Interaction between Eukaryotic Initiation Factors 1A and 5B Is Required for Efficient Ribosomal Subunit Joining*

Michael G. Acker†, Byung-Sik Shin§, Thomas E. Dever§, and Jon R. Lorsch††

From the †Department of Biophysics and Biophysical Chemistry, Johns Hopkins University School of Medicine, Baltimore, Maryland 21205 and the §Laboratory of Gene Regulation and Development, NICHD, National Institutes of Health, Bethesda, Maryland 20892

Eukaryotic initiation factor 5B (eIF5B) is a GTPase that facilitates joining of the 60 S ribosomal subunit to the 40 S ribosomal subunit during translation initiation. Formation of the resulting 80 S initiation complex triggers eIF5B to hydrolyze its bound GTP, reducing the affinity of the factor for the complex and allowing it to dissociate. Here we present a kinetic analysis of GTP hydrolysis by eIF5B in the context of the translation initiation pathway. Our data indicate that stimulation of GTP hydrolysis by eIF5B requires the completion of early steps in translation initiation, including the eIF1- and eIF1A-dependent delivery of initiator methionyl-tRNA to the 40 S ribosomal subunit and subsequent GTP hydrolysis by eIF2. Full activation of GTP hydrolysis by eIF5B requires the extreme C terminus of eIF1A, which has previously been shown to interact with the C terminus of eIF5B. Disruption of either isoleucine residue in the eIF1A C-terminal sequence DIDDI reduces the rate constant for GTP hydrolysis by ~20-fold, whereas changing the aspartic acid residues has no effect. Changing the isoleucines in the C terminus of eIF1A also disrupts the ability of eIF5B to facilitate subunit joining. These data indicate that the interaction of the C terminus of eIF1A with eIF5B promotes ribosomal subunit joining and possibly provides a checkpoint for correct complex formation, allowing full activation of GTP hydrolysis only upon formation of a properly organized 80 S initiation complex.

The ultimate goal of translation initiation is to assemble a ribosomal complex with an initiator methionyl-tRNA (Met-tRNA),2 positioned at the AUG start codon of the mRNA in the P-site of the ribosome. In bacteria, this process requires three initiation factors (IFs) as well as the hydrolysis of GTP. IF1 directs fMet-tRNA, to the P-site of the small ribosomal subunit by blocking the A-site. IF2 is a GTPase involved in both fMet-tRNA binding and subunit joining. IF3 helps to ensure the fidelity of the mRNA/tRNA interaction in the ribosomal P-site and is ultimately involved in ribosome recycling (1).

In eukaryotes, translation initiation is significantly more complex, requiring at least 12 initiation factors (eIFs) and the hydrolysis of both ATP and GTP to assemble an 80 S complex capable of elongation. In the current model (2, 3), translation initiation begins with the formation of an eIF2-GTP-Met-tRNA, ternary complex (TC). TC is loaded onto the 40 S ribosomal subunit with the help of eIF1A and eIF3. The resulting 43 S complex then binds near the 5′-7-methylguanosine cap of an mRNA and is thought to scan the mRNA in search of an AUG start codon. Upon AUG recognition, eIF2 hydrolyzes its bound GTP with the help of the GTPase-activating protein eIF5. These events appear to be triggered by an AUG-dependent conformational change in the 43 S mRNA complex that leads to a destabilization of eIF1 binding (4). The movement or dissociation of eIF1 triggers irreversible GTP hydrolysis via the gated release of P, from the complex (5). At this point, a second GTPase, eIF5B, promotes joining of the 60 S subunit to the 40 S mRNA:Met-tRNA, complex (6). After subunit joining, eIF5B hydrolyzes its bound GTP, reducing the affinity of the factor for the 80 S ribosome (7, 8), and dissociates, leaving a translationally competent 80 S initiation complex.

