The Termination Phase in Protein Synthesis is not Obligatorily Followed by the RRF/EF-G-Dependent Recycling Phase

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Abstract

It is general wisdom that termination of bacterial protein synthesis is obligatorily followed by recycling governed by the factors ribosomal recycling factor (RRF), EF-G, and IF3, where the ribosome dissociates into its subunits. In contrast, a recently described 70S-scanning mode of initiation holds that after termination, scanning of 70S can be triggered by fMet-tRNA to the initiation site of a downstream cistron. Here, we analyze the apparent conflict. We constructed a bicistronic mRNA coding for luciferases and showed with a highly resolved in vitro system that the expression of the second cistron did not at all depend on the presence of active RRF. An in vivo analysis cannot be performed in a straightforward way, since RRF is essential for viability and therefore, the RRF gene cannot be knocked out. However, we found an experimental window, where the RRF amount could be reduced to below 2.5%, and in this situation, the expression of the second cistron of a bicistronic luciferase mRNA was only moderately reduced. Both in vitro and in vivo results suggested that RRF-dependent recycling is not an obligatory step after termination, in agreement with the previous findings concerning 70S-scanning initiation. In this view, recycling after termination is a special case of the general RRF function, which happens whenever fMet-tRNA is not available for triggering 70S scanning.

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Introduction

It is generally accepted that in eubacteria, termination of a cistron is followed by a ribosomal recycling factor (RRF)- and EF-G-mediated dissociation of ribosomes from mRNA termed as the recycling phase of protein synthesis, a process supported by IF3 [1–5]. RRF is an essential factor [6], universal in Eubacteria but absent in Achaea and Eukaryota [7]. X-ray and cryo-electron microscopic studies have shed light on the mechanism of dissociation of post-termination complexes [8–13]. It is thought that the recycling phase is an obligatory step following the termination phase in eubacteria and is required for providing 30S subunits for initiating the next round of translation of a cistron.

An obligatory recycling phase after termination cannot easily be reconciled with numerous hints over the last decades for a re-initiation, where 70S ribosomes rather than 30S subunits were conjectured to be involved [14–17], although no mechanistic features of a 70S re-initiation were reported. Recently, we have demonstrated a frequent initiation mode in bacteria, the so-called 70S-scanning initiation, according to which 70S ribosomes do not dissociate after termination but rather scan along the mRNA until reaching the initiation site of the downstream cistron of the same mRNA. 30S scanning was not observed; binding of fMet-tRNA triggers 70S scanning, which occurs in the absence of energy-rich compounds (e.g., GTP) and seems to be driven by unidimensional diffusion [18]. A recent report about 70S ribosomes
with tethered subunits preventing full dissociation into ribosomal subunits demonstrated that these ribosomes were able to synthesize active proteins [19].

The apparent conflict between these two mechanisms, RRF-dependent recycling versus 70S-scanning initiation, is analyzed here. If the recycling phase is an essential prerequisite for the subsequent initiation event, depleting the RRF pool should result in a collapse of protein synthesis. Here, we show in vitro and in vivo with suitable bicistronic mRNA constructs that RRF is not essential for the expression of a downstream cistron.

Results

The RRF/EF-G-dependent recycling phase is thought to be an obligatory and essential step after translation termination [20]. First, we tested whether the RRF—used in the following in vitro experiments—is active. To this end, we applied a test reported by Zavialov et al. [3], who used a 7-, 22-, and 75-fold excess of RRF in the presence of IF3 and EF-G. We incubated a 70S complex containing a deacylated tRNA in the P site with EF-G•GTP, IF3, and different amounts of RRF (5- and 15-fold excess of 70S ribosomes; Fig. 1). The addition of RRF triggered a clear dissociation effect, viz. the RRF preparation we used is active.

