and 37° for 2–8 days. (B) Wild-type (TKY631, p*TEF5 URA*), eEF1Bα-deficient (TKY767) with pRS316, and TKY767 transformed with a *TEF1* (*SBD* mutant) *URA3* plasmid strains were grown and assayed as in A.

(*SBD11*). Sequencing of the *TEF1* and *TEF2* genes from *SBD1-4* mutants demonstrated eEF1A mutants of R164K (*SBD1*), D156E (*sbd2*), D156N (*SBD3*), and T22S (*SBD4*). Thus, two mutants, R164K and D156N, were found in both screens. Interestingly, E122K and D156N were previously identified as allowing better growth at low temperatures of a strain lacking eEF1Bα when present as the third copy of eEF1A (KINZY and WOOLFORD 1995; CARR-SCHMID *et al.* 1999a). To confirm the results from the genomic DNA sequencing, site-directed mutagenesis was utilized to produce each mutation in a *TEF1 URA3 CEN* plasmid. All constructs were transformed into the eEF1Bα-deficient strain, as in Figure 4A, and promoted enhanced growth (Figure 4B), confirming that they can cause the *SBD* phenotype. Interestingly, growth of these strains with the three eEF1A-encoding genes was better suppressed at lower temperatures, even though the original *SBD* strains with the normal two eEF1A-encoding genes showed better growth at higher temperatures (Figures 2 and 3).

To look more closely at translation effects, sensitivity to translation inhibitors was monitored for all 11 *SBD* mutant strains, which lack eEF1Bα and have one wild type and one mutant copy of a chromosomal eEF1A gene. The results show that while there was a modest difference in the sensitivity to the drugs cycloheximide and hygromycin B, some *SBD* mutants partially suppressed the severe paromomycin-sensitive phenotype of the eEF1Bα-deficient strain. However, all strains expressing *SBD*s remained hypersensitive to paromomycin compared to the isogenic wild-type strain (Tables 2 and 3). To determine if any secondary phenotypes are conferred by the suppressor mutations when eEF1Bα is restored, the strains containing *SBD1-11* were transformed

TABLE 2

Drug sensitivity phenotypes of mutant strains isolated from a [*PSI*+] eEF1Bα-deficient mutant screen

Strains		Hygro (25 mM)	Cyclo (2 mM)	Paromo (1.3 M)
TKY235	WT eEF1Bα	2.5[a]	12.8	3.3
TKY298	eEF1Bα deficient	3.0	16.3	9.3
TKY372	*SBD1* *TEF2* R164K	2.3	15.5	4.0
TKY373	*sbd2* *TEF1* D156E	1.3	12.2	4.5
TKY374	*SBD3* *TEF2* D156N	3.5	15.2	8.0
TKY375	*SBD4* *TEF2* T22S	2.5	13.2	6.0

[a] Radius of inhibition of growth around the drug-containing filter in millimeters on YEPD.

TABLE 3

Drug sensitivity phenotypes of mutant strains isolated from a [*psi*−] eEF1Bα-deficient mutant screen

		−eEF1Bα		
		Hygro (25 mM)	Cyclo (2 mM)	Paromo (1.3 M)
TKY603	Wild type eEF1Bα	2.8[a]	9.0	1.0
TKY604	eEF1Bα deficient	4.0	14.8	9.3
TKY781	*SBD5* *TEF1* E122K	4.3	13.8	5.8
TKY782	*SBD6* *TEF2* D156N	3.5	11.8	4.3
TKY783	*SBD7* *TEF1* R164K	3.3	13.3	2.5
TKY784	*SBD8* *TEF2* A112T	2.8	11.8	2.0
TKY785	*SBD9* *TEF1* A117T	3.5	14.3	4.5
TKY786	*SBD10* *TEF1* A117V	3.5	12.5	4.5
TKY787	*SBD11* *TEF1* A117V T172A	4.5	12.5	3.5

[a] Radius of inhibition of growth around the drug-containing filter in millimeters on YEPD.

