Mutations in a GTP-binding Motif of Eukaryotic Elongation Factor 1A Reduce Both Translational Fidelity and the Requirement for Nucleotide Exchange*  

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A series of mutations in the highly conserved N\textsuperscript{135}KMD\textsuperscript{138}GTP-binding motif of the Saccharomyces cerevisiae translation elongation factor 1A (eEF1A) affect the GTP-dependent functions of the protein and increase misincorporation of amino acids in vitro. Two critical regulatory processes of translation elongation, guanine nucleotide exchange and translational fidelity, were analyzed in strains with the N153T, D156N, and N153T/D156E mutations. These strains are omnipotent suppressors of nonsense mutations, indicating reduced A site fidelity, which correlates with changes either in total translation rates in vivo or in GTPase activity in vitro. All three mutant proteins also show an increase in the $K_m$ for GTP. An in vivo system lacking the guanine nucleotide exchange factor eukaryotic elongation factor 1Bα (eEF1Bα) and supported for growth by excess eEF1A was used to show the two mutations with the highest $K_m$ for GTP restore most but not all growth defects found in these eEF1Bα-deficient-strains to near wild type. An increase in $K_m$ alone, however, is not sufficient for suppression and may indicate eEF1Bα performs additional functions. Additionally, eEF1A mutations that suppress the requirement for guanine nucleotide exchange may not effectively perform all the functions of eEF1A in vivo.

The G-protein superfamily members are defined by highly conserved sequence motifs and structural features and regulate a variety of critical cellular processes (1). The ability of G-proteins to transition between active and inactive forms based on whether GTP or GDP is bound, respectively, allows them to function as a molecular switch (2). This transition is modulated by accessory factors that stimulate either GTP hydrolysis (GTPase-activating proteins) or guanine nucleotide exchange (guanine nucleotide exchange factors (GEFs)).1 These factors can regulate the activity of the G-protein in response to molecular and cellular signals. Many mutations have been isolated, particularly in residues important for GTP binding, that affect the ability of the G-protein to interact with GTPase-activating proteins and GEFs or function as a target for the activities of these factors.

The eukaryotic translation elongation factor 1A (eEF1A) is a member of the G-protein family. The prokaryotic homolog, EF1A, was the first G-protein x-ray crystal structure solved (3). eEF1A is a GTPase whose activity is stimulated by binding of aminoacyl-tRNA, ribosomes, and most importantly the presence of a codon-anticodon match between the aminoacyl-tRNA and the A site codon of the ribosome-bound mRNA. Thus, the ribosome acts as a GTPase-activating protein for eEF1A. Correspondingly, eEF1A requires a GEF, eEF1B (4). Only the eEF1Bα subunit is required for nucleotide exchange, and in the yeast Saccharomyces cerevisiae eEF1Bα, like eEF1A, is an essential gene product (5). The regulation of eEF1A activity by GTPase-activating proteins and GEFs is critically important in efficient and accurate protein synthesis and consequently cell growth.

All G-proteins in the translation factor family share three well-conserved motifs, GXXGXXG, DXG and NKXD (6). Previous structural and mutational studies of the Escherichia coli EF1A protein have supported the important role of the N\textsuperscript{135}KMD\textsuperscript{138} motif. Structurally, this motif is important for binding and recognizing the guanine ring (7). Substitutions of Lys\textsuperscript{136} result in a dominant negative growth effect that is suppressed by overexpression of the prokaryotic GEF EF1B (8). Genetic analysis of the Lys\textsuperscript{136} mutant protein indicates that intragenic suppressors of the dominant negative phenotype cluster in the G-domain of EF1A and all result in reduced affinity for EF1B (9). Other dominant negative mutations in Asn\textsuperscript{135} to Asp and Ile also appear to function in a similar manner. A homologous mutation in S. cerevisiae eEF1A, N153D, is no longer functional as the only form of the protein (10) and also results in a conditional dominant negative growth phenotype.2 Thus, this motif is important in interacting with G-protein-associated factors. Double mutants altering both Asn\textsuperscript{135} and Asp\textsuperscript{138} can completely inactivate nucleotide binding by EF1A, consistent with effects on affinity (11). Thus, the importance of these residues of the motif element on nucleotide binding is clear. Mutational analysis of the conserved Asp\textsuperscript{138} in EF1A results in altered nucleotide specificity, resulting in a protein that now binds XTP with affinities near the GTP binding affinity of the wild-type protein (12, 13). The mutant protein does not affect translational fidelity in vitro, because there appears to be no major change in nucleotide affinity. The NKXD GTP-binding element is thus demonstrated to play im-