In the next experiment, we asked whether or not the translation of the second cistron in a bicistronic mRNA depends on the presence of RRF. A modified PURE system was applied [21], which allows protein synthesis in the presence or complete absence of the factors IF1, IF3, and RRF. Our PURE system is further characterized by a lowered total Mg$^{2+}$ concentration (8.5 instead of 13 mM), which yields a free Mg$^{2+}$ concentration of 2.3 mM Mg$^{2+}$. The low Mg$^{2+}$ concentration is responsible for an extremely slow equilibrium rate between 70S ribosomes and the subunits during the incubation of the translation assay (Fig. S4 in Ref. [18]); during the first 15 min of incubation, no equilibrium is observed between 70S and subunits.

We designed a bicistronic mRNA coding for luciferases from Renilla (Rluc) and firefly (Fluc), respectively (Fig. 2, top). The mRNA is similar to that used in Ref. [18], where we have shown that blocking either the expression of the first cistron or the intercistronic runway for scanning 70S ribosomes by hybridizing antisense oligo-DNAs strongly reduces the expression of the second cistron (Fig. 1 in Ref. [18]). Furthermore, we introduced a secondary structure hiding the Shine–Dalgarno (SD) sequence in front of the second cistron (Fig. 2). Such a secondary structure prevents 30S binding but can be resolved by scanning 70S ribosomes (see Fig. 2C of Ref. [18]). Another feature of the mRNA shown in Fig. 2 is a fusion of the second cistron Fluc with a part of the sequence of the regulatory protein SecM (SecM125–170), which blocks translating ribosomes on the mRNA [22]. Therefore, each ribosome will

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**Fig. 1.** Post-termination complexes containing a deacylated tRNA in programmed 70S ribosomes were incubated with the indicated components. When RRF was added (lanes 4 and 5) in 5 or 15 fold excess over the 70S ribosomes, dissociation occurs.
translate the Fluc cistron only once; afterwards, a recycling phase cannot take place (see Fig. 2B in Ref. [18], where the same Fluc construct was used; the control in Fig. S3B demonstrated that Fluc was present exclusively as peptidyl-tRNA). The SecM sequence is long enough to ensure that the entire Fluc protein can pass through the ribosome exit tunnel before stalling occurs, allowing an undisturbed Fluc folding.

Before we consider the expected outcome of the experiments using the mRNA shown in Fig. 2, we should consider an important difference between 30S and 70S initiation. In 70S ribosomes, mRNA is not located in an open cleft as in free 30S subunits but rather in a tunnel; therefore, free 70S ribosomes cannot directly initiate at internal initiation sites of an mRNA (like the Fluc initiation site) but must thread the mRNA from the 5′-end (see Fig. 2B of Ref. [18]).

When we add 70S ribosomes to our modified PURE system in the presence of the mRNA shown in Fig. 2, ribosomes will scan down to the initiation site of the first cistron and translate Rluc. If the RRF/EF-G/IF3-dependent recycling phase compulsorily follows the translation termination of the first Rluc cistron, all ribosomes will leave the mRNA and dissociate into subunits, resulting in a block of Fluc synthesis, because 30S-binding initiation is prevented by the secondary structure hiding the SD sequence. In contrast, if 70S scanning occurs after termination of the Rluc synthesis, 70S ribosomes will scan down the intercistronic region of the mRNA, resolve the secondary structure with the SD sequence, and translate the Fluc cistron, regardless of whether or not RRF is present.

Translation assays were performed with 70S ribosomes. Rluc and Fluc synthesis was monitored in the presence and absence of RRF, IF1, and IF3. In the absence of both initiation factors, negligible amounts of luciferases were synthesized (Fig. 2); in their presence, the yield of both luciferases was independent of the presence of RRF. The clear-cut results indicate that the RRF/EF-G recycling phase is not an obligatory step after translation of a cistron in our in vitro system.

To seek more evidence, we turned to in vivo systems and tried to manipulate the RRF amounts in the cells; due to its essential nature, the gene cannot be knocked out. By chance, we found an experimental window, through which this problem could be tackled. We worked with an Escherichia coli strain carrying a temperature-sensitive RRF. Originally isolated by the Kaji group (LJ14 strain; [27]), the strain used here was modified; it carries Tn10 conferring tetracycline resistance with ~25% linkage to the frr locus as selection marker [28].

