Eukaryotic translation elongation factor 2 (eEF2) catalyzes reverse translocation of the eukaryotic ribosome

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A ribosome contains three tRNA binding sites: the A-site for incoming aminoaeryl-tRNAs, the P-site for tRNA bound to the growing polypeptide chain, and the E-site for deacylated tRNA. The tRNA sites localize at the interface of two ribosomal subunits, the large subunit (LSU) and the small subunit (SSU).

Particular states of tRNAs on the ribosome are designated in relation to the position of the tRNA anticodon part on the SSU to the position of its acceptor part on the LSU. Protein synthesis requires codon-by-codon movement of the tRNA–mRNA complexes in the ribosome (1), which occurs as a transition of the complexes through hybrid tRNA states during translocation (2). Following the formation of peptidyl-tRNA in the A-site and deacylated tRNA in the P-site as a result of the transpeptidation reaction in the LSU, the ribosome fluctuates between classical (A/A:P/P) and hybrid (A/A:P/E or A/P:P/E) positions of the tRNAs (3). This pretranslocational state (PRE) is associated with a rotation of the SSU relative to the LSU (4, 5). Another large-scale ribosomal movement, swiveling of the SSU “head” domain, moves the anticodon ends of the tRNAs into the ap/P:pe/E states (6). Resolution of these conformational changes brings the ribosome into the posttranslocational (POST) state with the tRNAs in the P/P and E/E positions and sets a new codon in the vacant A-site.

Although translocation may proceed spontaneously, this process is extremely slow (7) unless catalyzed by a GTPase, translation elongation factor 2. Elongation factor 2 (termed EF-G in prokaryotes and eEF2 in eukaryotes) is a strictly conservative protein consisting of six domains that are grouped in two loosely associated superdomains (8). The first superdomain includes subdomain G’, GTPase domain I (or G domain), and domain II. The second superdomain includes domains III, IV, and V (9).

Elongation factor 2 in complex with GTP binds to the PRE ribosome and, regardless of its initial conformation, causes an appearance of the hybrid tRNA states (5, 10). After binding, the protein undergoes conformational changes that bring the second superdomain into the A-site of the SSU (11, 12), where it interacts via domain IV with the decoding center and tRNA–mRNA complex (12–14). This interaction disengages the codon–anticodon duplex from the decoding center and allows the SSU head to swivel (12, 14). Recent structural studies carried out on the bacterial (13, 15) and eukaryotic (12) trans-

nucleotide; GMP-PCP, guanylyl β,γ-ethylene diphosphonate; GMP-PNP, guanylyl iminodiphosphonate; ETA, exotoxin A; RRL, rabbit reticulocyte lysate; ADP-R, ADP-ribosylated; rfu, relative fluorescence unit; FAM, fluorescein amidite; MVHL, methionine-valine-histidine-leucine; MVVL, methionine-valine-valine-leucine; MV, methionine-valine.

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location intermediates show that elongation factor 2 induces a swiveled state of the SSU head domain, with the tip of domain IV stabilizing the ap/P conformation of the P-site ligand (12, 13, 15).

Mutations of the conservative histidine residue located at the tip of domain IV (H699 in yeast and H715 in humans) decrease the rate of translocation (16). The residue is modified into dihydridamide in archaea and eukaryotes by a set of conserved enzymes (17). Various bacterial toxins, such as diphtheria toxin (18), exotoxin A (17), or cholera toxin (19), perform ADP-ribosylation of this residue, which leads to translational inhibition (20) and cell death (18). For eEF2, it was shown that ADP-ribosylation affected the translocation step but did not influence eEF2 binding to the ribosome (21). Structural studies have also revealed that the modification does not alter the overall structure of eEF2 bound with the ribosome but rather impedes interactions of eEF2 domain IV with the decoding center of the SSU (22). Accordingly, it was proposed that ADP-ribosylation may directly interrupt the ability of eEF2 to stabilize the intermediate conformation of the tRNA ends during their movement through the SSU in the course of translocation (12).

The role of hydrolysis of GTP by elongation factor 2 in the catalysis of translocation is currently under active discussion (23). The questions that need to be clarified include how hydrolysis is related to the structural rearrangements of the ribosome and the movements of tRNA and whether the energy released in the reaction directly drives translocation.

