RNA (1998), *4*:973–983. Cambridge University Press. Printed in the USA.

Release factor RF-3 GTPase activity acts in disassembly of the ribosome termination complex

GUIDO GRENTZMANN,[1] PAUL J. KELLY,[2] SOUMAYA LAALAMI,[3] MASAHIRO SHUDA,[4] MATTHEW A. FIRPO,[1] YVES CENATIEMPO,[3] and AKIRA KAJI[4]

[1]Howard Hughes Medical Institute, University of Utah, Salt Lake City, Utah 84112, USA
[2]University of Utah, Salt Lake City, Utah 84112, USA
[3]ESA-CNRS 6031, Institut de Biologie Moléculaire et d'Ingénierie Génétique, Université de Poitiers, 86000 Poitiers, France
[4]Department of Microbiology, School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania 19104, USA

ABSTRACT

RF3 was initially characterized as a factor that stimulates translational termination in an in vitro assay. The factor has a GTP binding site and shows sequence similarity to elongation factors EF-Tu and EF-G. Paradoxically, addition of GTP abolishes RF3 stimulation in the classical termination assay, using stop triplets.

We here show GTP hydrolysis, which is only dependent on the simultaneous presence of RF3 and ribosomes. Applying a new termination assay, which uses a minimessenger RNA instead of separate triplets, we show that GTP in the presence of RF3 stimulates termination at rate-limiting concentrations of RF1. We show that RF3 can substitute for EF-G in RRF-dependent ribosome recycling reactions in vitro. This activity is GTP-dependent. In addition, excess RF3 and RRF in the presence of GTP caused release of nonhydrolyzed fmet-tRNA. This supports previous genetic experiments, showing that RF3 might be involved in ribosomal drop off of peptidyl-tRNA. In contrast to GTP involvement of the above reactions, stimulation of termination with RF2 by RF3 was independent of the presence of GTP. This is consistent with previous studies, indicating that RF3 enhances the affinity of RF2 for the termination complex without GTP hydrolysis. Based on our results, we propose a model of how RF3 might function in translational termination and ribosome recycling.

Keywords: GTPase; RF1; RF2; RRF; translation

INTRODUCTION

Translational termination has been defined as hydrolysis of the completed polypeptide chain from the terminal peptidyl-tRNA localized in the ribosomal P-site (Ganoza, 1966; Capecchi & Klein, 1969; Caskey et al., 1969; Tate & Brown, 1992; Grentzmann & Kelly, 1997). In prokaryotes, one of two release factors (RF1 or RF2) induces hydrolysis by recognition of a stop codon (Ganoza & Nakamoto, 1966; Capecchi, 1967). The two factors are codon specific. RF1 recognizes UAG and UAA, whereas RF2 recognizes UGA and UAA (Scolnick et al., 1968). In eukaryotes, one factor (eRF1) decodes all three stop codons (Goldstein et al., 1970a; Frolova et al., 1994). Stop signal recognition by RF1 or RF2 induces ribosomal peptidyl hydrolase to release the completed protein (Caskey, 1977). Release factors were identified using a simplified termination assay (Caskey et al., 1968) in which labeled f[^{35}S]met-tRNA$_f^{met}$ is bound to the ribosomal P-site via interaction with an AUG triplet. Incubation with a UAG, UAA, or UGA stop triplet in the presence of factor-containing cell fractions induced hydrolysis of formyl-methionine from its tRNA.

A third factor (RF3) is known to play a role in translational termination (Goldstein et al., 1970b), but its function has not yet been clarified. RF3 stimulates in vitro termination by RF1 and RF2 at low stop triplet concentration. At saturating stop triplet concentration, RF3 stimulates RF1 less efficiently (2-fold) than RF2 (10-fold) (Grentzmann et al., 1995). In both in vitro studies, termination stimulation by RF3 was observed in the absence of GTP. RF3 binds GTP, but GTP abolishes in vitro termination amplification by RF3 in the triplet assay (Milman et al., 1969). Furthermore, RF3 and GTP actively dissociated an RF1·UAA·ribosome

Reprint requests to: Guido Grentzmann, Howard Hughes Medical Institute, 6160 Eccles Building, University of Utah, Salt Lake City, Utah 84112, USA; e-mail: guido@howard.genetics.utah.edu.

Abbreviations: IF, initiation factor; EF, elongation factor; RF, release factor; eRF, eukaryotic release factor; RRF, ribosome recycling factor; PTH, peptidyl-tRNA hydrolase; fmet, formyl-methionine.

complex (Goldstein & Caskey, 1970), which led to the hypothesis that the RF3-GTPase activity plays a role in decomposition of the termination complex after release of the polypeptide chain. The amino acid sequences of the RF-3 genes in prokaryotes (*prfC*) and eukaryotes (*sup35*) revealed a GTP-binding site in both RF3 and eRF3 (Grentzmann et al., 1994; Mikuni et al., 1994; Zhouravleva et al., 1995), similar to those of translational elongation factors EF-Tu and EF-G. In eukaryotes, GTP is necessary in order to observe termination stimulation by eRF3 (Zhouravleva et al., 1995), and eRF3 has been shown to be a GTPase, which is dependent on ribosomes and eRF1 (Frolova et al., 1996). We investigated ribosome and factor dependencies for the GTPase activity of RF3 from prokaryotes.