![Fig. 2](https://example.com/fig2.png)

**Fig. 2.** Expression of the bicistronic mRNA coding for Rluc and Fluc in the PURE system containing 70S ribosomes and all necessary components, and IF1/IF3 and the ribosomal recycling factor RRF when indicated (molar ratio of RRF:70S = 1:1). IR, intercistronic region; the blue stretch of the IR contains a heteropolymeric sequence of 20 nt that is 21 nt away from the upstream Rluc stop-codon and 40 nt from the downstream Fluc initiation AUG codon [18].
We confirmed the observation of Hirokawa et al. [29] that after a shift from permissive (30 °C) to non-permissive temperatures (43 °C), temperaturesensitive RRF (RRF-ts) was strongly diminished within 2 h as demonstrated by Western blotting using antibodies against RRF (Fig. 3a, lane 2). The wild-type (WT) strain showed normal levels of RRF. Most surprisingly, after an overnight incubation even at the permissive temperature of 30 °C, the mutant frr-ts strain has lost its RRF in contrast to the WT strain (Fig. 2A, lanes 7 and 8 versus 5 and 6). A possible explanation is that the active conformer of RRF-ts requires transient interactions with the ribosome to maintain its active state. If so, it might lose both its active conformation and compact structure in the absence of significant protein synthesis during the stationary phase and is thus degraded.

In the next experiment, we tested how much time the RRF-ts strain needs to replenish the RRF factor pool again after a 30 °C overnight incubation. After diluting the overnight culture 100-fold in fresh LB medium, the incubation was continued at the permissive temperature of 25 °C. The density of the culture was measured at distinct time points and the amount of RRF assessed by Western blotting. The first faint RRF band begins to appear after 60 min, and only after 240 min, it reaches a level similar to that in the strain WT for frr (Fig. 3b and c). The onset of growth after the lag phase is observed only after 60 min, when the RRF levels exceed 20% of the WT levels, and after 240 min, the RRF-ts strain has reached the growth rate of the WT cells (Fig. 3c). The results indicate that RRF is virtually absent during the first 30 min, thus providing a time window of at least 25 min at 25 °C for testing protein synthesis under negligible concentrations of RRF in vivo.

We transformed both WT and RRF-ts strains with a bicistronic Rluc/Fluc reporter plasmid (sequence in Fig. 4, top), but this time lacking both the secondary structure hiding the SD sequence and the Fluc–SecM fusion used in the experiment shown in Fig. 2. After an overnight incubation at 30 °C, cells were diluted in fresh LB medium. IPTG was added immediately to induce expression of the luciferases, and incubation was continued at 25 °C for 25 min. At this time point, the RRF levels were at least 40-fold lower in the RRF-ts strain than in the WT strain (Fig. 4a, green bars). Despite a negligible amount of RRF, an arrest of protein synthesis was not observed in the RRF-ts strain. The expression ratio Fluc/Rluc was only moderately reduced by less than 30% (yellow-red bars in Fig. 4a, see the measured values in Fig. 4b). It is clear that an RRF-dependent recycling phase after translation of the first cistron is not a prerequisite for translating the downstream cistron.

One important aspect of the RRF-dependent recycling phase was thought to provide 30S subunits for the 30S mode of initiation[4,30]. If so, such a dramatic RRF reduction as that seen in Fig. 4a should deprive the cells for free ribosomal subunits. To test this point, we analyzed sister aliquots of mutant and WT cell lysates from the experiment shown in Fig. 4 in a sucrose gradient under conditions that hardly change the equilibrium between ribosomal subunits and 70S ribosomes at 0 °C [20 mM Hepes (pH 7.6) at 0 °C, 6 mM Mg2+, and 30 mM K+] [31]. The relative molar ratios of (70S plus polysomes):50S:30S are practically constant in WT cells (normal RRF amounts, Fig. 5a) and in cells deprived of RRF (Fig. 5b; see the comparison in Fig. 5c). Although protein expression is damped after stationary phase, these results do not support the view that the RRF-mediated recycling phase is an essential source for providing ribosomal subunits for the 30S mode of initiation.
Discussion