In addition to direct translocation (spontaneous and catalyzed by EF-G), spontaneous reverse translocation of bacterial ribosomes has been described in vitro (24–26). During the reaction, tRNAs shift by one codon from the P- and E-sites to the A- and P-sites, respectively (27). Reverse translocation requires a cognate deacylated tRNA in the E-site (24, 25) and proceeds through hybrid tRNA states similar to direct translocation (26).

An absence of data about reverse translocation of eukaryotic ribosomes prompted us to address this question using a mammalian translation system reconstituted from individual components. Surprisingly, we found that the reaction was induced by eEF2 and that its efficiency was increased in the presence of non-hydrolyzable GTP analogues. ADP-ribosylation of eEF2 inhibited reverse translocation, indicating the mechanism of action of the modification. Our findings provide the unique possibility to compare catalyzed direct and reverse translocation and elucidate a role of eEF-2 as a translocational enzyme. These results uncover a universal mechanism by which tRNA–mRNA complexes are moved within the ribosome and deepen our understanding of ribosome translocation.

**Results**

**POST ribosomes relocate backwards by three nucleotides in the presence of cognate deacylated tRNA and eEF2**

The process of reverse translocation has been described in detail in bacterial in vitro systems (24–26, 28); however, it has not been observed for eukaryotic ribosomes. To address this issue, we assembled eukaryotic ribosomal POST complexes on model mRNA (Fig. S1A) encoding the MVHL tetrapeptide in a reconstituted in vitro mammalian translation system (29, 30). The complexes were purified by sucrose density gradient from unbound translational components. The A-site of the POST ribosomes contained the UAA stop codon, the E-site was inhabited by the histidine CAU codon, and the P-site was occupied by MVHL–peptidyl-tRNA^Aeu bound to the leucine CUG codon. The POST state of the complexes was confirmed using the peptide release reaction in the presence of release factors eRF1 and eRF3 and by the ability of the complexes to undergo direct translocation in the presence of the UAA suppressor aminoacyl-tRNA and both elongation factors (Fig. S1, B and C).

Positions of the purified ribosomal complexes along the mRNA were detected using a primer extension reaction (toeprint), followed by capillary electrophoresis (Fig. 1A). The POST complexes produced two cDNA fragments: main (127 nucleotides (nt)) and additional (125 nt) (Fig. 1A). The latter appeared because of the presence of a stop codon in the A-site, which is known to adopt a compact conformation leading to mRNA retraction into the A-site (31, 32). This stop codon conformation is stabilized by eRF1, thus enhancing the 125-nt peak when it binds to the ribosome (30).

Next, we added a deacylated tRNA^His, cognate to the E-site codon, to the POST ribosomal complexes along with eEF2-GTP. As a result, we observed a shift of the main toeprint peak by 3 nt toward the 5’ end of the mRNA (a −3 nt peak) (Fig. 1, A and B). Similar data were obtained with eEF2-GTP for the POST complexes containing MV- and MVVL-peptidyl tRNAs and using deacylated tRNA^Met and tRNA^Val, respectively (Fig. S2A). In comparison, incubation of the POST complexes with eEF2 alone resulted in the appearance of a −1 peak in the toeprint (Fig. 1B), as described previously (30). Incubation with eEF2 and deacylated tRNA^Met, non-cognate to the E-site, did not cause the toeprint shift (Fig. 1B).

An antibiotic, hygromycin B, effectively inhibits translocation of mRNA and tRNAs on the ribosome in both bacteria (33) and eukaryotes (34). The compound has been shown to prevent reverse translocation of bacterial ribosomes (35). We found that hygromycin B blocked the toeprint shift induced by eEF2 and deacylated tRNA (Fig. S2B). Taking into consideration that the shift occurred in a triplet manner and that deacylated tRNA should be cognate to the E-site, similar to reverse translocation in prokaryotes (23, 24), we concluded that we observed reverse translocation of the eukaryotic ribosomes.

