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The Highly Conserved LepA Is a Ribosomal Elongation Factor that Back-Translocates the Ribosome

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SUMMARY

The ribosomal elongation cycle describes a series of reactions prolonging the nascent polypeptide chain by one amino acid and driven by two universal elongation factors termed EF-Tu and EF-G in bacteria. Here we demonstrate that the extremely conserved LepA protein, present in all bacteria and mitochondria, is a third elongation factor required for accurate and efficient protein synthesis. LepA has the unique function of back-translocating posttranslocational ribosomes, and the results suggest that it recognizes ribosomes after a defective translocation reaction and induces a back-translocation, thus giving EF-G a second chance to translocate the tRNAs correctly. We suggest renaming LepA as elongation factor 4 (EF4).

INTRODUCTION

The functions of the ribosome can be separated into four phases, each of which is governed by specific protein factors. The four phases are initiation, elongation, and termination of protein synthesis, and the recycling phase, during which the ribosomes are dissociated into their subunits so that the small subunit is ready to re-enter the subsequent initiation phase (for a review, see Nierhaus and Wilson, 2004). The details of the translation phases differ significantly between ribosomes from the three domains of life, viz. bacteria, archaea, and eukarya, with the exception of the elongation phase; the elongation phase is at the heart of protein synthesis and consists of a cycle of reactions (hence termed "elongation cycle"), during which the nascent polypeptide chain is prolonged by one amino acid. The elongation cycle is governed by two universal elongation factors, termed EF-Tu and EF-G in bacteria, and EF1 and EF2 in archaea and eukarya. EF-Tu transports an aminoacyl-tRNA (aa-tRNA) in the ternary complex aa-tRNA•EF-Tu•GTP to the decoding center of the ribosomal A site (A for aminoacyl-tRNA) on the small ribosomal subunit. After decoding, EF-Tu hydrolyzes GTP and leaves the ribosome as EF-Tu•GDP, whereas cognate aa-tRNA accommodates fully into the A site. The next step, peptide bond formation, does not require a translation factor. During this process, the peptidyl residue at the ribosomal P site (P for peptidyl-tRNA) is cleaved off of the peptidyl-tRNA and transferred to the aa-tRNA, resulting in the peptidyl-tRNA residing at the A site and being prolonged by one amino acid. The third step in the elongation cycle is the translocation reaction that is promoted by EF-G•GTP: the $(tRNA)_2$•mRNA complex is shifted by a codon length on the ribosome, moving the peptidyl-tRNA from the A to the P site and the deacylated tRNA from the P to the E site (E for exit). In higher fungi, such as yeast and *Candida albicans*, a third elongation factor, EF3, has been identified as being essential for viability. EF3 is an ATP-dependent E site factor because ATP hydrolysis by EF3 is necessary to open up the E site, which enables the E-tRNA to be released upon A site occupation (Triana-Alonso et al., 1995a).

In most bacteria, the *lepA* gene is the first cistron of a bicistronic operon. The second cistron, the leader peptidase or *lep* gene, encodes the signal peptidase Lep (March and Inouye, 1985a)—an integral membrane protein that is inserted into the inner cell membrane and cleaves off the N-terminal signal (leader peptide) from secreted and periplasmic proteins (Zwizinski and Wickner, 1980). Dibb and Wolfe (1986) reported that a LepA knockout in *Escherichia coli* has no phenotype under the various growth conditions tested. Curiously, these null results contrast with the fact that LepA is one of the most conserved proteins on this planet (amino acid identity in bacteria 55%–68%). Indeed, LepA is even more conserved than other essential bacterial translation factors such as initiation factor IF3 or guanine exchange factor EF-Ts (see Table S1 in the Supplemental Data). LepA is also present in the obligatory parasite *Mycoplasma*, which has a minimized bacterial genome containing only ~500 genes; in *Rickettsia*, the smallest free-living bacteria; and in mitochondria from all eukaryotes, ranging from yeast to human (Figure 1). The discrepancy between the knockout results

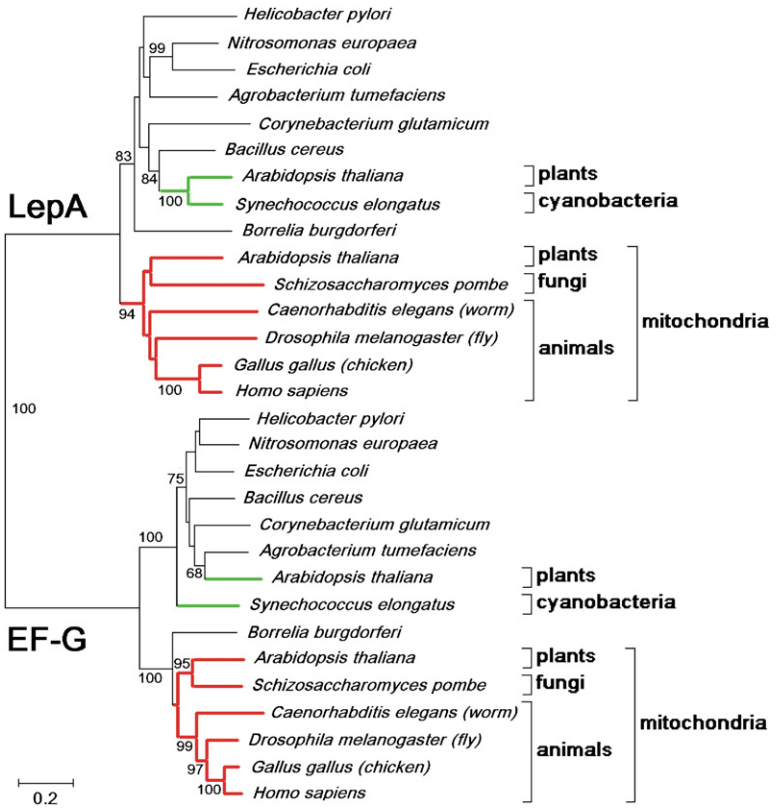


Figure 1. Phylogenetic Analysis of LepA and EF-G

An unrooted Neighbor Joining algorithm (NJ) tree of EF-G and LepA proteins enables reciprocal rooting of each subfamily. Branch lengths reflect the estimated amino acid substitutions per every site (see scale bar). Numbers on internal branches indicate statistical support of clades based on 1000 bootstrap samples. Note that the total branch lengths of both GTPase families are comparable. In both families mitochondrial eukaryotic proteins are monophyletic. Fly EF-G and LepA proteins branch with their animal orthologs (confirmation of the coelomata hypothesis). A prospective secondary LepA protein of *Arabidopsis* branches with chloroplasts. There are no signs of interdomain lateral gene transfer in the LepA family.

and high conservation of LepA prompted us to analyze the function of this protein.

E. coli LepA is a polypeptide of 599 amino acid residues with a molecular weight of 67 kDa. LepA has been shown to bind to the ribosome in vivo (Colca et al., 2003), but no function for this factor has been assigned. The amino acid sequence of LepA indicates that it is a G protein. As well as having high conservation with EF-G, LepA also exhibits a conspicuous similarity in terms of the domain structure with EF-G in that it contains equivalents to EF-G domains I to V, with the exception of domain IV; this domain is absent (see Figures 2A and 2B). In addition, LepA has a specific C-terminal domain (CTD), and since the majority of overproduced LepA (March and Inouye, 1985b) is found in the periplasmic membrane, one might speculate that this is related to the LepA-specific CTD. EF-G is a structural mimic of the ternary complex aa-tRNA•EF-Tu•GTP (Nissen et al., 2000), and according to this mimicry, domain IV of EF-G corresponds to the anticodon stem loop (ASL) region of the tRNA (Figure 2C). Domain IV of EF-G interacts with the decoding center at the A site of the ribosome, and removing domain IV abolishes the translocation activity of EF-G (Martemyanov and Gudkov, 1999, and references therein).

