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Dominant-negative mutant phenotypes and the regulation of translation elongation factor 2 levels in yeast

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ABSTRACT

The eukaryotic translation elongation factor 2 (eEF2), a member of the G-protein superfamily, catalyzes the post-peptidyl transferase translocation of deacylated tRNA and peptidyl tRNA to the ribosomal E- and P-sites. eEF2 is modified by a unique post-translational modification: the conversion of His699 to diphthamide at the tip of domain IV, the region proposed to mimic the anticodon of tRNA. Structural models indicate a hinge is important for conformational changes in eEF2. Mutations of V488 in the hinge region and H699 in the tip of domain IV produce non-functional mutants that when co-expressed with the wild-type eEF2 result in a dominant-negative growth phenotype in the yeast *Saccharomyces cerevisiae*. This phenotype is linked to reduced levels of the wild-type protein, as total eEF2 levels are unchanged. Changes in the promoter, 5′-untranslated region (5′-UTR) or 3′-UTR of the *EFT2* gene encoding eEF2 do not allow overexpression of the protein, showing that eEF2 levels are tightly regulated. The H699K mutant, however, also alters translation phenotypes. The observed regulation suggests that the cell needs an optimum amount of active eEF2 to grow properly. This provides information about a new mechanism by which translation is efficiently maintained.

INTRODUCTION

Translation is divided into three steps: initiation, elongation and termination. Initiation is completed when the initiator Met-tRNA$_i^{Met}$ and the 80S ribosome are positioned at the start codon of an open reading frame (ORF) [reviewed in (1)]. During the elongation step, all the subsequent amino acids are added until a stop codon is reached [reviewed in (2)]. Termination then occurs and the newly formed protein is released [reviewed (3)]. Translation is highly regulated at the level of *cis*-acting mRNA elements, translation factors and the ribosome [reviewed in (4)]. Regulation via the mRNA is controlled by elements in the 5′- and 3′-untranslated regions (5′- and 3′-UTR's), such as upstream ORFs or the poly(A) tail, respectively [reviewed in (5)]. Translation factors are modified by post-translational events such as phosphorylation, and some ribosomal proteins are regulated by feedback inhibition, phosphorylation or ubiquitination [reviewed in (6)].

The eukaryotic translation Elongation Factor 2 (eEF2) is a 93 kDa member of the G-protein superfamily. Following peptide bond formation, eEF2 catalyzes translocation of the deacylated tRNA in the P-site and peptidyl tRNA in the A-site into the E- and P- sites, respectively. Thus, the mRNA advances by three bases to ensure another cycle of elongation. In *Saccharomyces cerevisiae,* eEF2 is encoded by two genes, *EFT1* and *EFT2*. The encoded proteins are identical and one must be present for viability (7). Yeast eEF2 contains six structural domains arranged in two blocks, the N-terminal and C-terminal regions. The N-terminal region consists of domains I (or G), G′ and II while the C-terminal region has domains III, IV and V. Elucidation of the crystal structure of eEF2 showed a reorientation between the N-terminus and the C-terminus. This reorientation is promoted by a hinge region, residues 481–489, which undergoes a drastic conformational change when the apo eEF2 structure is compared with the sordarin bound eEF2 structure (8).

eEF2 is subjected to two post-translational modifications. *S.cerevisiae* eEF2 is phosphorylated on Thr57 by an endogenous kinase encoded by the *RCK2* gene (9). Rck2p is a Ser/Thr protein kinase homologous to the mammalian calmodulin kinases, which require phosphorylation for activation (10,11). eEF2 is phosphorylated in response to osmotic stress, resulting in lower protein synthesis rates (9). Phosphorylation reduces the activity of the protein by reducing the affinity for GTP, but not GDP, and decreasing ribosome binding (12).

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Additionally, a histidine in domain IV of eEF2 (H699 in yeast, and H715 in mammals) is converted to diphthamide. Diphthamide is the target for ADP ribosylation by Diphtheria toxin and *Pseudomonas aeruginosa* exotoxin A, both of which inactivate the protein (13). ADP ribosylation of eEF2 does not affect ribosome or nucleotide binding, suggesting inhibition after eEF2 has bound the ribosome (14). Yeast strains bearing mutations in H699 of eEF2 can affect cell growth and ADP ribosylation (15,16).

In the present work, we identified two dominant-negative mutants of *S.cerevisiae* eEF2. Mutagenesis of residues located in the hinge and tip of domain IV of eEF2, V488A and H699K, respectively, produced non-functional proteins. This lack of viability was not the result of the inability to express the mutants. The total eEF2 protein levels, however, were not detectably increased with the extra copy of the eEF2 mutant. Additionally, wild-type eEF2 overexpression plasmids did not increase total eEF2 levels, inferring that these levels are carefully maintained. The dominant-negative phenotype of the non-functional mutants is predominantly owing to reduced levels of wild-type functional eEF2. This regulation of eEF2 levels is not at the level of transcription as seen by promoter substitutions, via the *EFT2* 5′- or 3′-UTR, or the proteasome. The eEF2$_{H699K}$ mutant, however, also causes dominant effects on total protein synthesis, paromomycin sensitivity and translation elongation. The eEF2$_{V488A}$ mutant does not confer any dominant translation effects, indicating the translation phenotypes are specific to an alteration in the anticodon mimic loop and not general consequences of reduced wild-type eEF2 levels. These results suggest that an optimum amount of eEF2 is necessary for cells to grow and synthesize proteins efficiently. The effect of the mutants supports structural models of eEF2 function and indicates a potential function for H699, and the anticodon mimicry loop, in translation. The regulation of eEF2 levels may further represent a novel mechanism by which translation is controlled.