**Shift of POST complexes cannot be explained by a reassembly model**

To explain the shift of the toeprint signal via a pathway other than reverse translocation, a reassembly model was proposed that implied the de novo assembly of the shifted ribosomal complexes from the constituents coming from the solution. To distinguish these possibilities, we utilized cycloheximide, a eukaryote-specific antibiotic that competes with the acceptor end of deacylated tRNA for binding of the E-site of the LSU (36). According to our assumption, cycloheximide should not interfere with reassembly because the P-site in this case acquires deacylated tRNA directly from the solution, whereas it should interfere with reverse translocation, during which deacylated tRNA enters the P-site through the E-site (Fig. 2A). We
added cycloheximide to the POST complexes in the presence of eEF2 and deacylated tRNA^His and found that it blocked the \(-3\) nt shift that supports the reverse translocation model (Fig. 2B).

**eEF2 catalyzes reverse translocation**

Spontaneous reverse translocation of bacterial POST complexes has been demonstrated previously (24, 25). We therefore investigated the ability of eukaryotic ribosomes to perform spontaneous reverse translocation.

Specifically, increased Mg\(^{2+}\) concentration and the presence of polyamines have been shown to promote the spontaneous reverse translocation of prokaryotic ribosomes (24, 25). This is consistent with an ability of cations to increase the affinity of deacylated tRNA toward the E-site (37). We thus tested the effect of increased Mg\(^{2+}\) concentration in the absence or presence of 1 mM spermidine on reverse translocation in the eukaryotic system (Fig. 3). We found that, in the absence of eEF2, reverse translocation of the POST complexes did not occur at high concentrations of Mg\(^{2+}\) and spermidine. Even extremely high concentrations of Mg\(^{2+}\) and spermidine (up to 20 mM Mg\(^{2+}\) and 8 mM spermidine) did not induce spontaneous reverse translocation (Fig. S3). It is possible that spontaneous translocation was not observed in our system (during a similar time interval) because of the far lower concentration of ribosomal complexes utilized (nanomolar) in comparison with those in studies in a bacterial system (micromolar). Under such circumstances, a slow rate of spontaneous reaction (24–26) would likely be even further decreased. These results highlight an active role of eEF2 in catalysis of the eukaryotic reverse translocation.

**Condition requirements for reverse translocation**

We tested the dependence of the reverse and forward translocation on the concentration of eEF2 and observed that reverse translocation required up to a 20-fold excess of eEF2 over the ribosomal complexes (Fig. 4A), whereas direct translocation was effective at a 2:1 ratio (Fig. 4A). It should be noted that, during forward translocation, eEF2 binds to the PRE complex, capable of undergoing spontaneous conformational changes, including an intersubunit rotation of the ribosomal subunits.
During reverse translocation, eEF2 binds to the POST complex, which has a conformation of unrotated ribosomal subunits because no tRNAs with hybrid acceptor ends are present therein (5, 38). Earlier it has been shown by kinetic (39) and structural (40) data that EF-G prefers to bind a ribosome in the state of rotated subunits and exhibits a 7-fold higher dwell time of binding for this conformation (39). It means that EF-G better interacts with PRE than POST complexes, which correlates with our results (Fig. 4A). We believe that differences in eEF2 binding efficiency with ribosomal complexes determine its working concentrations. The authors of the previous studies on bacterial ribosomal complexes (24, 25) probably did not detect EF-G–dependent reverse translocation because the concentration of EF-G in the experiments was only 1.4–2-fold higher than that of the ribosomes.

In addition, we showed that non-hydrolyzable or slowly hydrolyzable GTP analogues such as GMP-PCP and GMP-PNP, able to stall elongation factor on the ribosome (22, 41), increased the efficiency of the reverse translocation reaction (Fig. 4B). Previously it had been shown that EF-G in complex with GMP-PCP was able to induce direct translocation (42). However, a recent kinetic analysis indicated a slower rate of the process compared with translocation in which GTP hydrolysis was allowed (39). The authors of the study proposed that in the presence of the non-hydrolyzable analogue, EF-G stabilized some intermediate state of ribosome ratcheting which was then