Prokaryotic and eukaryotic translation initiation are evolutionarily linked by at least two common initiation factors. eIF1A is orthologous to IF1 (9) and is thought to occupy the same site (A-site) on the eukaryotic ribosome as IF1 does on the prokaryotic ribosome (10–12). eIF5B shares at least 27% identity and 48% similarity with its ortholog IF2; however, there is some discrepancy as to their relative functions. Whereas IF2 is thought to facilitate the binding of fMet-tRNA, to the small ribosomal subunit (13, 14), this activity is assigned to eIF2 in eukaryotes. Recently, eIF5B has been shown to bind tRNA weakly in solution, although this binding was not specific for Met-tRNA, (15). On the ribosome, however, eIF5B stabilizes Met-tRNA, binding (8) and, like IF2, is directly involved in subunit joining (6, 14). eIF1 and IF3 share a similar shape and charge distribution and appear to be functional orthologs, binding very similar positions on the 40 and 30 S ribosomal subunits, respectively, consistent with their common roles in monitoring codon/anticodon fidelity (16, 17).

Although IF1 and IF2 have not been reported to interact directly in solution, mounting evidence suggests that the factors interact on the ribosome in the context of translation initiation. IF1 and IF2 can be cross-linked on the ribosome (18), and the binding of each factor to the 30 S subunit is enhanced by the presence of the other (19–21).

Recently, interactions between the C termini of eukaryotic factors eIF1A and eIF5B have been discovered using genetic, biochemical, and structural methods (22–24). Interestingly, both eIF1A and eIF5B possess C-terminal ends that are not present in their bacterial counterparts. Unlike IF1, the C terminus of eIF1A consists of a long unstructured region (11). The C terminus of eIF5B is structurally distant from its G domain and contains a single helical segment, H14, at its end that is not found in IF2 (25). Marintchev and colleagues (24) have identified the sequence DIDDI at the extreme C terminus of eIF1A as the interacting partner for the eIF5B C-terminal helical binding pocket. The lack of homologous C-terminal sequences in bacterial IF1 and IF2 is strong evidence that this interaction is specific to eukaryotes; however, the role of the interaction in translation initiation is not known.

We therefore set out to explore the functional relevance of the interaction between eIF1A and eIF5B in eukaryotic translation initiation.
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using an in vitro reconstituted system from S. cerevisiae. We have conducted a quantitative study of eIF5B activity in the context of translation initiation using wild-type and C-terminal mutant versions of eIF1A. eIF5B hydrolyzes GTP at a specific point in the initiation process, only after preceding required steps are completed. Alteration of either of the isoleucine residues in the C-terminal sequence of eIF1A, DDIIID, but not the aspartic acid residues reduces both the GTP hydrolysis and subunit joining activities of eIF5B without significantly affecting earlier steps of translation initiation. Disruption of the eIF5B C-terminal binding domain for eIF1A results in similar decreases in GTPase and subunit joining activity. Our data indicate that the eIF1A-eIF5B C-terminal interaction is important for efficient ribosomal subunit joining and subsequent GTP hydrolysis by eIF5B.

EXPERIMENTAL PROCEDURES

Initiation Factor Purification—eIF1 was purified as described previously (26). eIF2 and eIF5 were purified as described (5). eIF1A was expressed as an N-terminal His tag fusion protein and purified from E. coli using vector pDS047 (gift of the Hinnenbusch laboratory). Purification of eIF1A was as described for eIF5 (26) with minor modifications. Cleared lysate was applied to a Ni2+-chelated 5-mL HiTrap™ Chelating HP column (Amersham Biosciences). Eluted protein was then applied to a 5-mL HiTrap™ Heparin HP column (Amersham Biosciences) and eluted with a linear gradient from 0.05 to 1 M NaCl (20 mM HEPES-KOH, pH 7.6, 10 mM β-mercaptoethanol, 10% glycerol) over 60 ml. The His tag was cleaved overnight by the addition of Tev protease at a concentration of 0.33 units/mL. Cleaved protein was then applied to a 5-mL HiTrap™ Chelating HP column (Amersham Biosciences), and eIF1A lacking the His tag was eluted in the flow through. Fractions containing eIF1A were pooled and dialyzed against 20 mM HEPES-KOH, pH 7.6, 100 mM KOAc, 2 mM dithiothreitol, 10% glycerol.