MATERIALS AND METHODS

Strains and media

S.cerevisiae strains used in this study are listed in Table 1. *Escherichia coli* DH5α cells were used for plasmid preparation. Standard yeast genetic methods were employed (17). Yeast cells were grown in either YEPD (1% Bacto yeast extract, 2% peptone and 2% dextrose) or defined synthetic complete media (C or C$^-$) supplemented with 2% dextrose, 2% raffinose or 2% galactose as the carbon source (18). Yeast strains were transformed by the lithium acetate method (19).

Table 1. *S.cerevisiae* strains

Strain	Genotype	Reference
YEFD12 h	*MATa ade2 leu2 ura3 his3 trp1 eft1::HIS3 eft2::TRP1 pEFT1 URA3 CEN*	(15)
TKY675	*MATa ade2 leu2 ura3 his3 trp1 eft1::HIS3 eft2::TRP1 pEFT2-6×His LEU2 CEN*	(21)
TKY751	*MATa ade2 leu2 ura3 his3 trp1 eft1::HIS3 eft2::TRP1 pEFT2-HA LEU2 CEN*	This work
TKY918	*MATa ade2 leu2 ura3 his3 trp1 eft1::HIS3 eft2::TRP1 pEFT2-6×His URA3 2μ*	This work
YPG499	*MATa ura3-52 leu2-Δ0 his3-Δ200 trp1-Δ63 lys2-801 ade2-101*	(29)
CMM806	*MATa ura3-52 leu2-Δ0 his3-Δ200 trp1-Δ63 lys2-801 ade2-101 cim5-1*	(29)
KMY851	*MATα ura3-Δ5 his2-1 leu2-2 pre1-1 pre2-2*	K. Madura

DNA manipulations and mutagenesis

Recombinant DNA techniques were performed as described (20). Restriction endonucleases and DNA modifying enzymes were obtained from Roche or Gibco BRL. Mutations in *EFT2* were created utilizing the PCR based QuikChange Site-Directed Mutagenesis Kit (Stratagene). pTKB501 (eEF2^{HA}, provided by Dr M. Justice, Merck Research Laboratories) and pTKB612 [eEF2^{HIS}, (21)] were utilized as the templates for PCR. pTKB658 (eEF2$^{HIS}_{V488A}$) and pTKB696 (eEF2$^{HA}_{V488A}$) were created using primers 5′V488A (5′-CTCTGTCTCTCCAGCTGTGCAAGTCGCTGTCG-3′) and 3′V488A (5′- CGACACCGACTTGCACAGCAGGAGAGACAGAG-3′). pTKB701 (eEF2$^{HA}_{H699K}$) and pTKB704 (eEF2$^{HIS}_{H699K}$) were created using primers 5′H699K (5′-CATGCCGATGCTATCAAGAGAGGTGGTGGTCAAATC-3′) and 3′H699K (5′-GATTTGACCACCACCTCCCTTGATAGCATCGGCATG-3′). All mutations were confirmed by restriction digestion and DNA sequence analysis.

Cell growth

Growth of strains containing either eEF2 under the *GAL1* promoter or the empty vector control was performed by growing cells to an A$_{600}$ of 1.0 in C-Leu liquid media. Serial 10-fold dilutions (10 μl each) were spotted on C-Leu and C-Leu plus galactose media followed by incubation at 13, 24, 30 and 37°C for 3–7 days. For galactose induction experiments cells were grown to an A$_{600}$ of 0.5 in C-Leu plus raffinose, washed with water, and transfered to C-Leu plus galactose. Samples were taken for protein extraction, RNA extraction and flow cytometry prior to washing as well as following induction with galactose for 0.5, 1, 2, 3, 5, 7.5, 9, 12 and 24 h. Doubling times were determined by growing strains in C-Leu at 30°C starting with an A$_{600}$ of 0.1 and monitoring the A$_{600}$ for 12 h.

Sensitivity to translation inhibitors and protein synthesis rates

Halo assays for sensitivity to cycloheximide, paromomycin and hygromycin B were performed as described previously (22). Microtiter assays in liquid culture were performed for at least three independent colonies of each strain grown at 30°C in C-Leu to mid-log phase, diluted to A$_{600}$ of 0.1 and grown at 30°C in triplicate in 96-well microtiter plates with varying concentrations of paromomycin. Growth was monitored on a Bio-Tek Elx 800 microtiter reader, and reported as the mean of the triplicate A$_{600}$ at 24 h. For *in vivo* [^{35}S]methionine incorporation assays yeast strains were grown in liquid cultures (100 ml) in C-Met-Leu at 30°C to mid-log phase and assayed as described (22). All time points were analyzed in triplicate.

Western blot analysis

Cells were harvested by centrifugation, suspended in lysis buffer [50 mM Tris–HCl, pH 7.5, 50 mM NaCl, 1 mM DTT, 0.2 mM phenylmethylsulphonyl fluoride and 10%

glycerol] and lysed with glass beads (18). Total protein was determined by Bradford protein analysis (BioRad, Hercules, CA) and 1 μg was separated by SDS–PAGE and transferred to nitrocellulose membranes. Membranes were probed with polyclonal antibodies to yeast eEF2 (1:20 000 dilution), Pgk1p (1:10 000 dilution) or a monoclonal antibody to the hemaglutinin (HA) tag (1:250 dilution) and detected by a secondary antibody conjugated to peroxidase (1:7500 dilution; Amersham ECL plus).