The RRF-dependent recycling phase does not obligatorily follow termination

RRF can increase oligopeptide synthesis two- to threefold as shown in highly resolved in vitro tests using mRNAs coding for oligo-peptides with six or less residues [32–34]. Translation experiments with short peptides are designed for an extreme recycling activity and may not reflect physiological synthesis of proteins with an average length of 300 to 400 aa in bacteria. Concerning the synthesis of larger proteins, two different types of RRF effects were reported: (i) in vitro synthesis of the coat protein from the RNA phage R17 performed with fractions of S100 enzymes was stimulated up to 1.5-fold by addition of RRF fractions [32], a result not arguing for an essential role of the RRF recycling phase. However, these results might not be conclusive because the system was rather crude. Similar results were reported, when unaffected expression of β-galactosidase was observed in a strain harboring RRFts under semi-permissive conditions (39 °C), depriving the pool of active RRF and also arguing against an essential role of the RRF-dependent recycling phase. However, the residual RRF amounts in the cells were not checked [35]. (ii) It was reported that after termination, a re-initiation of protein synthesis might occur directly after the stop codon [32] or in any reading frame [27] under conditions of RRF deprivation (“unscheduled translation”). The observed effects were very weak; for example, expression of β-galactosidase lacking some N-terminal amino acids revealed a signal-to-noise ratio of 4 (Fig. 6 in Ref. [27]), whereas normal

Fig. 4. In vivo expression of the bicistronic mRNA shown above (a) in the WT and RRFts strains. After an overnight incubation of 30 °C for 19 h, the cell suspensions were diluted in fresh LB medium containing 1 mM IPTG for triggering the expression of the luciferases during an incubation of 25 °C for 25 min. (a) Comparison of the RRF amounts (green bars) with the lucerase ratio Fluc/Rluc (yellow-red bars). (b) Absolute values of the luciferases in WT and RRFts cells normalized via the input of A260 units of the S30 lysate (for details, see Materials and Methods).
β-galactosidase expression can achieve a 1000-fold ratio. Therefore, only a minority of ribosomes undergo "unscheduled translation".

These interesting but inconclusive indications prompted us to analyze the effects of RRF on protein synthesis under highly controlled in vitro conditions. We designed a bicistronic mRNA coding for Rluc and Fluc with the features described in the Results section. The features assure that the translation of the second Fluc cistron depends on the translation of the first Rluc cistron, because direct 30S-binding initiation of the Fluc cistron was prevented by hiding the SD sequence in the secondary structure that, however, could be resolved by scanning 70S ribosomes [18]. Consequently, Fluc expression would depend exclusively on 70S-scanning initiation.

At least three scenarios could be considered: (i) If the RRF/EF-G-dependent recycling phase were an obligatory and essential step after translation of a cistron (here Rluc), forcing all ribosomes to leave the mRNA and to dissociate into subunits, there would be no expression of the downstream cistron Fluc. (ii) If recycling would not be obligatory but at least would be able to efficiently compete with 70S scanning, Fluc expression would be sharply reduced in the presence of RRF. (iii) Finally, the mRNA construct is also a highly sensitive test of the "unscheduled translation": if in the absence of RRF the ribosomes would be quantitatively subjected to "unscheduled translation" in any frame as proposed, the fraction of scanning 70S ribosomes initiating the second Fluc cistron in a defined frame would be strongly reduced, thus also strongly impairing the amount of synthesized Fluc.

None of the predictions could be confirmed: in vitro translation in the highly defined PURE system demonstrated that the yield of both luciferases was practically the same, independent of presence or absence of RRF (Fig. 2).