N-terminally truncated eIF5B (residues 396–1002 (27)) was cloned into pTYB2 (New England Biolabs) and purified as described for eIF5, with some modifications. Post-chitin column fractions containing eIF5B were pooled and applied to a 5-mL HiTrap™ Heparin HP column (Amersham Biosciences) and eluted with a linear gradient from 0.05 to 1 M NaCl (20 mM HEPES-KOH, pH 7.6, 2 mM dithiothreitol, 10% glycerol) over 60 ml. Fractions containing eIF5B were pooled and applied to a HiTrap™ Q HP column (Amersham Biosciences), and eIF1A lacking the His tag was eluted in the flow through. Fractions containing eIF1A were pooled and dialyzed against 20 mM HEPES-KOH, pH 7.6, 100 mM KOAc, 2 mM dithiothreitol, 10% glycerol.

40 and 60 S Ribosomal Subunit Purification—40 S ribosomal subunits were purified as described (5) with the following additions. Gradient fractions containing 60 S ribosomal subunits were also pooled and subjected to buffer exchange similar to 40 S subunits, and the concentration of 60 S ribosomal subunits was calculated by measuring the absorbance at 260 nm and using the extinction coefficient, 4 × 10^4 cm^-1 M^-1.

Model mRNA and tRNA Preparation—Model mRNA and initiator tRNA^Met were both synthesized by T7 polymerase run-off transcription and purified by denaturing PAGE as previously described (see Refs. 28 and 5, respectively). Model mRNA was of the sequence 5’-GGAA(UUC)-UAUG(CU)_(10)-C-3’. [35S]Met-tRNA, and stoichiometric charged Met-tRNA, were prepared as previously described (29).

Fluorescence Assays—Fluorescence anisotropy analysis of mutant eIF1A binding to 40 S ribosomal subunits was performed by competition with wild-type eIF1A fluorescently labeled at its C terminus as previously described (30). Kinetic analysis of phosphate release was performed as previously described (5).

eIF5B GTPase Assays—Ternary complex (TC) was formed at a concentration of 3X by mixing 2.4 μM each UltraPure GTP-Mg2+, eIF2, and Met-tRNA, in 1X reaction buffer (30 mM HEPES-KOH, pH 7.4, 100 mM KOAc, 3 mM Mg(OAc)2, 2 mM dithiothreitol) and incubated for 15 min at 26 °C. Ternary complex was then added to an equal volume of 3X factors mix containing 1X reaction buffer, 1.2 μM 40 S ribosomal subunits, 2.4 μM eIF1, 1.2 μM eIF2A, and 2.4 μM mRNA and incubated for 2 min at 26 °C, forming 43 S complex. During this incubation, 3X initiation mix was formed by adding 0.6 mM [32P]GTP to 1.2 μM 60 S ribosomal subunits, 2.4 μM eIF5, and 3 μM eIF2 in 1X reaction buffer, and a sample was removed for correction of background GTP hydrolysis. To initiate the reaction, 1 volume of 3X initiation mix was added to two volumes of preformed 43 S complex, resulting in a final concentration of 1X for the reaction. For each time point, 2 μl of 1X reaction mix was quenched in 6 μl of 100 mM EDTA, pH 8.0. Samples from each time point were added onto 15% polyacrylamide-10B gel to separate [32P]GTP from the hydrolysis product [32P]Pi. After analysis by phosphorimaging, the fraction of [32P]GTP hydrolyzed was calculated. In the presence of WT eIF1A or eIF1A mutants behaving like WT, observed rate constants were obtained by fitting the data with a double exponential equation, A(1 – exp(–kt1)) + B(1 – exp(–kt2)). In the absence of eIF1A or in the presence of eIF1A mutants affecting GTP hydrolysis, observed rate constants were obtained by fitting the data with a single exponential equation, A(1 – exp(–kt)).