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1The abbreviations used are: GEF, guanine nucleotide exchange factor; eEF1A, eukaryotic Elongation Factor 1A (formerly EF-1α); eEF1Bα, eukaryotic Elongation Factor 1Bα (formerly EF-1β); EF1A, Elongation Factor 1A (formerly EF-Tu); EF1B, Elongation Factor 1B (formerly EF-Ts).

2A. L. Laitusis and T. G. Kinzy, unpublished observation.
portant roles in both the specificity and affinity of nucleotide binding in G-proteins.

We have utilized the basis of knowledge available for the NKXD GTP-binding element to study the effect of GTP binding and hydrolysis by eEF1A on the efficiency and accuracy of translation in the eukaryotic organism S. cerevisiae. Three mutant forms of the protein that alter Asn\(^{153}\), Asp\(^{156}\), and both residues of the N\(^{153}\)MKD\(^{156}\) element all result in reduced affinity for GTP, and two correspondingly can suppress the redefined synthetic complete medium (C or C\(^{+}\)) either YEPD (1% Bacto yeast extract, 2% peptone, 2% dextrose) or yeast genetic methods were employed (15). Yeast cells were grown in S. cerevisiae strains used in these studies are listed in Table I. Standard and were prepared by either transformation of a URA3 derivative of the heterozygous TEF5/tef5::TRP1 digested the TRP1 digesting the TRP1 N153T (JC5, tef2-17) and cloning the resulting mutant proteins and strains analyzed result in reduced A site fidelity as monitored that correspond to changes previously characterized in the D156N mutation of yeast eEF1A, unlike the feEF1A Mutations Affecting GEF Requirements and Fidelity

**EXPERIMENTAL PROCEDURES**

**Strains and Media—**E. coli DH5\(^{a}\) was used for plasmid preparation. S. cerevisiae strains used in these studies are listed in Table I. Standard yeast genetic methods were employed (15). Yeast cells were grown in either YEPD (1% Bacto yeast extract, 2% peptone, 2% dextrose) or defined synthetic complete medium (C or C\(^{-}\)) supplemented with 2% dextrose as a carbon source. Yeast were transformed by the lithium acetate method (16). Strains supported for growth by plasmid-borne eEF1A genes (TEF1 or TEF2) are derivatives of MC213 or MC214 prepared by plasmid shuffling (17). Strains deficient in eEF1B are derivatives of the heterozygous TEF5/tef2::TRP1 diploid JWY4175 (14) and were prepared by either transformation of a URA3-based plasmid containing the indicated tef2 allele into JWY4175 followed by sporulation and dissection or by transformation into the haploid JWY4229 (tef5::TRP1 pTEF5-LEU2) (18) followed by spontaneous loss of the TEF5 LEU2 helper plasmid.

**DNA Manipulations—**Restriction endonucleases and DNA modifying enzymes were obtained from Roche Molecular Biochemicals. Construction of the tef2 alleles on TRP1-based plasmids are described in Cavalieri and Merrick (10). The URA3-based plasmids were prepared by digesting the TRP1-based pRS314-4JC plasmids containing the indicated mutation: D156N (JC6, tef2-17), N153T/D156E (JC32, tef2-18), or N153T/JC5, tef2-19) with BamHI and SacI and cloning the resulting fragment into pRS316 (URA3 CEN) digested with BamHI and SacI. The resulting plasmids were pTKB293 (tef2-17, D156N), pTKB300 (tef2-18, N153T/D156E), and pTKB299 (tef2-19, N153T).

