Cell, Vol. 111, 1015–1025, December 27, 2002, Copyright ©2002 by Cell Press

Uncoupling of Initiation Factor eIF5B/IF2 GTPase and Translational Activities by Mutations that Lower Ribosome Affinity

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Summary

Translation initiation factor eIF5B/IF2 is a GTPase that promotes ribosomal subunit joining. We show that eIF5B mutations in Switch I, an element conserved in all GTP binding domains, impair GTP hydrolysis and general translation but not eIF5B subunit joining function. Intragenic suppressors of the Switch I mutation restore general translation, but not eIF5B GTPase activity. These suppressor mutations reduce the ribosome affinity of eIF5B and increase AUG skipping/leaky scanning. The uncoupling of translation and eIF5B GTPase activity suggests a regulatory rather than mechanical function for eIF5B GTP hydrolysis in translation initiation. The translational defect suggests eIF5B stabilizes Met-tRNA$_i^{Met}$ binding and that GTP hydrolysis by eIF5B is a checkpoint monitoring 80S ribosome assembly in the final step of translation initiation.

Introduction

The GTP binding protein (G protein) superfamily includes Ras, heterotrimeric G proteins, and several translation factors including elongation factor 1A (EF1A/EF-Tu), elongation factor 2 (EF2/EF-G), and initiation factor 2 and its ortholog eukaryotic initiation factor 5B (IF2/eIF5B). These proteins share a common core structure, the guanine-nucleotide binding domain (G domain). Comparisons of G domain structures in their active GTP-bound and inactive GDP-bound states identified two elements designated Switch I and Switch II that undergo marked conformational changes (Sprang, 1997). Switch I is characterized by an invariant Thr residue that helps coordinate the essential Mg^{2+} ion in the active GTP-bound state of the enzyme. Based on studies of Ras, the conserved Thr is thought to be essential for the dynamic properties of Switch I and the regulatory properties of G proteins (Spoerner et al., 2001). In addition, both Switch I and Switch II serve as interaction sites for effectors of G proteins (Sprang, 1997; Vetter and Wittinghofer, 2001).

Most G proteins are thought to function as regulatory switches. In the GTP-bound state, the G protein is active and interacts with downstream effectors, such as Ras interacting with Raf (Sprang, 1997); GTP hydrolysis switches the G protein to its inactive state and disrupts effector interactions. In contrast, mechanochemical or motor functions, in which GTP hydrolysis generates force required for mechanical work, have been proposed for G proteins dynamin and EF2. In the case of dynamin, mutations in Switch I that abolish GTPase activity and presumed associated conformational changes of the protein block endocytosis. This finding led to the idea that dynamin performs a mechanochemical function in vesicle scission (Marks et al., 2001); however, other studies proposed a traditional regulatory role for dynamin (Sever et al., 1999). Elongation factor EF2 was originally considered a classical regulatory GTPase. Binding of EF2•GTP to the ribosome was thought to induce a conformational change and promote ribosomal translocation following peptide bond formation. Subsequent hydrolysis of GTP would enable release of EF2 from the ribosome. However, kinetic analyses revealed that GTP hydrolysis by EF2 precedes ribosomal translocation, and the latter is followed by the release of EF2 (Rodnina et al., 1997). In addition, an interdomain cross-link designed to prevent a conformational change in EF2 impaired translocation, but not GTP hydrolysis (Peske et al., 2000). These findings led to the idea that EF2 is a motor protein that uses GTP-hydrolysis dependent conformational changes to drive ribosomal translocation (Rodnina et al., 1997).

In eukaryotic translation initiation, two G proteins, eIF2 and eIF5B, are necessary to assemble an 80S ribosome in which initiator Met-tRNA$_i^{Met}$ is base-paired with the AUG codon of an mRNA. The active, GTP-bound form of eIF2 binds Met-tRNA$_i^{Met}$ and delivers it to the 40S ribosomal subunit (reviewed in Hinnebusch, 2000). With the assistance of additional factors, this 40S•eIF2•GTP•Met-tRNA$_i^{Met}$ complex binds to an mRNA near the 5′ end and scans down the mRNA. Recognition of an AUG codon triggers GTP hydrolysis by eIF2 and release of the factor from the preinitiation complex. Thus, eIF2 functions as a classic G protein: in the active state, it binds Met-tRNA$_i^{Met}$ and following GTP hydrolysis, it switches conformation and releases the Met-tRNA$_i^{Met}$ to the ribosome. Following release of eIF2, eIF5B promotes joining of the 60S ribosomal subunit. Deletion of the *FUN12* gene encoding yeast eIF5B caused a severe slow-growth phenotype due to impaired translation initiation (Choi et al., 1998); and biochemical studies in mammals revealed that eIF5B promotes subunit joining (Pestova et al., 2000). Substitution of non-hydrolyzable GDPNP for GTP locked eIF5B on the 80S ribosome fol-

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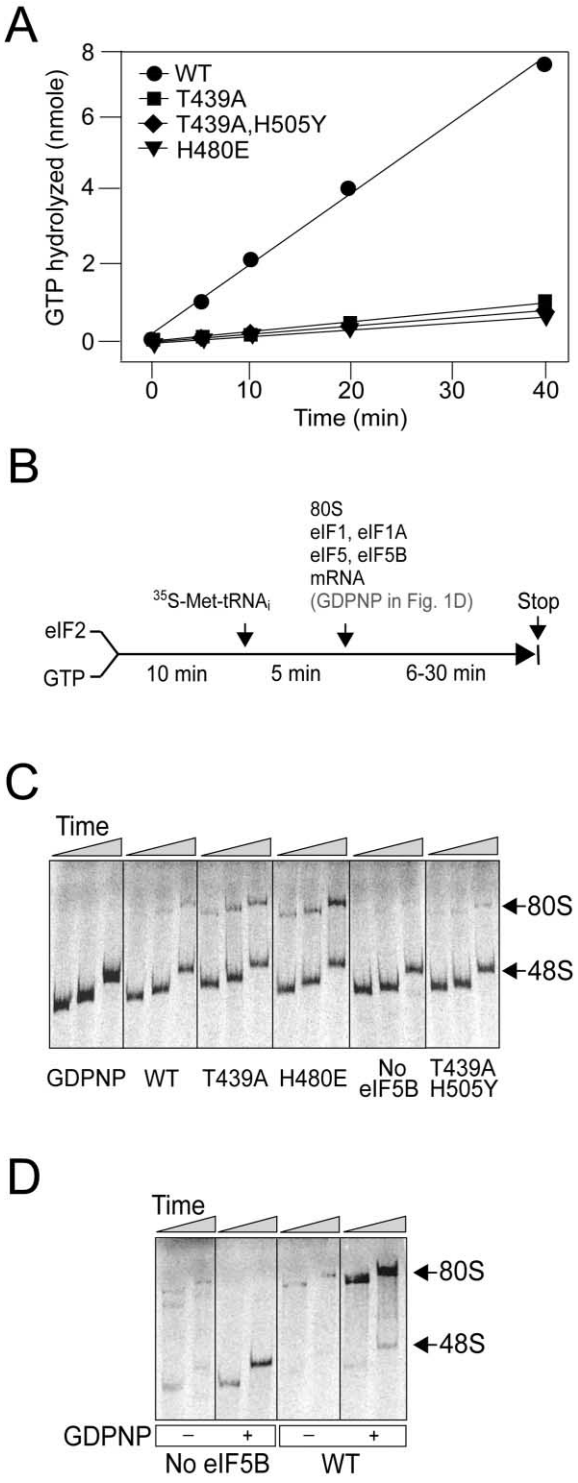


Figure 1. T439A Mutation in Switch I of eIF5B Impairs Ribosome-Dependent GTPase Activity, but Not Subunit Joining

(A) Ribosome-dependent GTPase assays. Equal amounts of purified, recombinant wild-type eIF5B (WT), eIF5B-T439A, eIF5B-T439A,H505Y, and eIF5B-H480E were incubated with [γ-^{33}P]GTP in the presence or absence of purified yeast 80S ribosomes. Aliquots from the reactions were analyzed at various time points by thin-layer chromatography, and the amount of phosphate released was quantified. The values were corrected by subtracting the GTPase activities observed for the various proteins in the absence of ribosomes. The results shown are the average of three independent experiments.