43 and 80 S Complex Formation Assays—43 S complex assays were conducted as previously described (4, 5, 28). 80 S complex assays were conducted as previously described (8) with minor modifications. A 3X concentration of TC was formed by adding saturating GTP (200 μM) to 2.4 μM eIF2 and 1.5 nm [35S]Met-tRNA, ([35S]Met-tRNA, is limiting to all components) in 1X reaction buffer and incubating for 15 min at 26 °C. One volume of 3X TC was added to one volume of 3X Factors mix (1.2 μM 40 S ribosomal subunits, 2.4 μM eIF1, 1.2 μM eIF1A, 2.4 μM mRNA in 1X reaction buffer) and incubated for 2 min at 26 °C, forming 43 S complex. Initiation mix was formed at 3X concentration with 1.2 μM 60 S ribosomal subunits, 2.4 μM eIF5, 1.5 μM eIF5B, and 6 mM GDPNP. Similar results were obtained with GTP (data not shown). 80 S complex formation was initiated by adding one volume of 3X initiation mix to two volumes of preformed 43 S complex, resulting in a final concentration of 1X for the reaction. Reaction samples (10 μl) were stopped by loading on a running native polyacrylamide gel. Gels were analyzed by phosphorimaging and quantitated for the fraction of [35S]Met-tRNA, localized in 80 S complexes. Free [35S]Met-tRNA, in solution is susceptible to deacylation (but is protected when incorporated into 43 and 80 S complexes). Deacylation is more likely to occur in experiments yielding reduced 80 S complex formation, and the resulting free [35S]Met-tRNA is not electrophoretically enter the gel due to its charge. This results in an artificially high fraction of [35S]Met-tRNA, in 80 S complexes due to the loss of counts from [35S]Met-tRNA, deacylation. In addition, this assay cannot distinguish between a reduction in 80 S complex formation and instability of formed 80 S complexes that leads.
and absence (9, 10) of eIF1A, data were fit to a double exponential equation as the single exponential fit yielded a 30-fold higher $x^2$ value, and there was clear systematic error in the fit. C, GTP hydrolysis by eIF5B occurs with a first phase rate constant of 0.036 ± 0.007 s$^{-1}$ in the presence of all components (●) but is significantly reduced in the absence of eIF1A (□) or eIF2 (▲, ○). Omitting eIF5 (△) or forming TC with GDPNP (■) also reduces GTP hydrolysis by eIF5B. GTP hydrolysis by eIF5B in the presence of ribosomal subunits alone occurs with a rate constant of 0.0017 ± (9 × 10$^{-4}$) s$^{-1}$ (○).

RESULTS
eIF5B Is Only Activated to Hydrolyze GTP after Previous Steps in the Pathway Are Complete—In the current model of translation initiation, GTP-bound eIF5B promotes 60 S subunit joining to the 40 S ribosomal subunit containing an initiator Met-tRNA base paired to the AUG start codon in its P-site (2). After formation of the 80 S complex, eIF5B hydrolyzes GTP, lowering its affinity for the ribosome and allowing the factor to dissociate. To test this model, we examined the GTP hydrolysis activity of eIF5B in vitro in the context of the translation initiation pathway. In the presence of 40 and 60 S ribosomal subunits alone, eIF5B hydrolyzes GTP with a rate constant of 0.0017 ± (9 × 10$^{-4}$) s$^{-1}$ (Fig. 1D). In order to specifically observe the GTPase activity of eIF5B during translation initiation, we first formed the 43 S complex consisting of unlabeled eIF2-GTP-Met-tRNA, ternary complex, mRNA, 40 S ribosomal subunits, eIF1, and eIF1A. We then initiated 80 S complex formation with the addition of 60 S ribosomal subunits, eIF5, eIF5B, and $^{32}$P-GTPγ and followed GTP hydrolysis by eIF5B (see reaction scheme in Fig. 1A). GTP dissociation from TC and GDP dissociation from eIF2 are slow (0.03 and 0.2 min$^{-1}$, respectively), and thus no GTP hydrolysis by eIF2 is measured under these conditions (29). In the presence of all components of our reconstituted system, GTP hydrolysis by eIF5B is biphasic, with a first phase rate constant ($k_{obs1}$) of 0.036 ± 0.007 s$^{-1}$ and an amplitude of 0.18 ± 0.02 and a second phase rate constant ($k_{obs2}$) of 0.0018 ± (3 × 10$^{-4}$) s$^{-1}$ and an amplitude of 0.77 ± 0.02 (total amplitude is fixed to 0.95) (Fig. 1B). The concentrations of each component were varied to determine conditions that give maximal rate constants. The addition of eIF5 to 43 S-mRNA complex earlier in the reaction prior to initiation with eIF5B, 60 S ribosomal subunits, and $^{32}$PGTPγ did not alter the GTP hydrolysis kinetics of eIF5B; nor did the addition of eIF3 at concentrations stoichiometric to the 43 S complex (data not shown).

