Functional Interactions between Yeast Translation Eukaryotic Elongation Factor (eEF) 1A and eEF3*

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The translation elongation machinery in fungi differs from other eukaryotes in its dependence upon eukaryotic elongation factor 3 (eEF3). eEF3 is essential in vitro and required for each cycle of the translation elongation process in vivo. Models predict eEF3 affects the delivery of cognate aminoacyl-tRNA, a function performed by eEF1A, by removing deacylated tRNA from the ribosomal Exit site. To dissect eEF3 function and its link to the A-site activities of eEF1A, we have identified a temperature-sensitive allele of the YEF3 gene. The F650S substitution, located between the two ATP binding cassettes, reduces both ribosome-dependent and intrinsic ATPase activities. In vivo this mutation increases sensitivity to aminoglycoside drugs, causes a 50% reduction of total protein synthesis at permissive temperatures, slows run-off of polyribosomes, and reduces binding to eEF1A. Reciprocally, excess eEF3 confers synthetic growth, increased drug sensitivity, and reduced translation in an allele specific fashion with an E122K mutation in the GTP binding domain of eEF1A. In addition, this mutant form of eEF1A shows reduced binding of eEF3. Thus, optimal in vivo interactions between eEF3 and eEF1A are critical for protein synthesis.

The process of protein synthesis is mediated by soluble protein factors, many of which are functionally similar between prokaryotic and eukaryotic systems (1). During elongation, eukaryotic elongation factor 3 (eEF3) is required to recruit the aminoacyl-tRNA to the A site of the ribosome. The eEF1B complex or EF-Tu, in eukaryotes and prokaryotes, respectively, are the nucleotide exchange factors functioning to maintain the active pools of their respective G-proteins. eEF2 in eukaryotes or EF-G in bacteria are the GTP-driven translocons that move the mRNA and peptidyl-tRNA following peptide bond formation. Most protein synthesis factors are highly conserved between single cell eukaryotes and metazoans. For example, human and Saccharomyces cerevisiae eEF1A are 81% identical. Fungi are different in their absolute requirement of a third factor, eEF3. eEF3 of S. cerevisiae is essential for cell-free translation systems (2, 3), every cycle of translation elongation (4), and cell viability (5). Apart from the YEF3 gene of S. cerevisiae (6) and the related, nonessential HEF3 gene (7), eEF3 has been identified and sequenced from other fungi like Candida albicans (8, 9), Cryptococcus neoformans (10), and Pneumocystis carinii (11). A structural homology of eEF3 has also been reported to be present in the chlorella virus CVK2 (12). Some evidence indicates there may be an eEF3-like ATPase activity tightly associated with ribosomes in prokaryotes (13). This ATPase, RbbA, cross-reacts with anti-eEF3 antibody and exhibits inhibition of polyphenylalanine synthesis as well as ribosome-associated ATPase activity in the presence of anti-eEF3 antibody.

The 1044-amino acid sequence of eEF3 reveals multiple structural motifs spanning the protein, some possessing homology to proteins of established functions related to translation (Fig. 1A). A 200-amino acid motif near the N terminus bears ~30% homology with the Escherichia coli ribosomal protein S5. rpS5 binds to the 16 S rRNA and is an important constituent of the “decoding center” (14). Recombinant peptide derived from the N terminus of eEF3 interacts with 18 S rRNA and inhibits the ribosome-dependent eEF3 ATPase activity (15). The 51-amino acid stretch at the C terminus comprises highly basic amino acids containing at least three lysine-rich clusters, which are absolutely required for binding to yeast ribosomes (16, 17). eEF3 also possesses a highly conserved sequence found in tRNA-binding proteins, termed ELVES, residing in the intervening region of the “A” and “B” motifs of the second ATP binding cassette and has been hypothesized to assist the removal of deacylated tRNA from the ribosome (14). Thus, the various sequence motifs of eEF3 exhibit conservation to proteins known to interact with tRNA and rRNA and may indicate similar functions for eEF3.