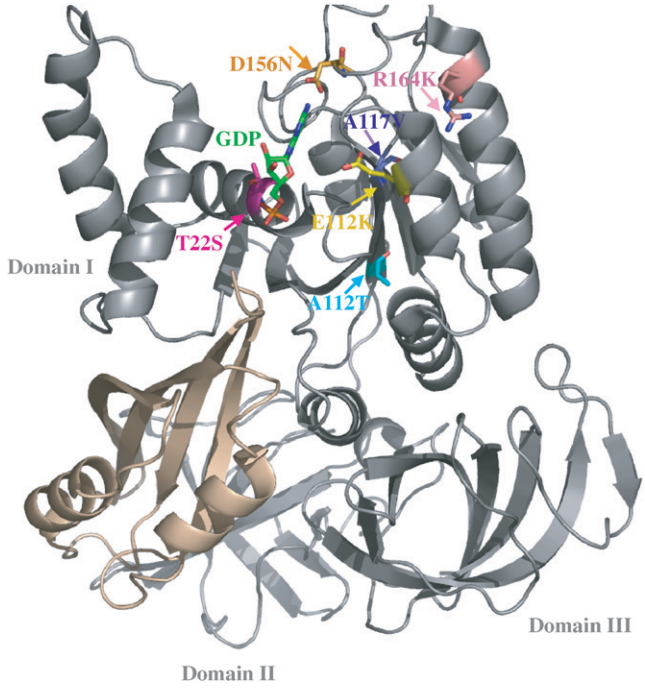

FIGURE 5.—X-ray crystal structure of *S. cerevisiae* eEF1A (shading) shows that the *SBD* mutations (arrows) T22 (S, magenta), A112 (T, cyan), A117 (V, T, purple), E122 (K, yellow), D156 (E, N, orange), and R164 (K, pink) that suppress the requirement for the eEF1Bα (brown) cluster in the GTP-binding domain (ANDERSEN *et al.* 2000). GDP is shown in green.

with a *TEF5 URA3* plasmid. None of the strains showed conditional or slow-growth phenotypes with eEF1Bα present (data not shown).

All *SBD* mutants in eEF1A map to the nucleotide-binding domain: *SBD* mutations that allow the eEF1A protein to function without its normally essential exchange factor are all located in domain I, the nucleotide-binding domain (Figure 5). Each mutation is located in the nucleotide-binding domain or in a very close proximity to at least one of the conserved sequence elements of the G-proteins. The mutations interact directly with either the nucleotide or the motifs that bind and stabilize it.

eEF1A mutants that suppress the eEF1Bα requirement are viable as the only copy of eEF1A: Strains that express only the *SBD* form of eEF1A were prepared by plasmid shuffling in a *tef1::LEU2 tef2*Δ strain. All mutants allowed viability with no growth defects at permissive temperatures compared to wild-type cells (Figure 6). A112T, and to a lesser extent D156E, show a cold-sensitive temperature defect. To assess effects on protein synthesis, sensitivity to different translation inhibitors was monitored (Table 4, first three columns). Interestingly, all the *SBD* mutant strains show at least a slight increase in hygromycin sensitivity. Cycloheximide sensitivity was unaffected in these strains. Strains harboring the R164K or A117V mutants showed paromomycin resistance, whereas T22S and A112T strains showed the greatest sensitivity to the drug.

***SBD* mutations do not have additive effects on suppression:** To determine if the eEF1A mutations conferring the *SBD* phenotype function through the same pathway, four double mutations were prepared. The T22S/A117V, R164K/A117V, A117V/A112T, and E122K/A112T double mutants were chosen on the basis of the distance between the side chains in the structure or the orientation of the side groups relative to each other (Figure 5). A strain with the double mutants present as a plasmid-borne extra copy and lacking eEF1Bα was assayed for growth at 24°, 30°, and 37°. The T22S/A117V mutant showed modest suppression of the loss of eEF1Bα, albeit at a lower level than seen for the single mutants. The R164K/A117V mutant showed little to no growth suppression. Interestingly, the A117V/A112T and E122K/A112T double mutants not only fail to suppress the requirement for eEF1Bα, but also show a dominant negative effect on cell growth (Figure 7). None of the double mutants were viable as the only form of eEF1A in a cell containing eEF1Bα (data not shown), indicating that they compromise functions beyond that tolerated by the cell.