In vivo analysis of the effect of RF3 expression in termination has been conducted by measuring efficiencies of stop signal suppressor tRNAs in *prfC*$^-$ strains. An in vivo study of RF3 inactivation, comparing more than 30 constructs in three different suppression systems, showed a decrease of termination efficiency only for UGA (RF2-specific) stop signals (Grentzmann et al., 1995). The observation was interpreted as a general stabilizing effect of RF3 on comparatively weaker ribosome complexes with RF2. In contrast, Mikuni et al. (1994) reported an RF3-deletion mutant that showed lower termination efficiency not only for UGA, but for all three stop codons. The different results might be due to different cellular backgrounds used for the in vivo experiments (Remes & Elseviers, 1980).

Recently we developed a new in vitro translational termination assay (Grentzmann & Kelly, 1997). The assay uses a "UUC AUG stop" minimessenger, instead of individual triplets, which presumably has a higher affinity for ribosomes. In this assay, binding of the AUG codon, by fmetRNA$_f^{met}$ to the P-site (Caskey et al., 1968), simultaneously programs the A- and E-sites of the ribosome. For example, when an fmet-tRNA$_f^{met}$ binds to the ribosomal P-site via UUC AUG UAA, termination can now be considered at the 0 order for UAA concentration at the A-site. The new assay allows study of the termination reaction at more physiological magnesium concentrations, lower than 10 mM. Kinetic patterns of the minimessenger assay are very comparable to the classical triplet assay, but about 10-fold lower release factor levels are sufficient to saturate the new assay system. In the present study, we used this assay to compare GTP-dependent RF3 termination stimulation of RF1 and RF2.

After translational termination by RF1 or RF2, the termination complex must be disassembled in order to recycle ribosomes, tRNA, mRNA, and release factors. In vitro release of tRNA and mRNA from the ribosome is catalyzed by elongation factor EF-G and the ribosome recycling factor (RRF, formerly called ribosome releasing factor) in presence of GTP (Hirashima & Kaji, 1972; Ogawa & Kaji, 1975; Janosi et al., 1996). RRF is essential for cell growth (Janosi et al., 1994). We tested whether RF3 could replace EF-G GTPase activity to allow ribosome recycling by RRF.

In this paper, we report a GTPase activity with RF3, which is only dependent on ribosomes, much comparable to the GTPase activity of EF-G. RF3 stimulates RF1-catalyzed termination dependent on GTP, whereas termination with RF2 is stimulated independently of the presence of GTP. RF3 can replace EF-G in the in vitro mRNA release from the ribosome by RRF, dependent on GTP. Excess of RF3 and RRF can result in decomposition of the translation complex, independent of previous termination by RF1 or RF2.

After completion of our experiments, similar results to parts of this work, describing RF3 GTPase and GTP-dependent stimulation of RF1-dependent termination by RF3, have been published (Freistroffer et al., 1997; Pavlov et al., 1997).

RESULTS

A guanosine triphosphatase activity dependent on RF3 and ribosomes

We wanted to establish which elements of the translational apparatus were essential for an in vitro GTPase activity in the presence of RF3. Table 1 shows the results of measuring GTPase activity with RF3. Ribosomes, ribosomes with RF1 or RF2, as well as complete termination complex did not exhibit GTPase activity (Table 1; lines 1–5), nor did RF3 alone (Table 1, line 6). GTP was hydrolyzed only in the presence of RF3 and ribosomes (Table 1, lines 7–13). In contrast to the eukaryotic factor (eRF3) (Frolova et al., 1996), RF3 did not require RF1 or RF2 for GTPase activity (Table 1, compare line 7 and lines 10, 11). Other components of

TABLE 1. Ribosome dependent GTPase of RF3.[a]

Added factors	GTP hydrolysis (pmol)
1) Ribosomes	1
2) Ribosomes + RF1	2
3) Ribosomes + RF1 + mRNA + fMet-tRNA	2
4) Ribosomes + RF2	1
5) Ribosomes + RF2 + mRNA + fMet-tRNA	1
6) RF3	1
7) Ribosomes + RF3	45
8) Ribosomes + RF3 + mRNA	49
9) Ribosomes + RF3 + mRNA+ fMet-tRNA	58
10) Ribosomes + RF1 + RF3	46
11) Ribosomes + RF2 + RF3	60
12) Ribosomes + RF1+ mRNA+ fMet-tRNA + RF3	35
13) Ribosomes + RF2+ mRNA+ fMet-tRNA + RF3	56

[a]Reactions contained 2 pmol of ribosomes or termination complex, 20 punits of RF1 or RF2, and 1 unit of RF3 as indicated, and were performed for 15 min at 37 °C [80 mM Tris, pH 7.2, 100 mM KCl, and 8 mM $Mg(OAc)_2$].

the termination reaction, mRNA or fmet-tRNA, did not have significant influence on the GTPase activity (Table 1, lines 8, 12 and 9, 13). To confirm RF3 dependence of the GTPase activity, an RF3 dose–response curve was established (Fig. 1). Ribosome-dependent RF3 GTPase activity was linearly dependent on the amount of RF3 under the experimental conditions used. Although the linear dose–response relation gives supplemental evidence that RF3 is the GTP-hydrolyzing entity, we cannot formally exclude that the GTPase center is in the ribosome.