The rate constant for the second phase of GTP hydrolysis is similar to the rate constant of initiation-independent GTP hydrolysis by eIF5B in the presence of 40 and 60 S subunits (but the absence of other factors) and could arise from interactions of eIF5B with 80 S ribosomes formed spuriously in a factor-independent manner. Likewise, eIF5B-GTP could encounter 80 S ribosomes formed previously in the translation initiation pathway, resulting in the hydrolysis of eIF5B-bound GTP at the slower initiation independent rate. Alternatively, biphasic kinetics is not unprecedented for steps involving the C terminus of eIF1A (31) and may represent alternate conformations of eIF1A in the initiating ribosomal complex, each capable of activating GTP hydrolysis by eIF5B although at different levels. As described below, we only observe changes to $k_{obs1}$, not $k_{obs2}$, when the initiation process is interrupted or the eIF1A C terminus is modified, suggesting that the fast phase is relevant to the initiation process. Consequently, we focus on $k_{obs1}$. 

FIGURE 1. GTP hydrolysis by eIF5B is dependent on the earlier steps of translation initiation. A, reaction scheme for GTP hydrolysis experiments. TC made with unlabeled GTP is mixed with 40 S ribosomal subunits, eIF1, eIF1A, and mRNA. After 2 min, GTP hydrolysis is initiated with 60 S ribosomal subunits, eIF5, eIF5B, and GDPNP, and samples are quenched at varying times. Only hydrolysis by eIF5B is observed, because exchange of GTP from TC or GDP from eIF2-GDP is very slow. B, GTP hydrolysis by eIF5B in the presence (●) and absence (□) of eIF1A. In the presence of eIF1A, data were fit to a double exponential equation as the single exponential fit yielded a 30-fold higher $x^2$ value, and there was clear systematic error in the fit. C, GTP hydrolysis by eIF5B occurs with a first phase rate constant of 0.036 ± 0.007 s$^{-1}$ in the presence of all components (●) but is significantly reduced in the absence of eIF1A (□) or eIF2 (▲, ○). Omitting eIF5 (△) or forming TC with GDPNP (■) also reduces GTP hydrolysis by eIF5B. GTP hydrolysis by eIF5B in the presence of ribosomal subunits alone occurs with a rate constant of 0.0017 ± (9 × 10$^{-4}$) s$^{-1}$ (○).
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| TABLE 1  | eIF1A-ΔDIDDI behaves like wild-type eIF1A in early steps of translation initiation |
|-----------|---------------------------------------------------------------------------------|
|           | eIF1A wild type | eIF1A-ΔDIDDI |
| $K_c$ for 40 S subunits (ns) | 21 ± 2.3 | 17 ± 1.3 |
| Without eIF1 | 3 ± 1.3 | 2.5 ± 1.3 |
| With eIF1 | 0.064 ± 0.008 | 0.12 ± 0.04 |
| $K_c$ for 43 S complex formation (s⁻¹) | 102 ± 12 | 45 ± 13 |
| $K_c$ for, GTP hydrolysis by eIF1A (s⁻¹) | 0.1 ± 0.01 | 0.1 ± 0.01 |
| Initiated by TC | 12 ± 1 | 12 ± 1 |
| Initiated by eIF5 | 6.7 ± 0.3 | 5.8 ± 0.4 |

* Reported errors are errors of curve fits except $K_{obs}$, for 43 S complex formation, which is the mean deviation.