Here we demonstrate that LepA represents a third essential bacterial elongation factor with a novel function in translation that rationalizes the high conservation of this factor. We find that LepA is a G protein with uncoupled ribosome-dependent GTPase activity rivaling that of EF-G in terms of turnover rate. Our analysis reveals a surprising and unique function for LepA, namely its ability to induce ''back-translocation'' of tRNAs on the ribosome. Collectively, our results might suggest that LepA recognizes ribosomes with mistranslocated tRNAs and induces a back-translocation.

RESULTS

In Silico Analyses of LepA: Conservation and Domain Structure

LepA is one of the most highly conserved proteins known; the amino acid identity of LepA among bacterial orthologs ranges from 55% to 68%, which compares well with the corresponding values for EF-Tu, EF-G, and IF-2, which are 70%–82%, 58%–70%, and 35%–49%, respectively (Table S1). Database searches revealed that LepA orthologs could be found in all bacteria and nearly all eukaryotes. It is only missing in eukaryotes that have lost mitochondria and have only retained mitochondrial remnants without ribosomes like *Encephalitozoon cuniculi* or *Giardia lamblia* (Knight, 2004). In all plants with completely sequenced genomes (rice, mouse-ear cress, and red algae), we found two forms of LepA. Whereas one form branches with other mitochondria LepA sequences in our phylogenetic analysis, the second form branched with cyanobacterial orthologs, indicating its subcellular

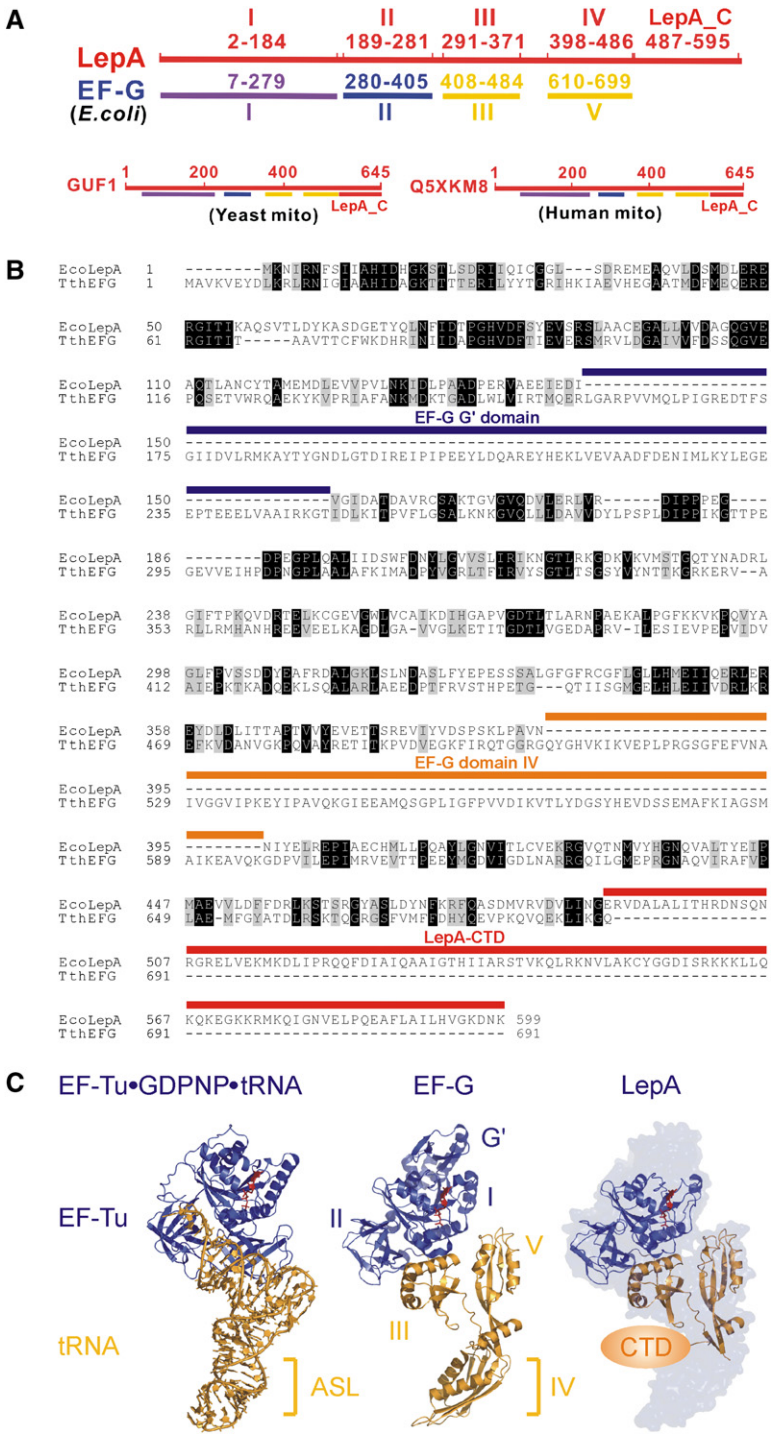


Figure 2. In Silico Analysis of LepA Compared with EF-G

(A) Domain structures of LepA and orthologs in comparison with EF-G. Red, LepA (*E. coli*) and orthologs Guf1 (yeast mitochondria) and Q5XKMB (human mitochondria); different colors, corresponding EF-G domains. LepA has five potential structural domains (I, II, III, IV, and LepA_C) according to the amino acid sequence (2–595), which have high consensus with *E. coli* EF-G domains I (purple), II (blue), III, and V (yellow).

(B) Alignment of *E. coli* LepA with EF-G from *T. thermophilus*. Black and gray boxes indicate amino acid identity and similarity, respectively. G' subdomain (blue) and domain IV (orange) are lacking in LepA, whereas LepA contains a specific C-terminal domain (CTD, red).

(C) Comparison of the crystal structure of the ternary complex aa-tRNA•EF-Tu•GTP (PDB1TTT) with that of EF-G (PDB1WDT) and a homology model for *E. coli* LepA. The domains of EF-G are indicated with roman numerals, except for the G' subdomain of domain I. Note that the EF-G domain IV corresponds to the anticodon stem loop (ASL) of the aa-tRNA within the ternary complex and that LepA lacks the G' domain and domain IV, but has a LepA specific CTD.

targeting to chloroplasts in plants (see Figure 1). This suggests that LepA is essential for bacteria, mitochondria, and plastids.

The enormous conservation in LepA covers the entire protein (see Figure S1 in the Supplemental Data). The first four domains are strongly related to the EF-G domains I, II, III, and V, with the last CTD being unique (Figures 2A and 2B). This domain arrangement of LepA is found in bacteria and mitochondria from yeast to human. Due to the high conservation between EF-G and LepA, it is possible to generate a homology model for LepA based on the known EF-G structure. From the representation seen in Figure 2C, it is obvious that LepA lacks the G' subdomain of EF-G domain I as well as the complete domain IV.

In Vivo Analyses of LepA

The *E. coli LepA* gene was cloned into pET14b and transformed into an *E. coli* BL21(DE3) strain, and thus, expression of LepA could be induced with isopropyl-beta-D-thiogalactopyranoside (IPTG) (see Experimental Procedures). Even without induction, we observed a prolonged lag phase, and the viable cells (BL21_LepA in Figure 3) turn earlier into the stationary phase than the control expression of EF-G does. Induction of LepA expression by addition of 1 mM IPTG severely affected growth, as indicated by an early entry into the stationary phase, whereas overexpression of EF-G as a control shows a growth behavior similar to wild-type. SDS-PAGE analysis reveals "leaky" expression of uninduced BL21_LepA and strong expression of both induced LepA and EF-G (data not shown). After a 10-fold dilution in the presence of IPTG, the strain overexpressing LepA hardly grows at all, whereas the control EF-G strain again shows almost wild-type growth (Figure 3). These results demonstrate that overexpression of LepA is toxic to the cell.