eEF3 possesses both intrinsic and ribosome-dependent ATPase and GTPase activities by virtue of a repeated bipartite nucleotide binding domain characteristic of the ATP-binding superfamily of proteins possessing ATP binding cassettes (ABCs) or Walker boxes (reviewed in Ref. 18). eEF3, Gen20p, and UvrA, unlike other members of the ABC family of proteins, are a subfamily of soluble proteins and do not function as membrane bound translocators, the larger part of this family (18). Rather, eEF3 relies upon its ATP hydrolytic activity to perhaps induce a conformational change within the ribosome, facilitating the exit of deacylated tRNA from the E-site and subsequent binding of charged aa-tRNA to the A-site (19). The
two ABCs are distinct, as the first has the classic 70–90-amino acid spacing between the A and B motifs, whereas the second has a 188-amino acid insertion. However, eEF3 does bind two molecules of ATP in a cooperative manner (20). A protein lacking amino acids 689–793, including the A motif of the second ATP binding cassette, however, maintains intrinsic ATPase activity, indicating for this function both cassettes are not essential (19). More subtle mutations in the conserved glycines (G469V and G701V), and lysines (K469R and K707R), in the A motif of the ATP binding cassette (GXXXGKS/G) abolish ribosome-dependent ATPase activity, polyphenylalana-nine synthesis, and cell growth (21). However, little is known about the function or consequences of altered ATPase activity in vivo or in the individual steps of eEF3 function. Overall, the many distinct sequence motifs of the eEF3 protein may perform different functions; however, there appears to be an interaction among different domains to govern the overall catalytic action of the eEF3 protein.

Previous studies show that, functionally, eEF3 stimulates the delivery of only cognate aa-tRNA by eEF1A at the A site (22, 23), in a manner that appears to be codon-dependent (24). The role of eEF3 in an allosteric interaction between the A and E sites of the ribosome has been demonstrated (19). Further, there is evidence that eEF3 is an E site factor controlling the removal of deacylated tRNA to facilitate binding of the cognate (aa-tRNA-GTP-eEF1A) ternary complex to the A site. In fact, eEF3 binds eEF1A in vitro (25), although the functional consequences on elongation and eEF1A function remain unknown. In the current study we report that mutation of Phe-650 to Ser in the intervening region of the two ATP binding cassettes of S. cerevisiae eEF3 is conditionally lethal at 37 °C. The F650S mutant strain demonstrates reduced global translation, slower run-off of polyribosomes, reduced intrinsic and ribosome-stimulated ATPase activities, and reduced interaction with eEF1A. The eEF3 mutant strain of yeast does not affect +1 ribosomal frameshifting or nonsense suppression, but has moderate effects on sensitivity to translation inhibitors. eEF3 also exhibits alterations in both genetic and physical interactions with a G-domain mutation in eEF1A in a highly allele-specific fashion. The present study demonstrates a direct allele-specific genetic interaction between the two translation elongation factors and shows intact and functional nucleotide binding domains of both proteins are necessary for their physical association. Taken together these results provide in vivo evidence supporting a functional link between these two factors.