To probe which steps in translation initiation are required to observe GTP hydrolysis by eIF5B, we blocked the pathway at different points. In the absence of eIF2 or eIF1A, Met-tRNA cannot be loaded onto the 40 S ribosome, and eIF5B GTP hydrolysis is reduced to background levels (Fig. 1, B and C). Formation of a ternary complex in the presence of the slowly cleavable GTP analog GDPNP or the elimination of eIF5 from the system prevents eIF2 from hydrolyzing its bound nucleotide and thus from dissociating from the 43 S complex. In both cases, the rate of GTP hydrolysis by eIF5B is reduced when the 40 S subunits alone also results in a 2-fold increase in this rate constant, providing further support to the idea that the second phase represents background GTP hydrolysis by eIF5B. Adding increasing amounts of eIF1A-ΔDIDDI in the presence of a constant amount of wild-type eIF1A inhibits GTP hydrolysis by eIF5B with 50% inhibition at a mutant/wild-type ratio of 1:1, consistent with equal affinity of the factors for 40 S subunits. At a ratio of 4:1, GTP hydrolysis by eIF5B is reduced to the level with eIF1A-ΔDIDDI alone (data not shown).

To further explore the function of the eIF1A C-terminal sequence DIDDI, a peptide of the sequence DIDDI was chemically synthesized and added in trans to eIF5B GTP hydrolysis experiments containing either wild-type eIF1A or eIF1A-ΔDIDDI. The presence of synthetic DIDDI at concentrations up to 50 μM did not significantly affect the rate of GTP hydrolysis by eIF5B with either eIF1A sample (data not shown), suggesting that, at least at experimentally achievable conditions, its function requires a direct tether to eIF1A.

Evidence from Wagner and co-workers (24) specifically points to Ile₁⁴⁰ at the C terminus of eIF1A as playing an important role in the interaction with eIF5B. To confirm this and identify the specific influence of each of the five C-terminal residues in activating GTP hydrolysis by eIF5B during initiation, we replaced wild-type eIF1A with each single alanine substitution mutant in the eIF5B GTP hydrolysis assay. eIF1A mutants replacing Asp with Ala at position 139, 141, or 142 (eIF1A-ΔDADDI, -ΔADDI, or -ΔDADI) promote wild-type levels of GTP hydrolysis by eIF5B (Fig. 2C). However, eIF1A mutants replacing Ile with Ala at position 140 or 143 (eIF1A-ΔDADDI or -ΔDIDDA) result in a decrease in the observed rate constant for eIF5B GTP hydrolysis to the levels of eIF1A-ΔDIDDI and -5Aala mutants (Fig. 2, compare D with B). Thus, the two isoleucine residues in this region, but not the aspartates, influence GTP hydrolysis by eIF5B during translation initiation.

**eIF1A Is Involved in eIF5B-promoted 80 S Complex Formation**—Using the reaction scheme in Fig. 1A, it was possible to monitor 80 S complex formation on a native polyacrylamide gel by following the incorporation of radiolabeled [³⁵S]Met-tRNA, (Fig. 3A). In the presence of wild-type eIF1A, we observed ~45% of the Met-tRNA, in 80 S complexes at completion, triple the amount when 80 S complexes are formed with eIF1A-ΔDIDDI present (Fig. 3B). As in the eIF5B GTP hydrolysis experiments, incorporation of eIF1A-5Aala or the single Ile to Ala substitution mutant eIF1A-ΔDIDDA leads to a decrease in 80 S complex levels similar to that with eIF1A-ΔDIDDI. However, when the single Asp to Ala substitution mutant eIF1A-ΔDADAI is included, 80 S complex levels are the same as those with wild-type eIF1A (Fig. 3B). Similar to eIF5B GTPase activity, 80 S complex formation was only decreased by the observed rate constant is $3.2 \times 10^{-4} \pm (2 \times 10^{-5})$ s⁻¹, a reduction of ~100-fold (Fig. 2B). When eIF1A-ΔDIDDI is substituted for wild-type, the observed rate constant for eIF5B GTP hydrolysis decreases ~20-fold to 0.00131 ± (4 \times 10^{-5}) s⁻¹ (versus 0.036 s⁻¹ for wild type), although the steps prior to and including GTP hydrolysis by eIF2 in the 43 S-mRNA complex are not significantly slowed (Table 1). Substitution of eIF1A-5Aala for WT eIF1A results in a similar decrease in the observed rate constant for GTP hydrolysis (Fig. 2B).
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The 80 S complex formation activity of eIF5B-ΔH14 was then analyzed. The presence of wild-type eIF5B results in ~45% of the Met-tRNAi incorporated into 80 S complexes (Fig. 4, B and C). Substituting eIF5B-ΔH14 for wild-type reduces the amount of Met-tRNAi in 80 S complexes by more than 4-fold to ~10%, similar to the level of 80 S complex formation in the absence of eIF5B.