GTP stimulation of termination is dependent on the presence of RF3

RF3 amplifies in vitro termination in the classical triplet assay (at limiting concentration of stop triplets) by diminishing the apparent K_m of RF1 and RF2 for the terminating ribosome (Goldstein et al., 1970b). RF3 amplification in the triplet assay is abolished by addition of GTP (Goldstein & Caskey, 1970). Nevertheless, the sequence of the RF3 gene suggests a GTP binding site in the protein and it was reasonable to expect that GTP-binding or hydrolysis would be part of the major function of RF3. Abolishing stimulation of termination by GTP might be due to an artifact, specific to the classical triplet assay. We therefore used an assay with UUC AUG UAA as the mRNA (Grentzmann et al., 1997) to test the effect of RF3 and RRF in translational termination (Fig. 2). Experiments were performed at limiting concentrations of RF1, which releases basal levels of formyl-methionine (between 50 and 100 fmol). Stimulation of termination, as determined by formyl-methionine release, was then examined. In the applied conditions, RF3 stimulated RF1 termination slightly in the absence of guanine nucleotides (Fig. 2a,b) or in the presence of GDP or GMPPNP (Fig. 2g,h). This increase was not seen in the presence of GMP (Fig. 2c,d). In contrast, a threefold stimulation by RF3 was seen in the presence of GTP (Fig. 2i,j). Stimulation of termination by GTP in the absence of RF3 was not significant (Fig. 2a,i) and GTP stimulation of termination was strictly dependent on the presence of RF3 (Fig. 2b,j).

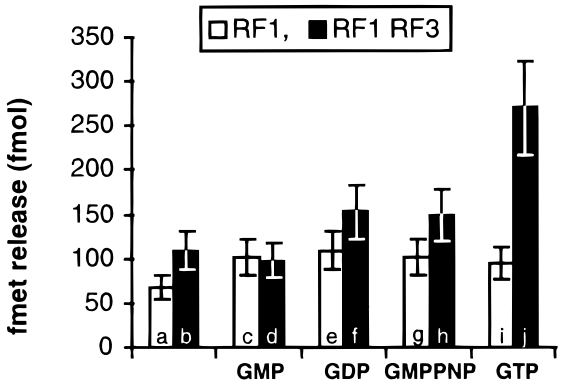

FIGURE 2. Stimulation of RF1-mediated in vitro termination by GTP and RF3. a, RF1; b, RF1 and RF3; c, RF1 and GMP; d, RF1, RF3, and GMP; e, RF1 and GDP; f, RF1, RF3, and GDP; g, RF1 and GMPPNP; h, RF1, RF3, and GMPPNP; i, RF1 and GTP; j, RF1, RF3, and GTP. For the effect of RF3, compare the height of the black and the white bar in each set. Reaction mixtures were incubated at 30 °C for 13 min and contained in 10 μL: 0.5 pmol f[^{35}S]met-tRNA$_f^{met}$·UUC AUG UAA·ribosome complex, 5 punits RF1, 0.2 units RF3, 1 pmol of RRF and 160 μM GMP, GDP, GTP, or GMPPNP as indicated [8 mM Mg(OAc)$_2$, 100 mM KCl, 80 mM Tris, pH 7.5]. Released fmet in fmol. Free fmet at time zero (50 fmol) was subtracted from all values. Standard deviation from the averaged value was less than 20%.

Comparison of RF3 stimulation of termination by RF1 or RF2

We recently established kinetic parameters for termination by RF1 and RF2 using the minimessenger RNA termination assay (Grentzmann & Kelly, 1997). In the classical stop triplet assay, RF3 stimulates binding of RF1 and RF2 at low stop triplet concentration in the absence of GTP, as determined by enhanced formyl-methionine release (Goldstein et al., 1970b). Moreover, addition of GTP abolishes RF3 stimulation (Goldstein & Caskey, 1970). In order to determine the effects of RF3 and RF3/GTP in termination assays catalyzed by RF1 or RF2 using minimessenger RNA, we compared the kinetics of in vitro termination reactions under various conditions (Fig. 3). At 24 °C, under the experimental conditions, RF3 did not significantly stimulate RF1 in the absence of GTP (Fig. 3A). On the other hand, RF3 did stimulate termination by RF2 (Fig. 3B), independent of GTP. The reaction velocity as well as the final level of fmet-tRNA hydrolysis were stimulated, which corresponds to previously established results with stop