EXPERIMENTAL PROCEDURES

Yeast and Bacterial Strains, Growth, and Drug Sensitivity Assays—S. cerevisiae strains and their genotypes used in this study are listed in Table I. E. coli DH5α was used for plasmid preparation. Procedures for cell growth and genetic manipulations were according to standard protocols (26). Yeast cells were grown in either YEPD (1% Bacto-yeast extract, 2% peptone, 2% dextrose) or in defined synthetic complete medium (C or C−) supplemented with 2% dextrose as the carbon source unless noted. Yeast were transformed by the lithium acetate method (27). Temperature sensitivity was assayed by growing strains containing wild type (TKY97) or the mutant form of eEF3 (TKY599) to an A600 of 1.0. Serial 10-fold dilutions (5 μl each) were spotted on YEPD, followed by incubation at 13, 24, 30, and 37 °C for 3–7 days. Phenotypic suppression of a nonprogrammed +1 frameshift allele (met2−1 and his4−713) were determined by spotting 10 μl of the same dilutions onto complete medium lacking methionine or histidine, respectively, and incubating for 5 days at 30 and 33 °C. Halo assays for sensitivity to cycloheximide, paromomycin, and hygromycin B were as previously described (28). Sensitivity to paromomycin in conditions where eEF3 is overexpressed relative to wild type levels was determined in liquid culture for at least two independent colonies of each strain grown at 30 °C in C-Ura to mid-log phase, diluted to A600 of 0.05, and grown in triplicate in a 96-well microtiter assay plates with varying concentrations of paromomycin. Plates were incubated with shaking at 30 °C and growth monitored as the mean of the triplicate A600 at 22 h.

Isolation and Cloning of the F650S Mutant Allele of S. cerevisiae YEF3—A screen for temperature-sensitive (Ts+) alleles of YEF3 was performed by passage of pYEF3 through XL1-Red E. coli (Stratagene), transformation of TKY554, and replica plating on 5-fluoroorotic acid to monitor for slow growing colonies at 34 °C. The mutated YEF3 plasmid was recovered from yeast and transformed into E. coli DH5α, and isogenic wild type and mutant eEF3 strains were constructed by transforming pTKB594 (YEF3 TRP1) or pTKB595 (yeF3 F650S TRP1) into yeast TKY554 (29). Loss of the YEF3 TRAP3 plasmid was monitored by growth on 5-fluoroorotic acid, producing strains TKY597 (wild type) and TKY599 (F650S).

Translation Assays—β-Galactosidase-based assays for nonsense suppression were programmed by ribosomal frameshifting efficiencies on the yeast L-A virus signal were performed. Nonsense suppression assays were performed on strains TKY597 and TKY599 using a URA3 wild type lacZ control plasmid (pUKS1154tail) or a URA3 plasmid with an in-frame UAA nonsense codon in lacZ (pUKS1151tail (UAAX)) as described previously (30). For −1 frameshifting assays, URA3-based pT125 (0 frame) and pT124 (LA virus −1 frame) plasmids were used (31). Total yeast translation was monitored by in vivo [35S]methionine incorporation as previously described (30) using the indicated MET2 strains. Yeast polyribosome preparation was performed as previously described (32) with the following specifications. Yeast cultures were grown at 30 °C to A600 of 0.5–1.0, divided, and extracted with and without cycloheximide added to the cells and lysis buffer. Cell extracts (40 μg total) were layered on 35 ml of 7–47% sucrose gradient and centrifuged for 4 h at 27,000 rpm in a SW28 rotor. The A600 was monitored and recorded using a model 185 density gradient fractionator (ISCO, Inc., Lincoln, NE).

ATP Hydrolysis—His6-tagged wild type and F650S mutant eEF3 proteins were purified from the strains TKY702 and TKY707, respectively, on Ni2+ HiTrap chelating column (Amersham Biosciences). Yeast extracts for GST pull-down analysis were prepared by glass bead lysis in TEDG buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM DTT, 0.2 mM PMSF) with 20% methanol. The protein was eluted with buffer B (50 mM KPO4, pH 7.6, 300 mM KCl, 1 mM DTT, and 0.2 mM PMSF) with 20% methanol. The protein peak was dialyzed into buffer B (20 mM Tris, pH 7.4, 50 mM KCl, 1 mM DTT, 0.1 mM EDTA, and 100 mM KCI). ATP hydrolysis was performed as was described previously (29). Briefly, the standard assay mixture contained 0.5 μmol units of yeast ribosomes and 150 μg (γ32P)ATP. Hydrolysis was allowed to proceed for 5 min at 30 °C, and P, release determined. ATP hydrolysis levels were calculated after subtracting the background for buffer alone.