(B) Experimental scheme of the 80S formation assay. As originally described by Algire et al. (2002), eIF2 was mixed with saturating amounts of GTP (0.5 mM) for 10 min to allow exchange of eIF2-bound GDP for GTP, followed by addition of [^{35}S]Met-tRNA$_i^{Met}$. After 5 min incubation to allow ternary complex formation, 80S, eIF1, eIF1A, eIF5, an unstructured model mRNA template (5′-GGAA $(UC)_7UAUG(CU)_{10}C$), and eIF5B (150 nM for wild-type and mutants) were added simultaneously to initiate 80S complex formation. Following incubation at 26°C for up to 30 min, aliquots were mixed with 10× loading dye and loaded directly onto a running 4% polyacrylamide gel. In these assays, subunit joining activity was indirectly assessed by monitoring [^{35}S]Met-tRNA$_i^{Met}$ bound to 48S complexes (40S ribosome, eIF2•GTP•Met-tRNA$_i^{Met}$, mRNA, and additional factors) and 80S complexes.

(C) Phosphorimage of a native gel examining the ability of eIF5B mutants to stimulate 80S complex formation. Progress of 80S complex formation was monitored in reactions containing wild-type eIF5B (WT), eIF5B-T439A, eIF5B-H480E, eIF5B-T439A,H505Y, or no eIF5B by stopping the reactions at 6, 12, and 30 min. In the first image, reactions lacked eIF5B and non-hydrolyzable GDPNP was substituted for GTP in ternary complex formation, resulting in accumulation of the 48S translation initiation intermediate. The staggering of the bands is due to the fact that the samples were loaded at different times onto a running gel. The positions of 80S and 48S complexes are indicated.

(D) GDPNP chase experiment. Reactions containing wild-type or no eIF5B, as indicated, were conducted as described in (B) (note the modified protocol): in the third incubation step GDPNP (10 mM), where indicated, was included at a 50-fold molar excess over the starting GTP concentration (200 μM).

lowing subunit joining, demonstrating that the eIF5B ribosome-dependent GTPase activity is not necessary for subunit joining (Pestova et al., 2000). However, the 80S ribosomes formed in the presence of GDPNP were not competent for translation. Thus, GTP hydrolysis by eIF5B may be required for essential mechanical work to convert the 80S product of subunit joining to a translationally competent state. Alternatively, GTP hydrolysis may function as a regulatory switch necessary for eIF5B release following subunit joining. Consistent with a mechanical role for GTP hydrolysis, studies on bacterial IF2, an ortholog of eIF5B, suggested that GTP hydrolysis facilitated adjustment of Met-tRNA$_f^{Met}$ on the ribosome (La Teana et al., 1996). In addition, kinetic studies indicated that IF2 functions like EF2 with rapid GTP hydrolysis and slow P_i release that is rate-limiting for subsequent steps (Tomsic et al., 2000). Intriguingly, these kinetic analyses challenged the importance and function of GTP binding and hydrolysis by IF2, as these activities did not appear to be critical for translation initiation (Tomsic et al., 2000). Following subunit joining and eIF5B release, translation initiation is completed and the 80S ribosome is poised to begin translation elongation.

The X-ray structure of *M. thermoautotrophicum* (*M. therm.*) eIF5B revealed a four domain protein resembling a molecular chalice with the first three domains forming the cup of the chalice and connecting through a long α helix to domain IV, the base of the chalice (Roll-Mecak et al., 2000). The G domain (domain I) and the closed antiparallel β barrel domain II of eIF5B are structurally homologous to the G and second domains, respectively, of elongation factors EF1A and EF2 (Roll-Mecak et al.,

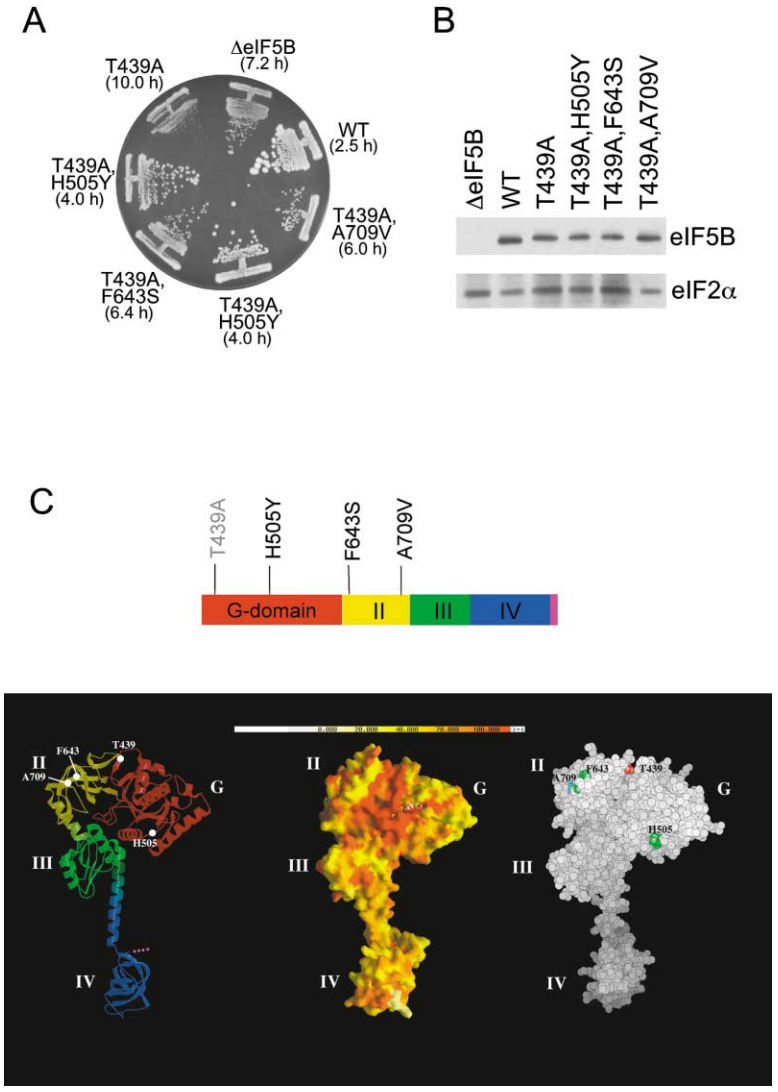


Figure 2. Intragenic Suppressors of the eIF5B-T439A Mutant

(A) Growth rate analysis of yeast expressing various forms of eIF5B. The ΔeIF5B strain J130 was transformed with the plasmid YCplac33 (ΔeIF5B), or the same plasmid containing the indicated wild-type (WT) or mutant eIF5B genes. Transformants were streaked on SD medium supplemented with the required nutrients and incubated at 30°C for 7 days. The doubling times during exponential growth in liquid SD medium are shown in parentheses.

(B) Western blot analysis of eIF5B expression. Whole-cell extracts prepared from transformants described in (A) were subjected to immunoblot analysis using anti-eIF5B or anti-eIF2α antiserum, as described previously (Choi et al., 2000). Immune complexes were visualized using enhanced chemiluminescence.