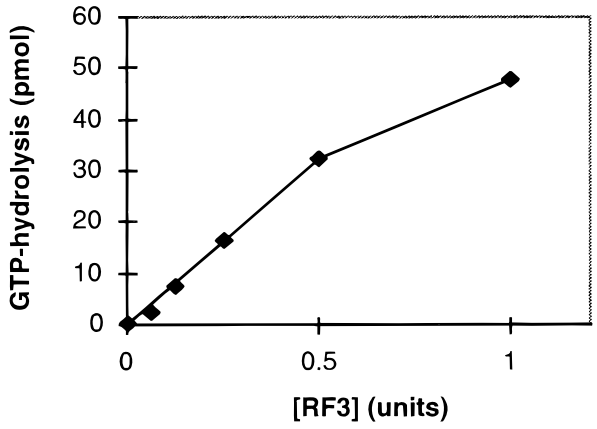

FIGURE 1. Ribosome-dependent GTPase of RF3. Reaction mixtures contained 2 pmol of ribosomes or termination complex, 20 punits of RF1 or RF2 and RF3 as indicated, in a total volume of 10 μL. GTP hydrolysis was measured after 15-min incubation at 37 °C [80 mM Tris, pH 7.2, 100 mM KCl, and 8 mM Mg(OAc)$_2$].

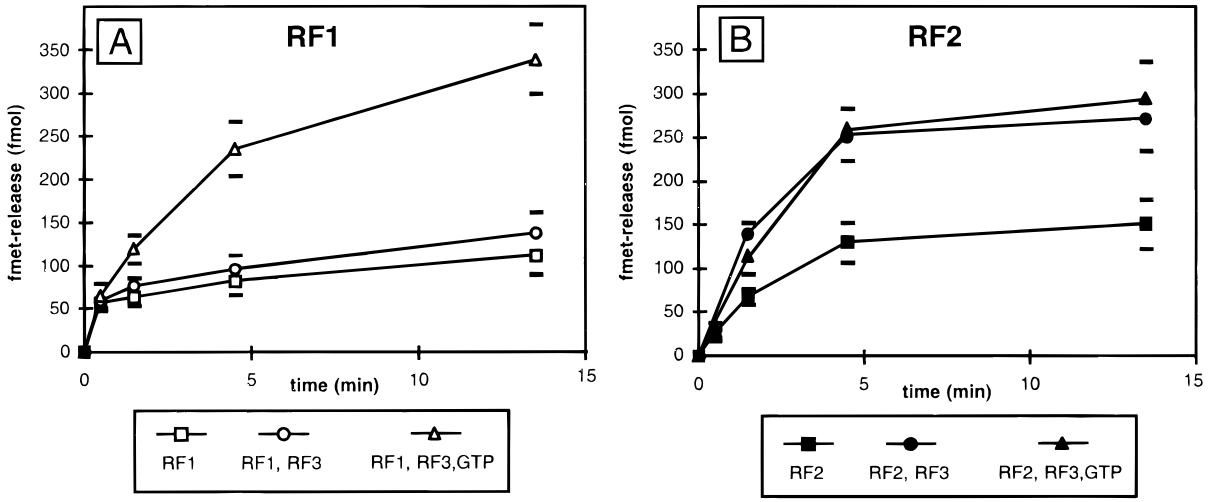

FIGURE 3. Differential RF3 effect on translational termination by RF1 or RF2. The reaction mixture contained in 10 μL: 0.5 pmol f[^{35}S]met-tRNA$_f^{met}$·messenger·ribosome complex, 5 punits RF1 or RF2, 0.2 units RF3, and 160 μM GTP, as indicated and was incubated at 24 °C (Caskey et al., 1971). Free fmet at time zero (50 fmol) was subtracted from all values. Standard deviation, indicated by black bars, was about 10%.

triplets at saturating concentrations (Grentzmann et al., 1995). In contrast to the classical assay, addition of GTP and RF3 strongly enhanced RF1 termination (Fig. 3A). GTP-dependent RF1 stimulation by RF3 increased the reaction efficiency more than twofold. In addition, GTP did not abolish GTP-independent stimulation of RF2 by RF3 (Fig. 3B). Results with minimessenger RNAs, containing RF1 (UAG)-and RF2 (UGA)-specific stop codons, reproduced the observation that GTP-dependent RF3 stimulation was specific for termination with RF1 and, even at minimal concentrations of RF2, no GTP-dependent stimulation by RF3 could be observed (data not shown).

In the presence of GTP, RF3 and RRF disassemble the translation complex

The GTPase activity of RF3 (Table 1) was very much reminiscent of the GTPase activity of EF-G (Conway & Lipmann, 1964). EF-G has been shown to be required for ribosome recycling in vitro, previously defined as the dissociation of mRNA and tRNA from the terminated ribosome, in the presence of RRF and GTP (Hirashima & Kaji, 1972). We therefore asked whether RF3 might be able to release mRNA from the ribosome in the presence of RRF and GTP (Table 2). We used an assay that shows release of the ^{32}P-labeled UUC AUG UAA minimessenger RNA (*mRNA) (Table 2A), ^{35}S-fmet (*fmet), as well as nonhydrolyzed ^{35}S-fmet-tRNA$_f^{met}$ (*fmet-tRNA) (Table 2B), in the presence of RF1 or RF2, RF3 or EF-G, RRF, and GTP. Using the minimessenger RNA, we were able to reproduce previously published results on release of naturally occurring mRNA dependent on EF-G and RRF in the presence of GTP (Ogawa & Kaji, 1975) (Table 2A, line 1, compare to line 6).