GST and His6 Pull-downs of eEF1A and eEF3—Yeast extracts for GST pull-down analysis were prepared by glass bead lysis in TEDG buffer (10 mM Tris-HCl, pH 7.4, 2 mM EDTA, 5 mM DTT, 50 mM KCl, 1 mM PMSF) from eEF1A wild type (MC214) or mutant (TKY229, TKY252, and TKY688) strains expressing either GST alone (pMA210) or GST-eEF3 (pTKB540) fusion proteins under the GAL4 promoter and induced by growth in C-Ura + galactose (25). Reactions containing 50 μg of total protein (determined by Bradford reagent, Bio-Rad) and 20 μl of a 50% glutathione-Sepharose 4B slurry (Sigma) in a 200-μl final volume in KETN 150 buffer (150 mM KCl, 1 mM EDTA, 20 mM Tris, pH 8.0, 0.5% Nonidet, 1 mM PMSF) were mixed at 4 °C for 1 h. Beads were washed three times with KETN buffer (either 150 mM KCl or 300 mM KCl), samples resolved by SDS-PAGE, and proteins detected with a polyclonal antibody to yeast eEF1A by ECL (Amersham Biosciences). Ni2+–NTA pull-down of eEF1A with His6-tagged eEF3 were performed on extracts from strains TKY702 and TKY707, expressing wild type and F650S His6–tagged eEF3 expressed from a 2-μg TRP1 plasmids as the only form of eEF3, respectively, as described previously (33).

RESULTS AND DISCUSSION

Characterization of a Novel Temperature-sensitive Allele of YEF3—To apply a genetic approach to understanding eEF3 function, a screen for randomly generated Ts− alleles of the YEF3 gene was performed. A mutation lethal at the nonpermissive temperature of 37 °C (Fig. 1B), substituting phenylalanine 650 with serine, was identified. Additionally, there are two substitutions, N153F and V332L, in both the wild type and mutant plasmid-borne alleles relative to the published (6) and annotated yeast genome sequences. These two substitutions likely reflect polymorphisms between yeast strains and are in residues of low conservation between fungal eEF3 sequences (10). To help determine the mechanism causing the Ts− phe-
TKY599 strain was grown to mid log-phase in YEPD at 30°C. Motifs: an rpS5-like domain (lanes 2 and 4) and two Walker type ATP binding motifs (lanes 3 and 5). The cross-talk between these proteins (reviewed in Ref. 34), and the presence of this mutation in the intervening region of the two motifs could potentially affect the proper alignment of these two domains. Thus, it was predicted that the F650S mutation would interfere with hydrolytic activity. As seen in Fig. 2, wild type eEF3 hydrolyzes 0.82 nmol of ATP/pmol of eEF3. The presence of ribosomes stimulates the hydrolysis 2-fold. In comparison, F650S eEF3 protein shows only background levels of hydrolysis in the presence or absence of ribosomes. Thus, the mutation severely compromises an eEF3 activity, ATP hydrolysis, previously shown to be critical for elongation in vitro.

A Strain Expressing the F650S eEF3 Mutant Shows Altered Sensitivity to Translation Elongation Inhibitors but Not Reduced Translational Fidelity—To identify changes in protein synthesis caused by the F650S mutation, the wild type and mutant strains were tested for sensitivity to translation inhibitors. Altered sensitivity to aminoglycoside drugs such as paromomycin and hygromycin B typically correlates with reduced translational fidelity (35, 36), whereas cycloheximide is a general translation elongation inhibitor. At the permissive temperature, the F650S mutant eEF3 strain showed increased sensitivity to paromomycin and hygromycin, but not cycloheximide (Table II). To further analyze potential effects on fidelity, the ability of the wild type and mutant strains to suppress two chromosomally encoded frameshift mutations (met2-1 and his4-713) was monitored by the ability of strains to grow on medium lacking the corresponding amino acid. Compared with the wild type, the mutant strain did not grow better on C-Met or C-His media at either permissive or semipermissive temperatures, indicating that the mutant allele does not reduce fidelity to allow phenotypic suppression of a +1 frameshift mutation (data not shown). These results were corroborated by several quantitative measurements of translational fidelity. Suppression of the –1 frameshift signal from the yeast L-A virus or a UAA stop codon was determined using lacZ-based assays of read-through. There was no significant increase in the β-galactosidase activity levels of either the –1 frameshift signal (6.4 ± 1% for wild type versus 7.7 ± 2% for the mutant), or UAA stop codon read-through (0.15 ± 0.02% for wild type versus 0.12 ± 0.04% for the mutant) reporter system. Thus, even though paromomycin sensitivity usually correlates with
altered translational fidelity in yeast, this effect is not manifest on frameshifting or nonsense suppression.