Polyribosome analysis

Yeast polyribosome analysis was performed as described previously (23) with the following specifications. Yeast cultures were grown in C-Leu at 30°C to mid-log phase, divided, and extracted with and without cycloheximide added to the cells and lysis buffer [10 mM Tris–HCl, pH 7.5, 100 mM NaCl, 30 mM $MgCl_2$, 200 μg/ml heparin and 0.2% diethyl pyrocarbonate]. Cell extracts (30 A_{260}) were layered on 35 ml 7–47% sucrose gradients and centrifuged for 4 h at 27 000 r.p.m. in a Beckman SW28 rotor. The A_{254} was monitored using a Model 185 density gradient fractionator (ISCO, Inc., Lincoln, NE).

Northern blot analysis

Cells were harvested by centrifugation and total RNA was isolated as described (24). RNA was separated on a 1% formaldehyde agarose gel, transferred to Hybond plus membranes overnight by capillary action and crosslinked to the membrane with a Spectrolinker XL-1000 (Spectronics). Hybridization and detection were performed as per the ExpressHyb protocol (Clonetech). ^{32}P-labeled probes for yeast *EFT2* and *ACT1* were prepared with the Rad Prime Labeling System kit (Invitrogen). Northern blots were detected using a Phosphorimager Typhoon 9410 (Molecular Dynamics) and quantified with the ImageQuant program (Molecular Dynamics).

RESULTS

eEF2$_{V488A}$ and eEF2$_{H699K}$ mutations confer dominant-negative phenotypes

Based on the X-ray crystal structure of eEF2, the functions of the domain III hinge region and the tip of domain IV were addressed with mutations targeting residues V488 and H699 (Figure 1). The hinge region (amino acids 481–489) is 93% identical in eukaryotes and undergoes a conformational change between the crystal structures of apoeEF2 in comparison with eEF2 bound to the anti-fungal drug sordarin (8). The hinge region is hypothesized to be essential for the conformational change of eEF2. H699 is located at the tip of domain IV and is post-translationally modified to diphthamide. Diphthamide is the site of ADP ribosylation by Diphtheria toxin and *Pseudomonas* exotoxin A (13). This tip has been shown to be in close proximity to the decoding site of the ribosome by cryoelectron microscopy, suggesting it is needed for preventing frameshifting of the mRNA (25). To differentiate between the wild-type and mutant eEF2 proteins, the mutants were expressed with either a HA or 6× His tag. The HA tag does not interfere with function as eEF2^{HA} can complement the loss of wild-type eEF2 (Figure 2A) and had identical sensitivity to

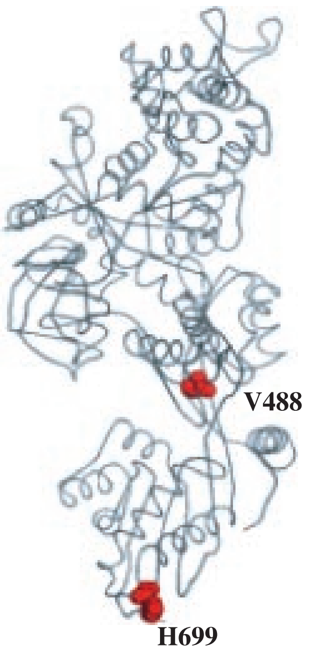

Figure 1. Structure of yeast eEF2 indicates the location of V488 in the polylinker region and H699 at the tip of domain IV. The structure was produced with the PyMOL program (37), using PDB 1NOV coordinates (8).

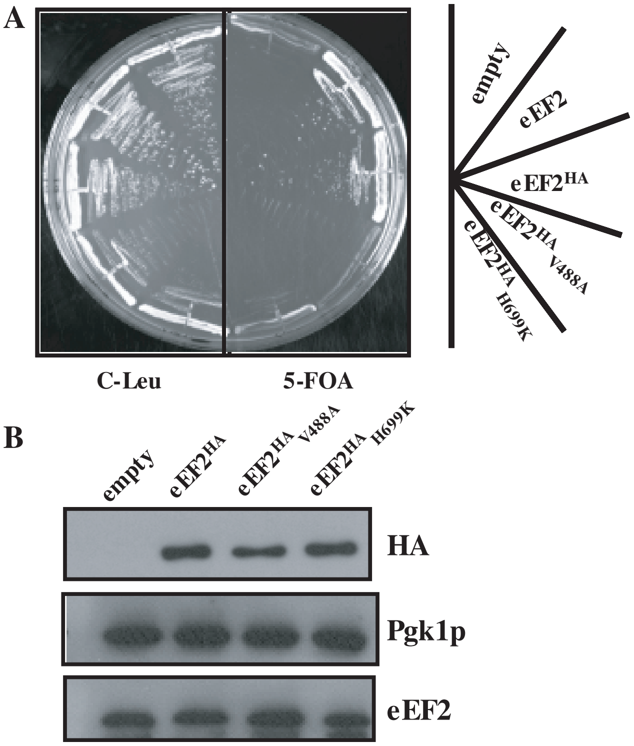

Figure 2. eEF2$_{V499A}$ and eEF2$_{H699K}$ are unable to function as the only form of eEF2 *in vivo* and confer a dominant-negative phenotype. (**A**) YEFD12h transformed with pRS315 (empty), pTKB615 (eEF2), pTKB501 (eEF2^{HA}), pTKB696 (eEF2$^{HA}_{V488A}$) and pTKB701 (eEF2$^{HA}_{H699K}$) were streaked on C-Leu or 5-FOA and incubated from 3 to 7 days at 30°C. (**B**) Western blot analysis against the HA tag shows that the mutant proteins are expressed. Lanes are empty vector, eEF2^{HA}, eEF2$^{HA}_{V488A}$ and eEF2$^{HA}_{H699K}$. Protein extracts were prepared, resolved by SDS–PAGE, and detected with antibodies against HA, eEF2 and Pgk1p (loading control).