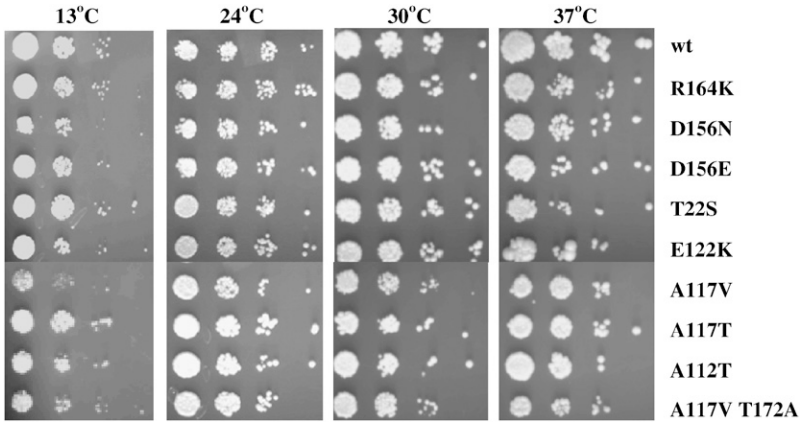

FIGURE 6.—Strains expressing wild-type eEF1A (MC213 p*TEF1 TRP1*) or *SBD* mutants (TKY789, 791, 846-852) from a plasmid as the only form were grown in YEPD and diluted to an A_{600} of 1.0. Ten-fold serial dilutions were spotted onto YEPD plates and grown at 13°, 24°, 30°, and 37° for 2–8 days.

***SBD* mutations can support viability as the only form of eEF1A in the absence of eEF1Bα:** To establish the consequence of the full loss of eEF1Bα and a mutant form of eEF1A less dependent on the normally essential activity of this exchange factor, strains were constructed lacking the chromosomal eEF1A and eEF1Bα genes. Strains were prepared by recombination of a *tef5::TRP1* deletion cassette in the strains bearing the R164K, A117V, T22S, A112T, or D156N *SBD* mutations as the only form of eEF1A on a plasmid. All the strains produced viable cells that lack the eEF1Bα-encoding gene as detected by DNA and protein analysis. The strains showed no growth defect compared to the wild-type strain (Figure 8) or to the strains harboring the same mutations as the only form but with eEF1Bα present (compare to Figure 6).

To determine the effects of these strains on translation, *in vivo* [^{35}S]methionine labeling assays were performed. A112T and T22S strains showed a 25 or 35% reduction, respectively, in total translation both in the presence and in the absence of eEF1Bα. However, R164K and A117V strains translated as efficiently as wild type in the presence of eEF1Bα. In the absence of eEF1Bα, A117V showed a 25% decrease in total protein synthesis, while R164K showed a 25% increase (Table 5). Drug sensitivity assays were performed to determine the

TABLE 4

Drug sensitivity phenotypes of the strains expressing *SBD* mutants as the only form of eEF1A

		+eEF1Bα			−eEF1Bα:
Strains		Hygro (50 mM)	Cyclo (2 mM)	Paromo (1.3 M)	Paromo (1.3 M)
TKY102	Wild type eEF1Bα	15[a]	33	14.7	NA
TKY789	*SBD1/SBD7* *TEF1* R164K	17.3	31	9.7	20
TKY846	*sbd2* *TEF1* D156E	18.7	27.7	14.7	ND
TKY847	*SBD3/SBD6* *TEF2* D156N	18.7	31.7	16.7	22.3
TKY848	*SBD4* *TEF2* T22S	19	30	18.7	21
TKY850	*SBD5* *TEF1* E122K	18	30	16.3	ND
TKY849	*SBD8* *TEF2* A112T	21.3	33.7	22.3	22.3
TKY851	*SBD9* *TEF1* A117T	18	29.7	14	ND
TKY852	*SBD10* *TEF1* A117V	18	33.3	12	16.7
TKY791	*SBD11* *TEF1* A117V/T172A	18.3	31	17	ND

NA; not applicable. ND; not determined.
[a] Radius of inhibition of growth around the drug-containing filter in millimeters on YEPD.