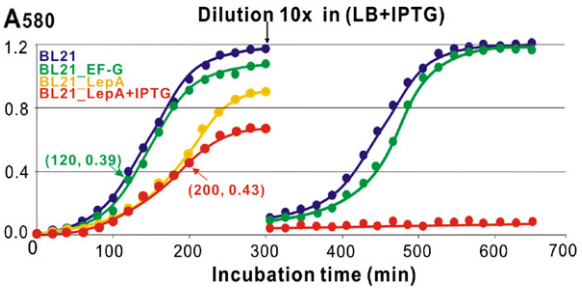


Figure 3. Overexpression of LepA Blocks Growth

The strain BL21(DE3) containing the plasmid pET+LepA stops growth soon after IPTG induction (red, BL21_LepA) in contrast to wild-type or overexpression of EF-G (BL21_EF-G). The yellow curve shows the growth of BL_LepA without induction. The arrows indicate the addition of IPTG (1 mM). After 10-fold dilution in the presence of IPTG, the strain overexpressing LepA does not resume growth, in contrast to wild-type and the strain overexpressing EF-G.

In Vitro Analyses of LepA: GTPase Activity and Assays Related to Translocation

The high similarity between the domain structure of LepA and EF-G prompted us to test the ribosome-dependent GTPase activity of LepA. EF-G is known to have the strongest ribosome-dependent GTPase activity among all characterized G proteins involved in translation. When the ribosome stimulation of GTP cleavage is not coupled to protein synthesis, it is referred to as being uncoupled GTPase activity. Figure 4A demonstrates that LepA not only exhibits uncoupled GTPase activity, but also that this activity is stimulated by ribosomes to the same extent as that of EF-G.

Next we tested whether or not LepA can affect peptide bond formation. To this end we constructed three functional states of the ribosome: (1) the Pi state, with only one tRNA on the ribosome, namely the peptidyl-tRNA analog AcPhe-tRNA^{Phe} at the P site (i for initiation, since this state compares with the 70S initiation complex); (2) the pretranslocational complex (PRE) state, with a [^{32}P] tRNA$_f^{Met}$ at the P and an Ac[^{14}C]Phe-tRNA at the A site; and (3) the posttranslocational (POST) state with the same tRNAs, but now located at the E and P sites, respectively. Puromycin, an analog of the 3′ end of an aa-tRNA that binds to the A-site region of the peptidyl-transferase center, reacts quantitatively with the Pi and POST states (0.72 and 0.73, respectively, in Figure 4B), but not with PRE state ribosomes (0.00) as expected. Surprisingly, in the presence of LepA the POST state does not react with puromycin anymore (0.01), whereas the Pi state still does (0.71; PM reaction in Figure 4B). At the same time, LepA does not affect the amount of tRNAs bound to the programmed ribosomes (binding values for both tRNAs are the same in the presence and absence of LepA). Dipeptide analyses also support the puromycin results, suggesting that the addition of LepA to a POST state ribosome prevents dipeptide formation by precluding binding of aa-tRNA to the A site (Figure S2).

Figure 4C shows an additional detail: in the presence of GTP, LepA works catalytically, in a similar fashion as EF-G, saturating at 0.4 molecules per 70S ribosome (the corresponding number for EF-G is 0.3). However, in the presence of the nonhydrolysable GTP analog GDPNP, the LepA action becomes stoichiometric, saturating at ~1 molecule per 70S ribosomes. Therefore, GTP cleavage seems to be required for dissociation of LepA from the ribosome, and thus, the factor behaves like a typical G protein (reviewed by Bourne et al., 1991). In order to test whether the rates of EF-G-dependent translocation and LepA-dependent back-translocation are comparable, we performed kinetics at 30°C in order to slow down the reactions. Single turnover conditions were applied (LepA and EF-G in a 5- and 10-molar excess of ribosomes, respectively, in the presence of GDPNP). Figure 4D shows that both reactions occur with similar rates, indicating that the LepA-dependent reaction can be incorporated into an elongation cycle without a significant delay of protein synthesis.

A possible explanation for the puromycin and dipeptide observations is that LepA induces a back-translocation of the POST state to the PRE state, since the occupation of the A site by the AcPhe-tRNA after back-translocation would prevent both the puromycin reaction and binding of a cognate ternary complex to the A site (dipeptide formation). In contrast, the Pi state cannot be back-translocated because the A site of the ribosome cannot be occupied in a stable fashion without a tRNA in the adjacent P site (Rheinberger et al., 1981). This explains why the Pi state remains puromycin-reactive even in the presence of LepA. To verify this back-translocation hypothesis we employed three strategies: (1) chemical probing of the tRNA positions through analysis of diagnostic base protections in the 16S rRNA, (2) monitoring a POST state-specific 23S rRNA conformation marker by Pb^{2+} cleavage,

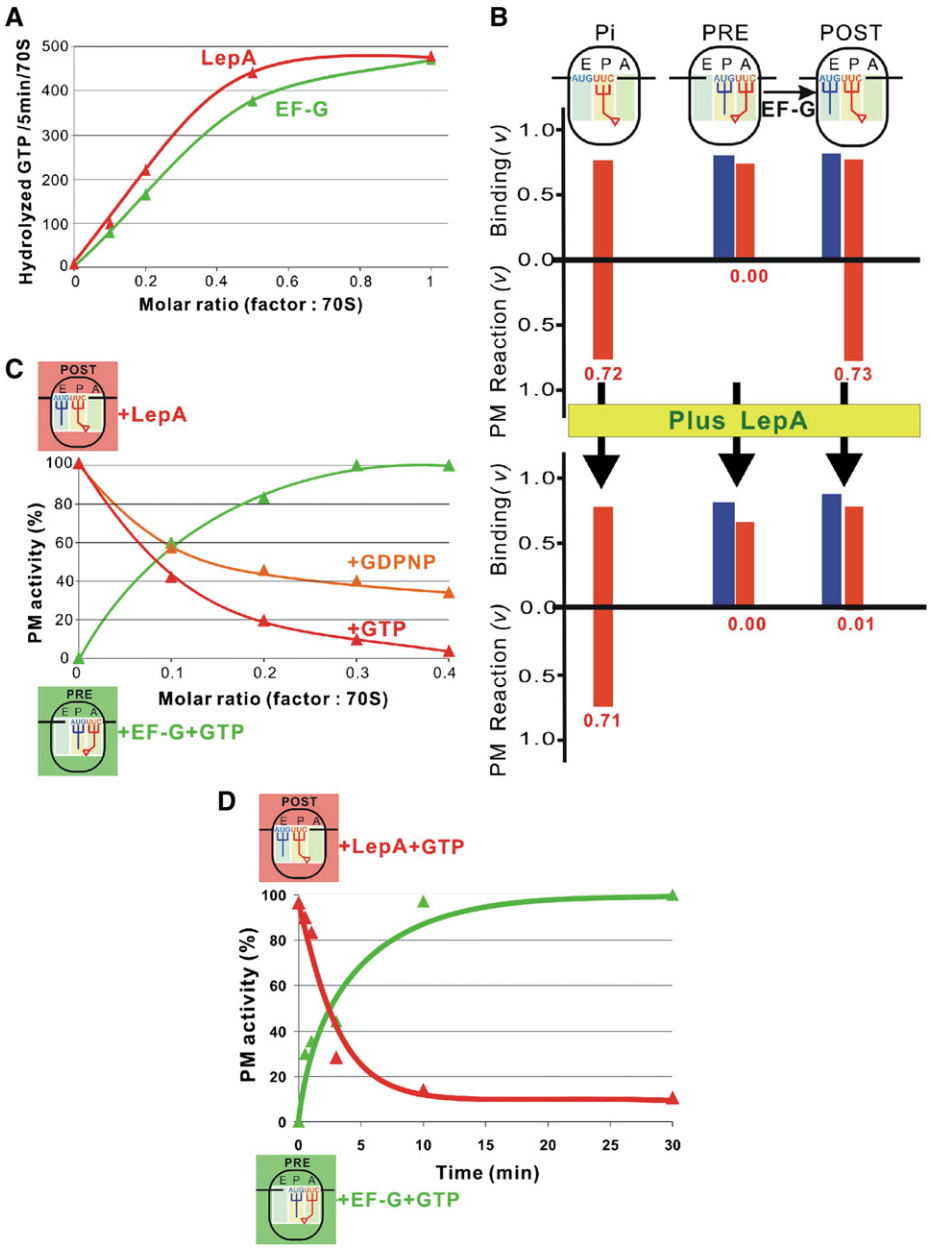


Figure 4. In Vitro Assays with LepA

(A) Uncoupled ribosome-dependent GTPase activity of EF-G (green) and LepA (red). The GTPase activity is given as GTP hydrolyzed in 5 min per ribosome, and plotted as a function of the molar ratio of factor to ribosome.