The following conclusions can be drawn from these results: (i) an RRF-dependent recycling is not an obligatory step after translation termination of a cistron. (ii) "Unscheduled translation" does not play an important role in the absence of RRF. (iii) 70S scanning is majority after termination of Rluc–cistron translation. (iv) Assuming a frequent RRF/EF-G recycling of 70S ribosomes after the synthesis of Rluc, 70S ribosomes will dissociate into subunits, and thus, less 70S ribosome will be available for the scanning process and the translation of the downstream Fluc cistron. Observing identical amounts of both Rluc and Fluc in the presence or absence of RRF means that 70S scanning is the prevailing event after translation of the Rluc cistron under our experimental conditions. These surprising results prompted us to check again the effects of RRF on protein synthesis in vivo in virtual absence of RRF.

It has been reported that RRFts is degraded at non-permissive temperature and is present at permissive temperature at lower amounts than RRF in the WT strain [29]. In extending these observations, we found that RRFts was practically absent after an overnight incubation at permissive temperature (stationary-phase cells, Fig. 3a). After diluting the overnight cell suspension in fresh LB medium and continuing incubation at permissive temperature, RRFts did not recover for more than 30 min (Fig. 3b), which opened an experimental window for an analysis in vivo. The essential outcome of our analysis was that at least 40-fold reduction of the cellular RRF amount severely block growth (Fig. 3c) but impaired protein synthesis only moderately by about 30% (Fig. 4a and b). Forcing the RRF level down to 2.5% of the WT amounts did not affect the relative amounts of ribosomal subunits in vivo (Fig. 5).
We observed in vitro and in vivo that the absence of RRF only slightly affects protein synthesis. These results suggest that the RRF-dependent recycling phase does not necessarily follow translation termination in bacteria. A recent report [18] indicates that—instead of recycling—70S ribosomes stay on the mRNA and scan to the initiation site of the downstream cistron.

**Which RRF activity might be responsible for the factors’ essential feature?**

An extensive literature about RRF has been accumulated over the last five decades. Revisiting the published RRF data in depth will be presented elsewhere. Here, we will give only few arguments suggesting a broader, functional spectrum of RRF, where a possible RRF-depending recycling of post-termination ribosomes is not obligatorily.

The first hint comes from an assay testing the activity of RRF. In this assay, the conversion of polysomes to monosomes/70S ribosomes is monitored [1]. Efficient conversion required the removal of the peptidyl group from the ribosome-bound tRNA by puromycin [36]. Therefore, the RRF assay includes a treatment with puromycin before the conversion is triggered by RRF and EF-G. The resulting 70S complexes carrying deacylated tRNAs are considered to be models for post-termination ribosomes ready for the recycling phase. This assumption is not valid for most ribosomes, because either they are vacant ribosomes or will have a sense codon at the A site in contrast to post-termination ribosomes, which have a stop codon at the A site. Therefore, 70S ribosomes carrying a deacylated tRNA at the P site (P-tRNA) are a target for RRF and EF-G.

A genetic screen gathered further evidence; it revealed a suppressor mutation of the temperature-sensitive phenotype of the peptidylhydrolase (Pth)ts. The mutation was located in the promoter region of the RRF gene frr [37] and caused low amounts of RRF. Pth is another essential gene in bacteria [38] and hydrolyses peptidyl-tRNAs rather than fMet-tRNA, which had accidentally fallen from the ribosome [39,40] or are still present on stalled ribosomes (see next paragraph). It follows that low amounts of RRF can prevent the accumulation of peptidyl-tRNA in the cytosol in a Pthts background. This observation suggests that RRF—directly or indirectly—is involved in peptidyl-tRNA release from the ribosome in cooperation with EF-G and Pth. Targeting stalled ribosomes would be an important if not an essential RRF function.