translational inhibitors (Table 3). The 6× His tagged eEF2 was shown previously to be functional as the only form of eEF2 (21). Alanine was selected for mutagenesis of V488 to minimize the side chain while H699K was demonstrated in previous work to confer a dominant growth phenotype (16). Plasmids expressing HA tagged $eEF2^{HA}_{V488A}$ or $eEF2^{HA}_{H699K}$ were transformed into yeast strain YEFD12h. The mutations result in non-functional eEF2 proteins, as shown by their inability to grow on media containing 5-FOA (Figure 2A). The lack of function is not owing to the lack of expression of the mutants as monitored by western blot analysis with an HA antibody (Figure 2B). The 6× His tagged wild-type or mutant eEF2 proteins could not be recognized by an anti 6× His monoclonal antibody, but expression was confirmed by Ni-NTA pulldown and Coomassie staining of the SDS–PAGE gel (data not shown). Surprisingly, the total eEF2 protein levels were essentially identical when the empty vector or an additional eEF2 expressing plasmid were present, indicating the inability to overexpress the protein (Figure 2B). To monitor the amount of co-expressed HA tagged mutant eEF2, eEF2 and HA antibody recognition was standardized via western blot analysis. Subsequent quantitative analysis of the recognition of total eEF2 (eEF2 antibody) and HA-tagged eEF2 (HA antibody) demonstrated that $eEF2^{HA}$ constituted ~60% of total eEF2 while $eEF2^{HA}_{V488A}$ constituted 44%. The co-expression of the mutant forms with wild-type eEF2 resulted in a slow-growth phenotype on C-Leu (Figure 2A), indicating a dominant-negative phenotype. In a systematic mutagenesis study of H699 the dominant phenotype of $eEF2_{H699K}$ was noted, however, the mechanism of the effect is undetermined (16). The growth effect was not the result of the HA tag on the mutant, as growth defects were also observed with an additional copy of either untagged and 6× His tagged $eEF2_{V488A}$ or $eEF2_{H699K}$ (data not shown). The phenotype observed on solid media is also evident in liquid media, where the doubling times of strains expressing a mutant form of eEF2 increased up to 2-fold (Table 2).

The dominant-negative phenotype of eEF2 mutants is due to similar effects on eEF2 levels except differential effects on translation functions

The dominant-negative phenotype observed for the two eEF2 mutants could be due to either reduced levels of functional (wild-type) protein or obstruction of eEF2's function in translation. To address these possibilities we examined the effect of $eEF2_{V488A}$ and $eEF2_{H699K}$ expression on translation activity. Strains expressing wild-type eEF2, an empty vector, or an extra plasmid with wild-type or a mutant form of eEF2 were examined for their effect on total protein synthesis by [^{35}S]methionine incorporation. No significant effect on total translation was observed with co-expression of wild-type eEF2 or $eEF2_{V488A}$ (Figure 3A). Co-expression of $eEF2_{H699K}$, however, showed a consistent 20% decrease in total protein synthesis >60 min of growth (Figure 3A). Effects on protein synthesis were also monitored by sensitivity to the translation inhibitors cycloheximide, paromomycin and hygromycin B. Co-expression of wild-type or mutant forms of eEF2 caused no changes in cycloheximide or hygromycin B resistance, while paromomycin sensitivity was uniquely increased for cells co-expressing $eEF2_{H699K}$ (Table 3). The altered drug sensitivity was quantified in a liquid microtiter growth assay in the presence of increasing concentrations of these compounds. Similar to the halo assay, sensitivity to cycloheximide and hygromycin B were not altered (data not shown). Paromomycin sensitivity was increased ~5-fold only with co-expression of $eEF2_{H699K}$ (Figure 3B).

Polyribosome profile analysis was used to determine whether the elongation step of protein synthesis was affected. Standard polyribosome profiles of extracts prepared in the presence of cycloheximide showed no significant difference in the levels of free 40S or 60S ribosome subunits, 80S monoribosomes or translating ribosomes in the polyribosome region between strains co-expressing wild-type or either eEF2 mutant (Figure 3C, top panel). When cycloheximide is excluded from the preparation of the extracts, ribosomes continue elongating on the mRNA, complete translation, and move to lower order polyribosomes or the 80S peak (26). A strain expressing $eEF2_{H699K}$ shows a higher amount of polyribosomes in the absence of cycloheximide than strains co-expressing wild-type or $eEF2_{V488A}$ (Figure 3C, lower panel). Thus, $eEF2_{H699K}$ uniquely shows a reduction in most probably elongation, although other post-initiation events such as termination or ribosome recycling cannot be ruled out with this assay. The total protein synthesis, drug sensitivity and polyribosome analysis results suggest that the effects conferred by $eEF2_{H699K}$ are consistent with an inhibition of translation elongation. Thus, the different results obtained with the mutants show that the more severe dominant phenotype of $eEF2_{H699K}$ is probably caused by dominant effects on translation and reduced active eEF2, while $eEF2_{V488A}$ shows a less severe dominant growth effect owing to only reduced active eEF2 levels.

Table 2. Doubling times of YEFD12h with a plasmid expressing the indicated form of eEF2

YEFD12h +	Doubling time (min)
Empty vector	99 ± 6
eEF2	95 ± 5
$eEF2^{HA}$	124 ± 7
$eEF2^{HA}_{V488A}$	137 ± 4
$eEF2^{HA}_{H699K}$	210 ± 2

Doubling times were determined by growing strains in C-Leu at 30°C starting with an A_{600} of 0.1 and monitoring the A_{600} over 12 h. All the eEF2 plasmids have the authentic *EFT2* promoter. The results are the average of three experiments.