The assay was then used to study whether RF3 could replace EF-G (Table 2A). Up to 60% of the mRNA was released (*mRNA) in the presence of RF3, RRF, and GTP after termination by RF2 (Table 2A, line 2). This activity was dependent on GTP (Table 2A, compare line 2 and line 3) but not GDP (line 4). Furthermore, mRNA release depended on RRF (Table 2A, line 5) and RF3 (Table 2A, line 6). Unexpectedly, release of mRNA with RF1 was minimal, even in the presence of RF3, GTP, and RRF (Table 2A, line 7). Subsequent filter binding experiments showed that, under the assay conditions, RF1 efficiently binds the minimessenger by itself and to the ribosome, whereas RF2 did not and excess of nonradioactive mRNA competed with RF1 binding of the ^{32}P-labeled minimessenger (data not shown). To prevent rebinding of released labeled minimessenger RNA, in one series, 1 nmol of nonradioactive mRNA was added (+n.r.RNA). The presence of excess, nonlabeled mRNA increased release of labeled mRNA in the presence of RF1 by RF3 and RRF to significant amounts, compared to background values [Table 2A, lines 7 to 9 (+n.r.RNA)].

Significant amounts of mRNA were released by RF3, RRF, and GTP, even in the absence of RF1 or RF2 (Table 2A, line 10). Again, this activity was dependent on the presence of GTP (Table 2A, line 11), RRF (Table 2A, line 12) and RF3 (Table 2A, line 13). In contrast to established data for ribosome recycling with EF-G and RRF, hydrolysis of fmet-tRNA was not a prerequisite for mRNA release through RF3 and RRF. Because fmet release was strictly dependent on the presence of RF1 or RF2 (Table 2B, *fmet), free nonhy-

TABLE 2. Ribosomal recycling with EF-G or RF3 in the presence of RRF.[a]

Added factors	Release of A *mRNA	A *mRNA (+n.r.RNA)	B *fmet	B *fmet-tRNA
1) RF2, EF-G, GTP, RRF	70		57	9
2) RF2, RF3, GTP, RRF	60		60	
3) RF2, RF3, RRF	10		64	
4) RF2, RF3, GDP, RRF	17		67	n.d.
5) RF2, RF3, GTP	14		70	
6) RF2, GTP, RRF	8		68	
7) RF1, RF3, GTP, RRF	5	20	48	
8) RF1, RF3, GTP	1	5	38	n.d.
9) RF1, GTP, RRF	0	1	36	
10) RF3, GTP, RRF	48		4	41
11) RF3, RRF	1		4	1
12) RF3, GTP	6		2	5
13) GTP, RRF	2		2	1

[a]Release of (**A**) [^{32}P]mini-messenger RNA (*mRNA) and (**B**) [^{35}S]fmet (*fmet) and nonhydrolyzed [^{35}S]fmet-tRNA (*fmet-tRNA), by translational termination and ribosome recycling, dependent on RF1 or RF2, EF-G or RF3, GTP and RRF. In one series of termination by RF1 (lines 7, 8, 9), 1 nmol of nonradioactive mRNA (+n.r.RNA) was added to compete for RF1 rebinding of labeled messenger-RNA, after recycling. One picomole of f[^{35}S]met-tRNAfmet·[^{32}P]UUC AUG UAA·ribosome complex was incubated together with 200 punits RF2 or 40 punits of RF1, 5 units RF3 or 12 pmol EF-G, 6 pmol RRF, and 160 μM GTP or GDP as indicated, at 30 °C for 10 min in 50 μL final volume. For experiments with EF-G, 0.2 mM phosphoenolpyruvate and 3 μg of pyruvate kinase were added (Ogawa & Kaji, 1975). Released f-met and mRNA are expressed in 1/100 pmol. Backgrounds of free fmet (50 fmol) and free mRNA (150 fmol) in absence of factors were subtracted. Standard deviations were between 5 and 15%.

drolyzed fmet-tRNA was detected after the reaction in absence of RF1 or RF2 (Table 2B, *fmet-tRNA, line 10). Termination-independent mRNA release, as shown in Table 2, might be due to excess concentrations of RF3 and RRF and not of in vivo significance. On the other hand, our observation is supported by previous in vivo studies, indicating that an RF3$^-$ mutant diminishes intracellular levels of free peptidyl-tRNA (Grentzmann, 1994).