**eEF3 Mutant Shows a Global Reduction in Translation at the Elongation Step**—To monitor the effects of the F650S mutation on total protein synthesis, [³⁵S]methionine incorporation was monitored at the permissive temperature of 30 °C. A strain expressing the mutant eEF3 protein shows an ~50% decrease in total translation over 60 min of growth (Fig. 3A). This is similar to the level of reduction seen for a strain bearing the F650S eEF3 mutant (Fig. 3B), which also dramatically affects cell growth (37, 38). To identify the step in protein synthesis affected, standard polyribosome profiles were analyzed for the wild type and mutant eEF3 strains at the permissive temperature and following a 2-h shift to 37 °C. No significant differences between the profiles of wild type or F650S mutant strain extracts were noted (Fig. 3C for 30 °C and data not shown). A similar analysis of the E286K eEF1A mutant, which also shows a significant reduction in total translation, similarly shows no significant polyribosome profile alterations (Fig. 3D for 30 °C and data not shown). Consequently, to look more specifically at the elongation step, identical experiments were performed at the permissive temperature of 30 °C except that cycloheximide was omitted from all steps of the standard experimental protocol. Because cycloheximide freezes ribosomes on the mRNA, the lack of this drug would allow ribosomes to continue elongating and run-off. Slower run-off would be seen as enhanced retention of polyribosomes, thus providing a better monitor of effects on elongation. The F650S mutant eEF3 strain exhibits a much higher population of polyribosomes as compared with the wild type eEF3 strain (Fig. 3C, -CH), indicating a retardation of the disassembly of the ribosome and hence a slower translation elongation rate. Similarly, analysis of the eEF1A E286K mutant strain shows a significant increase in polyribosomes at 30 °C compared with wild type yeast (Fig. 3D, -CH).

The F650S Substitution in eEF3 Reduces Its Physical Interaction with eEF1A—It has been shown previously (25) that eEF1A and eEF3 physically interact. To decipher the cause of the translation defect seen in the F650S eEF3 mutant, plasmids expressing His₆-tagged wild type and F650S eEF3 proteins were prepared, and shown to function in place of the untagged wild type eEF3 with essentially identical growth to their untagged counterparts (data not shown). Using a Nid²–NTA pull-down from total cell extracts, the native eEF1A associates with wild type His₆ eEF3 (Fig. 4). When the His₆ F650S eEF3 mutant is expressed as the only form of eEF3, however, it shows essentially no binding of eEF1A (Fig. 4). Thus, these results demonstrate that the mutation of Phe-650 to Ser in eEF3 can reduce the physical interaction with eEF1A in vivo as well as elongation, indicating the physiological significance of this interaction.