**Western Blot Analysis—**Yeast strains containing wild-type eEF1A (MC214) or one of the three mutant forms of eEF1A: N153T (TKY226), D156N (TKY228), and N153T/D156E (TKY229) were grown in liquid YEPD to an A\(_{600}\) in mid-log phase (0.4 to 1.0 units) and extracts prepared by glass bead lysis. Approximately 0.5 \(\mu\)g of total protein, as determined by Bradford protein analysis (Bio-Rad) were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. Membranes were probed with polyclonal antibodies to yeast eEF1A (1:5000 dilution) and Rfa1p (1:1000 dilution, kindly provided by Dr. Steven Brill, Rutgers University) and colorometrically detected for multiple time points by a secondary antibody conjugated to alkaline phosphatase (1:10,000 dilution, Bio-Rad) to assure a linear response.

**Temperature Sensitivity, Translational Fidelity, and Growth of tef2 Strains—**Temperature and cold sensitivity were assayed by growing a strain containing wild-type eEF1A (MC213) or a mutant form of eEF1A as the only form of the protein: D156N (TKY278), N153T (TKY280), and N153T/D156E (TKY282) in YEPD to an A\(_{600}\) of 1.0. Serial 10-fold dilutions (5 \(\mu\)l each) were spotted on YEPD followed by incubation at 13, 23, 30, and 37 \(^\circ\)C for 3–7 days. Phenotypic suppression of the lys2-801 (UAG) mutation was determined by spotting 10 \(\mu\)l of the same dilutions on complete medium lacking lysine and incubating for 2–5 days at 30 \(^\circ\)C. Paromomycin-induced misreading was similarly assayed on complete medium or medium lacking lysine containing 0.5 mg/ml paromomycin. Strains containing a tef5::TRP1 null allele and supported for growth by the plasmid-borne TEF5 (JWY4229), an eEF1B-deficient strain suppressed by wild-type eEF1A (TKY283), or an eEF1B-deficient strain suppressed one of the three eEF1A mutants D156N (TKY279), N153T/D156E (TKY287), or N153T (TKY302) were assayed for growth and fidelity defects as described above. Double times of the eEF1B-deficient strains and the eEF1B-containing parental strain were determined by measuring the growth in liquid culture of at least two independent isolates of each strain. Cultures grown for 1 day in YEPD at 30 \(^\circ\)C were diluted to an A\(_{600}\) of approximately 0.1 in fresh YEPD and grown at 30 \(^\circ\)C with vigorous shaking. Optical density (A\(_{600}\)) was assayed approximately every 2 h. Cultures were diluted into fresh YEPD when the A\(_{600}\) reached mid-log phase (0.4 to 0.6 units) to allow continued monitoring.

**Drug Sensitivity—**2-ml cultures of each strain were grown at 30 \(^\circ\)C in YEPD to mid-log phase. At least two independent colonies were assayed for each mutant allele tested. For each culture 0.3 ml was spread plated onto YEPD plates, and 10 \(\mu\)l of each drug were pipetted onto sterile BBL \(\frac{1}{4}\)-inch diameter-paper discs. The concentrations of drugs used were 1 \(\mu\)g cycloheximide, 25 \(\mu\)g hygromycin B, and 0.4 \(\mu\)g paromomycin. A maximum of two filters were placed on each plate, and the plates were incubated for 2–3 days at 30 \(^\circ\)C. Sensitivity to each drug was measured by the radius of growth inhibition in mm around each disc.

**Nononsense Suppression Assays—**Nonsense suppression assays were
performed on strains containing wild-type (MC214) or one of the mutant forms of eEF1A: N153T (TKY226), D156W (TKY228), and N153T/D156E (TKY229) and the URA3 wild-type lacZ control plasmid pUKC815tail (lacZ under the PGK1 promoter with the PGK1 transcription terminator) or URA3 plasmids with in-frame nonsense codons in lacZ, pUKC819tail (UGA), pUKC817tail (UAU), and pUKC818tail (UAG). The mutant and wild-type strains containing each plasmid were grown overnight at 30 °C in medium lacking uracil to mid-log phase. At least four samples for each strain, and plasmids were analyzed in duplicate using the 2-nitrophenyl-β-D-galactopyranoside assay previously described (19).