(C) Location of Switch I and suppressor mutations in eIF5B. Upper image: Schematic of N-terminally truncated eIF5B. The GTP binding domain and domains II, III, and IV are labeled and shaded to reflect the color of the domains in the structure presented in the lower image. Lower image: Structural model of yeast eIF5B, using the *M. therm*. eIF5B X-ray structure as a template. *M. therm*. eIF5B and the C-terminal region of *S. cerevisiae* eIF5B (residues 397–1002) share 35% sequence identity, suggesting a common 3-D structure (Sander and Schneider, 1991). Left: Ribbons diagram of yeast eIF5B (nucleotide binding view). The four domains are labeled and color coded as follows: GTP binding domain (red), domain II (yellow), domain III (green), and domain IV (blue). Due to weak amino acid sequence similarity two α helices at the C terminus of the protein, following domain IV, could not be modeled as indicated by the magenta dots. The locations of Switch I and suppressor mutations are labeled. Middle: Surface representation of the yeast eIF5B model, color coded according to amino acid conservation (on a gradient < 40% identity, white; < 70%, yellow; 100%, red). Right: Atom representation of the yeast eIF5B model with the T439 Switch I residue colored red, the H505 and F643 residues shaded green, and the A709 residue in blue.

2000, 2001). Comparison of the structures of the GTP- and GDP-bound forms of eIF5B revealed that modest active site conformational changes upon GTP binding are amplified by an articulated lever mechanism (Roll-Mecak et al., 2000). The strategy used by eIF5B to amplify these small conformational changes has surprising similarities to that used by the motor myosin and raised the possibility that eIF5B might use the energy of GTP hydrolysis to perform mechanical work on the ribosome. Alternatively, these domain movements may be necessary to switch eIF5B from an active state with high ribosome affinity to an inactive state that dissociates from the ribosome. To distinguish between mechanical and regulatory roles for eIF5B, we conducted a mutational and biochemical analysis of eIF5B. Our results indicate that the energy of GTP hydrolysis is not critical for essential mechanical functions of eIF5B, but instead activates a regulatory switch required for eIF5B release from the ribosome following subunit joining.

Results

Mutation of the Conserved Thr in Switch I of eIF5B Impairs Cell Growth

Switch I residues E_{434}AGGIT_{439}Q of yeast eIF5B including the universally conserved Thr-439 were individually mutated to Ala. As the poorly conserved N terminus of yeast eIF5B is not required in vivo or in vitro (Choi et al., 2000), an N-terminally truncated form of eIF5B (residues 396–1002) was utilized for all of the studies in this report. Plasmid-borne wild-type and Switch I mutant versions of eIF5B were introduced into a ΔeIF5B strain lacking the *FUN12* gene encoding yeast eIF5B. Yeast expressing an eIF5B-T439A (Thr-439 to Ala) mutant as the sole source of eIF5B exhibited a severe slow-growth phenotype with a doubling time 4-fold higher than wild-type (10 hr versus 2.5 hr). Mutations in the residues flanking Thr-439 were without effect (G436A, G437A) or caused a less severe slow-growth phenotype (E434A, I438A). Interestingly,

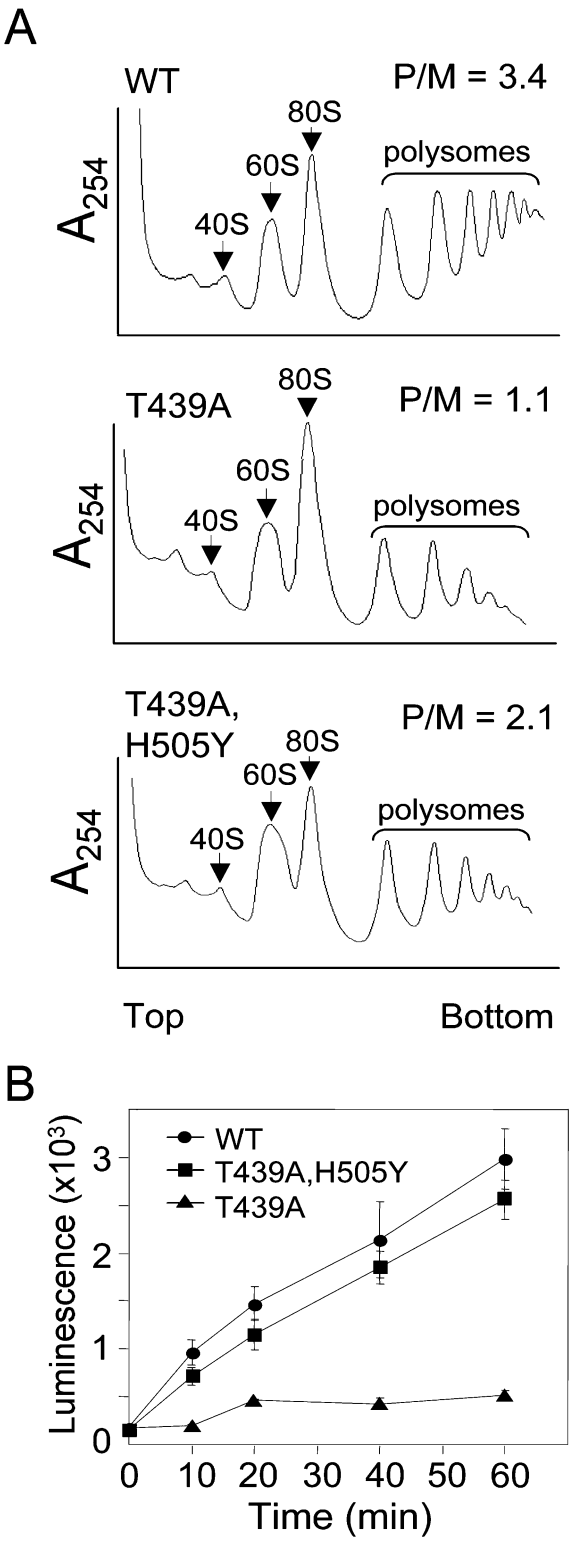


Figure 3. Impaired Translation in Yeast Expressing eIF5B-T439A and Restoration of Translational Activity by the eIF5B Suppressor Mutation

(A) Analysis of polysome profiles in wild-type, eIF5B-T439A, and eIF5B-T439A,H505Y strains. Whole-cell extracts from yeast strain J130 expressing the indicated eIF5B wild-type (WT) or mutant protein were resolved by velocity sedimentation in 7 to 47% sucrose gradients as described by Asano et al. (2000). Gradients were fractionated while scanning at A_{254}, and the positions of the 40S and 60S subunits, 80S ribosomes, and polysomes are indicated. Ratios of polysomes to monosome (P/M) were calculated by measuring the area in the combined polysome fractions and in the 80S peak. (B) Analysis of translation of an electroporated luciferase reporter mRNA. Yeast strain J130 expressing wild-type eIF5B (WT), eIF5B-T439A, or eIF5B-T439A,H505Y, as indicated, was electroporated with in vitro synthesized, capped, and polyadenylated luciferase mRNA. Cells were incubated at 30°C and assayed for luciferase activity at the indicated time points as described by Masison et al. (1995). The luciferase activity was calculated as luminescence units per μg of total protein, and the results shown are the average of three independent experiments.

yeast expressing eIF5B-T439A grew more slowly than cells lacking eIF5B (see Figure 2A). Thus, the eIF5B-T439A mutant is not simply inactive, but interferes with an eIF5B-independent translation initiation pathway operative in ΔeIF5B cells.

Switch I T439A Mutation Impairs the GTPase Activity, but Not the Ribosome Joining Function of eIF5B

Biochemical analyses revealed that the T439A mutation did not impair GTP or GDP binding to eIF5B (data not shown). In contrast, the ribosome-dependent GTPase activity of eIF5B-T439A was below background levels (Figure 1A). Likewise, an eIF5B-H480E mutation, which alters a conserved Switch II residue corresponding to the catalytically important Gln-61 of human Ras, reduced the GTPase activity to below background levels (Figure 1A).