**Translation Elongation Factor eEF3 Shows Genetic Interactions with eEF3**—Based on the above results and prior models linking the functions of eEF1A and eEF3, we sought reciprocal genetic support for this model by testing the effect of overexpression of either protein in a cell bearing a mutant form of the other. Overexpression of eEF1A in eEF3 wild type yeast results in reduced growth, even at permissive temperatures (39), and consequently is unable to suppress the Ts defect in eEF3 mutant cells (data not shown). The effect of excess eEF3 was monitored on strains bearing a series of alleles expressing mutant forms of eEF1A with alterations of the E40K, E122K, E122Q, D130N, T142I, N153T, D156N, or N153T/D156E residues in the GTP binding domain I and residues E286K, D295K, or E317K in domain II near the proposed aa-tRNA binding site. MK22 (MC214, TKY225, circles) and F650S (TKY226, squares) eEF3 strains (A) or wild type (TKY221, circles) and E286K (TKY222, triangles) eEF1A strains (B) were measured by growing the culture to mid log phase in C–Met and labeled for varying times with [³⁵S]methionine. Incorporation is expressed as cpm/500 unit of cells. C and D, polyribosome extracts of wild type (TKY597) and F650S (TKY598) eEF3 strains (C) or wild type (MC214) and E286K (TKY588) eEF1A strains grown at 30 °C (D) were prepared and analyzed in the presence (+CH) and absence (–CH) of cycloheximide by 7–47% sucrose gradients. Altered sensitivities of wild type and F650S eEF3 expressing S. cerevisiae strains to translation inhibitors (Fig. 2). The F650S mutation in yeast eEF3 results in loss of ATPase activity. One pmol of His₆-tagged wild type (wt) or F650S mutant (F650S) eEF3 proteins were analyzed for ATP hydrolysis. Both intrinsic (–) as well as yeast ribosome-stimulated (+) ATPase activities were monitored. The nanomoles of Pi released from [γ-³²P]ATP are shown after subtracting the hydrolysis in the presence of buffer alone. The results are an average of three experiments and the standard deviation shown.
Slow growth defect in strains overexpressing eEF3 was observed specifically for the E122K mutant strains (TKY113 or TKY252), but no other eEF1A mutants, at all temperatures tested (Fig. 5A and E) and data not shown. To determine whether the growth defect correlates with a change in the protein synthesis activities of the two proteins, the effect on sensitivity to the translation inhibitor paromomycin was determined for wild type, E122K, and N153T/D156E eEF1A strains with and without excess eEF3. As shown in Fig. 5B, the E122K mutant shows a more than 10-fold increase in paromomycin sensitivity in the presence of excess eEF3, with a 50% reduction in growth achieved at 0.45 mg/ml paromomycin compared with 5.84 mg/ml for a wild type strain. This effect is not seen for another mutation in the GTP binding domain of eEF1A that is unaffected by excess eEF3 (N153T/D156E, Fig. 5C). In all strains, however, the level of eEF3 overexpression was the same (Fig. 5D and data not shown). These results clearly show an allele-specific interaction between eEF1A and eEF3 in vivo. To determine whether the F650S eEF3 mutant compromised for eEF1A binding was consequently unable to show the synthetic growth defect, His_6-tagged wild type and mutant eEF3 were overexpressed in strains expressing wild type and E122K eEF1A. Although the wild type His_6-tagged eEF3, unlike untagged eEF3, shows a slight slow growth defect in a wild type cell (Fig. 5E), the reduced growth of the E122K eEF1A mutant cells in the presence of excess His_6-tagged eEF3 is much more severe. Overexpression of His_6-tagged F650S eEF3 was unable to produce the synthetic slow growth phenotype in yeast with the E122K form of eEF1A. Thus, the genetic link between eEF1A and eEF3 is reciprocal and dependent on the ability of the two proteins to interact.

A Specific G-domain Mutant Form of eEF1A Impairs Binding to eEF3. To determine whether the synthetic slow growth defect of eEF1A E122K and excess eEF3 is through an alteration in total translation, [35S]methionine incorporation was monitored at the permissive temperature of 30 °C for strains expressing wild type or the E122K mutant form of eEF1A in the presence and absence of excess eEF3. As shown in Fig. 6A, wild type yeast show a slight increase in total translation in the presence of excess eEF3 (closed circles), consistent with the lack of reduced growth or altered sensitivity to translation inhibitors (Fig. 5, A and B) and potentially indicating eEF3 activity or levels are limiting relative to eEF1A. A strain bearing the E122K mutation shows a 28% reduction in total translation (open diamonds). In the presence of excess eEF3, however, this effect is even more pronounced, a reduction of 55% relative to wild type and 38% relative to the E122K mutant without excess eEF3 (closed diamonds). Compared with the E286K mutant unaffected by eEF3 overexpression but with a 32% reduction in total translation, a modest 20% reduction in total translation is observed with excess eEF3 in this mutant (Fig. 6B, triangles).