In fact, it has been reported that RRF participates in the release of peptidyl-tRNAs from stalled ribosomes in the elongation phase [41]. Another report directly demonstrated a peptidyl-tRNA release in vivo [42] carrying long peptides (about 350 residues) on stalled ribosomes. The authors also demonstrated that Pth can hydrolyze peptidyl-tRNA on stalled ribosomes, when a sense codon is at the A site. Possibly, Pth can also hydrolyze peptidyl-tRNA on the ribosome, when a peptidyl-tRNA resides at the A site.

IF3 and RF3 seem to cooperate with RRF as indicated in the following observations. Growth defects of a Pthts strain at non-permissive temperature could be healed not only by a reduced RRF synthesis as expected but also to a lesser extent with a deleted RF3 gene; restoration of viability of the Pthts strain was optimal with both reduced RRF synthesis and deletion of the RF3 gene [37]. A similar full restoration of the Pthts phenotype was reported from a strain containing a functionally compromised IF3 together with the mentioned insertion in the promoter of the RRF gene, causing low cellular levels of RRF [43]. Furthermore, the same authors demonstrated that overexpression of IF3 could heal the temperature-sensitive phenotype of RRFts [43]. Curing the temperature-sensitive feature of RRFts indicates that IF3 is linked to an essential RRF function in addition to its involvement in the RRF post-termination recycling activity. It follows that the RRF-dependent rescue of stalled ribosomes consists of the triad RRF, EF-G•GTP, and IF3.

There are two more systems, which can hydrolyze peptidyl-tRNA on the ribosome preferentially on stalled ribosomes. The factor ArfB (YaeJ) is able to hydrolyze peptidyl-tRNAs at the P site codon independently [44,45]. The same is true for the cooperative action of ArfA (YdhL) and RF2, which however preferentially cleave peptidyl-tRNA, when the A site does not contain a codon [45,46]. Therefore, ArfA and RF2 are an alternative for the tmRNA/SmB rescue system, which requires a peptidyl-tRNA at the P site and a codon-free A site [47].

All three systems, viz. Pth, ArfB, and ArfA/RF2, are targeting stalled ribosomes and generating 70S complexes with a deacetylated P-tRNA and a sense codon at the A site, which are ready targets for the suggested RRF/EF-G/IF3 rescue system possibly essential for the viability of the bacterial cell. The post-termination complex is similar, because it also carries a deacetylated P-tRNA but rather a stop codon at the A site. We suggest that the apparent conflict between recycling and 70S scanning can be reconciled in the following way. If an fMet-tRNA for any reason is not available to chase the deacetylated P-tRNA of a post-termination complex and trigger 70S scanning, the stalled post-termination complex will be recycled (Fig. 6). Even if fMet-tRNA is available for 70S scanning, there is a case for RRF/EF-G recycling. We have seen 70S scanning on intercistronic regions with a length of up to ~60 nt, and more than 70% of the intercistronic regions are shorter than 30 nt [18]. It seems that 70S scanning...
may work for the most of genes. However, the successful 70S scanning depends on optimal SD sequence in front of AUG codon (see Fig. S7 of Ref. [18]). This result indicates that 70S scanning is difficult to manage without SD sequence even if the intercistronic region is below ~60 nt. Moreover, we have not yet analyzed the maximum distance of 70S scanning. How 70S scanning and the RRF/EF-G•GTP-dependent recycling compete after the termination event still remains to be elucidated.

Materials and Methods

Buffers, ribosomes, mRNAs

H$_2$O M$_6$N$_{30}$SH$_4$, 20 mM Heps-KOH (pH 7.6) at 0 °C, 6 mM Mg(Ac)$_2$, 30 mM NH$_4$(Ac), and 4 mM β-mercaptoethanol; H$_2$O M$_{4.5}$K$_{150}$SH$_4$ Spd$_2$Spm$_{0.05}$, 20 mM Heps-KOH (pH 7.6) at 0 °C, 4.5 mM Mg(Ac)$_2$, 150 mM KAc, 4 mM β-mercaptoethanol, 2 mM spermidine, and 0.05 mM spermine; SDS sample buffer, 60 mM Tris–HCl (pH 6.8), 2% SDS, 10% glycerol, 200 mM β-mercaptoethanol, and 0.05% bromophenol blue.