Termination dependence of ribosome recycling with RF3 and RRF

To establish that RF3 replaces the role of EF-G in the release of ribosomes by RRF, RF3 dose–response curves for mRNA release were determined (Fig. 4). Release of mRNA increased with the amount of added RF3 in complete absence of EF-G. This establishes that RRF-dependent mRNA release can use RF3 instead of EF-G. To verify termination dependence of ribosome recycling by RF3 and RRF, we established the RF3 dose–response curves at lower concentration

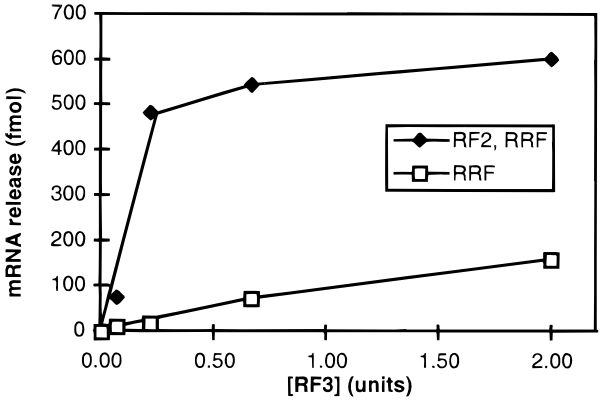

FIGURE 4. Release of mRNA by RRF and RF3. RF3 dose response relative to RF2. One picomole of f[^{35}S]met-tRNA$_f^{met}$·[^{32}P]UUC AUG UAA·ribosome complex was incubated at 30 °C for 10 min together with 2 pmol RRF, 160 μM GTP in 50 μL, with or without 200 punits RF2. Released mRNA is expressed in fmol. Free mRNA (150 fmol) present in the absence of RF3 and RRF was subtracted from all values.

of RRF than in initial experiments (Table 2), and in the absence or presence of release factor RF2. Under these conditions, release of mRNA from ribosomes was dependent on the presence of RF2. Even at concentrations of RF3 (0.22 units) allowing maximal ribosome recycling, termination-independent recycling was not significant. To further confirm release of mRNA by RRF from ribosomes in the presence of RF3, a dose–response curve of mRNA release, regarding RRF, was established (Fig. 5). Again, mRNA release increased with added amounts of RRF.

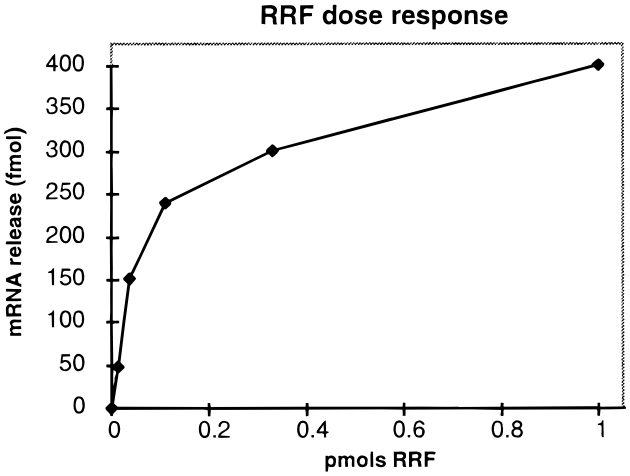

FIGURE 5. Release of mRNA by RRF and RF3. RRF dose response. One picomole of f[^{35}S]met-tRNAfmet·[^{32}P]UUC AUG UAA·ribosome complex was incubated at 30 °C for 10 min together with 1 unit of RF3, 160 μM GTP in 50 μL with increasing amounts of RRF [8 mM Tris, pH 7.2, 40 mM NH_4Cl, and 8 mM $Mg(OAc)_2$]. Released mRNA in fmol. Free mRNA (150 fmol) in absence of RF3 and RRF was subtracted from all values.

Presence of RRF does not significantly alter termination stimulation by RF3

Because RRF, in the presence of GTP, was shown to interact with RF3 in dissociation of the ribosome complex after termination, we studied the effect of coupling ribosomal disassembly to the termination process (Fig. 6). Addition of RF3 (Fig. 6a,b) or RRF (Fig. 6a,e) only slightly stimulated RF1 termination in the absence of GTP, although their effects seemed to be additive (Fig. 6b,f and e,f). Most importantly, stimulation by RF3 in the presence of GTP was threefold or more and was not enhanced significantly by the presence of RRF (compare Fig. 6c,d and g,h). Again, separate experiments at minimal concentration of RF2, in presence of RRF, did not allow observation of a GTP-dependent termination stimulation by RF3 (data not shown).

DISCUSSION

GTP-dependent activities of RF3

Because RF3 contains a GTP binding site, the issue arises whether GTP binding or hydrolysis plays a role in RF3 function. The work described here identifies GTP-dependent activity of RF3 in three different in vitro assays.

We demonstrate that prokaryotic RF3 does indeed show a GTPase activity that is only dependent on the simultaneous presence of ribosomes. This correlates with established conditions for the GTPase activity of EF-G (Conway & Lipmann, 1964). We also show GTP-dependent RF3 stimulation of RF1 activity (Figs. 2, 3). GMP, GDP, or a noncleavable GTP analogue GMP-PNP did not significantly stimulate amplification by RF3, indicating that it is not the conformation of GTP-bound RF3, but hydrolysis of GTP through RF3 that drives GTP-dependent termination amplification. Finally, we show that RF3 can replace the function of EF-G in ribosome recycling in vitro (Table 2; Figs. 4, 5), which is GTP-dependent. It is possible that RF3 and GTP might dissociate RF1 from the posttermination complex, thereby facilitating recycling of RF1. Dissociation of RF1 by RF3 and GTP may explain why GTP inhibits termination stimulation by RF3 in the classical assay, where two independent triplets bind fmet-tRNA (AUG) and RF1 or RF2 (stop triplet). This way, the RF3 GTPase activity might dissociate the RF1,2·stop-triplet·ribosome complex before hydrolysis of the fmet-$tRNA_f^{met}$ can happen.