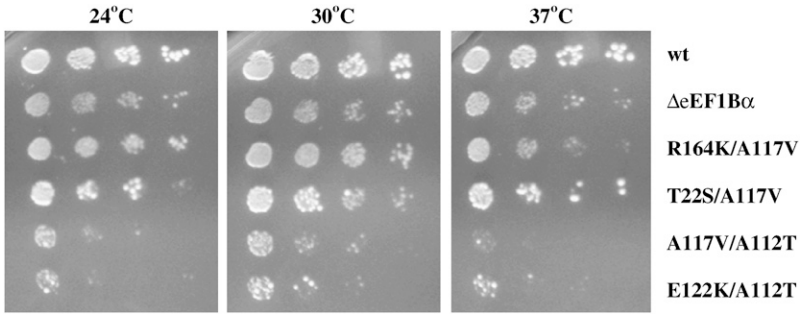

FIGURE 7.—Wild type (TKY631, p*TEF5 URA3*, eEF1Bα-deficient (TKY767) with pRS316 and TKY767 expressing the indicated *TEF2 SBD* double-mutant plasmid strains were grown in C-Ura and diluted to an A_{600} of 1.0. Ten-fold serial dilutions were spotted onto C-Ura plates and grown at 24°, 30°, and 37° for 2–8 days.

paromomycin sensitivity of these strains. In the absence of eEF1Bα, all the mutant strains except A112T were more sensitive to paromomycin compared to the presence of eEF1Bα (Table 4). To measure the effect of these strains on the accuracy of translation, we performed *in vivo* dual luciferase assays. Previously used *lacZ* reporters were compared to dual luciferase reporters for the analysis eEF1A mutants and all mutants reproduced same trends in nonsense or frameshift suppression (data not shown). While A112T and T22S strains showed higher nonsense suppression rates by 2.5- to 2.7-fold compared to wild type, R164K and A117V strains showed no effect (Table 5). No mutation affected missense suppression (Table 5).

DISCUSSION

While the guanine nucleotide exchange factor of eEF1A, eEF1Bα, is essential for cell viability, recent studies showed that eEF1A is able to dissociate GDP without its GEF although with a 700-fold slower rate (PITTMAN *et al.* 2006). This slow rate of exchange is not sufficient for cell viability but can be partially compensated for by an extra copy of eEF1A (KINZY and WOOLFORD 1995). Cells lacking eEF1Bα with an extra copy of wild-type eEF1A exhibit slower-growth phenotypes, a 50% reduction in total translation, and reduced translational fidelity. Interestingly, *SBD* mutations in eEF1A likely cause a more favorable conformation of eEF1A for nucleotide exchange, allowing it to function efficiently without eEF1Bα and thus suppress most of the eEF1Bα deficiency phenotypes. Two genetic screens were performed to isolate the suppressors of the eEF1Bα deficiency, and although the screens were developed specifically to avoid eEF1A mutations by the presence of three eEF1A-encoding genes, both screens exclusively yielded eEF1A mutations. Since the same mutation was selected more than once on multiple occasions, it appears that eEF1A mutations are the major, if not the sole, suppressors of the lethal effects of the loss of eEF1Bα.

An analysis of three different strains containing the *SBD* mutants provided unique insights into the requirement for eEF1Bα. The initial eEF1Bα-deficient strains isolated had one wild type and one *SBD* mutant eEF1A-encoding gene and thus normal eEF1A protein levels. Previous studies of suppression of the requirement for eEF1Bα used strains with the two chromosomal wild-type eEF1A-encoding genes and an eEF1A mutant on a plasmid (KINZY and WOOLFORD 1995) and showed a significant difference in the temperature effects. While the *SBD* mutant strains with two eEF1A genes suppress growth defects at 37° (Figures 2 and 3), eEF1Bα-deficient strains from this (Figure 4) or prior work (KINZY and WOOLFORD 1995; CARR-SCHMID *et al.* 1999a) with three eEF1A genes show enhanced growth at low temperatures. This likely relates to the effects of excess eEF1A on the actin cytoskeleton, which results in reduced growth at elevated temperatures (MUNSHI *et al.* 2001). The new strains lacking eEF1Bα and both chromosomal eEF1A genes and thus expressing only the *SBD* form of eEF1A indicate that it is in fact possible to suppress all the deficiencies of the loss of the nucleotide exchange factor with little to no effect on growth (Figure 8).