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**Figure 2.** Cycloheximide supports a reverse translocation model for POST complex relocation. A, schematic of cycloheximide influence on ribosome reassembly and reverse translocation. B, toeprinting analysis of the POST complexes (PC) in the presence of eEF2 and tRNA_His with or without cycloheximide. Experiments were replicated at least three times. Error bars represent the standard deviation of the mean.
slowly resolved in the forward direction under the influence of increased Mg$^{2+}$ concentration (39). Considering possible factors that might drive the process in the opposite direction, we found that reverse translocation required an excessive concentration of cognate deacylated tRNA, similar to that demonstrated for bacterial systems (Fig. 4C) (24, 25). In addition to the ability of such excess to compensate for the low affinity of deacylated tRNA toward the ribosome (24, 25), we suggest that it might also affect the PRE/POST state equilibrium. In particular, it has been shown that deacylated tRNA in the E-site stabilizes the unrotated conformation of the PRE complex, with the A- and P-tRNAs in the classical states (5). Furthermore, we also have previously shown the same mechanism of stabilization regarding eukaryotic termination complexes, in which deacylated tRNA in the P-site was produced by peptidyl-tRNA hydrolysis (43).

In summary, these data suggest that during reverse translocation, eEF2 stabilized some intermediate state of translocation that then was driven to the PRE state by an increased deacylated tRNA concentration.

**ADP-ribosylation of eEF2 prevents reverse translocation**

Recent data indicate that the most rate-limiting step during translocation is the movement of the tRNA anticodon ends, associated with swiveling of the SSU head (26, 44, 45). Elongation factor 2 facilitates the movement in two ways. First, the protein induces bond breakage between the decoding center and the codon–anticodon duplex in the A-site, which allows the SSU head to swivel (14, 22). Second, the protein stabilizes hybrid ap/P and ep/E tRNA conformations in the swiveled head of the SSU (12, 13). In the POST ribosome, the A-site is empty, and spontaneous head swiveling is allowed (12, 22); therefore, we supposed that the stabilization of the swiveled head intermediate determined an ability of eEF2 to induce reverse translocation.

It has been proposed that ADP-ribosylation of the domain IV diphthamide could affect the eEF2 interaction with hybrid tRNAs during forward translocation (12). We thus decided to test the influence of ADP-ribosylation on reverse translocation. First, we assessed the ability of ADP-ribosylating protein, a catalytic domain of exotoxin A (ETA) from Pseudomonas aeruginosa, to repress luciferase biosynthesis in the rabbit reticulocyte lysate (RRL) system. The toxin inhibited translation of luciferase in the presence of its substrate, NAD$^+$ (Fig. 5A). Further, we found that reverse translocation was also blocked in the presence of both ETA and NAD$^+$ (Fig. 5B). ADP-ribosylation of eEF2 under such conditions was confirmed by native PAGE (Fig. S4, A and B) (20). An ability of eEF2-ADPR to bind POST complexes was shown by Western blotting of the POST complexes purified in a sucrose density gradient (Fig. S4D). Notably, ADP-ribosylation of eEF2 also blocked reverse translocation in the presence of GMP-PNP, confirming that the mechanism of inhibition is not linked to the overall affinity of eEF2 to the ribosome (21) (Fig. S4C). Therefore, the inhibition of reverse translocation by ADP-ribosylation showed that interaction of eEF2 domain IV with the SSU is involved in the catalysis of this process.

**Discussion**

In this study, we demonstrated reverse translocation of the eukaryotic ribosomal complexes. Specifically, we showed that eEF2 induced a movement of a peptidyl-tRNA from the P-site to the A-site in the presence of cognate deacylated tRNA in the E-site (Fig. 1B). In the absence of eEF2, reverse translocation did not occur, even under conditions of high concentrations of magnesium and spermidine (Fig. 3).

In addition, we demonstrated that eEF2 in complex with GMP-PCP was also able to induce reverse translocation (Fig. 4). This indicates that hydrolysis of GTP is not necessary for the catalysis of the reaction and assumes that the factor in the GTP state stabilizes some intermediate of translocation, which can then resolve in the forward or backward direction. This hypothesis is supported by the fact that deacylated tRNA in high concentration accompanied by eEF2 shifts the intermediate state of translocation to the PRE state (i.e. in a backward direction), which can be explained by binding of an additional tRNA in the E-site of the PRE complex (5, 43) (Fig. 4C).