To analyze the subunit joining activity of wild-type and mutant forms of eIF5B, we used the reconstituted yeast translation initiation system of Algire et al. (2002) (Figure 1B). In the absence of eIF5B, few 80S complexes were formed (Figure 1C, fifth image) consistent with the role of eIF5B to promote ribosomal subunit joining. Incubation of eIF2 with non-hydrolyzable GDPNP in place of GTP also blocked 80S complex formation (Figure 1C, first image) consistent with the requirement for GTP hydrolysis by eIF2 prior to subunit joining. Addition of wild-type eIF5B enhanced the rate of ribosomal subunit joining at least 3–10-fold (Figure 1C, and data not shown). Addition of eIF5B-T439A or eIF5B-H480E greatly stimulated observable 80S complex formation (Figure 1C). As both eIF5B-T439A and eIF5B-H480E lacked detectable GTPase activity, these proteins may promote the 80S joining step and then fail to be released from the ribosome. Accordingly, the presence of eIF5B on the 80S ribosome may stabilize Met-$tRNA_i^{Met}$ binding. These results are consistent with the previous observation that GTP hydrolysis is not required for eIF5B-catalyzed ribosomal subunit joining in mammals (Pestova et al., 2000).

To test the idea that the lower levels of 80S complex formed with wild-type eIF5B versus eIF5B-T439A reflected the release of wild-type eIF5B and the Met-$tRNA_i^{Met}$ from the ribosome following GTP hydrolysis, we modified the protocol for the 80S joining assay. In the first step of the assay eIF2 was incubated with GTP, then a 50-fold molar excess (relative to GTP) of non-

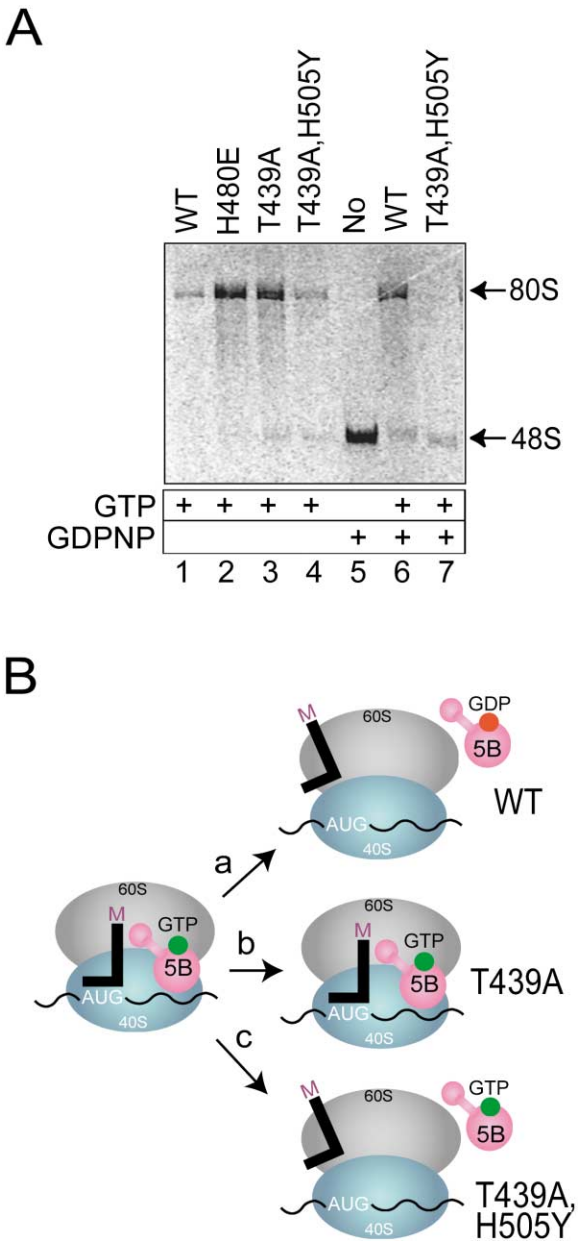


Figure 4. Analysis of Subunit Joining Activity of eIF5B-T439A,H505Y

(A) GDPNP chase experiment. Lanes 1–4: 80S formation assays were conducted as described in Figure 1B using the indicated eIF5B wild-type and mutant proteins. Reactions were incubated for 30 min at 26°C following addition of ribosomes and accompanying factors. Lane 5: Non-hydrolyzable GDPNP substituted for GTP throughout the reaction and no eIF5B was added. Thus, 48S complexes accumulate and there is no subunit joining. Lanes 6–7: Reactions were conducted as described in Figure 1B (note the modified protocol): in the third incubation step GDPNP (10 mM) was included at a 50-fold molar excess over the starting GTP concentration (200 μM). The positions of 48S and 80S complexes are indicated.

(B) Model to explain the results of the subunit joining experiments: stabilization of Met-tRNA$_i^{Met}$ binding to 80S ribosomes by eIF5B. Wild-type eIF5B, eIF5B-T439A, and eIF5B-T439A,H505Y all promote subunit joining; however, the fate of eIF5B, GTP, and Met-tRNA$_i^{Met}$ differs for the three proteins. Following subunit joining, Met-tRNA$_i^{Met}$ is bound to the ribosomal P site and eIF5B is bound near the GTPase activating center of the ribosome with domain IV traversing across the top of the A site (Left). ("a") GTP hydrolysis by wild-type eIF5B results in release of the factor from the ribosome, and Met-tRNA$_i^{Met}$ binding is unstable. We propose that Met-tRNA$_i^{Met}$ binding is stabilized in vivo by rapid binding of the first elongator tRNA. ("b") eIF5B-T439A (or eIF5B-H480E) is unable to hydrolyze GTP and the factor is locked on the ribosome where it stabilizes Met-tRNA$_i^{Met}$ binding. ("c") eIF5B-T439A,H505Y is unable to hydrolyze GTP; however, the mutations reduce the ribosomal affinity and the factor is released in the absence of GTP hydrolysis. Accordingly, Met-tRNA$_i^{Met}$ binding to the 80S complex is unstable as proposed in ("a") following release of wild-type eIF5B.

hydrolyzable GDPNP was added together with eIF5B (Figure 1B). Addition of GDPNP enhanced by ~9-fold the amount of 80S complexes observed with wild-type eIF5B (Figure 1D). This result is consistent with the idea that locking eIF5B on the ribosome by blocking GTP hydrolysis, stabilizes Met-tRNA$_i^{Met}$ binding. In assays lacking eIF5B, addition of GDPNP resulted in accumulation of 48S complexes (Figure 1D). As GTP hydrolysis by eIF2 is required for its release, the GDPNP locks eIF2 and Met-tRNA$_i^{Met}$ on the 48S complex.

Isolation of Intragenic Suppressors of the eIF5B-T439A Mutation

To gain further insight into the roles of Switch I and GTP hydrolysis for eIF5B function, we screened for intragenic suppressors of the eIF5B-T439A mutation. Three independent suppressor mutations were identified (Figure 2A): His-505 to Tyr (H505Y) in the G domain, and Phe-643 to Ser (F643S), and Ala-709 to Val (A709V) in domain II. The H505Y mutation was the most effective suppressor; however, all three suppressors conferred better growth rates than the vector control (Figure 2A). As shown in Figure 2B, eIF5B-T439A and the suppressor eIF5B-T439A,H505Y were expressed to the same level, similar to wild-type eIF5B.