The slow-growth phenotype of an eEF1Bα-deficient strain is suppressed in the presence of *SBD* mutants of eEF1A. However, while *SBD* strains with one wild-type and one *SBD* mutant eEF1A gene exhibit modest differences in the sensitivity to the drugs cycloheximide

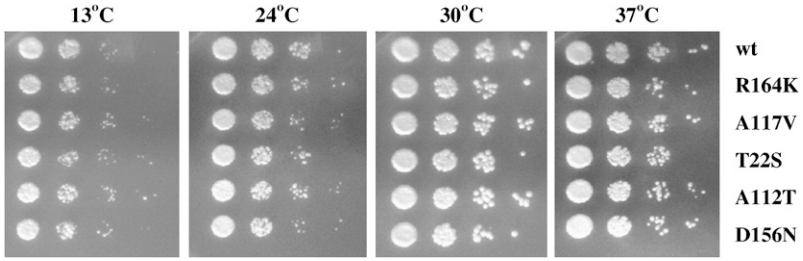

FIGURE 8.—A wild-type strain (MC213 p*TEF1 TRP1*) and strains TKY961–965 obtained by transforming the *tef5::TRP1* fragment into TKY789, 847–849, and 852 and selected by growth on C-Trp were grown in YEPD and diluted to an A_{600} of 1.0. Ten-fold serial dilutions were spotted onto YEPD plates and grown at 13°, 24°, 30°, and 37° for 2–8 days.

TABLE 5

Total translation and nonsense and missense effects of the *SBD* mutant strains in the presence and the absence of eEF1Bα

	Total translation relative to wild type (% change)	Nonsense suppression (UAA) (R_{test}/R_{con})[a]	Missense suppression (R_{test}/R_{con})[a]
Wild type	100	0.029 ± 0.01	0.01 ± 0.004
R164K + eEF1Bα	100	ND	ND
R164K − eEF1Bα	125	0.034 ± 0.01	0.012 ± 0.004
A117V + eEF1Bα	100	ND	ND
A117V − eEF1Bα	75	0.028 ± 0.01	0.008 ± 0.003
T22S + eEF1Bα	65	ND	ND
T22S − eEF1Bα	65	0.073 ± 0.02	0.01 ± 0.004
A112T + eEF1Bα	75	ND	ND
A112T − eEF1Bα	75	0.079 ± 0.02	0.012 ± 0.003
D156N + eEF1Bα	125	ND	ND
D156N − eEF1Bα	100	ND	ND

ND, not determined.

[a] R_{test}/R_{con}: the activity ratio derived from lysates expressing test cassettes divided by the activity ratio of the control reporter. Standard deviations were calculated using a minimum of four samples.

and hygromycin B, some *SBD* mutants only partially suppress the severe paromomycin-sensitive phenotype of the eEF1Bα-deficient strain. These results indicate that *SBD* mutations cannot completely recover the paromomycin sensitivity phenotype, suggesting reduced A-site fidelity. Strains expressing only the *SBD* form of eEF1A with eEF1Bα show widely varying sensitivity or resistance to paromomycin compared to the isogenic wild-type strain. In the absence of eEF1Bα, however, all the eEF1A mutant strains show higher sensitivity to paromomycin than a wild-type strain with eEF1Bα. A112T and T22S mutants exhibit translational readthrough of a UAA stop codon, correlating with their higher paromomycin sensitivity and reduced translation rates. The fact that not all phenotypes are suppressed underscores the additional translational roles of eEF1Bα other than nucleotide exchange, especially translational fidelity and, potentially, the proposed channeling of aa-tRNA to eEF1A (Andersen *et al.* 2000).

Examination of the crystal structure of the eEF1A:eEF1Bα complex revealed a series of important hints for understanding the suppression of the GEF requirement as well as the mechanism of guanine nucleotide exchange. The established role of eEF1Bα is to accelerate the rate of GDP release following GTP binding to eEF1A. Structurally, eEF1Bα is suggested to displace the Mg^{+2} ion associated with the nucleotide, stimulating GDP release (Andersen *et al.* 2001; Pittman *et al.* 2006). The *SBD* forms of eEF1A are located in the nucleotide-binding domain. Analysis of the *SBD* mutations mapped onto the structure of eEF1A indicate that they are near the P-loop or the NKXD motif. The P-loop is essential for phosphate binding of the nucleotide while the NKXD motif binds to the base of the nucleotide. Together with the functional data, two classes of mutants were identified: class I mutants A112T and T22S and class II mutants R164K and A117V.