Furthermore, using ADP-ribosylated eEF2, which inhibited reverse translocation (Fig. 5), we propose that this intermediate state could be a state of swiveled SSU head because ADP-ribosylation occurs at domain IV of eEF2, the location involved in stabilization of the ribosome conformation with swiveled SSU head (12, 22), and probably disrupts interactions important for such stabilization. The question remains of how the domain IV of eEF2 and tRNA can bind the A-site simultaneously after peptidyl-tRNA movement from the P- to the A-site during reverse translocation, especially in the presence of GTP analogues. Perhaps a recent study (11), which found high interdomain mobility of EF-G and its ability to bind the ribosome, containing tRNA in the A-site in a compact form, gives the answer.
Finally, we propose the following model for reverse translocation of the eukaryotic ribosomes (Fig. 6, Movie S1). Binding of eEF2 stabilizes the swiveled head conformation of the ribosome and the ap/P state of tRNA through an interaction of eEF2 domain IV with the peptidyl-tRNA in the P-site and the decoding center. If the E-site contains cognate deacylated tRNA, then it adopts the pe/E-state. Then eEF2 either dissociates or changes conformation, and the SSU head swivels in the reverse direction. This allows tRNA–mRNA duplexes to adopt the P/E and A/P states that then may evolve into the classical P/P and A/A states. The third deacylated tRNA may engage the E-site and prevent forward translocation.

The biological role of eukaryotic reverse translocation is obscure. Taking into consideration the reaction requirements (e.g. eEF2 stable binding), it is unlikely that the process could compete with forward translocation under normal conditions. However, in exceptional cases, for example in the presence of the fungal antibiotic sordarin (52), eEF2 can be stabilized on the ribosome, increasing chances of reverse reaction. This indicates that reverse translocation may contribute to the antibiotic activity.

Figure 4. Dependence of reverse translocation on eEF2 and deacylated tRNA concentration. A, toeprinting analysis of reverse translocation and forward translocation obtained in the presence of tRNA^His and Ser-tRNA^{UAU}/eEF1, respectively, and different amounts of eEF2. B, toeprinting analysis of reverse translocation obtained in the presence of tRNA^His and eEF2 supplemented with GTP, GMP-PNP, or GMP-PCP. Asterisks mark the characteristic ~1 peak of eEF2-induced ribosome conformation changes. PC, POST ribosomal complex. C, toeprinting analysis of the MVHL POST complexes in the presence of eEF2 and different concentrations of tRNA^His. Experiments were replicated at least three times. Error bars represent the standard deviation of the mean.
We also propose that diphthamide can play an important role in reverse translocation catalysis. A diphthamide residue is much larger than the histidine from which it is originated and carries additional positive charge (17). These features could help the residue to protrude into the P-site and interact with tRNA during binding of eEF2 in the A-site of the ribosome. However, this hypothesis needs to be clarified using eEF2 diphthamide mutants.

Nevertheless, our data provide the unique possibility to compare catalyzed forward and reverse translocations and elucidate key features of eEF-2 that confer the directionality and a high rate to the translocation reaction in living cells.

We showed that the translocation reaction with POST complexes required a large excess of eEF2 (Fig. 4A). At lower physiological concentrations, the protein productively reacted only with the PRE ribosomes (Fig. 4A). Moreover, considering the model of reverse translocation in the opposite direction (Fig. 6 and Movie S1), it may be assumed that the dissociation of deacylated tRNA from the E-site of the PRE complex should result in translocation in the forward direction. Recently, a structural study has shown that the dissociation occurs upon binding of a complex of eEF2 and GTP, which stabilizes the rotated conformation of the PRE ribosome that is incompatible with deacylated tRNA in the E site (46). This also agrees with a recent

**Figure 5. ETA and NAD⁺ prevent eEF2-induced reverse translocation.** ETA inhibits translation in cell lysate and reverse translocation in vitro. A, analysis of the efficiency of luciferase (Luc) biosynthesis in RRL in the presence of ETA and NAD⁺. B, toeprinting analysis of the movements of the POST complexes (PC) in the presence of tRNA™ and eEF2 with or without ETA and NAD⁺. rlu, relative luminescence unit. Experiments were replicated at least three times. Error bars represent the standard deviation of the mean.