The promoter, 5′-UTR or 3′-UTR do not confer the inability to increase eEF2 levels

In order to determine the role of the promoter and UTR elements on the ability to increase eEF2 levels, a series of altered *EFT2* expression constructs were prepared. Both *CEN* (low copy) and 2μ (high copy) plasmids expressing eEF2 from *EFT2* were used (Figure 4A). The constructs expressed eEF2 under the *EFT2* or *TEF5* constitutive promoters, and were epitope-tagged to assure that the plasmid-borne alleles were expressed. The 3′-UTR's from *EFT2* were substituted by HA or 6× His tags. The untagged *EFT2* plasmid with the authentic 5′- and 3′-UTR's, and the *EFT2* constructs with the HA or 6× His tag replacing the 3′-UTR all produced equal amounts of protein when present as the only form of eEF2 (data not shown). Plasmids were transformed into yeast

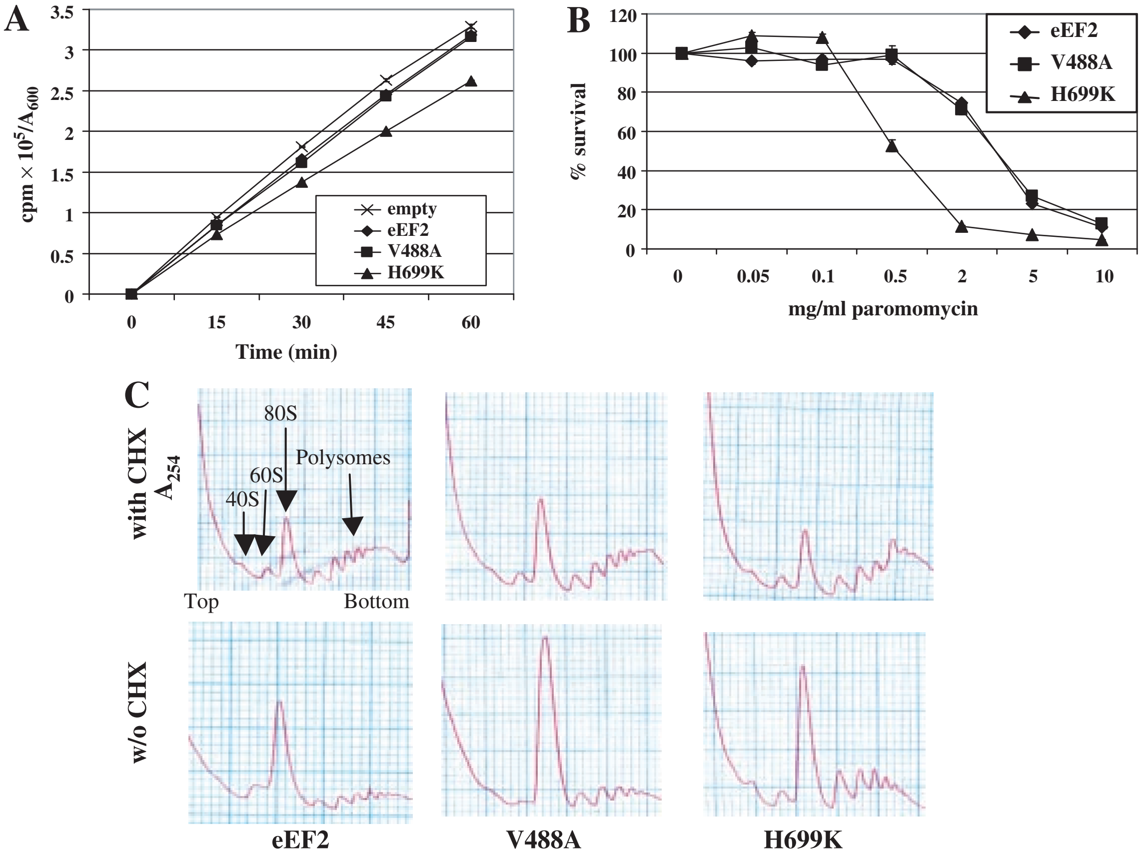

Figure 3. Strains expressing eEF2$_{H699K}$ affect translation at the elongation step. (**A**) Strain YEFD12h expressing no additional copies of eEF2 (pRS315, crosses), eEF2 (pTKB612, diamonds), eEF2$_{V488A}$ (pTKB658, squares) or eEF2$_{H699K}$ (pTKB704, triangles) were grown in C-Met-Leu to mid-log phase. [^{35}S]methionine was added and total protein synthesis was measured at each time point by TCA precipitation. (**B**) eEF2$_{H699K}$ confers dominant sensitivity to paromomycin. YEFD12h transformed with eEF2 (pTKB501, diamonds), eEF2$_{V488A}$ (pTKB696, squares) or eEF2$_{H699K}$ (pTKB701, triangles) were grown to mid-log phase in C-Leu medium, diluted to $A_{600} = 0.1$ in C-Leu in the presence of various concentrations of paromomycin (in triplicate) in 96-well microtiter plates, and grown at 30°C with constant shaking. Growth was monitored by A_{600} on a Bio-Tek Elx microtiter plate reader and expressed as percent survival relative to untreated cells. (**C**) Polyribosome analysis shows eEF2$_{H699K}$ confers a defect in translation elongation. Cells were grown at 30°C to mid-log phase, harvested in the presence (with CHX) or absence (w/o CHX) of cycloheximide and extracts were prepared and layered on a 7–47% sucrose gradient. Gradients were centrifuged, fractionated and the A_{254} monitored.