(B) tRNA binding and puromycin reaction with ribosomal complexes in different functional states (shown schematically) before and after incubation with LepA and GTP. The data are normalized to reactions per one ribosome (ν). LepA does not influence the amounts of bound tRNAs (upright bars), but it abolishes the puromycin reaction (hanging bars) of the POST state rather than that of the Pi state. Red tRNAs and columns, Ac[^{14}C]Phe-tRNA; blue tRNAs and columns, [^{32}P]tRNA$_f^{Met}$. As a control, the experiment was also performed in the presence of nonlabeled AcPhe-tRNA, added either to (1) the PRE state (no chasing of the Ac[^{14}C]-Phe-tRNA at the A site was observed: 0.65 and 0.69 Ac[14C]Phe-tRNA at the A site per ribosome in the absence and presence of nonlabeled AcPhe-tRNA), or (2) during back-translocation, when the nonlabeled AcPhe-tRNA was added to the purified POST state before addition of LepA to induce back-translocation (0.56 and 0.54 of Ac[14C]Phe-tRNA per 70S in the absence and presence of nonlabeled AcPhe-tRNA after back-translocation).

(C) Puromycin reaction of the PRE state after incubation with various amounts of EF-G•GTP (green curve) and the POST state after incubation in the presence of various amounts of LepA with GTP and GDPNP (red and orange, respectively). In the presence of GDPNP, quantitative blockage of the puromycin reaction is only achieved at a molar ratio of LepA:70S = 1:1, whereas with GTP, full blockage is already seen at a ratio of (0.3–0.4):1.

(D) Comparison of kinetics of EF-G-dependent translocation and LepA-dependent back-translocation. The reactions were performed at 30°C under single turnover conditions in the presence of GDPNP (0.5 mM; molar ratios of EF-G and LepA to 70S were 5:1 and 10:1, respectively).

and (3) direct testing of the ribosome movement on the mRNA with a toeprint assay.

A-site-bound tRNAs protect a set of characteristic bases in the 16S and 23S rRNA from chemical modifications (Moazed and Noller, 1989, 1990). In order to unravel the ribosomal location of AcPhe-tRNA in our various complexes, we screened two known A-site tRNA footprints in 16S rRNA. At position A1408 in the decoding center, AcPhe-tRNA in the PRE state ribosome produced the expected A-site protection, which however was lost upon translocation to the P-site in the POST state (Figure 5A). Significantly, the addition of LepA•GTP to the POST state re-established the A1408 footprint. An essentially identical tRNA footprinting pattern was observed at position U531 of 16S rRNA (data not shown). These data are compatible with the notion that AcPhe-tRNA reoccupies the A site upon LepA addition to POST state ribosomes.

Furthermore, the 50S subunit also shows structural evidence for a LepA-promoted back-translocation. Previously we demonstrated that the 50S conformation of the posttranslocational ribosome is different to that of the pretranslocational ribosome—a difference that could be monitored by site-specific Pb^{2+} cleavage of 23S rRNA (Polacek et al., 2000). A diagnostic cleavage was detected at position C2347, which was significantly enhanced in the POST state compared with the PRE state. Figure 5B demonstrates that LepA brings the strong signal observed in the POST state down to the level of the PRE signal, suggesting that upon binding of LepA•GTP, the ribosome adopts a PRE configuration.

Additionally, we confirmed the back-translocation ability of LepA using a toeprinting assay. In this assay, the programming mRNA carries a complementary [^{32}P]-labeled DNA primer annealed to the 3′ end, located downstream of the ribosome. The primer is prolonged by reverse transcription until the polymerase clashes with the ribosome. In this way, the length of the transcript provides a measure of the distance between the primer and the ribosome. During translocation the ribosomes move by a codon length toward the primer position, and thus, the reverse transcript becomes shorter by three nucleotides (Hartz et al., 1990). Conversely, the transcript will be longer by three nucleotides after the putative back-translocation. A translocation of a PRE state shows a decrease in the length of the reverse transcript by three nucleotides, while the addition of LepA•GTP to a POST state increases the length of the transcript to that of the PRE state again (Figure 5C), proving that LepA is a back-translocator.

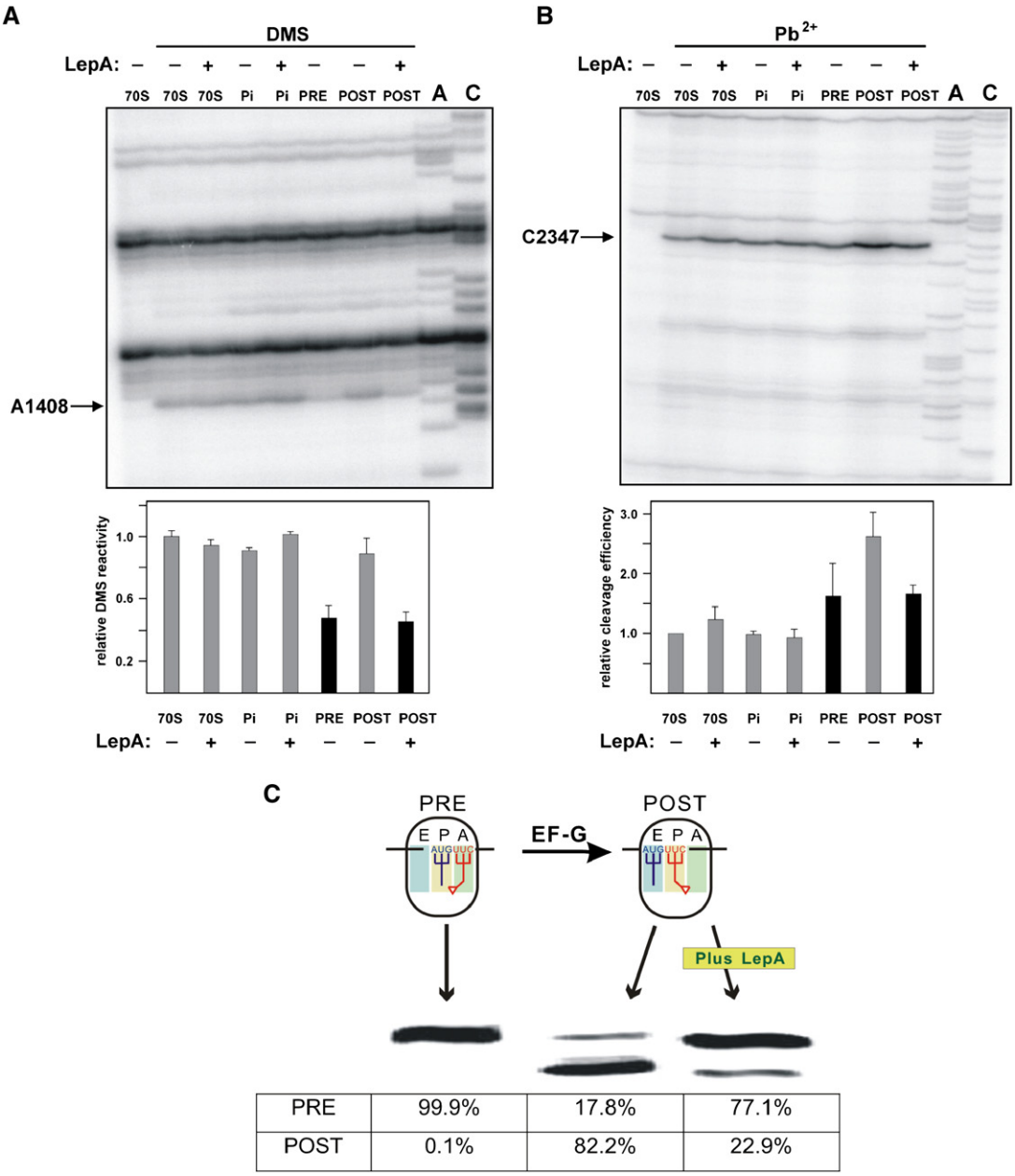
An alternative possibility is that we are not in fact observing a back-translocation, but rather a complete release of AcPhe-tRNA from the POST state coupled with quantitative rebinding of the tRNA to the A site to form a PRE state. To exclude this, the back-translocation experiment was repeated in the absence and presence of a 2 molar excess of nonlabeled AcPhe-tRNA over ribosomes. If LepA triggers a release of Ac[^{14}C]Phe-tRNA from the P site and rebinding at the A site, then the presence of nonlabeled AcPhe-tRNA would reduce the ribosome-bound Ac[^{14}C]Phe-tRNA dramatically. However, no reduction in the [^{14}C] label during the back-translocation was observed (numbers given in the legend to Figure 4B).