The isolation of ribosomes, the use of the highly resolved PURE system, and the construction of the bicistronic mRNAs coding for Rluc and Fluc used in vitro (Fig. 2) have been constructed in the following way: Rluc and Fluc-SecM were amplified from plasmids “pET23c R-IR-F” and “pET23c Fluc-SecM” using the primers “T7 promoter” and “Rluc-rev” or “Fluc-fw” and “T7 terminator” (see Ref. [18]). Primers Rluc-rev and Fluc-fw were 5’-phosphorylated prior to amplification. The two genes were ligated and cloned using BglII and EcoRI into pET23c (Novagen); the resulting plasmid was named pET23c R-Fluc-SecM. The intercistronic region with the hidden SD sequence (IR-HSD) was introduced as double-stranded oligonucleotides providing 5’ overhangs compatible to
BamH1 and NcoI restriction sites via ligation into pET23c R-Fluc-SecM. Oligonucleotides used:

IR-HSD sense (5′-GATCCACCCACCCCCACCGGCAAG

GATCGAGGCGAGCTGGA

CCACCAACCCACCCCCCTCTCATAGGA

GAACAT3′) and IR-HSD antisense (5′-CATGGTAGTT

CTCCTAATGGAGAGGGGTGGGGTG

GGGTGAGCTCGCTGATCGTCTGCGGGGTTG

GGGTGGGTTG3′).

The plasmid construction for the expression of the luciferases from the bicistronic mRNA used in vivo (Fig. 4) has been described [18].

Testing the activity of RRF

We incubated 10 pmol 70S with 20 pmol tRNA^Phe in the presence of poly(U) (final concentration 8 mg/ml) in buffer H_2O_Mg_2+Mg_2+Spd_2Spd_3, at 37 °C for 15 min in a reaction volume of 10 μl (post-termination state). Then, 40 pmol EF-G, 150 pmol IF3, 0.5 mM GTP, and RRF at different amounts (0 or 50 or 150 pmol) were added, increasing the reaction volume to 100 μl of the same buffer; an incubation at 37 °C for 20 min followed. The reaction mixtures were loaded on 10–30% sucrose gradients in binding buffer and centrifuged at 24,000 rpm for 20 h in a SW40 rotor. The ribosome profiles were monitored at A_260.

Experiments with a strain harboring a RRF<sup>ts</sup>

Generation of E. coli LJ14.1 (Tn10) and LJ14.2 (Tn10 flr<sup>ts</sup>) was described earlier [28]. E. coli LJ14.1 and LJ14.2 (WT and RRF<sup>ts</sup> strains, respectively) were grown overnight in LB medium (ampicillin 25 mg/L and tetracycline 12.5 mg/L) at 30 °C. After determining the OD<sub>600</sub>, the suspension was diluted (starting OD<sub>600</sub> = 0.05) with 400 ml LB medium with LB medium (ampicillin 25 mg/L and tetracycline 12.5 mg/L), and incubation continued at 25 °C. At various time points (Fig. 3B and C), aliquots were taken (100 ml at the beginning, later 50 ml) and the cells harvested. SDS-sample buffer was added and proteins were denatured at 95 °C for 5 min. The samples (corresponding to 1 OD<sub>600</sub>) were loaded onto 15% polyacrylamide gels for assessment of the RRF amounts by Western blot. The Western blot was performed as described [18] using polyclonal antibodies against RRF and the ribosomal protein L2 as input control and for normalizing the RRF bands.

Expressing Renilla and firefly luciferases in vitro

Construction of the bicistronic mRNA coding for Rluc and Fluc (shown in Fig. 2, top) and in vitro expression in a modified PURE system have been described in Ref. [18].