The location of the suppressor mutations were mapped on the structure of eIF5B. Using the X-ray structure of eIF5B from *M. therm*. as a template, a model of yeast eIF5B (Figure 2C) was determined using the program MODELLER (Sali and Blundell, 1993). The three suppressor mutations H505Y, F643S, and A709V map to the ventral surface of eIF5B (Figure 2C, lower right image), the same side as the GTP binding pocket. Interestingly, residues on the ventral face of the G domain and domain II of eIF5B are among the most highly conserved residues in this factor (Figure 2C, lower middle image). Cryoelectron microscopic (cryo-EM) images of EF1A (Stark et al., 1997), EF2 (Agrawal et al., 1998), and eukaryotic eEF2 (Gomez-Lorenzo et al., 2000) bound to the ribosome revealed that the ventral surfaces of domains I and II of these GTPases forms the ribosome binding interface for these factors. Assuming a similar ribosome binding interface for eIF5B (given the structural similarities between these regions of eIF5B, EF1A, and EF2; Roll-Mecak et al., 2000, 2001), we propose that the H505Y, F643S, and A709V mutations suppress the eIF5B-T439A mutation by altering the ribosome binding properties of eIF5B.

Switch I Suppressor Mutation Restores General Translation, but Not eIF5B GTPase Activity

The H505Y mutation, the most effective suppressor of the eIF5B-T439A growth defect (Figure 2A), was se-

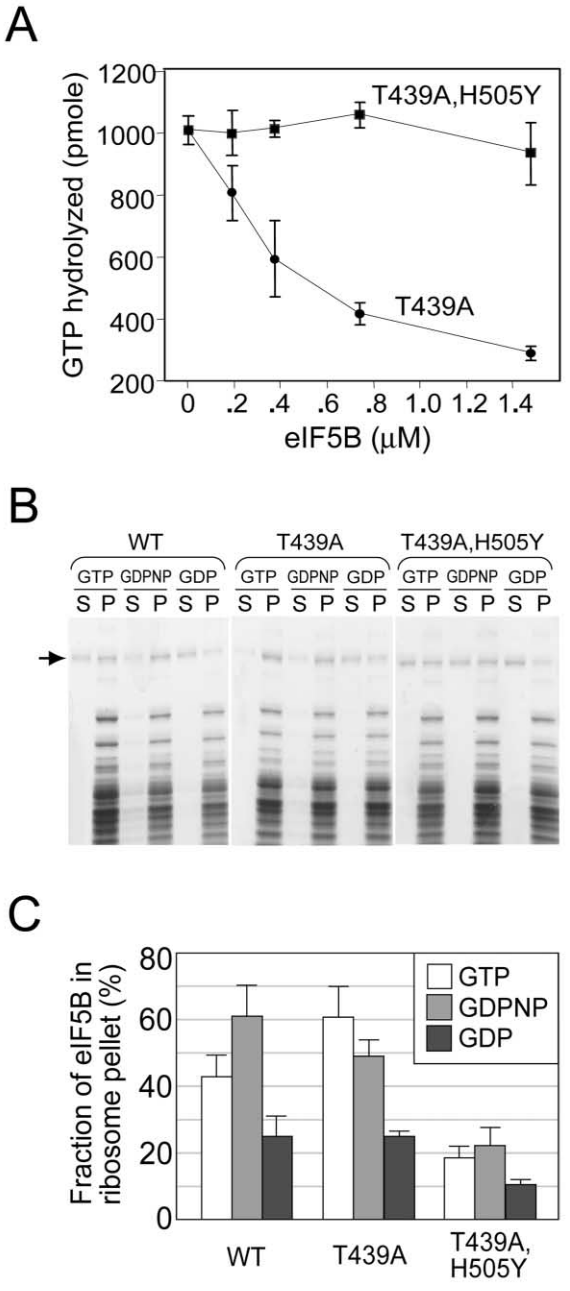


Figure 5. Reduced Ribosomal Binding Affinity of the eIF5B-T439A,H505Y Mutant

(A) eIF5B GTPase competition assay. Increasing amounts of purified eIF5B-T439A or eIF5B-T439A,H505Y, as indicated, were added to GTPase reactions containing [γ-^{32}P]GTP, limiting amounts of 80S ribosomes (2 pmol), and saturating amounts of purified wild-type eIF5B (7.4 pmol). Reactions were incubated at 30°C for 30 min, and the extent of GTP hydrolysis was monitored by thin-layer chromatography and quantified using a phosphoimager. The results shown are the average of three independent experiments.

(B) Ribosome binding assay. Purified eIF5B (WT), eIF5B-T439A, or eIF5B-T439A,H505Y was mixed with purified yeast 80S ribosomes in the presence of GTP, GDPNP, or GDP as indicated and then loaded on a 10% sucrose cushion. Following centrifugation the supernatant (S) and ribosomal pellet (P) fractions were analyzed by SDS-PAGE. The arrow marks the position of eIF5B, the lower molecular weight bands are ribosomal proteins.

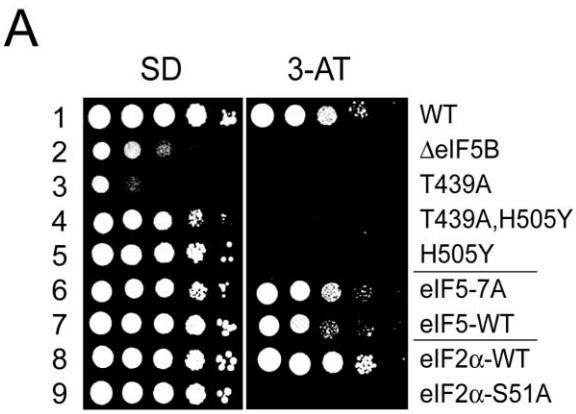
(C) The amount of eIF5B recovered in the supernatant and pellet fractions was determined by quantitative densitometry, and the fraction of total recovered eIF5B present in the ribosomal pellet was calculated. The data presented are the average of at least three independent experiments.

lected for more in depth analysis. The H505Y mutation failed to suppress the H480E mutation indicating that H505Y is a specific suppressor of Switch I mutations in eIF5B (data not shown). In addition, yeast expressing eIF5B-H505Y grew like wild-type (see Figure 6A). As the T439A mutation impaired the ribosome-dependent GTPase activity of eIF5B (Figure 1A), the simplest mechanism of suppression would be for the H505Y mutation to restore GTPase activity. However, as shown in Figure 1A, eIF5B-T439A,H505Y lacked detectable GTPase activity. In addition, eIF5B-T439A,H505Y bound GTP and GDP with similar affinities as wild-type eIF5B and eIF5B-T439A (data not shown). Thus, the H505Y mutation suppressed the growth defect associated with the eIF5B-T439A mutation without restoring GTPase activity.

Two assays were employed to examine the effects of the eIF5B mutations on general translation in yeast. Polysome profile analysis revealed that the eIF5B-T439A mutation resulted in a severe loss of polysomes and corresponding increase in inactive 80S monosomes (compare polysome/monosome ratio of 3.4 for the wild-type strain to 1.1 for the eIF5B-T439A strain; Figure 3A). Thus, the translation defect caused by the eIF5B-T439A mutation affected the majority of mRNAs in the cell and not simply a few key growth-regulatory mRNAs. Consistent with the notion that the H505Y suppressor mutation restored the ability of eIF5B-T439A to promote general translation, the polysome to monosome ratio in the suppressor strain increased nearly 2-fold (2.1 for eIF5B-T439A,H505Y versus 1.1 for eIF5B-T439A; Figure 3A). A second test of general translational activity was to monitor expression from an electroporated luciferase reporter mRNA. The eIF5B-T439A mutation impaired translation of the luciferase mRNA (Figure 3B), and the H505Y suppressor mutation restored translational activity to near wild-type levels (Figure 3B). Based on these results, we conclude that eIF5B-T439A,H505Y promotes general translation initiation in vivo despite severely impaired GTPase activity.