In Vitro Analyses of LepA: Effects on Synthesis of Active Protein

We have demonstrated previously that bacterial coupled transcription-translation systems can produce large amounts of protein (e.g. 4 mg/ml green fluorescent protein [GFP]), but under standard conditions (30°C incubation) the active fraction (50% ± 20%) is unsatisfactorily low (Dinos et al., 2004; Iskakova et al., 2006). The experimental setup is that the total protein amount is assessed via SDS-PAGE, since the reporter protein GFP does not overlap in a Coomassie stained gel with any other protein present in the cell lysate. This enables the GFP band to be scanned and an accurate determination of the total amount to be made. In parallel, the same samples are loaded onto native gels and the active amount is revealed via the fluorescence of the GFP band (Figure 6A), thus allowing a precise assessment of the active fraction.

In the presence of increasing amounts LepA, the total GFP amount increases and peaks at a ratio of 0.1 molecules LepA added per 70S. Further addition of LepA leads to a rapid reduction in the GFP production, eventually blocking the total synthesis completely at a molar ratio of LepA:70S = ($\geq$0.5):1, in agreement with the toxic effects of overproduced LepA in vivo (Figure 3). In contrast, the native gel reveals that the active GFP amount increases to attain the same levels as the total GFP amount at LepA stoichiometries of $\geq$0.2 LepA per 70S. In other words, addition of LepA promotes the synthesis of fully active proteins (also demonstrated with luciferase, data not shown). We conclude that LepA not only increases the total protein yield, but more importantly improves the activity of the produced protein.

The best-known drugs that induce misincorporations are aminoglycosides, such as paromomycin, which bind directly in the decoding center to impair the tRNA selection process (Ogle et al., 2001). Since LepA was shown to increase the active fraction of proteins, we asked whether LepA could also correct paromomycin-induced translational errors. Figure 6B, left panel, demonstrates that paromomycin severely decreases GFP synthesis, blocking it completely at 2 μM. On the native gel, the fluorescence of GFP was even more strongly reduced by paromomycin addition, indicating that paromomycin causes a drop in the active GFP fraction. In the presence of LepA, a similar paromomycin-dependent reduction in the active GFP fraction is observed (Figure 6B, right panel), indicating that LepA cannot counteract the paromomycin-induced errors. Equivalent results were obtained with both other aminoglycosides, such as streptomycin and neomycin, which also bind to the decoding center of the A site, and with the E site antibiotic edeine (data not shown).



PRE	99.9%	17.8%	77.1%
POST	0.1%	82.2%	22.9%

Figure 5. LepA Induces Back-Translocation

(A) Primer extension analysis of DMS-modified 16S rRNA from various ribosomal complexes in the absence (–) or presence (+) of LepA•GTP. The band of the diagnostic A-site tRNA footprint at A1408 of 16S rRNA is indicated by an arrow. A and C denote dideoxy-sequencing lanes. Quantification of the DMS reactivity at A1408 in different ribosomal complexes is shown below the gel. The DMS reactivity at A1408 in the empty ribosome (70S) was taken at 1.00. Values shown represent the mean and the standard deviation of two independent DMS probing experiments.

(B) Primer extension analysis of Pb^{2+}-cleaved 23S rRNA. The cleavage efficiency at C2347 of 23S rRNA (arrow) was monitored in various ribosomal complexes in the absence (–) or presence (+) of LepA•GTP. The characteristic cleavage enhancement at C2347 in the POST sate disappears upon LepA addition. A and C denote dideoxy-sequencing lanes. The Pb^{2+} cleavage efficiency of vacant ribosomes (70S) was taken as 1.00. Values shown represent the mean and the standard deviation of two independent Pb^{2+} cleavage experiments.

(C) Toeprint assay with PRE and POST states. The PRE state (lane 1) was translocated with EF-G and GTP and the resulting POST state was purified by pelleting through a sucrose cushion. The purified POST state was then toeprinted either directly (lane 2) or after an incubation in the presence of LepA and GTP (lane 3). The relative amounts of the PRE and POST states given in percentages were obtained by scanning the respective bands.

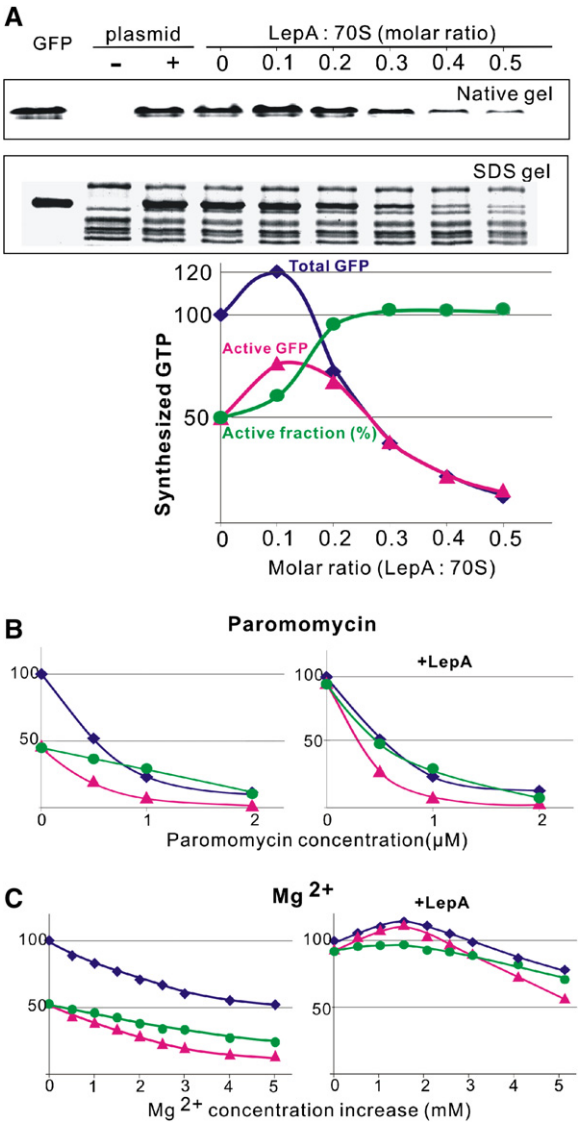


Figure 6. LepA Effects on GFP Synthesis in a Coupled Transcription-Translation System In Vitro

(A) Addition of various amounts of LepA. One aliquot of the reaction mixture was applied to a native gel and the fluorescence was measured (upper panel, amount of active GFP); a sister aliquot was developed in an SDS-gel (middle panel, total amount of GFP). The total amount of GFP synthesized in the absence of LepA was designated as 100%, the relative amounts of total and active GFP were determined, and the active fraction was calculated (lower graph).