Table 3. Drug sensitivities of YEFD12h with a plasmid expressing the indicated form of eEF2

YEFD12h +	1 mM Cycloheximide	800 mg/ml Paromomycin	100 mM Hygromycin B
Empty vector	39 ± 1 mm[a]	12 ± 2	9 ± 0
eEF2	40 ± 1	12 ± 1	10 ± 1
eEF2^{HA}	41 ± 1	12 ± 1	10 ± 1
eEF2$^{HA}_{V488A}$	41 ± 2	12 ± 1	10 ± 1
eEF2$^{HA}_{H699K}$	43 ± 1	21 ± 1	10 ± 1

[a] Sensitivity was determined by measuring the diameter of inhibition of growth (in mm) around a filter disk containing 10 µl of the indicated drug. Cells were plated on C-Leu and grown at 30°C. All the eEF2 plasmids have the authentic *EFT2* promoter. The results shown are the average of three experiments.

lacking both chromosomal genes encoding eEF2 but with a *CEN EFT2* plasmid to allow survival. Cells were maintained in selective media, and the total amount of eEF2 protein was analyzed by western blot analysis (Figure 4B). eEF2 protein levels were unchanged in the presence of a plasmid with the *EFT2* gene including the authentic promoter and the 5′- and 3′-UTR's, or with a low or high copy number plasmid with eEF2 expressed from the *TEF5* promoter (Figure 4B). Additionally, the eEF2^{HA} *CEN* constructs lacking the *EFT2* 3′-UTR but with the *EFT2* promoter and 5′-UTR resulted in unchanged levels of eEF2. The inability to overexpress the eEF2 protein is not owing to a defect in the expression of the plasmid-borne eEF2 gene, as western analysis with the HA antibody confirms that the eEF2^{HA} is expressed (data not shown). eEF2^{HIS} expression was confirmed by Ni-NTA pulldown and Coomassie staining of the SDS–PAGE gel (data not shown). These results show that eEF2 levels remain unchanged despite altering the promoter or 5′- or 3′-UTR's.

Overexpression was also attempted by expressing eEF2^{HIS} under the *GAL1* galactose inducible promoter in the absence of the *EFT2* 5′- and 3′-UTR's (Figure 5A). This plasmid complemented for the loss of wild-type eEF2 plasmid in media containing galactose (data not shown). Growth of the transformed strain on C-Leu media with glucose as the carbon source was essentially wild-type. A slight slow growth phenotype was observed when the same cells were shifted to C-Leu plus galactose (Figure 5B). This result suggests that excess eEF2 was transiently expressed and has a negative impact on cell growth seen only early in the induction stage and not when steady state is reached. To confirm that eEF2 was transiently overexpressed from this plasmid, western blot analysis was

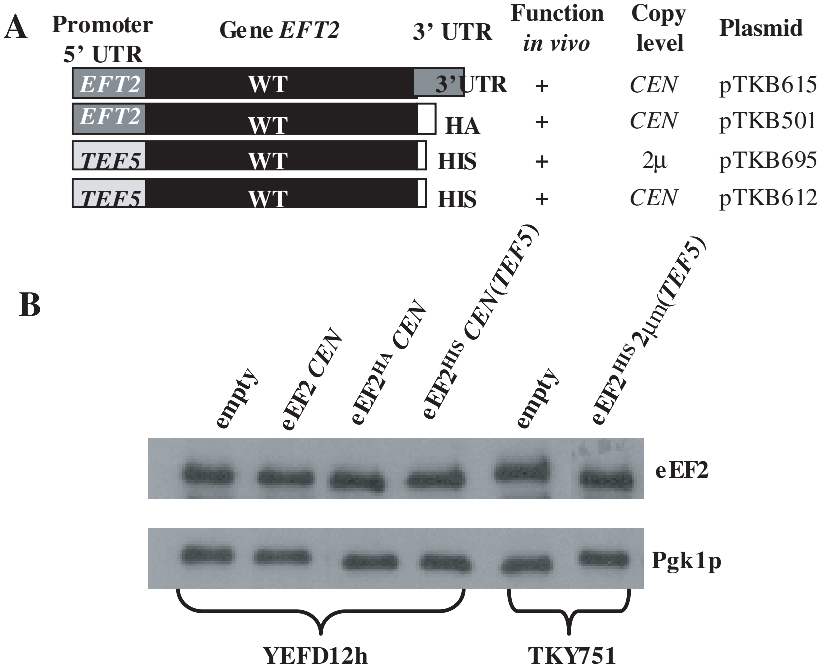

Figure 4. eEF2 protein levels are not controlled by the *EFT2* promoter, 5′- or 3′-UTR. (**A**) Cartoon of eEF2 constructs on low (*CEN*) and high (2μ) copy number plasmids with *EFT2* or *TEF5* promoters and 5′-UTR or the *EFT2* 3′-UTR is replaced with a 6× His or HA tag. (**B**) Plasmids expressing empty vector, eEF2 *CEN* (*EFT2* promoter), eEF2^{HA} *CEN* (*EFT2* promoter) and eEF2^{HIS} *CEN* (*TEF5* promoter) were transformed into YEFD12h, and eEF2^{HIS} 2μ (*TEF5* promoter) was transformed into TKY751. Protein extracts were prepared, resolved by SDS–PAGE, and detected with antibodies against eEF2 and Pgk1p (loading control). eEF2 expression is expressed relative to Pgk1p levels.