eIF5B-T439A,H505Y Promotes Ribosomal Subunit Joining Similar to Wild-Type eIF5B

We next examined the ability of eIF5B-T439A,H505Y to promote ribosomal subunit joining. As shown in Figure 4A, the amount of 80S complexes formed with eIF5B-T439A,H505Y was similar to that obtained with wild-type eIF5B and substantially ($\sim$2.5-fold) less than those formed in the presence of eIF5B-H480E or eIF5B-T439A. In addition, wild-type eIF5B and eIF5B-T439A,H505Y catalyzed 80S formation at the same rate (Figure 1C), indicating that the GTPase activity of eIF5B does not accelerate the observed rate of subunit joining. Because eIF5B-T439A,H505Y and eIF5B-T439A lacked detectable GTPase activity, yet behaved differently in the 80S complex formation assay, we postulated that the H505Y mutation facilitated release of eIF5B from the ribosome



B

GCN4-lacZ expression

	R	DR	R
WT	10	125	27
ΔeIF5B	8	7	154
T439A	6	5	150
T439A,H505Y	2	3	68
H505Y	2	7	48

Figure 6. Impaired *GCN4* Translational Control in eIF5B Mutant Strains

(A) Three sets of isogenic strains were grown to saturation and 4 μl of serial dilutions (of OD_{600} = 1.0, 0.1, 0.01, 0.001, and 0.0001) were spotted on minimal medium supplemented with essential nutrients (SD) or medium containing 3-aminotriazole (3-AT). Plates were incubated 4 days at 30°C. The strains in rows 1–5 are derivatives of the ΔeIF5B strain J130 transformed with empty vector (ΔeIF5B) or plasmids expressing wild-type eIF5B (WT), eIF5B-T439A, eIF5B-T439A,H505Y, or eIF5B-H505Y, as indicated. The strains in rows 6–7 are *GCN2*$^+$ derivatives of strains KAY36 (*tif5-FL-7A*; eIF5-7A) and KAY39 (*TIF5-FL*; eIF5-WT) described previously (Asano et al., 1999). The strains in rows 8–9 are *GCN2*$^+$ derivatives of strains H1816 (eIF2α-WT) and H1817 (eIF2α-S51A) described previously (Dever et al., 1993).

(B) Analysis of *GCN4-lacZ* expression. The wild-type *GCN4-lacZ* plasmid p180 (Hinnebusch, 1985) or a derivative in which an extended version of uORF1 overlaps the *GCN4* AUG start codon (pM226, Grant et al., 1994) were introduced into derivatives of strain J130 expressing wild-type eIF5B (WT), the indicated eIF5B mutant, or no eIF5B (ΔeIF5B). Cells were grown and β-galactosidase activities were determined as described previously (Hinnebusch, 1985), except that longer growth periods were required to obtain sufficient quantities of cells from the slow-growing ΔeIF5B and eIF5B-T439A strains. R, cells were grown under nonstarvation conditions in SD minimal medium where *GCN4* expression is repressed; DR, cells were grown under amino acid starvation conditions (SD + 10 mM 3-aminotriazole) where *GCN4* expression is derepressed. The β-galactosidase activities are the averages of three to six independent transformants and have standard errors of 30% or less.

in the absence of GTP hydrolysis. Accordingly, eIF5B-T439A,H505Y failed to stabilize Met-tRNA$_i^{Met}$ binding following subunit joining like eIF5B-T439A and thus appeared to function like wild-type eIF5B (Figure 4B). In order to test whether eIF5B-T439A,H505A can be released from the ribosome in the absence of GTP hydrolysis, we examined this mutant in the 80S joining assay using the modified GDPNP-chase protocol (Figure 1B). In contrast to the results with wild-type eIF5B, addition of GDPNP did not stabilize 80S complexes formed by eIF5B-T439A,H505Y (Figure 4A, compare lane 4 versus 7 and lane 1 versus 6). The increased levels of 48S complexes in assays containing eIF5B-T439A,H505Y likely reflects Met-tRNA$_i^{Met}$ rebinding to eIF2•GDPNP complexes.

Reduced Ribosomal Binding Affinity of eIF5B-T439A,H505Y

As both eIF5B-T439A and eIF5B-T439A,H505Y lacked GTPase activity (Figure 1A) and overexpression of these proteins impaired the growth of yeast expressing wild-type eIF5B (data not shown), we hypothesized that the eIF5B mutants competed with wild-type eIF5B for binding to the ribosome. We used a GTPase competition assay to indirectly assess the ribosome binding affinity of the eIF5B mutants. The GTPase assays contained a limiting amount of ribosomes, saturating levels of wild-type eIF5B, and increasing amounts of eIF5B-T439A or eIF5B-T439A,H505Y. Addition of eIF5B-T439A, but not eIF5B-T439A,H505Y, effectively blocked the ribosome-stimulated GTPase activity of wild-type eIF5B (Figure 5A). These results are consistent with the idea that the H505Y suppressor mutation lowers the ribosome binding affinity of eIF5B.

To directly assess ribosome binding affinity, wild-type and mutant forms of eIF5B were mixed with 80S ribosomes in the presence of GDP, GTP, or GDPNP and then analyzed using a sucrose cushion assay. Both wild-type eIF5B and eIF5B-T439A bound strongly to ribosomes in the presence of GTP or GDPNP, whereas the binding was significantly reduced in assays containing GDP or no nucleotide (Figures 5B and 5C, and data not shown). The binding of wild-type eIF5B to ribosomes was highest in assays containing non-hydrolyzable GDPNP suggesting that reduced binding in the presence of GTP was due to nucleotide hydrolysis during the assay. The ribosome binding affinity of eIF5B-T439A,H505Y was reduced under all conditions compared to wild-type eIF5B and eIF5B-T439A. Moreover, the binding of eIF5B-T439A,H505Y to ribosomes in the presence of GTP was comparable to the low level binding observed by wild-type eIF5B and eIF5B-T439A in the presence of GDP (Figure 5C). These results indicate that the H505Y mutation lowers the ribosome binding affinity of eIF5B, and they are consistent with the idea that this mutation enables eIF5B release from the ribosome following subunit joining in the absence of GTP hydrolysis.

Impaired *GCN4* Translational Control Response in Yeast Expressing eIF5B Alleles Lacking GTPase Activity

The observation that the eIF5B-T439A,H505Y mutant restored general translation yet lacked GTPase activity is inconsistent with a crucial mechanochemical role for the factor. Therefore, we sought to identify a regulatory role for eIF5B. If GTP hydrolysis and ribosomal release by eIF5B insured proper function in the translation initia-

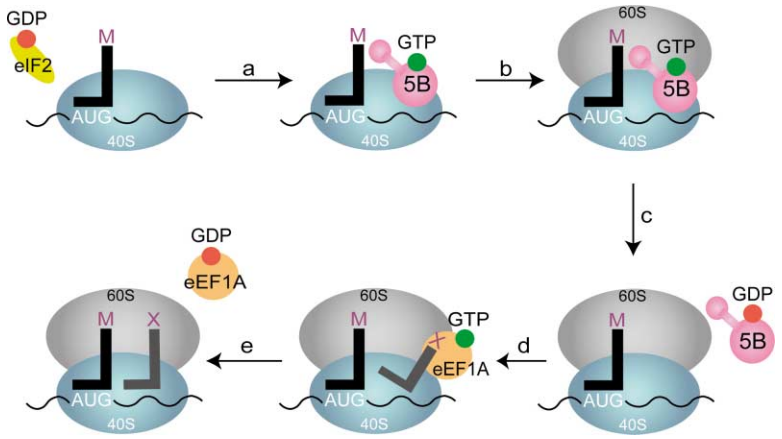


Figure 7. Model Depicting the eIF5B GTPase Regulatory Switch in Translation Initiation