**DISCUSSION**

Translation initiation in eukaryotes is dependent on GTP hydrolysis by eIF5B (6, 7). Our data indicate that full activation of GTP hydrolysis by eIF5B requires prior assembly of the 40 S ribosome, stabilizing the bound Met-tRNAi, while facilitating subunit joining. Upon 80 S complex formation, eIF5B can hydrolyze its GTP, allowing the factor to dissociate from the 80 S complex, which is now capable of elongation (6–8).

In recent years, eIF1A and eIF5B have been shown to interact in vitro via their respective C termini (22–24). Our data indicate that this interaction plays a functionally important role in translation by enhancing both the subunit joining and subsequent GTP hydrolysis activities of eIF5B. Specifically, the five most C-terminal isoleucine residues (DIADDI) of eIF1A are important for these activities. eIF1A mutants that alter either of the C-terminal isoleucine residues reduced both 80 S complex formation and GTP hydrolysis activity of eIF5B without significantly affecting the previous steps in the initiation pathway. Likewise, mutation of the eIF1A-binding pocket of the eIF5B C terminus prevents the binding of the factor to the complex, allowing the factor to dissociate from the 80 S complex, which is now capable of elongation (6–8).

**FIGURE 2. eIF1A C-terminal isoleucine residues are required for maximal GTP hydrolysis by eIF5B.** A, GTP hydrolysis by eIF5B is biphasic in the presence of wild-type eIF1A (●). but shows single exponential kinetics with ΔDIADDI-eIF1A (○, Δ). GT hydrolysis by eIF5B with wild-type eIF1A (●, k_{obs} = 0.036 ± 0.007 s^{-1}), C-terminal ΔDIADDI (●, k_{obs} = 0.00131 ± (4 × 10^{-5}) s^{-1}), 5-alanine substitution mutant (●, k_{obs} = 0.0045 ± (4 × 10^{-5}) s^{-1}), or no eIF1A (●, k_{obs} = 3.2 × 10^{-5} ± (2 × 10^{-5}) s^{-1}). C-terminal eIF1A mutants substituting Ala for Asp (DIADDI (●), DIADDI (●), and DIADDI (○)) display GTP hydrolysis rates similar to WT eIF1A (●). ×, no eIF1A. D, C-terminal eIF1A mutants substituting Ala for Ile (DADDI (●), and DIDA (○)) reduce GTP hydrolysis rates to the level of the ΔDIADDI and 5-alanine mutants. ●, WT eIF1A; ×, no eIF1A.

eIF1A isoleucine variants, whereas aspartic acid mutants displayed wild-type levels of 80 S complexes (Fig. 3B) (data not shown).