In Vivo Methionine Incorporation—Strains containing wild-type eEF1A (MC213) or the mutant forms D156N (TKY278), N153T (TKY280), and N153T/D156E (TKY282) were converted to MET2 by transformation of a polymerase chain reaction fragment containing the wild-type MET2 gene and homologous recombination. Liquid cultures (100 ml) were grown in medium lacking methionine at 30 °C to an A600 of 0.5–0.7. At the zero time point, 50 μl cold methionine and [35S]methionine to a final concentration of 1 μCi/ml (7.9 μCi/ml, 293.0 MBq/ml, NEN Life Science Products) were added to each culture. At 10-min intervals the optical density (A600) of the cultures were determined, and 1-ml aliquots were removed to monitor labeled methionine incorporation by cold trichloroacetic acid precipitation. Ice-cold 50% trichloroacetic acid (0.2 ml) was added to each aliquot, and aliquots were incubated on ice for 10 min, heated to 70 °C for 20 min, and filtered through Whatman GF/C filters. Filters were washed with 10 ml of 5% trichloroacetic acid (4 °C) and 10 ml of 95% ethanol, dried, and counted in a scintillation counter. All time points were analyzed in triplicate.

RESULTS

Mutations were targeted to the N153KMD156 GTP-binding consensus element of yeast eEF1A in an attempt to alter the nucleotide specificity (10). Based on homology to other G-proteins that had successfully been modified for their nucleotide specificity (12, 20–22), as well as the pattern of hydrogen bonds and salt bridges between the GTP moiety and eEF1A, a series of substitutions were prepared in Asn153 and Asp156 (10). Three of the resulting mutants were utilized in a series of in vivo studies to correlate in vitro changes in eEF1A function with in vivo growth and translation effects. These mutant alleles alter the first and last residues of the N153KMD156 element from Asp156 to Asn (N153T, TKY280), and Asn153 to Thr and Asp156 to Glu (D156N, TKY278), and Asn153 to Thr and Asp156 to Gln (D156W, TKY228). The mutant proteins all show increased misincorporation in an in vitro translation assay (10). By utilizing monitors of altered translation and fidelity, we initiated a study to determine the consequences of these mutants in vivo.

Because overexpression of eEF1A can result in changes in cell growth (18) and fidelity, three Western blot analysis was performed to rule out the possibility that the N153KMD156 mutations in eEF1A might result in an enhanced stability or expression. Strains containing chromosomal disruptions of both eEF1A genes (tef1Δ tef2A) were prepared with either plasmid-borne wild-type TEF2 (MC213) or a mutant form of eEF1A, tef2-17 (D156N, TKY278), tef2-19 (N153T, TKY280), or tef2-18 (N153T/D156E, TKY282). Yeast grow at essentially wild-type levels are still below the cellular content of GTP (100 s of μM).

and little or no increase in sensitivity to hygromycin B (Table II). However, all three strains alter sensitivity to paromomycin, either increasing (D156N) or reducing (N153T and N153T/D156E) sensitivity to the compound. This result indicates that these strains may have altered translational fidelity (23, 24). In vitro analysis of the purified mutant proteins indicated that all three increase misincorporation of leucine in the poly(U)-directed polyphenylalanine synthesis assay (10). To determine whether changes in A site fidelity also occurred in vivo, we utilized quantitative reporter constructs to assay for nonsense suppression. Using in-frame lacZ constructs containing one of the three stop codons, nonsense suppression was monitored relative to expression of the wild-type lacZ protein. Fig. 2A shows that all three mutants increase in the level of β-galactosidase activity in a strain containing a nonsense reporter construct. When expressed as the fold wild type read through level for each stop codon (Fig. 2B), it is clear that all three mutations are omnipotent suppressors.

There are several mechanisms that might cause the reduced fidelity at the A site conferred by these mutations. One mechanism in vivo is an increase in the speed of translation. As shown in Fig. 1, this increase would not be due to increased eEF1A protein but would have to result in a change in the activity of the protein. To monitor a general increase in protein synthesis, total methionine incorporation was determined for strains containing either wild-type eEF1A or one of the three NKXD mutant forms of the proteins. Fig. 3 (A and B) indicate that no change in total translation occurs in strains containing the D156N or N153T mutations. The N153T/D156E mutant strain, however, shows a statistically significant increase in the rate of incorporation. (Fig. 3C).