To understand the basis of the altered functional interaction between the E122K mutation in eEF1A and eEF3, a series of strains expressing wild type and E122K, N153T/D156E and E286K mutant forms of eEF1A were transformed with plasmids expressing GST or a GST-eEF3 fusion protein. Following pull-down of GST or GST-eEF3 from cell extracts with glutathione beads, the amount of associated eEF1A was determined by Western blot analysis. Wild type eEF1A is associated with GST-eEF3 and not with GST alone (Fig. 7, wt). Similar analysis of the E122K mutant form of eEF1A showed an equivalent low
with glutathione beads and pull-downs performed. The pellet (grown in C
GST or a GST-eEF3 fusion under a galactose-inducible promoter and
E286K (TKY588) mutant strains transformed with plasmids expressing
type (MC214) and E122K (TKY252), N153T/D156E (TKY229), and
Cell extracts were prepared from eEF1A wild
blotting with a polyclonal antibody to eEF1A.
10% of input) were separated by SDS-PAGE and analyzed by Western
of input), unbound supernatant (S, 5% of input) and total extract (E,
10% of input) were separated by SDS-PAGE and analyzed by Western
with a polyclonal antibody to eEF1A.
level of association with either GST or GST-eEF3, indicating
essentially no binding of eEF3 (Fig. 7, E122K). Two other
eEF1A mutants in domain I (N153T/D156E) or domain II
(E286K) showed no significant difference in the ability to as-
semble with GST-eEF3 (Fig. 7). This demonstrates those
eEF1A mutants that reduce total translation (E286K) or other
mutations in the GTP binding domain (N153T/D156E) are not
sufficient to alter this association. Taken together, these
results are consistent with the interpretation that the sensitivity
of the G-domain E122K eEF1A mutant to eEF3 levels is man-
ifest in subtle defects in protein synthesis and the interaction
between the two proteins and further indicate a necessary
balance between the activity of the two proteins.

**DISCUSSION**

One of the interesting motifs and functional requirements for
eEF3 is the tandem ATP binding cassettes. The location of the
F650S mutation between the two cassettes may affect the
interaction between these two ATP binding sites. Work on
other members of the ABC family has indicated the require-
ment for two ATPase domains, as well as the collaboration of
the two domains in ATP hydrolysis (reviewed in Ref. 34). The
loss of ATPase activity in this mutant form of eEF3 may pro-
vide insight into the communication required between the
two domains. Prior studies that inactivated either of the ATP
binding domains by mutations in the highly conserved residues of
the Walker A motif, GXXXXGK, support the essential role of
ATP hydrolysis. A mutation of Gly-463 to Val appears at least
partially functional in vivo, although the analysis was limited
to slow growth on media to monitor complementation of the
wild type protein (21). A comparative analysis between this
ATP binding site mutation and the F650S mutation may pro-
vide insight into the unique characteristics in vivo of altering
the critical ATPase activity of eEF3 by completely different
mechanisms.

Although the mutant form of eEF3 studied in this work does
not affect nonsense suppression or maintenance of reading
frame, it was not isolated for these characteristics. The in vitro
data on the role of eEF3 in favoring binding of cognate aa-tRNA
(22) and the link to eEF1A function support a potential role in
accuracy at the A-site. It is clear, however, that simply reduc-
ing the activity of eEF3 in protein synthesis is not sufficient to
alter fidelity as determined here. Genetic screens designed to
select mutant forms of eEF3 that affect fidelity may shed light
on the proposed role of eEF3 on this process. A direct and
highly specific role in misincorporation is still possible. Further
in vitro analysis of the F650S eEF3 and E122K eEF1A mutants
and their functional interaction during elongation may explain
whether the observed paromomycin sensitivity could point to-
ward altered misreading of sense codons. However, it is of note
that mutations in eEF1A that alter misincorporation also alter
other forms of fidelity (30), in particular nonsense suppression,
so this high specificity would be a novel and unanticipated
finding.