**Disrupting the eIF1A Binding Site on eIF5B Affects eIF5B-mediated Processes in Translation Initiation**—The binding site for the C terminus of eIF1A is located in domain IV of eIF5B, a distance of >50 Å from the catalytic G domain responsible for GTP binding and hydrolysis (25). Our data, however, suggest that disrupting this interaction significantly affects GTP hydrolysis by the factor as well as 80 S complex formation. In light of this new information, it was necessary to determine if disrupting the eIF5B binding domain for eIF1A would also affect the eIF5B-mediated steps of translation initiation. To this end, we designed and purified eIF5B-ΔH14, a truncation of the C terminus at helix H14 (numbering from archaeal structure), which along with helix 13 forms the binding pocket for the C terminus of eIF1A (24).

eIF1A-ΔH14 displayed a background rate of GTP hydrolysis (in the presence of ribosomal subunits alone) the same as wild-type eIF1A (data not shown), indicating that it was not grossly misfolded. During translation initiation, wild-type eIF5B hydrolyzes GTP with a rate constant of 0.036 ± 0.007 s^{-1} (Fig. 4A). In contrast, eIF5B-ΔH14 eliminates translation initiation-associated GTPase activity, hydrolyzing GTP with a single rate constant of 0.0006 ± (3 × 10^{-5}) s^{-1}, similar to the level in the absence of eIF5B (k_{obs} = 0.0006 ± (3 × 10^{-5}) s^{-1}). These data indicate that mutations in domain IV of eIF5B distant from the G domain can influence the GTPase activity of the factor and further support the conclusion that the interaction between eIF1A and eIF5B is important for the activity of the latter.
resulted in reduced 80 S complex formation and GTP hydrolysis activity, similar to C-terminal eIF1A mutants.

GTP hydrolysis by eIF5B is not required for subunit joining (8) but enables the 80 S ribosome to proceed to peptide elongation by releasing eIF5B, yet mutations that affect the eIF1A-eIF5B C-terminal interaction reduced 80 S complex formation, suggesting that this interaction is important for efficient subunit joining. A decrease in the amount of 80 S complex could result from reduced recruitment of 60 S subunits to the 43 S mRNA complex in the absence of the eIF1A-eIF5B interaction. The eIF1A-eIF5B interaction could modulate the positions of the two factors in such a way as to provide the correct binding surface for the 60 S ribosomal subunit, the absence of which would decrease subunit joining efficiency. Alternatively, the C-terminal interaction between eIF1A and eIF5B could facilitate the dissociation of eIF1A before, during, or perhaps even after subunit joining (22–24). eIF1A mutants lacking the appropriate C-terminal binding sequence DIDDD could fail to leave the initiating ribosome at the appropriate time, thereby blocking the recruitment of the 60 S ribosomal subunit and interfering with the activation of GTP hydrolysis by eIF5B. Finally, since our assay for subunit joining follows [35S]Met-tRNAi, a reduction in 80 S complex formation could also result from the destabilization of the Met-tRNAi in the 80 S complex when the eIF1A-eIF5B interaction is disrupted.

Recent cryoelectron microscopy reconstructions of initiating ribosomes in bacteria indicate that a conformational change in the C terminus of IF2 takes place in the presence of IF1 (32, 33). Despite lacking the C-terminal structures involved in direct binding that their eukaryotic counterparts possess, IF1 and IF2 are localized on the ribosome in a manner that would allow the C-terminal interaction to take place upon correct initiation complex formation.

The simplest model to explain the decrease in the GTPase activity of eIF5B is that it is the direct effect of the subunit joining defect caused by the mutations in the C terminus of eIF1A or eIF5B, if subunit joining does not take place efficiently, eIF5B is not activated to hydrolyze its
bound GTP. However, we cannot rule out the possibility that eIF1A directly activates GTP hydrolysis by eIF5B via the interaction at their C termini. Such an activation would be very interesting, since the G-domain of eIF5B is quite distant from the C terminus (>50 Å), connected in solution only by a long α-helix. Shin et al. (8) have proposed that eIF5B serves to check the accuracy of 80S complex formation, surveying the organization of the newly formed complex and hydrolyzing GTP when it is found to be correct. This checkpoint could be accomplished by the activation of the eIF5B GTPase by the C terminus of eIF1A. Binding of the C terminus of eIF1A to eIF5B could signal that subunit joining involves eIF5B-eIF1A interaction.

**Acknowledgments**—We thank Mikkel Algire, Sarah Mitchell, Chad Slawson, Julie Takacs, and Alan Hinnebusch for comments on the manuscript.

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