(B) GFP synthesis as in (A), but in the presence of various concentrations of the aminoglycoside paromomycin. Left panel, no LepA; right panel, in the presence of LepA (0.3 mole per mole of 70S).

(C) Same as (B), but with increasing Mg^{2+} concentrations. "0" indicates the intrinsic Mg^{2+} concentration of 12 mM.

The fidelity of protein synthesis is very sensitive to changes in magnesium concentration, such that an increase of only 5 mM (from 12 to 17 mM) reduces the total synthesis of GFP to 40% and the active fraction from 50% to 25% (Figure 6C, left panel). Addition of LepA dramatically alters the picture: with a Mg^{2+} increase up to 3 mM, the total synthesis is not reduced; in fact, a small but significant increase is observed (up to 120%) and the active fraction is maintained at ~100% (Figure 6C, right panel). It follows that the dominant effect of LepA is seen at a Mg^{2+} increase of 2–3 mM, where the total protein synthesis is doubled with an active fraction of virtually 100%.

DISCUSSION

The conserved domain structure of LepA, in which the first four domains correspond to the EF-G domains I, II, III, and V, coupled with the presence of a fifth unique LepA domain (Figure 2), makes it easy to trace LepA through the three domains of life. We used LepA domain III and IV (corresponding to III and V from EF-G) as well as the unique CTD as probes in order to avoid false positives caused by EF-G or the corresponding factor EF2 in archaea and the cytoplasm of eukarya. We found LepA orthologs in all bacteria and eukaryotes with mitochondria, but not in archaea. This observation suggests that LepA does not contribute to eukaryotic cytoplasmic translation, but is probably essential for correct mitochondrial translation. LepA is probably also ubiquitous in chloroplasts, since we found LepA with apparent chloroplast import sequences to be nuclear encoded in the three plant genomes that have been completely sequenced, viz. the dicotyledon *Arabidopsis thaliana*, the monocotyledon *Oryza sativa* and the red alga *Cyanidioschyzon merolae* (data not shown). We note that LepA phylogeny largely reflects the canonical species phylogeny and shows no signs of interdomain horizontal gene transfer (HGT). In this respect, LepA behaves like ribosomal proteins rather than tRNA synthetases that frequently undergo HGT (Wolf et al., 1999).

The lack of EF-G domain IV in the LepA structure is intriguing. Our finding that LepA is a back-translocator fits with the early suspicion that domain IV of EF-G has a "door-stop" function, i.e., by occupying the decoding region of the A site after the tRNAs have been translocated from A and P sites to the P and E sites, respectively, domain IV of EF-G prevents a back-movement of the tRNAs (Nierhaus, 1996a; see also Figure 2C). In this respect, LepA would reduce the activation barrier between PRE and POST states in a way similar to EF-G, but due to the absence of domain IV, it catalyzes a back-translocation rather than a canonical translocation.

In addition to lacking EF-G domain IV, LepA also lacks the G' subdomain (Figures 2B and 2C). It has been speculated that the function of G' might be to promote the GDP-GTP exchange, as EF-Ts does for EF-Tu (Czworkowski et al., 1994). However, the GDP-GTP exchange on EF-G can also be explained without the help of an additional factor or G' subdomain (Nierhaus, 1996b). Despite the absence of the G' subdomain, LepA shows uncoupled GTPase activity in the presence of 70S, paralleling that of

EF-G (Figure 4A). This argues against the assumption that this subdomain is involved in GDP-GTP exchange.

The first experimental hint for the back-translocation activity of LepA came from two separate functional tests, the puromycin reaction and dipeptide formation. Both Pi and POST states with an AcPhe-tRNA donor at the P site usually act as equally good substrates for peptide bond formation using puromycin, or an aa-tRNA, as an A site acceptor. The essential point is that LepA prevents peptide bond formation exclusively of the POST state, while leaving the Pi state unaffected (Figure 4B and Figure S2). The most likely interpretation for this is that LepA induces a back-translocation by shifting the tRNAs from the E and P sites back to the P and A sites, respectively, whereas a ribosome with a single tRNA at the E site cannot be moved either forward or backward. After a back-translocation, the A site is now filled with AcPhe-tRNA; this prevents binding of both puromycin and aa-tRNA, and thus prevents peptide bond formation with both substrates.

This interpretation was substantiated by three structural assays monitoring (1) the tRNA occupancy of the A site via protection of diagnostic rRNA bases of the A site, (2) the functional state—PRE or POST—of the ribosome via conformation-specific Pb^{2+} cleavage, and (3) the movement of the ribosome on the mRNA via toeprinting. Protection of residues A1408 and U531 of the 16S rRNA is indicative of the presence of a tRNA at the A site (Moazed and Noller, 1990). POST state ribosomes have an empty A site and therefore show no A-site tRNA footprints; however, upon administering LepA•GTP to such a POST state, protection of these A site-specific positions was observed, thus arguing for the reoccupation of the A site by the peptidyl-tRNA (Figure 5A). Pb^{2+} cleavages occur within distinct binding pockets of RNAs and are therefore very sensitive to conformational changes. Cleavage at position C2347 of 23S rRNA is strong in the POST state and weak in the PRE state (Polacek et al., 2000), and LepA reduces the cleavage level of the POST state to that of the PRE state (Figure 5B). Finally, the toeprinting assay (Hartz et al., 1991) was used to directly demonstrate the back-movement of the ribosome on the mRNA by one codon upon the addition of LepA to the POST state (Figure 5C). Such a back-translocation cannot take place in a Pi state: a single tRNA on the ribosome cannot move from the P to the A site since the resulting complex (A site occupied, P site free) is unstable (Rheinberger et al., 1981). The fact that LepA functions only with the POST state, rather than with the Pi state, means that the function of this factor depends on the ribosome having an occupied E site. This requirement is a strong indication that the E site also exists in mitochondrial ribosomes, for which the number of tRNA binding sites has not yet been assessed.

The mitochondrial membrane potential depends on the respiratory activity of the mitochondria (Petit et al., 1990), which in turn might influence the intraorganelle ionic strength, creating a requirement for LepA. We note, however, that this must be true only under specific and yet unknown conditions, because a knockout of the LepA ortholog GUF1 in yeast mitochondria exhibits no clear phenotype (Kiser and Weinert, 1995). Be that as it may, the extreme conservation of both the domain structure and the amino acid sequence in all currently available sequences of mitochondrial LepA orthologs signals that an important function for this protein must also exist in this organelle.

Although LepA seems to work like a typical G protein (Figure 4C), one note of caution must be added: the binding of LepA to the ribosome was monitored in the absence of nucleotides and in the presence of GTP or GDPNP via pelleting the ribosomes through a sucrose cushion and determining the presence of LepA in an SDS gel. We observed 0.20, 0.19, and 0.51 LepA bound per 70S ribosome, respectively. In a second experiment, back-translocation of a purified POST state was analyzed using a toeprinting assay in the presence of LepA with and without GTP. Surprisingly, we observed that LepA promoted back-translocation, even in the absence of GTP; however, the level was up to 50% of that observed in the presence of GTP (data not shown). One explanation might be that, even in the absence of nucleotide, a fraction of the LepA molecules have retained the GTP conformation. Whether this "apo" LepA can work catalytically has yet to be determined. Such a scenario would go some way toward explaining why a 40% reduction of the puromycin reactivity was observed in the presence of GDPNP when the LepA concentration was only 10% of that of the ribosomes (Figure 4C): while half of this discrepancy can be accounted for by the fraction of ribosomes in the POST state (60%), the rest may result from the possible catalytic action of apo LepA.

How can we reconcile the in vivo and in vitro effects of LepA to provide a complete molecular description of its function? One possible explanation is that LepA slows down the translational rate, thus improving both cotranslational folding of proteins and the active fraction of the synthesized proteins. We cannot exclude this possibility, but at the moment we favor an alternative scenario as follows.