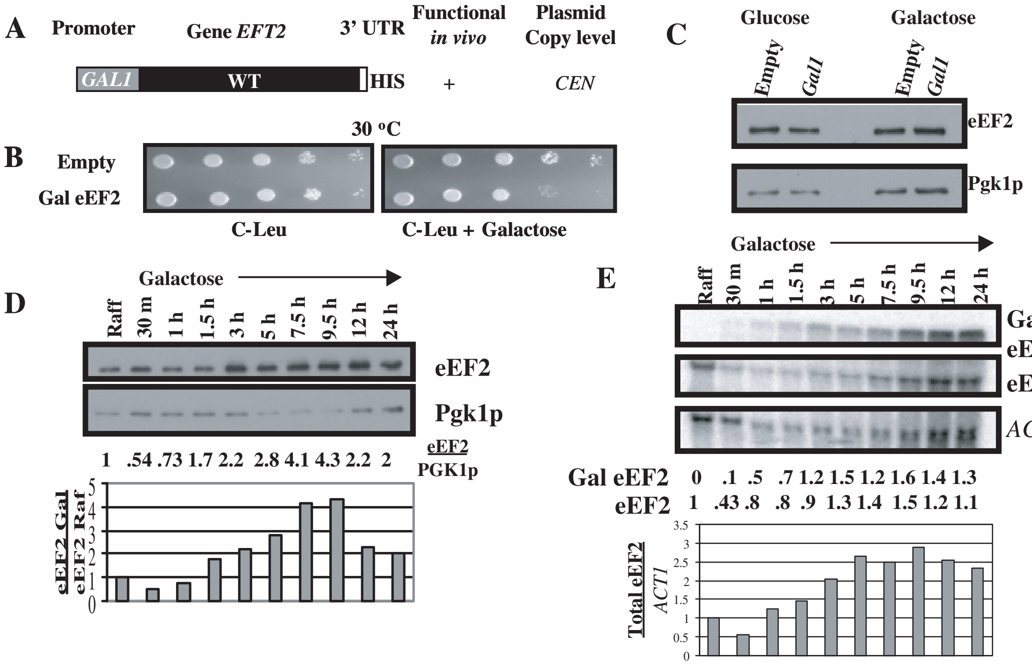

Figure 5. Transient overexpression of eEF2 demonstrates protein levels are reduced before mRNA levels. (**A**) Construct in which eEF2 is expressed under the *GAL1* galactose inducible promoter. (**B**) YEFD12h was transformed with pRS315 (empty) and pTKB763 (*GAL1* eEF2^{HIS}). Cells were grown to mid-log phase at 30°C in C-Leu liquid media then spotted as 10-fold serial dilutions on C-Leu and C-Leu plus galactose. (**C**) Protein extracts were prepared from the strains as in (B), grown to steady state in the absence and presence of galactose. Total proteins were resolved by SDS–PAGE and detected with antibodies against eEF2 and Pgk1p. The fold expression is calculated relative to Pgk1p levels. (**D**) YEFD12h with pTKB763 (*GAL1* eEF2^{HIS}) was grown to mid-log phase in C-Leu plus raffinose media and a sample taken. The culture was harvested, washed with water and resuspended in C-Leu plus galactose. Subsequently samples were taken for each time point, protein extracts prepared, resolved by SDS–PAGE and detected with antibodies against eEF2 and Pgk1p. The numbers indicate eEF2 expression relative to Pgk1p levels. The bar graph shows eEF2 expression levels at each galactose time point relative to expression in raffinose media. (**E**) RNA extracts were prepared from the same time points as (D), resolved by a formaldehyde agarose gel and detected with probes against *EFT2* and *ACT1* (loading control). The numbers indicate mRNA expression from each eEF2-expressing plasmid relative to *ACT1*. The bar graph shows total *EFT2* mRNA expression relative to *ACT1* mRNA.

performed on cells grown in liquid C-Leu plus galactose for 2 days to reach steady state. In this case no growth defect was observed and eEF2 levels were unchanged compared with the empty vector control (Figure 5C). Expression was subsequently induced from the *GAL1* promoter and eEF2 protein levels were determined over time. Cells where the extra copy of eEF2 is expressed from the *GAL1* promoter were grown on C-Leu plus raffinose to repress expression, transferred to C-Leu plus galactose to induce expression, and eEF2 protein levels were monitored from 0 to 24 h by western blot analysis (Figure 5D). The eEF2 protein levels transiently increased 4-fold at 9.5 h, but subsequently reduce by half at the longest time point.

To examine whether the effect observed at the protein level was also observed at the mRNA level, total mRNA was prepared from matched samples and the eEF2 mRNA levels monitored (Figure 5E). eEF2 mRNA produced from the authentic eEF2 promoter versus the *GAL1* promoter was differentiated by differences in 3′-UTR lengths. The *GAL1* expression plasmid utilizes a cryptic transcription terminator and thus creates a specific but larger mRNA. The results from the western blot analysis (Figure 5D) show this mRNA is translated, although the longer mRNA could potentially alter other events such as stability. The *GAL1* induced eEF2 mRNA levels rose, peaked at 9.5 h and remained elevated for a longer period than the protein levels. The initial appearance of the *GAL1*-dependent eEF2 mRNA correlated with a slight reduction in the *EFT2* and *ACT1* mRNA levels owing to the carbon source change, which quickly returned to wild-type. Since excess eEF1A alters the cell cycle progression of yeast (27) we determined the effect of transient overexpression of eEF2 on the cell cycle. Flow cytometry analysis of matched samples from all time points of the galactose induction experiment showed no change in G1 and G2/M distribution when compared with cells transformed with an empty vector (data not shown).

Based on the lack of overexpression with promoter, 5′ and 3′-UTR alterations, we hypothesized that the regulation of eEF2 levels is probably post-transcriptional. A major mechanism that regulates protein levels involves degradation by the 26S proteasome [reviewed in (28)]. To address the effect of the proteasome on the regulation of eEF2 levels, the proteasome-deficient *cim5–1* and *pre1-1*, *pre2-2* mutants were used. The *cim5-1* mutation lies in the 19S proteasome regulatory subunit, while the *pre1-1*, *pre2-2* mutant affects the 20S catalytic subunit. These mutations have been shown to decrease proteasome-dependent protein degradation (29). The different subunit locations of these mutants allow for a study that is independent of the proteasome regulatory or catalytic function. pTKB695 ($EFT2^{HIS}$, 2μ, *TEF5* promoter) or the empty vector was transformed into the proteasome mutant and control strains. eEF2 protein levels and growth were unchanged (data not shown), indicating the inability to overexpress eEF2 is proteasome-independent.