Because these three mutant supports growth even though they show molecular defects in vitro, we were interested in using these mutations to test the hypothesis that mutant forms of eEF1A with reduced nucleotide affinity could suppress the requirement for guanine nucleotide exchange in vivo. This hypothesis is based on our previous finding that although yeast normally require the guanine nucleotide exchange factor eEF1Bα, encoded by the TEF5 gene, the presence of a third copy of an eEF1A gene (either TEF1 or TEF2) allows viability (14). Further, some mutant forms of eEF1A more effectively suppress the requirement for eEF1Bα. Biochemical analysis indicates that all three mutations increase the K0.14 μM for wild-type eEF1A compared with 13.1 μM for D156N, 10.3 μM for N153T/D156E, and 6.0 μM for N153T (10). We hypothesized that although all three mutant forms of eEF1A show a dramatic increase in the Km for nucleotide, these levels are still below the cellular content of GTP (100 s of μM).

Thus, the effect of these mutations to reduce GDP binding would be more dramatic in vivo. This change would reduce the requirement for eEF1Bα and suppress some of the negative

3 N. Durko and T. G. Kinzy, unpublished observation.

FIG. 1. Mutations in the NKXD GTP-binding element of eEF1A do not affect the stability or level of the protein. Strains expressing only the plasmid-borne wild-type TEF2 (MC213) or a mutant allele encoding eEF1A, tef2-17 (D156N, TKY278), tef2-19 (N153T, TKY280), or tef2-18 (N153T/D156E, TKY282) were grown to mid-log phase at 30 °C, and total proteins were extracted. Equal amounts of protein, as determined by Bradford assay, were run on a Laemmli gel, transferred to nitrocellulose, and probed with polyclonal antibodies to Rs1p (as a loading standard, top panel) and yeast eEF1A (bottom panel).
growth effects normally seen when the requirement for eEF1Ba is suppressed by an extra copy of a wild-type eEF1A gene (14).

Plasmids containing a wild-type eEF1A gene or one of the three mutants were prepared on a URA3-based low copy plasmid. The plasmids were placed into a strain of yeast with the chromosomal eEF1B gene (14). Other characteristics of eEF1Bα-deficient strains suppressed by excess eEF1A are cold- and temperature-sensitive growth and sensitivity to translational inhibitors. The D156N and N153T/D156E mutants very effectively suppress the cold-sensitive defect of a strain lacking eEF1Bα, clearly better than wild-type eEF1A or the N153T mutation, the latter of which is 10.3 K for D156N and 10.0 K for N153T/D156E, as derived from poly(U) assays (13, 14). The graphs express the cpm/A∞0 at the indicated time point (t) over the cpm/A∞0 at t0.

An eEF1Ba-deficient strain containing the N153T mutation showed poor suppression of the eEF1Ba requirement (Fig. 4, third panel) and a doubling time of 3.7 h, higher than the 3 h for a strain suppressed by wild-type eEF1A. Thus two mutants with the highest Ks values for GTP (13.1 μM for D156N and 10.3 μM for N153T/D156E), as derived from poly(U) assays using 15 pmol of eEF1A and an Eadie-Hofstee plot (10), best suppress the requirement for eEF1Ba.

Other characteristics of eEF1Ba-deficient strains suppressed by excess eEF1A are cold- and temperature-sensitive growth and sensitivity to translational inhibitors. The D156N and N153T/D156E mutants very effectively suppress the cold-sensitive defect of a strain lacking eEF1Ba, clearly better than wild-type eEF1A or the N153T mutation, the latter of which is dead at 13 °C (Fig. 4, first panel). eEF1Ba-deficient strains

### Table II

**Drug sensivities of strains containing mutations in the NKXD GTP-binding element of yeast eEF1A**

The sensitivity was determined by the radius of inhibition of growth (in mm) surrounding a filter disc containing the indicated drug on a lawn of cells of the indicated strain. The results shown are the averages of three experiments.

| Strain  | Mutation                  | Paromomycin (0.4 mM) | Cycloheximide (1 mM) | Hygromycin (25 mM) |
|---------|---------------------------|----------------------|----------------------|--------------------|
| TKY101  | Wild-type eEF1A           | 0.7                  | 6.3                  | 3                  |
| TKY225  | D156N                     | 3.7                  | 7.7                  | 3.7                |
| TKY229  | N153T/D156E               | 0                    | 8.7                  | 3.3                |
| TKY226  | N153T                     | 0                    | 8.3                  | 3.3                |