We have demonstrated that LepA improves the fidelity of translation and induces back-translocation of POST state ribosomes, which suggests that there is a link between translocation and activity of the synthesized protein. EF-G-dependent translocation is probably not successful in 100% of all cases, particularly at higher Mg^{2+} concentrations, at which the ribosome may not reach the canonical POST state. Translocation of tRNAs occurs at the interface between the small and large subunits and involves a ratchet-like movement of one subunit relative to the other (Frank and Agrawal, 2001). It has long been known that Mg^{2+} and ionic strength influence subunit interaction (Hapke and Noll, 1976), and therefore it is easy to envisage that high ionic strength could hinder EF-G action and/or induce a defective POST state in the ribosome. This defective posttranslocation state might have two consequences. (1) It may result in a suboptimal display

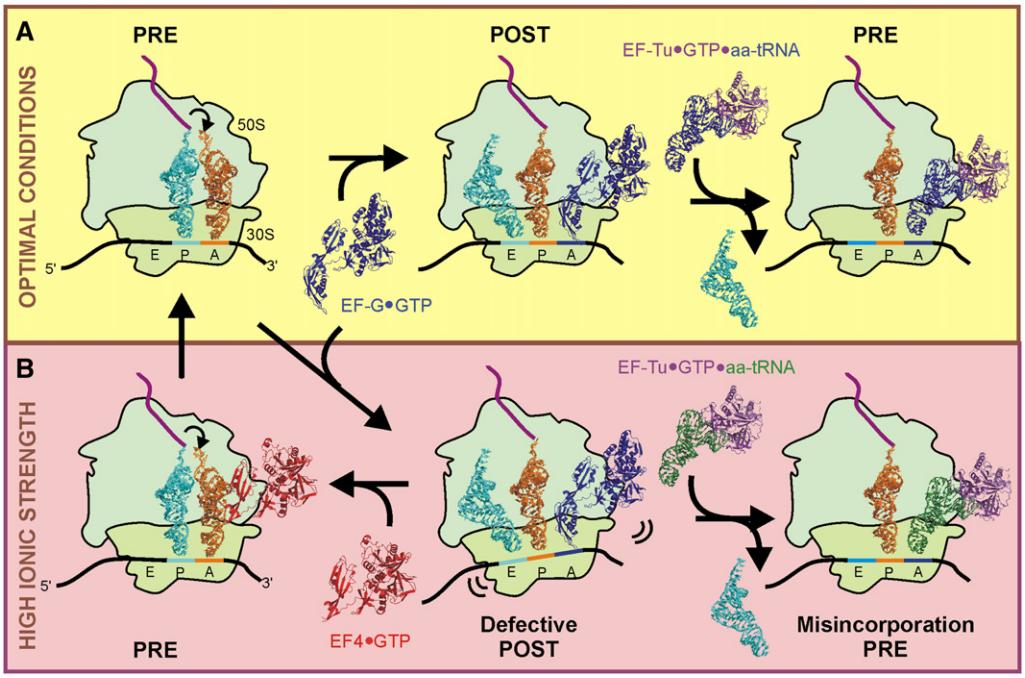


Figure 7. Model for LepA (EF4) Function

(A) Under optimal growth conditions the translocation has a very low rate of error, and therefore, EF4 is not so important under such conditions. Translocation involves the movement of tRNAs at the A and P sites (PRE state) to the P and E sites (POST state). This reaction is catalyzed by elongation factor G (EF-G, blue) and GTP. After dissociation of EF-G, the A site is now free for binding of the next ternary complex aa-tRNA•EF-Tu•GTP (blue tRNA to blue A site codon), which leads to release of the E-tRNA (cyan).

(B) In the rare case that EF-G malfunctions, a defective translocation complex may result. This is likely to occur more frequently under conditions of high ionic strength. The consequences of the defective translocation complex are 2-fold: (1) ribosomes may incorrectly display the A site codon, allowing binding of near-cognate ternary complexes and therefore misincorporation, as illustrated by the right-hand pathway (binding of green tRNA to blue codon); (2) under extreme conditions, the ribosome may even become stuck, thus precluding continued translation. The defective translocation state is recognized by EF4•GTP (red), which induces a back-translocation, allowing EF-G a second chance to catalyze a correct POST state. In this way EF4 reduces translational errors and relieves stuck ribosomes.

of the A-site codon in a way that promotes misincorporation. This is evident from the coupled transcription-translation system, in which the addition of low amounts of LepA leads to dramatic increases in the accuracy of GFP synthesis, particularly in conditions of high ionic strength (Figures 6A and 6C). (2) In some cases, such as under the influence of high Mg^{2+} concentrations, a ribosome might even become stuck during the course of a translocation reaction. In this respect it should be noted, however, that the increased misincorporation seen at higher Mg^{2+} concentrations (Figure 6C) cannot alone explain the lethal effect, since *E. coli* strains harboring a RAM mutation in S4 or S5 exhibit 10-fold higher misincorporation rates, i.e. rates equivalent to those induced by streptomycin, but are still viable (Zimmermann et al., 1971).

Based on our findings that LepA binds to POST-state ribosomes, we suggest that LepA should be renamed as elongation factor 4 (EF4). Collectively, our results enable a model for EF4 action to be proposed: at low concentrations of EF4 ($\leq$0.3 molecules per 70S ribosome), EF4 specifically recognizes improperly translocated ribosomes, back-translocates them, and thus provides EF-G with a second chance to catalyze a proper translocation reaction (Figure 7). At higher concentrations ($\sim$1 molecule per 70S), EF4 loses its specificity and back-translocates every POST ribosome, thereby putting the translational machinery into a nonproductive mode. This is seen by the inhibition of the coupled transcription-translation system at high concentrations of EF4 ($\geq$1.5 μM, equivalent to 1.5 μM ribosome concentration in the Rapid Translation System (RTS; Figure 6A), and it explains the toxicity of overexpressed EF4 in vivo (Figure 3). From these results it is clear that the intracellular level of EF4 must be precisely tuned and regulated to restrict it to the narrow beneficial concentration window. We finally note a potential application for EF4 derived from results of the coupled transcription-translation system, namely that the addition of a small, defined amount of EF4 to bacterial lysates significantly improves the protein output, combining both high yield and full activity of the synthesized protein. This illustrates not only the importance of EF4 for protein synthesis in the bacterial cell, but paves the way to the development of more efficient in vitro transcription-translation systems.

EXPERIMENTAL PROCEDURES

Materials

The materials used were described in Marquez et al. (2004).

Protein Sequence and Homology Analysis

Database searches for orthologs of *E. coli* and yeast LepA and EF-G were carried out using BLASTP with standard parameters and protein databases of organisms with completely sequenced genomes that were downloaded from the Integr8 web site (http://www.ebi.ac.uk/integr8/). Orthology was assigned based on best reciprocal hits. Additionally, the INPARANOID database (http://inparanoid.cgb.ki.se) and the NCBI nonredundant protein database (ftp://ftp.ncbi.nih.gov/blast/db/nr.00.tar.gz and ftp://ftp.ncbi.nih.gov/blast/db/nr.01.tar.gz) were screened for additional homologs that were tested for orthology by phylogenetic analysis. Subcellular localization of proteins was predicted using CHLOROP and MITOP. Multiple alignments were constructed using MAFFT software (http://www.biophys.kyoto-u.ac.jp/~katoh/programs/align/mafft/) for each common structural domain of EF-G and LepA separately, because domain order is only partially conserved between these proteins. Only regions with sufficient sequence similarity for unambiguous alignment were considered. Alignments for individual domains were concatenated and used for phylogenetic analysis. Phylogenetic analysis was carried out with MEGA software (version 3.1, http://www.megasoftware.net/). Pairwise sequence distances were obtained by Maximum Likelihood estimation on the basis of the JTT substitution rate matrix with the assumption of a uniform distribution of rates across sites. Phylogenetic trees were reconstructed using the Neighbor Joining algorithm. Statistical support values for internal branches of the tree were obtained from 1000 bootstrap samples and their analyses. Trees were calculated on a reduced set of organisms (Figure 1) and on a large set of organisms (data not shown). The essentials of the phylogenetic tree already emerged during the analysis of the smaller data set. Due to the higher number of taxa, the second data set offers an enhanced resolution of internal branching patterns in the bacterial subtree and confirms results from the first data set.