**Figure 6. Model of eEF2-catalyzed reverse translocation.**
kinetics study in which dissociation has been observed prior to the formation of the POST complex (44). The empty E-site allows deacylated tRNA in the P-site to adopt the hybrid P/E state. Therefore, initially, the directionality of translocation is biased at the stage of eEF2 binding because of the higher affinity of eEF2 to the PRE ribosomal complex and because of the dissociation of deacylated tRNA from this complex upon eEF2 binding. The stage after eEF2 binding seems to be similar for translocation in both directions. eEF2 facilitates transition through the main barrier of translocation; i.e. a movement of tRNA anticodon ends. Using hygromycin B (Fig. S2B), we showed the significance of the barrier for the reverse reaction. Blocking of reverse translocation upon ADP-ribosylation of eEF2 (Fig. 5) revealed that the mechanism of the barrier facilitation includes interactions of domain IV and the anticodon end transition intermediate, associated with SSU head swiveling. Taking into consideration a slower rate of forward translocation in the presence of GMP-PCP (39) along with a possibility of the reverse reaction as shown in our study, we conclude that GTP hydrolysis is needed to detach eEF2 from this intermediate state, providing a high rate of the translocation reaction in living cells.

During our experiments, we observed that addition of eEF2 and deacylated tRNA to the POST complexes increased the abundance of −1 and −2 peaks (Figs. 1B, 2B, and 4, B and C, and Fig. S2). The recent kinetics studies of translocation induced by EF-G (23) and the structural study of translocation induced by eEF2 (14) indicate that, during forward translocation, ribosomes proceed through different conformational states associated with the intermediate degrees of rotation of both the SSU head and body. We suppose that the additional toeprint peaks (−1 and −2) can reflect these intermediate conformations. Considering our model of reverse translocation (Fig. 6), we suggest that the −1 peak can be linked to the eEF2-induced head swiveling that, accompanied by SSU body rotation, can give rise to the −2 peak.

In summary, our data indicate that the ribosomal conformation as stabilized by eEF2 constitutes a universal intermediate in the transition of tRNA−mRNA complexes within tRNA-binding sites of the ribosome. eEF2, acting as an actual translocase enzyme, further catalyzes the process in either direction, operating on the reversible ribosomal machine. Preferential translocation of PRE ribosomes is determined by the higher affinity of eEF2 to this ribosomal complex.

Experimental procedures

Ribosomal subunits and translation factors

The 40S and 60S ribosomal subunits as well as the rabbit translation factors eIF2, eIF3, eIF1H, and eEF2 were purified from a rabbit reticulocyte lysate as described previously (30, 43). The human translation factors eIF1, eIF1A, eIF4A, eIF4B, ΔeIF4G, ΔeIF5B, and eIF5 were produced as recombinant proteins in Escherichia coli strain BL21 with subsequent protein purification on nickel-nitrilotriacetic acid–agarose and ion exchange chromatography (30, 47).

In vitro transcription of mRNA and tRNA

mRNAs and tRNAs were transcribed by T7 RNA polymerase from MVHL-stop, MVVL-stop, actin-5′ UTR-luciferase, and corresponding tRNA plasmids. mRNA plasmids for the eukaryotic translation system contained a T7 promoter, four CAA repeats, the β-globin 5′ UTR, and corresponding amino acid codons (MVHL and MVVL), followed by a stop codon and a 3′ UTR comprising the rest of the natural β-globin coding sequence (Fig. S1A) (30). For run-off transcription, mRNA plasmids were linearized using XhoI and tRNA plasmids with BstOI.