**Fig. 2.** Strains containing mutations in the NKXD GTP-binding element of eEF1A show increased suppression of all three stop codons. Strains containing wild-type (MC214) or one of the mutant forms eEF1A, tef2-17 (D156N, TKY225), tef2-19 (N153T/D156E, TKY229), and tef2-19 (N153T, TKY226), and the URA3 wild-type lacZ control plasmid pUKC815tail or a URA3 plasmid with an in-frame nonsense codon in lacZ; pUKC819tail (UGA), pUKC817tail (UAA), and pUKC818tail (UAG) were assayed for β-galactosidase production by a 2-nitrophenyl-β-d-galactopyranoside assay. A shows the β-galactosidase units, and B indicates the fold readthrough of each stop codon relative to the strain expressing wild-type eEF1A.

**Fig. 3.** Strains containing a mutation in the NKXD GTP-binding element show either no effect or a slight increase in total protein synthesis. Strains expressing only the plasmid-borne wild type (MC213, ●) or a mutant form of eEF1A: A, tef2-17 (D156N, TKY278, ■); B, tef2-19 (N153T, TKY280, ○); C, tef2-18 (N153T/D156E, TKY282, ▲) were grown in medium lacking methionine at 30 °C to an A∞0 of 0.5–0.7. 50 mM cold methionine and 1 μCi/ml [35S]methionine (7.9 mCi/ml, 293.0 MBq/ml, NEN Life Science Products) were added to each culture, and both the A∞0 and cold trichloroacetic acid precipitable radioactivity were determined at each time point. The graphs express the cpm/A∞0 at the indicated time point (t) over the cpm/A∞0 at t0.
suppressed by wild-type eEF1A show dramatic increases in sensitivity to translation elongation inhibitors, such as paromomycin, from 0 to 6 mm of inhibition of growth in a lawn growth assay (Table III). The D156N and N153T/D156E mutants not only suppress the growth defects at 30 °C but also restore the drug sensitivity of the eEF1Ba-deficient strain to strain containing either mutant grows at essentially the same rate as the wild-type strain at 37°C (30 °C and diluted to an A600 = 1.0. 5 ml of serial dilutions were spotted onto YEPD plates, followed by incubation at 37 °C (fourth panel), 30 °C (third panel), 24 °C (second panel), and 15 °C (first panel) for 3–7 days.

**DISCUSSION**

The NKXD GTP-binding consensus element is a hallmark of all G-proteins and a site of much of the nucleotide specificity of G-proteins. The three mutations of N153T, D156N, and N153T/D156E all retained the ability to bind GTP, although with a much higher K_m (10). These mutants allow for the in vivo analysis of the effect of reduced nucleotide binding on the requirement for guanine nucleotide exchange. Additionally, because the cellular concentration of GTP is hundreds of micromolar, the K_m for the three mutant forms of eEF1A described are all still an order of magnitude less than the physiological concentration of GTP (25). Thus, these mutants remain functional as the only form of the protein. The function, however, may allow for growth but does not necessarily prevent phenotypes associated with altered translation. These mutants also address the effect of changes in guanine nucleotide binding or GTP-dependent reactions on accurate translation. Strains expressing one of the three mutant alleles show characteristic changes in sensitivity to translation inhibitors consistent with altered elongation, such as a slight increase in sensitivity to cycloheximide and changes in sensitivity to paromomycin. The latter phenotype correlates with altered translational fidelity (19, 23, 24, 26).