The modified PURE system

The system, as well as the purified His-tagged IF1 and IF3, was provided by Dr. T. Ueda, Tokyo. The expression in the PURE system was performed according to Ref. [48] with the following modifications. The final concentration of ribosomes in the reaction was 0.5 μM. The amount of translation factors was accordingly reduced; our modified PURE system lacked IF1, IF3, and RRF; if not indicated. T7 polymerase, as well as CTP and UTP were omitted from the reaction mixture; GTP and ATP were present in 2 mM each. The final Mg2+ concentration was decreased to 8.5 mM. Note that the free Mg2+ concentration in our PURE system was about 2.5 mM in the presence of ATP and GTP (each 2 mM), which binds about 1 to 1.5 mM Mg2+ per mM NTP [49]. This is near to the in vivo conditions [50]. The reaction was incubated for 2 h at 30 °C.

Expressing Renilla and firefly luciferases in WT and RRF<sup>ts</sup> strains

We thawed 60 μl competent E. coli cells (WT and RRF<sup>ts</sup>) on ice and then transformed them with the plasmids carrying the bicistronic mRNA coding for Rluc and Fluc (1 μl with approximately 10 ng of DNA); these cells were mixed and well kept on ice for 5 min. Electroporation was performed with 1.5 kV, 200 Ω resistance, and C = 25 μF capacity by applying pulsed electrical field for about 4–5 ms, which causes pore formation and enables foreign plasmid to enter into the cells. Then, 0.5 ml fresh LB medium were added into the cuvettes and incubated at 30 °C for 1 h in 1.5 ml Eppendorf tubes. We plated 0.1 ml culture on LB plates with ampicillin 25 mg/L and tetracycline 12.5 mg/L and incubated it overnight for single colonies to grow.

E. coli cells (WT and the RRF<sup>ts</sup>) strain were grown overnight in 30 ml LB medium containing ampicillin (25 mg/L) and tetracycline (12.5 mg/L) at 30 °C. After determining the OD<sub>600</sub>, the suspension was diluted with 200 ml LB medium (0.2 OD<sub>600</sub>) containing ampicillin (25 mg/L), tetracycline (12.5 mg /L), and IPTG (1 mM) and incubated at 25 °C for 25 min. The cells were harvested, resuspended in H_2O_Mg_2+Spd_2Spd_3, supplemented with lysozyme (0.4 mg/ml) and 10 μl DNase (RNase free), and subjected to three freeze–thaw cycles to lyse the cells. The membranes were removed by two low-speed centrifugation steps (9000 g for 5 min and 9.000 g for 30 min). Then, 5 μl of the resulting S30 (diluted 100-times) was taken for measuring dual luciferase activity by Dual-Glo Luciferase Assay System (Promega); the chemiluminescence was measured in a Centro Microplate-Luminometer LB 960 (Berthold Technologies).

Expression values of Rluc and Fluc in WT and the RRF<sup>ts</sup> strain were normalized via the A<sub>260</sub> input into the reaction mixture. The RRF amount was determined by a Western blot as described [18] using polyclonal antibodies against RRF and the ribosomal protein L2 as input control and for normalizing the RRF bands.

Sucrose gradients of the cell lysates (S 30)

We loaded 1 A<sub>260</sub> unit of S30 of E. coli cells (WT and RRF<sup>ts</sup>) onto 10–30% sucrose gradient made in H_2O_Mg_2+Spd_3 buffer. A centrifugation with the swing-out rotor SW55 was performed (at 39,000 rpm for 20 min, and the gradient was recorded by gradient runner (LKB). Gradient runner is an instrument that measures the absorbance of samples at A<sub>260</sub>, while the sucrose gradient is pumped out. Based on the absorbance value, the curve was plotted and the peak areas were analyzed with the software Origin8 (OriginLab).
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Abbreviations used:
RRF, ribosomal recycling factor; Rluc, Renilla luciferase; Fluc, firefly luciferase; SD, Shine–Dalgarno; RRFts, temperature-sensitive RRF; WT, wild-type; Pth, peptidyl-dihydrolase; Pths, temperature-sensitive Pth.

References


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