This work shows that the physical interaction between
eEF1A and eEF3 is altered by mutations that compromise the
activity of the two proteins, but in a highly specific manner
with regards to eEF1A. The bank of mutations studied in this
work were either selected to affect translational fidelity (37) or
designed as point mutations to affect the affinity and specificity
of GTP binding (30, 40). The E122K mutation, the only one
affected by eEF3 levels, was originally isolated as a dominant
+1 frameshift suppressor of the met2-1 reporter allele (37).
The mutation, although located in the GTP binding domain I
(41), is in a fourth loop surrounding the bound nucleotide
separate from the well conserved GTP binding consensus ele-
ments such as the NKXD motif altered in several other domain
I mutations tested. Structurally, Glu-122 does stabilizes the
base packing of Lys-156 of the NKXD consensus sequence (33).
It is also not the strongest of the dominant +1 frameshift
suppressors isolated, as the two best suppressors lie in the
proposed aa-tRNA binding pocket of domain II. The E122K
mutation also shows unique characteristics as the only form
of eEF1A, including dramatic effects on programmed –1 riboso-
mal frameshifting, an event linked to A-site occupancy (28), as
well as less dependence on catalyzed nucleotide exchange by
eEF1Bα (30).

The effect of the combination of eEF1A E122K and excess
eEF3 on paromomycin sensitivity provides a strong in vivo link
to A-site function and fidelity. The determination that the
F650S mutant fails to cause the synthetic growth defect in the
E122K mutant indicates either ATP hydrolysis or perhaps the
ability to bind eEF1A are critical for this phenotype. It is
possible that, if eEF1A and eEF3 work cooperatively at the
A-site, the loss of this interaction may reduce translation.
Thus, excess eEF3 in the eEF1A mutant may lead to nonpro-
ductive interactions of the protein, perhaps with the ribosome.
Additionally, it is of interest that the effects identified link
through the nucleotide binding domains of both proteins, but
outside the consensus elements of either. The total translation
rate, most likely through the elongation cycle, is accelerated in
the presence of excess of eEF3 in the cells with wild type
eEF1A, indicating the protein may be limiting. However, the
reverse is seen in E122K eEF1A mutant cells. Thus, excess
eEF3, if unable to properly or efficiently interact with eEF1A,
is deleterious to the cell. The exact ratios of eEF1A and eEF3
required for an efficient interaction, and the effect of aa-tRNA
and nucleotide on this association, remain to be determined.

It remains unclear why fungi require the function of eEF3.
There is speculation that an eEF3-like function remains in
metazoans, perhaps associated with or part of the ribosome,
even though a homologous protein has not been found (42).
There is an ATPase activity associated with metazoan ribo-
somes, which may perform the same function as eEF3 in re-
lease of the deacylated tRNA from the E-site (20, 43, 44). Thus,
the critical functional interaction with eEF1A may provide a
criterion for establishing whether such a conserved function
exists. Alternatively, the physical association of eEF3 and
eEF1A may be less important than the allosteric effects of
E-site release of deacylated tRNA on the A-site. Because
eEF1A is exceptionally well conserved, 81% identical between
yeast and mammals compared with 64% identity for eEF2 or
43% identity for eEF1Bα, a unique eEF3 binding site is not
obvious. The unique characteristics of the F650S mutation in
eEF3 now provide an avenue toward a genetic dissection of

![Image](image65x647_to_299x738)
eEF3 function in vivo, understanding the link between the two ATPase domains, and the identification of other factors that interact with eEF3.

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