Following GTP hydrolysis, eIF2 is released from the 40S ribosomal subunit leaving the Met-tRNA$_i^{Met}$ base-paired to the AUG codon in the P site. ("a") eIF5B binds to this complex, likely traversing the top of the A site, and possibly contacting the Met-tRNA$_i^{Met}$. ("b") Binding of eIF5B promotes 60S subunit joining and formation of an 80S complex. ("c") Proper subunit joining triggers GTP hydrolysis by eIF5B, and the GDP form of eIF5B is then released due to decreased ribosomal affinity. According to our data from the 80S formation assay, the Met-tRNA$_i^{Met}$ in the P site of the 80S ribosome is unstable following eIF5B release. ("d") We propose that the rapid binding of the first elongating tRNA species, as part of an eEF1A•GTP•aminoacyl-tRNA ternary complex, to the A site stabilizes the Met-tRNA$_i^{Met}$ in the P site. ("e") Following GTP hydrolysis, eEF1A is released.

tion pathway, then it might be expected that yeast expressing eIF5B-T439A,H505Y would display a defect in translational accuracy or fidelity despite the ability of the mutant protein to restore general translational activity. Translation of the yeast *GCN4* mRNA, encoding a transcriptional activator of amino acid biosynthetic enzymes genes, is sensitive to perturbations in general translation initiation (Hinnebusch, 1996, 2000). Phosphorylation of eIF2α, or mutations that lower eIF2 activity, derepress *GCN4* expression enabling yeast to grow under histidine starvation conditions imposed by the drug 3-aminotriazole (3-AT). In contrast, cells expressing non-phosphorylatable eIF2α or lacking eIF5B cannot derepress *GCN4* expression and thus fail to grow on medium containing 3-AT (Figure 6A, compare rows 1, 2, 8, and 9). Likewise, cells expressing eIF5B-T439A, eIF5B-T439A,H505Y, or eIF5B-H505Y failed to grow under starvation conditions (Figure 6A, rows 3–5). Interestingly, eIF5B-H505Y lacked detectable ribosome-dependent GTPase activity (data not shown). Thus, the inability of these three mutants to support growth on 3-AT medium correlates with the loss of eIF5B GTPase activity. A mutation in the translation initiation factor eIF5 that caused a similar partial impairment of growth on SD medium as observed for the eIF5B-T439A,H505Y mutant showed no growth defect under starvation conditions (Figure 6A, row 6). Thus, the impaired growth under starvation conditions is specifically linked to lack of eIF5B GTPase activity. A *GCN4-lacZ* reporter was used to quantify *GCN4* expression. In wild-type cells, *GCN4-lacZ* expression was low under nutrient rich conditions and increased approximately 12-fold under amino acid starvation conditions (Figure 6B). This high level expression of *GCN4-lacZ* under starvation conditions was blocked in strains lacking eIF5B or expressing eIF5B-T439A, eIF5B-T439A,H505Y, or eIF5B-H505Y (Figure 6B), consistent with the inability of these mutant strains to grow on 3-AT medium.

The *GCN4* mRNA contains four short upstream open reading frames (uORFs) in its leader that restrict the access of ribosomes to the authentic *GCN4* start codon. Translational control of *GCN4* is dependent on ribosomes translating the first uORF followed by regulated reinitiation at the subsequent uORFs (Hinnebusch, 1996). Failure to translate uORF1 impairs *GCN4* expression (Hinnebusch, 1996). We used a mutated *GCN4-lacZ* construct in which uORF1 has been moved and its stop codon mutated such that uORF1 overlaps the *GCN4* ORF to assess the efficiency of translation initiation at the uORF1 AUG codon (Figure 6B, right image). On this mutant *GCN4-lacZ* mRNA ribosomes will scan the leader, translate uORF1, and then terminate 3′ of the *GCN4* AUG start codon. As ribosomes cannot extensively scan in a 3′ to 5′ direction to access the *GCN4* start codon, *GCN4-lacZ* expression was low in strains expressing wild-type eIF5B (Figure 6B). In strains lacking eIF5B or expressing eIF5B-T439A, eIF5B-T439A,H505Y, or eIF5B-H505Y, *GCN4-lacZ* expression from this uORF1-extended construct was increased approximately 2–5-fold (Figure 6B). This suggests a 2–5-fold increase in leaky scanning (ribosomes scanning over the uORF1 start codon without initiating translation) in the eIF5B mutant strains. Thus, the lack of GTPase activity and enhanced dissociation of the eIF5B-T439A,H505Y mutant from the ribosome following subunit joining impairs the efficiency of translation initiation (as revealed by an increase in leaky scanning) and indicates that GTP hydrolysis and subsequent release of eIF5B in the final step of translation initiation may serve as a checkpoint to insure proper 80S complex assembly.

Discussion

GTPase Regulatory Switch in eIF5B Modulates Ribosome Binding

Whereas most G proteins are thought to function as regulatory switches, recent studies suggested mechanochemical functions for the GTPases dynamin and EF2. The results of this study demonstrate that GTP hydrolysis by eIF5B is essential for translation initiation; however, this requirement is overcome when eIF5B contains mutations that lower its ribosomal affinity. We propose that GTP binding and hydrolysis by eIF5B fulfills a regulatory function governing ribosome binding by the factor. The eIF5B-T439A,H505Y mutant lacked detectable

GTPase activity, but promoted near wild-type growth and in vivo translational activity. Further biochemical analysis of this mutant revealed that the H505Y suppressor mutation lowers ribosome affinity, supporting the idea that GTP hydrolysis by eIF5B is required to release the factor from the ribosome following subunit joining. Finally, the defect in *GCN4* translational regulation and enhanced leaky scanning in strains expressing eIF5B-T439A,H505Y indicates that the GTPase activity of eIF5B is necessary for efficient subunit joining at AUG codons. A simple, cyclic model for eIF5B function can be proposed: eIF5B•GTP binds to the 40S subunit and promotes 60S joining, subsequent GTP hydrolysis switches eIF5B to a conformation with low ribosomal affinity, and the factor dissociates from the ribosome (Figure 7, steps "a–c"). Accordingly, the primary function of the domain movements in eIF5B that accompany GTP hydrolysis is to modulate the ribosomal affinity of the factor.

A recent kinetic analysis by Tomsic et al. (2000) revealed that IF2 hydrolyzes GTP rapidly upon ribosome binding and P_i release is slow. In addition, it was concluded that IF2 GTPase activity was not required for IF2 release following subunit joining. These results led to speculation that IF2 does not follow the switch mechanism observed for many GTPases, and that the IF2 GTPase activity is required for a function distinct from protein synthesis. Our data demonstrate that the GTPase activity of eIF5B is critical for protein synthesis. The T439A mutation impaired GTPase activity and caused a severe defect in general protein synthesis (Figure 3). The reasons for the discrepancy between our data and those of Tomsic et al. (2000) are not yet clear but could be because, as they suggested, the GTP hydrolysis-dependent step was not rate limiting in their in vitro assays. Alternatively, it is possible that the functions of eIF5B and IF2 are distinct.

Two of our results strongly indicate that GTP hydrolysis is required for release of eIF5B from the ribosome. First, eIF5B-H480E and eIF5B-T439A, which are defective GTPases, function better than wild-type eIF5B in the 80S formation assay (Figures 1C and 4A). We propose that these mutant forms of eIF5B are defective for release from the ribosome and thus stabilize or protect the Met-$tRNA_i^{Met}$ in the 80S complex. Our results agree with the studies of Pestova et al. (2000) showing that eIF5B is locked on the 80S ribosome following subunit joining in reactions containing GDPNP. Similarly, Luchin et al. (1999) reported that an H448E mutation in *E. coli* IF2, which corresponds to the eIF5B-H480E mutant, severely impairs GTPase activity and release of the factor from the ribosome following subunit joining. Interestingly, our proposal that eIF5B stabilizes Met-$tRNA_i^{Met}$ binding to the ribosome is consistent with the finding that the slow-growth phenotype of yeast strains lacking eIF5B is partially suppressed by overexpression of Met-$tRNA_i^{Met}$ (Choi et al., 1998). Second, the H505Y mutation, which suppressed the growth and general translation defects of the eIF5B-T439A mutant, lowered the ribosomal affinity of eIF5B (Figure 5). We propose that eIF5B-T439A,H505Y is functional in protein synthesis because the factor binds normally to the 40S subunit (perhaps via contacts between domain IV of eIF5B and eIF1A bound to the 40S subunit, Choi et al., 2000), promotes subunit joining, and is then released from the ribosome without GTP hydrolysis.