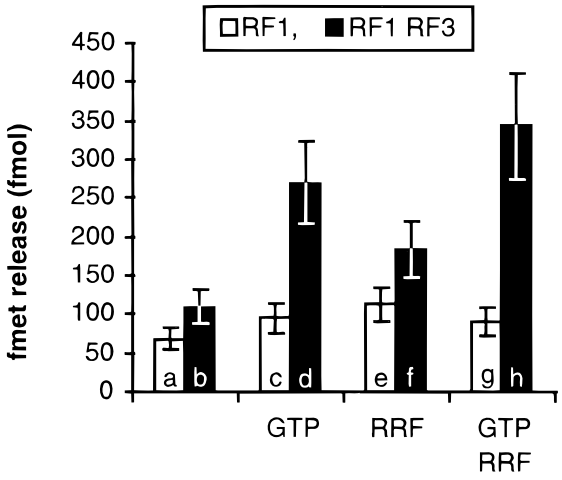

FIGURE 6. Effect of RF3 and GTP on termination in the presence and absence of RRF. a, RF1 only; b, RF1 and RF3; c, RF1 and GTP; d, RF1, RF3, and GTP; e, RF1 and RRF; f, RF1, RF3, and RRF; g, RF1, GTP, and RRF; h, RF1, RF3, GTP, and RRF. For the effect of RF3, compare the height of the black and the white bar in each set. Reaction mixtures were incubated at 30 °C for 13 min and contained in 10 μL: 0.5 pmol f[^{35}S]met-$tRNA_f^{met}$·UUC AUG UAA·ribosome complex, 5 punits RF1, 0.2 units RF3, and 1 pmol of RRF, as indicated [8 mM $Mg(OAc)_2$, 100 mM KCl, 80 mM Tris, pH 7.5]. Released fmet is expressed in fmol. Free fmet at time zero (50 fmol) was subtracted from all values. Standard deviation from the averaged value (±) was around 20%.

Different effects of RF3 on the function of RF1 and RF2

We show in this paper GTP-dependent RF3 stimulation of RF1 activity (Figs. 2, 3). Because RF3 can replace the function of EF-G in ribosome recycling in vitro (Table 2; Figs. 4, 5), it is possible that RF3 and GTP dissociate RF1 from the posttermination complex, thereby facilitating recycling of RF1. Our results and conclusions with RF-1 are in concordance with data published by Freistroffer et al. (1997).

In contrast, RF3 stimlation of termination by RF2 was independent of the presence of GTP (Fig. 3). We suggest that this GTP-independent stimulation is due to stabilization during termination complex formation as previously described (Goldstein et al., 1970b). Our explanation is in concordance with a recent report that nucleotide-free RF3 exhibits positive cooperativity with RF2 (Pel et al., 1998). Because we did not observe GTP-dependent stimulation by RF3 of RF2, we have no evidence that indicates RF2 recycling by RF3 and GTP. This is in contrast to the observation of Freistroffer et al. (1997) that RF3 can stimulate termination with RF2 in a GTP-dependent manner by recycling of RF2. It is possible that Freistroffer et al. did not see increased RF-2 binding affinity by RF3, if in their system RF2 recycling and not RF2 binding was the rate-limiting step of the termination reaction. A possible explanation is that Freistroffer's ribosome·mRNA·RF2 complex is additionally stabilized through the Shine–Dalgarno sequence only 18 nt upstream from the termination codon.

If in vitro RF-2 recycling to be rate-limiting needs an adjacent SD sequence, this suggests that release factor recycling might not be a rate-limiting step in regular

translation termination at 37 °C under physiological conditions (with the exception of coupled translation). This hypothesis is supported by the facts that at 37 °C the deletion of RF3 only supresses termination of the RF2-specific stop codon UGA (Grentzmann et al., 1995), yet RF3 is not essential at 37 °C (Grentzmann et al., 1994; Mikuni et al., 1994; Fraser et al., 1995). The situation might be inversed and recyling could be rate-limiting at lower temperatures, because growth of an RF3$^-$ mutant significantly decreases at low temperatures (Grentzmann, 1994; Grentzmann et al., 1994).

Different effects of RF3 on the function of RF1 and RF2 might be less surprising in light of the fact that the ribosomal binding sites of RF1 and RF2 overlap but are not identical. Ribosomal proteins L7/L12 are essential for RF1 and RF2 binding (Brot et al., 1974), but ribosomal protein L11 is necessary only for RF1 binding. RF2 binds with higher affinity to ribosomes depleted of L11 (Tate et al., 1983, 1984, 1986) and RF-2 termination is no longer amplified by RF3 (McCaughan et al., 1984). The clearcut biochemical difference between the effect of RF3 on RF1 and RF2 presented in this paper supports previous in vivo experiments, which indicate that the deletion of the RF3 gene preferentially suppresses termination directed by RF2 (Grentzmann et al., 1995). On the other hand, our finding that RF3 functions as a GTPase for ribosome recycling with RRF may explain why, in certain in vivo experiments, a differential effect of RF3 on RF1 and RF2 was not so apparent (Yanofsky et al., 1996).