The homology model for *E. coli* LepA (Figure 2C) was generated based on the sequence alignment (Figure 2B) and the crystal structure for *Thermus thermophilus* EF-G (PDB1WDT) using the Protein Homology / analogY Recognition Engine (PHYRE) (http://www.sbg.bio.ic.ac.uk/~phyre/). Figure 2C was created using the PyMOL Molecular Graphics System (2002) from DeLano Scientific, San Carlos, CA, USA (http://www.pymol.org).

LepA, Strains

E. coli LepA gene was cloned from genomic DNA using PCR primers that introduce NdeI and BamHI restriction sites for cloning into the expression vector pET14b (Novagen). The cultures of *E. coli* BL21(DE3) strain, or this strain transformed with either pET14b or pET+LepA, were grown overnight with 150 rpm shaking at 37°C. Cells were diluted 1:200 and grown for 2–3 hr at 37°C. When the optical density reached an A_{580} of ~0.4, the cells were induced with 1 mM IPTG.

Preparation of Purified Components for In Vitro Assays

Reassociated 70S ribosomes were prepared according to Blaha et al. (2000). MF-mRNA described in Triana-Alonso et al. (1995b) and encoding Met-Phe [sequence: GGG(A4G)3AAAAUGUUC(A4G)3AAAU] was prepared according to Schäfer et al. (2002). EF-Tu and EF-G with C-terminal His-tags were isolated from *E. coli* as described previously for EF-Tu (Boon et al., 1992) with the following changes: the cells were induced by a cell density of 0.5 A_{560} and incubated further for 4 hr. The cells were pelleted and resuspended (1 ml/g) in a buffer containing 20 mM Hepes-KOH (pH 7.6 at 0°C), 60 mM NH_4Cl, 7 mM $MgCl_2$, 7 mM β-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride (PMSF), and additionally, in the case of EF-Tu, 50 μM GDP. The cells were disrupted with the microfluidizer (model M-110L; Microfluidics). After a centrifugation step (30,000 × g for 45 min) the supernatant was treated according to method 2 in Boon et al. (1992) (EF-Tu elution from the Ni^{2+}-column at 80 mM and EF-G at 250 mM imidazole) followed by a final dialysis against a buffer containing 20 mM Hepes-KOH (pH 7.6 at 0°C), 6 mM $MgCl_2$, 150 mM KCl, 1 mM DTE, 10 μM GDP, and 10% glycerol. Crude or specific tRNAs were purchased from Sigma and charged according to Marquez et al. (2004). tRNA binding and dipeptide assays and translation of model-mRNAs were performed as described (Dinos et al., 2004), with the final conditions of our standard buffer as follows: 20 mM Hepes-KOH (pH 7.6 at 0°C), 4.5 mM $Mg(acetate)_2$, 150 mM NH_4acetate, 4 mM β-mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine.

GTPase Activity

The GTPase assays were as described previously (Connell et al., 2003), except that reactions were set up to maintain the condition of the standard buffer with a final ribosome concentration of 0.2 μM, a final protein concentration of 0.02–0.2 μM, and a [γ-^{33}P] GTP concentration of 50 μM.

Preparation of Defined Ribosomal Complexes

Pi, PRE, and POST complexes were made as described previously (Marquez et al., 2004). The Pi complex consisted of reassociated 70S ribosomes programmed with MF-mRNA and Ac[^{14}C]Phe-tRNA^{Phe} in the P site. The PRE complex consisted of reassociated 70S ribosomes programmed with MF-mRNA, a [^{32}P]deacyl-tRNA$_f^{Met}$ in the P site, and Ac[^{14}C]Phe-tRNA^{Phe} in the A site, and they were subsequently translocated by EF-G to yield the POST complexes. The complexes (1 ml) were then sedimented through a sucrose cushion (1 ml 10% sucrose in standard buffer) at 65,000 × g for 18 hr, 4°C in a TL-100 ultracentrifuge (Beckman) to remove nonbound mRNA and tRNA in the case of Pi and PRE complexes, or EF-G in the case of POST complexes.

Puromycin Assay

Defined ribosomal complexes (0.2 μM) in the standard buffer were incubated with or without 0.06 μM LepA and 250 μM GTP at 37°C for 10 min. A puromycin reaction followed as described previously (Marquez et al., 2004).

Structural Probing

Prior to tRNA footprinting, 5 pmol of ribosomal complexes were incubated in 16.3 μl standard buffer at 37°C for 10 min. Chemical probing with dimethyl sulfate (DMS) at 0°C for 30 min was performed as described (Bayfield et al., 2001). Modification with 1-cyclohexyl-2-morpholino-carbodiimidemetho-p-toluensulfonat (CMCT) was initiated by the addition of 8.15 μl CMCT solution (84 mg/ml standard buffer) and was performed at 37°C for 15 min. $Pb(OAc)_2$ cleavage of 5 pmol ribosomal complexes was performed in 18 μl standard buffer for 5 min at 25°C as described (Polacek et al., 2000). Primer extension products of modified rRNAs (Polacek and Barta, 1998) were separated on 6% polyacrylamide gels and quantified using a Molecular Dynamics Storm PhosphorImager.

Toeprint

The following mRNA was used: GGCAA**AGGAGGU**AUUAUUA***AUG UUCAAACGAUCAAUCUACGUA***UAAUAAAAGAAAAGAAAAGAAAAGAAAAGAAAAGGA*CAUCACAGAUUAACG*; this contains a Shine-Dalgarno sequence (bold underlined) and codes for MFKSIRYV (bold italic). The mRNA was annealed to a ^{32}P-5′-end-labeled primer (underlined italics) as described in Hartz et al. (1988) and then used to program ribosomes for PRE and POST complexes. Briefly, 200 pmol reassociated 70S were incubated with 5 pmol mRNA:primer and 400 pmol each of tRNA$_f^{Met}$ and Ac-Phe-tRNA^{Phe} in standard buffer. Aliquots of the reaction mixture with 5 pmol 70S were withdrawn before and after EF-G-dependent translocation reaction and used for toeprinting assays. The remaining posttranslocational mixture of 275 μl

was centrifuged through a 1 ml 10% sucrose cushion in standard buffer (65,000 × g for 18 hr). The pellet was resuspended in 90 μl standard buffer and aliquoted into 15 μl portions.

7.5 pmol POST complexes in 15 μl were incubated for 30 min at 37°C with 5 times excess of LepA and 200 times excess GTP (0.1 mM) and used for the toeprinting assay. The end-labeled primer on the mRNA was extended by 100 units of MuMLV reverse transcriptase (Fermentas) in the presence of dNTPs, each 135 μM in standard buffer at 37°C for 15 min. The reaction was stopped by 20 μl of loading buffer (9 M urea, 90 mM TRIS [pH 8.3] at room temperature, 90 mM boric acid, 15 mM EDTA, 0.05% xylene cyanol, 0.05% bromophenol blue) and heated at 95°C for 5 min. Toeprint reactions were analyzed on 8% urea-PAGE (8 M urea). The gels were quantified using a Molecular Dynamics Storm PhosphorImager.

Coupled Transcription-Translation Assay and Quantification of Fidelity

The assay and quantification were described previously (Dinos et al., 2004), except that each reaction volume of 10 μl contained both 0.1 μl of the plasmid solution with the GFP gene after the T7 promoter (pIVEX2.2-GFPcyc3; 1 μg/μl) and 1.4 μl with LepA and/or antibiotics.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.cell.com/cgi/content/full/127/4/721/DC1/.

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