ADP-ribosylation of eEF2

The catalytic domain of P. aeruginosa ETA (48) was a kind gift from A. Stepanov and S. Dmitriev. A reaction mixture containing 1 μM eEF2, 0.13 μM ETA toxin, and 0.1 mM NAD+, all in buffer A100 (20 mM Tris-HCl, 100 mM KCl, 6 mM β-mercaptoethanol, and 10% glycerol), was incubated for 10 min at 37 °C. The obtained ADP-ribosylated eEF2 (eEF2-ADPR) was used in reverse translocation reactions. In control experiments, eEF2 was treated in the same way, excluding addition of ETA protein or NAD+. The eEF2 ribosylation efficiency was about 80%.

In vitro translation in RRL

Luciferase translation from the template containing an actin 5′ leader followed by the luciferase coding sequence was performed in RRL as described previously (49). An effect of ETA on translation was assessed using the same toxin and NAD+ concentrations as described for in vitro eEF2 ribosylation.

Ribosomal POST complex assembly and purification

Mammalian ribosomal POST complexes were assembled as described previously (30). Briefly, 74 nM mRNA was incubated for 30 min in buffer A (20 mM Tris acetate (pH 7.5), 100 mM KAcO, 2.5 mM MgCl2, and 2 mM DT T) supplemented with 400 units RNase inhibitor, 1 mM ATP, 0.25 mM spermidine, 0.2 mM GTP, and 75 μg of total tRNA (acylated with all or individual amino acids) with 150 nM 40S and 60S purified ribosomal subunits, 250 nM each eIF2, eIF3, eIF4A, eIF4B, eIF1, eIF1A, eIF5, ΔeIF4G, and ΔeIF5B, 400 nM eEF1H, and 100 nM eEF2 and then centrifuged in a Beckman SW55 rotor for 95 min at 4 °C and 50,000 rpm in a 10−30% (w/w) linear sucrose density gradient prepared in buffer A with 5 mM MgCl2. Fractions corresponding to the POST complexes according to optical density were combined, diluted 3-fold with buffer A containing 1.25 mM MgCl2 (to a final concentration of 2.5 mM Mg2+), and used in the toeprint analysis.

Toeprint analysis of the ribosomal complexes

Aliquots containing 14 nM POST complexes were incubated with 200 nM eEF1, 67 nM Ser-tRNAAGA, and 27–270 nM eEF2 or eEF2-ADPR, supplied with 0.2 mM GTP, GMP-PNP or GMP-PCP and 67 nM different deacylated tRNAs (with or without 67 nM cycloheximide) for 20 min at 37 °C, and analyzed using a primer extension protocol as described previously (50, 51). Primer extension was performed with a 5′ FAM−labeled primer, 5′-FAM-GCATTGCGAGGACCGGG-3′, complementary to
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&-globin mRNA nucleotides 197–214. cDNAs were separated by electrophoresis using standard GeneScan® conditions on an ABI Prism® Genetic Analyzer 3100 (Applied). The percentage of the translocated complex was calculated using the following equations: (rfu of 130-nt peak/rfu of 130-nt peak + rfu of 127-nt peak) × 100 and (rfu of 124-nt peak/rfu of 124-nt peak + rfu of 127-nt peak) × 100 for reverse and forward translocations, respectively.

Peptide release assay

The peptide release assay was conducted as described previously (30) with minor modifications as follows. Aliquots containing 14 pm POST complexes assembled in the presence of \[^{35}S\]Met–tRNA were incubated at 37 °C for 3 min with or without 6.7 pm eRF1 and 2.7 pm eRF3. Ribosomes and tRNA were pelleted with ice-cold 5% TCA supplemented with 0.75% (w/v) casamino acids and centrifuged at 14,000 × g at 4 °C. The amount of released \[^{35}S\]-containing peptide was determined by scintillation counting of supernatants using an Intertechnique SL-30 liquid scintillation spectrometer.

Ribosomal complex binding assay

Aliquots containing 14 pm POST complexes were incubated with 10 pm eEF2-ADPR in the presence of 0.2 pm GDP or GDP-PCP at 37 °C for 15 min (in a total volume of 500 µl). Reaction mixtures were applied to the 10–30% (w/w) linear gradients were fractionated into 14 equal fractions, followed by precipitation in 10% TCA. The protein pellets were dried and analyzed by Western blotting using antibodies against eEF2 and ribosomal protein L9.

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