For the class I mutants, T22 of the P-loop directly interacts with the α-phosphate of GDP via an amide group while A112 is located on the β-sheet between a β-sheet that connects to the P-loop and a second that connects to the NKXD element. Both A112 and T22 are conserved in *E. coli* and *Thermus aquaticus* EF-Tu and human eEF1A, pointing out their importance in the G-domain. In the absence of its GEF, the spontaneous GDP dissociation of eEF1A is 85 times more rapid than that of EF-Tu (Gromadski *et al.* 2002; Pittman *et al.* 2006); this higher GDP dissociation rate of eukaryotic eEF1A may be what allows *SBD* mutants to be functional as the only form of eEF1A. Class I mutant strains show a 2.5- to 2.7-fold increase in nonsense suppression while class II mutants do not affect suppression. The P-loop is essential for the molecular switch between active and inactive forms of the proteins and the disturbance of the P-loop is suggested to be the major reason for the decreased affinity of GDP (Vetter and Wittenghofer 2001). Class I mutations also showed a 25–35% decrease in total translation rates with or without eEF1Bα. The presence of eEF1Bα thus does not alter translation efficiency.

A117 of class II is in the upper tip of the same β-sheet containing A112. The observation that A117V shows a doublet on SDS–PAGE gel may suggest a conformational change. This is consistent with the effect of the known nucleotide-dependent changes in conformations of G-proteins (Vetter and Wittenghofer 2001; Spoerner *et al.* 2005). A117 is conserved in *E. coli* and *T. aquaticus* EF-Tu and in human eEF1A, whereas R164 is conserved only in yeast and in human eEF1A. In prokaryotic systems, this residue is a leucine, which may

indicate the importance of a charged residue in this position for the eukaryotic systems.

eEFSec is the Sec-specific counterpart of eEF1A that incorporates selenocysteine into the protein. eEFSec is predicted to function independently of a guanine nucleotide exchange factor (Copeland 2003). While the residues of the both class I and class II mutations are identical in prokaryotic elongation factor SelB, none of the residues are conserved in eEFSec. This may indicate the importance of these residues such that eEFSec does not require exchange activity.

Double mutants that altered amino acids near both the P-loop and the guanine base were not viable as the only form of eEF1A and did not function as efficient suppressors of the requirement for eEF1Bα. This suggests that the effects on nucleotide release or binding surpass the threshold needed for cell survival. This finding also indicates that the suppression of the exchange factor is the common result of all *SBD*s but likely is achieved through separate alterations of the consensus elements.

This study presents the eEF1Bα function as dispensable for the cell, likely as long as nucleotide exchange or GDP release rates can be maintained above a certain threshold. Some of the G-proteins, including translation factors eRF3 and eEF2, do not depend on separate nucleotide exchange factors. However, some studies suggest that the ribosome acts as a GEF for prokaryotic RF3 and EF-G (Zavialov *et al.* 2005). Further determination of the GDP release and nucleotide exchange rates will also identify the step of the suppression during the G-protein cycle and provide more information on this novel exchange mechanism and its relationship to other G-proteins that lack a GEF.

Although eEF1B has two subunits in *S. cerevisiae*, only eEF1Bα has guanine nucleotide exchange activity. Furthermore, comparison of the crystal structure of the eEF1A:eEF1Bα complex to the prokaryotic EF-Tu-GDPNP-aa-tRNA structure has led to the suggestion that eEF1Bα may help to channel aa-tRNA to eEF1A (Andersen *et al.* 2000). The eEF1Bαγ complex is also proposed to have other regulatory functions in yeast, as both subunits are suggested to play a role in the oxidative stress response pathway (Olarewaju *et al.* 2004). The resulting phenotypes of the *SBD* strains may also be caused by the impairment of the additional functions of the eEF1Bα. These results suggest that GEFs for the elongation factors have gained more complexity and perhaps more functions in the cell throughout evolution. Further studies will determine the additional functions of these proteins.

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