Biochemical analysis of these three mutant forms of yeast eEF1A provided evidence that all three result in codon misreading using the in vitro polyphenylalanine synthesis assay. Additional forms of translational fidelity that are assessable in vitro include nonsense suppression and programmed and nonprogrammed ribosomal frameshifting. Previously, mutations in yeast eEF1A have been demonstrated to affect frameshifting at nondirected (17) and directed signals (19, 27). A strain expressing any one of the three mutant alleles shows no change in the ability to retrotranspose the yeast Ty1 element, indicative of no alteration in programmed +1 frameshifting (data not shown). To more closely address misreading at the A site, as is monitored in the leucine misincorporation assay, nonsense suppression was monitored for strains containing a single copy plasmid expressing wild-type eEF1A or one of the three mutations (N153T, D156N, and N153T/D156E). All three mutant strains, while showing normal growth rates, demonstrate increased readthrough of all three stop codons (Fig. 2). These results demonstrate a correlation between changes in translational fidelity in vitro and in vivo. The small differences in the extent of the changes are not surprising because the in vitro system used does not require the exchange factor eEF1B and other potential cellular functions for eEF1A are not a complication. Thus, two A site events in translational fidelity are similarly affected in these mutants, both of which require the proper identification of the A site codon.

The question arises as to the molecular defect in eEF1A resulting in the misreading. The N153T mutation shows a dramatic increase in the intrinsic GTPase activity of eEF1A (from 0.6 to 2.8 pmol phosphate/pmol protein), an effect consistent with eEF1A failing to wait for the GTPase activation signal from the formation of a codon-anticodon pair. The N153T/D156E double mutation shows a statistically significant increase in total protein synthesis (Fig. 3). This is a 2-fold change, but it should be noted that this change is relative to a strain containing a single copy of the eEF1A gene and not the normal complement of two chromosomal eEF1A genes (TEF1 and TEF2). Thus, when the level of eEF1A is slightly lower than normal, this effect may be more dramatic. Models of translational fidelity and rates would predict that increasing the speed of translation would reduce fidelity (28, 29). Consistent with this model, we have also found that an eEF1Ba mutant strain with reduced total translation shows enhanced translational fidelity (18).

The cause of the reduced translational fidelity by the D156N mutant is less apparent, although this mutation differs from the previous two in conferring sensitivity and not resistance to paromomycin. One possibility is that this mutant protein, which shows no difference in stimulation of the GTPase activity in the absence or presence of poly(U) with Phe-tRNA and ribosomes (9.0 pmol phosphate/pmol protein versus 9.3, respectively), is stimulating GTP hydrolysis in a ribosome-dependent manner but without sensing a codon-anticodon interaction. Alternatively, this mutation may affect a function of eEF1A not assayed, such as the affinity for the ribosome or actin binding. Guanine nucleotide binding to Dictyostelium eEF1A decreases the affinity for actin 7–7.5-fold (30), and thus the D156N mutant may alter the equilibrium between free and actin-bound eEF1A.

The altered nucleotide affinity of the three mutants also provides a tool to dissect the requirement for catalyzed guanine nucleotide exchange by the eEF1Ba subunit. The D156N and N153T/D156E mutants share an alteration of Asp^156 and the highest K_m value for GTP. Furthermore, an eEF1Ba-deficient strain containing either mutant grows at essentially the same
rate at 30 °C as cells expressing wild-type eEF1β. These eEF1A mutations, however, do not restore the temperature-sensitive defect of an eEF1β-deficient strain (Fig. 4). Thus, further analysis of mutations, either in eEF1A or other cellular factors, may help illuminate the nature of the temperature-sensitive defect of eEF1β-deficient cells. Perhaps the remaining defect indicates that there is another role for eEF1β. Alternatively, the level of eEF1A expression or activity required for suppression of the requirement for eEF1β may have a negative effect on the cell or one of the other proposed functions of eEF1A such as actin binding (31). Suppression of the requirement for eEF1B is not seen for the N153T mutation. This mutant has the lowest K_m for GTP (other than the wild-type protein); thus there may be a threshold of affinity required for efficient suppression of the need for catalyzed guanine nucleotide exchange. Alternatively, this mutant also shows the lowest V_max (1.1 pmol of Phe compared with 2.4 pmol for wild-type eEF1A), the highest misincorporation in vitro, and the highest intrinsic GTPase. Thus, perhaps the suppression of the requirement for eEF1β requires a form of eEF1A with the ability to release GDP efficiently without compromising eEF1A activity. The ability to dissect the function of a key G-protein such as eEF1A in a strain lacking a key regulatory factor such as the catalyzed guanine nucleotide exchange factor eEF1β is an excellent system to understand G-protein regulation.

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