DISCUSSION

eEF2 plays a key role in the essential process of protein synthesis by translocating tRNA's from the ribosomal A- and P-sites to the P- and E-sites, respectively, and allowing a new round of peptide bond formation to occur. As with other factors involved in translation, eEF2 is regulated by mechanisms that alter the outcome of protein synthesis. eEF2 is post-translationally modified by phosphorylation of Thr57. The effect of the diphthamide modification on H699, the target site for ADP ribosylation, is not well understood. eEF2 is also a target for natural products, binding the compound sordarin in a hinge region altered between conformations.

V488 is located in the hinge region of eEF2. This region consists of amino acids 481–489 and undergoes substantial changes in conformation in the presence and absence of the inhibitor sordarin (8). These structures show that eEF2 probably changes conformation during its function by a reorientation of the C-terminal domains III, IV and V relative to domains I, G′ and II. Cryoelectron microscopy structures of the yeast ribosome with eEF2 in the presence of sordarin show changes relative to the apo eEF2, supporting the need for eEF2 to change conformation to perform its function (25). Similar changes in conformation have been observed for other G-proteins (30). $eEF2_{V488A}$ is a stable but non-functional eEF2 protein, highlighting the dependence of this region for eEF2 function *in vivo*.

Mutations in H699 inhibit the modification of this residue to diphthamide (16). $eEF2_{H699K}$ is a stable, but non-functional protein *in vivo*. This residue is located at the tip of domain IV, which is proposed to mimic the anticodon arm of tRNA. The cryoelecton microscopy structure of the yeast ribosome in the presence of eEF2 and sordarin demonstrates that the tip of domain IV is in close proximity to the ribosome decoding site mRNA (25). Based on this location, it is hypothesized that this tip is involved in stabilizing codon–anticodon pairing during translocation, thus preventing fidelity errors. The structure of ADP ribosylated eEF2 and its binding affinity to the ribosome further suggest the importance of this region in the function of eEF2 (14). Here we have shown that expressing $eEF2_{H699K}$ in the presence of the wild-type eEF2 conferred not only the slow growth phenotype observed for the non-functional $eEF2_{V488A}$, but also resulted in dominant effects that decrease total protein synthesis and slow elongation. The sensitivity to paromomycin observed may be a further indication of altered translation at the level of fidelity (31,32). Thus, our *in vivo* results support the hypothesized function of the tip of domain IV in efficient and accurate translation.

In both cases the mutant protein is a portion of the total eEF2 pool, which remains fixed. This results in a reduction in the level of wild-type eEF2 protein. Thus, the reduced pool of wild-type eEF2 contributes to the dominant-negative slow growth phenotype. Given that the expression levels of the $eEF2_{V488A}$ and $eEF2_{H699K}$ mutants are essentially the same (Figure 2B), the difference in the inhibition of growth is probably caused by dominant effects on protein synthesis. Thus, $eEF2_{V488A}$ predominantly causes reduced functional eEF2 protein levels while $eEF2_{H699K}$ causes a more significant effect owing to both a decrease in wild-type eEF2 protein levels and a dominant-negative effect on protein synthesis.

Despite the different effects seen by the co-expression of $eEF2_{V488A}$ and $eEF2_{H699K}$, one thing they have in common is the reduced level of wild-type eEF2 protein. eEF2 protein levels are also unchanged between a wild-type strain and those also expressing one of a series of wild-type eEF2 constructs. This result is not owing to the inability to express the plasmid-borne genes, as shown by methods that recognize the tagged forms of eEF2. Using either low or high copy plasmids or changing the promoter, 5′- or 3′-UTR did not allow eEF2 overexpression. Thus, a post-transcriptional control mechanism regulates eEF2 levels. Our studies have shown previously that overexpression of the other translation elongation factors have different effects. eEF1A overexpression results in a slow growth phenotype, however, this phenotype is due to effects on the actin cytoskeleton and is not linked to protein synthesis (27). Overexpression of eEF1Bα also resulted in a slow growth phenotype without affecting actin or protein synthesis. Overexpression of eEF1Bγ has no effect on cell growth (22), while excess eEF3 enhances growth (22,33). Thus, overexpression can be achieved for all the elongation factors except eEF2. A recent report has shown that in mouse adipocytes in which the insulin receptor has been knocked out, eEF2 protein but not mRNA levels decrease (34). This supports a mechanism that regulates the eEF2 protein levels independent of transcription. Thus, eEF2 levels are probably regulated by a feedback mechanism similar to that observed with other components of the translational machinery such as ribosomal proteins (RPs) [reviewed in (35)]. Some RPs bind their own mRNA to regulate their expression via a post-transcriptional mechanism in splicing or translation, such as yeast rpL30 (36).

The inability to overexpress eEF2 and the dominant-negative phenotypes obtained with the non-functional mutants infers the need for an optimum amount of wild-type eEF2 in the cell for proper function. However, the differing ability of the two mutants to alter protein synthesis shows that H699 plays an important role in protein synthesis. Further analysis of domain IV mutants in the anticodon mimic region will define the precise role of this critical region in eEF2 function. It is clear from the regulation of eEF2 by phosphorylation (9) and protein levels regulation (this work) that eEF2 function is an important regulatory step in gene expression. The regulation of eEF2 protein levels provides a new mechanism by which protein synthesis is controlled during elongation.

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