Two GTPases Regulate the Fidelity and Efficiency of Translation Initiation

It was previously established that the GTPase activity of translation factor eIF2 governs the fidelity of AUG codon selection (Huang et al., 1997). Our work identifies a second checkpoint in translation initiation in which the GTPase activity of eIF5B insures accurate 80S ribosome assembly and efficient AUG codon selection. A model depicting these two checkpoints is presented in Figure 7. An eIF2•GTP•Met-$tRNA_i^{Met}$ ternary complex binds to the 40S ribosomal subunit, this complex then binds near the 5′ end of an mRNA and begins scanning. Base-pairing between the anticodon of the Met-$tRNA_i^{Met}$ in the 40S complex and an AUG codon triggers GTP hydrolysis by eIF2 and leads to release of eIF2•GDP (reviewed in Hinnebusch, 2000). Mutations in eIF2 or eIF5 that enhance the GTPase activity of eIF2 or that enable eIF2 to dissociate from Met-$tRNA_i^{Met}$ in the absence of GTP hydrolysis reduce the fidelity of AUG codon selection allowing initiation at non-AUG codons (Huang et al., 1997). Similarly, our work reveals that deletion of eIF5B, or mutations in eIF5B that block the GTPase activity of the factor and enable eIF5B release from the ribosome in the absence of GTP hydrolysis, increase leaky scanning. In most cases, ribosomes stop scanning when they reach the first AUG codon on an mRNA; however, if the start codon is in a poor sequence context ribosomes may leaky scan past the AUG without initiating translation (Kozak, 1999). We propose that eIF5B binds to a 40S subunit after eIF2 is released, stabilizes Met-$tRNA_i^{Met}$ binding, and promotes 60S subunit joining. Formation of a functional 80S ribosome triggers GTP hydrolysis by eIF5B and its release from the ribosome poised to elongate. In the absence of eIF5B, Met-$tRNA_i^{Met}$ dissociates from the 40S subunit, which then resumes scanning to the next AUG codon. The increased leaky scanning in cells expressing eIF5B-T439A may result from sequestration of the factor in inactive 80S complexes, thereby lowering the effective concentration of eIF5B in the cell and mimicking the situation in ΔeIF5B cells. Finally, whereas wild-type eIF5B does not hydrolyze GTP and dissociate from the ribosome until a functional 80S forms, eIF5B-T439A,H505Y dissociates prematurely without GTP hydrolysis resulting in release of the Met-$tRNA_i^{Met}$ and presumably resumed scanning by the 40S complex. Thus, GTP hydrolysis by eIF5B may serve as a checkpoint to insure accurate 80S ribosome assembly and efficient transition of the ribosome to translation elongation.

Experimental Procedures

Mutagenesis and Screening for Intragenic Suppressors

Random mutagenesis of the plasmid pC1293 encoding FLAG-tagged-ΔN(28-396)-eIF5B-T439A was performed using the bacterial mutator strain XL1-Red (Stratagene). The mutated plasmids were introduced into the ΔeIF5B strain J130 (*MATa ura3-52 leu2-3 leu2-112 fun12*::*hisG*) (Choi et al., 2000) and fast growing transformants were selected. From ~50,000 yeast transformants, 34 fast growing revertants were identified: an H505Y mutation was isolated 8 times, F643S and A709V mutations were isolated one time each, and 24 lacked the T439A mutation and were not analyzed further.

Purification of Proteins and Ribosomes

GST-eIF5B(396–1002) fusion proteins were expressed in yeast strain H1511 (*MAT*α *ura3-52 leu2-3 leu2-112 trp1-*Δ*63 GAL*$^+$) using the galactose-inducible vector pEG-KT (Mitchell et al., 1993). Cells were lysed in phosphate-buffered saline containing protease inhibitor mixture (complete protease inhibitor cocktail [Roche] and 1 μg/ml each of aprotinin, leupeptin, and pepstatin), and proteins were purified on glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech) and cleaved with thrombin to remove the GST.

80S ribosomes were purified from *S. cerevisiae* strain F353 (*MAT*α*ura3-52 trp1 leu2-*Δ*1 his3-*Δ*200 pep4::HIS3 prb1-*Δ*1.6 GAL*$^+$) using the method of Gomez-Lorenzo et al. (2000) with minor modifications. In the final step, 80S fractions collected from sucrose gradients were pelleted by centrifugation in a Type70Ti rotor for 24 hr at 25,000 rpm, and the ribosomal pellets were dissolved in buffer containing 20 mM HEPES [pH 7.4], 50 mM potassium acetate, 2.5 mM magnesium acetate, and 2 mM DTT. The concentration of ribosomes was determined using the conversion factor 1 A_{260} = 20 pmole of 80S.

GTPase Assay

20 μl reactions containing 10 pmol eIF5B, 14 pmol 80S, 1 mM GTP (containing 5 μCi [γ-^{33}P] GTP), and GTPase buffer (20 mM Tris [pH 7.5], 50 mM ammonium acetate, 2 mM DTT, and 2.5 mM magnesium acetate) were incubated at 30°C. Reactions were stopped by mixing 2 μl aliquots with an equal volume of 1 M perchloric acid, 3 mM potassium phosphate, and the products were analyzed by thin-layer chromatography on PEI-cellulose in 0.85 M potassium phosphate [pH 3.4] and quantified using a phosphorimager. GTPase activities were corrected by subtracting out the ribosome-independent activity observed for each mutant. However, the GTPase activity observed in reactions containing only 80S ribosomes was not subtracted because it exceeded the activity observed with some eIF5B mutants. (Due to this background, we cannot assess single-round GTPase activities of the eIF5B mutants.)

Ribosome Binding Assay

Binding of eIF5B to 80S ribosomes was measured based on the method described by Moreno et al. (1998) with minor modifications. Fifty μl binding reactions containing 25 pmol eIF5B and 28 pmol 80S ribosomes in binding buffer (20 mM Tris [pH 7.5], 50 mM ammonium acetate, 2 mM DTT, 10 mM magnesium acetate, and 2 mM guanine nucleotide) were incubated for 5 min at room temperature, layered on top of ice-cold 50 μl sucrose cushions (10% sucrose in binding buffer), and centrifuged at 4°C for 13 min at 90,000 rpm in a TLA 120.2 rotor. The unbound fraction was sampled by taking 50 μl from the supernatant, and the bound fraction was obtained by dissolving the ribosomal pellet in the remaining 50 μl of the reaction mixture. Aliquots of the supernatant and pellet fractions were fractionated by SDS-PAGE, stained with Coomassie brilliant blue, and the amount of eIF5B was quantified using NIH Image software (v. 1.62). In assays lacking ribosomes, ~10% of eIF5B was recovered in the pellet fraction, and the results were corrected by subtracting this background.

Acknowledgments

We thank members of our laboratories for helpful discussions; Dan Masison and Reed Wickner for advice and reagents for the electroporation experiments; and Alan Hinnebusch and an anonymous reviewer for helpful suggestions and comments on the manuscript. S.K.B. is an investigator in the Howard Hughes Medical Institute. This work was supported in part by NIH grants GM61262 (S.K.B.) and GM62128 (J.R.L.). D.M. and A.R.-M. were supported by National Science Foundation Graduate Fellowships.

Received: July 16, 2002
Revised: November 11, 2002

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