We find that our observation that RF3 stimulates RF2 in a GTP-independent manner is in accordance with the data of Freistroffer et al. (1997), but contrary to their conclusion that RF2 stimulation by RF3 is exclusively due to recycling. In experiments that studied the influence of RF3 on the k_{cat}/K_m of RF2, Freistroffer et al. showed increase of the maximum rate of RF2-dependent hydrolysis of peptidyl-tRNA by RF3. This corresponds to our data on RF2 stimulation by RF3 in the absence or in presence of GTP (Fig. 3). Because the experiments of Freistroffer et al. were only done in the presence of GTP, it is not clear whether their observation is GTP-dependent or not. However, stimulation of hydrolysis is only seen at saturating, and not at low RF2 concentrations, in the presence of constant amounts of termination complex (4 pmol). We would expect the opposite situation if recycling of RF2 was the reason for RF3 stimulation. In fact, the intracellular concentration of release factors is high enough to saturate the termination complexes present in the cell (Adamski et al., 1994). Therefore, experiments using high concentrations of RF2 seem to be the most representative to translation termination in vivo. In summary, the k_{cat}/K_m experiments of Freistroffer et al. suggest to us amplification of RF2 termination by RF3 in vivo, independent of ribosome recycling. On the other hand, their conclusion that RF3 stimulates RF2 exclusively by recycling contradicts their previous report showing that an RF3$^-$ mutant specifically suppresses RF2 termination in vivo (Grentzmann et al., 1995).

RF3 replaces EF-G in ribosome recycling with RRF in vitro

RF3 is not essential at 37 °C in *Escherichia coli* (Grentzmann et al., 1994; Mikuni et al., 1994). The observed similarity between RF3 and elongation factors led to the hypothesis that RF3 might be replaceable by EF-Tu or EF-G at 37 °C, inviting speculation on the function of RF3 (Laalami et al., 1996; Nakamura et al., 1996; Buckingham et al., 1997). EF-G enables ribosome recycling by RRF in vitro in the presence of GTP (Hirashima & Kaji, 1972). We show here that RF3 GTPase activity can replace EF-G GTPase activity in in vitro ribosome recycling. Release of the mRNA and tRNA after termination provides physical evidence supporting the involvement of RF3 in the dissociation of the translation complex after termination. Eventually, a translocation step catalyzed by RF3 and GTP after termination could release RF1 or RF2 from the A-site and, at the same time, transport the terminal tRNA to the E-site in much the same way EF-G does during chain elongation.

Finally, we find an in vitro activity of RF3 for dissociation of mRNA and fmet-tRNA in the absence of RF1 or RF2 (Table 2). This activity shows an RF3 dissociation function in the absence of translation termination and is observed only at high factor concentration. This observation is in concordance with the fact that an RF3$^-$ mutant has previously been characterized as a suppressor of a *pth* (peptidyl-tRNA hydrolase) thermosensitive strain (Grentzmann, 1994). Peptidyl-tRNA hydrolase is responsible for hydrolyzing peptidyl-tRNA that has been prematurely dropped off the ribosome during translation (Menninger, 1979). At nonpermissive temperatures, *pth* temperature-sensitive mutants die, presumably due to accumulation of peptidyl-tRNA. If RF3 plays a role in drop off, an RF3 mutant might suppress this thermosensitivity by lowering the amount of prematurely released peptidyl-tRNA. Further genetic experiments on the involvement of RRF and RF3 in ribosomal drop off, as well as in vitro measurements of peptidyl-tRNA release from ribosomes paused at stop signals or sense codons confirm that RF3 and RRF are able to stimulate peptidyl-tRNA release from ribosomes (Heurgué-Hamard et al., 1998)

Our data showing release of a short messenger RNA in the presence of RRF may appear to be contradictory to the recently published report of Pavlov et al. (1997) suggesting no release of short synthetic mRNA by RRF. On the other hand, a very recent report from Janosi et al. (1998) has proven the concept of RRF functioning in mRNA release from the posttranslational complex in vivo. The major difference between our experiments and those of Pavlov et al. is that their mRNA

contains a strong Shine and Dalgarno sequence only a few nucleotides away from the termination codon. Reinitiation of translation after termination, due to a Shine–Dalgarno sequence near a stop codon, is a phenomenon that has been reported previously as coupled translation in prokaryotes (for review see Nomura et al., 1984; Oppenheim & Yanofsky, 1980). To prove a role of RF3 in ribosome recycling, physical evidence for mRNA release from the ribosome is necessary. Our somewhat simpler in vitro assay is independent of a Shine–Dalgarno sequence and allows observing mRNA release by RRF as one expects with most naturally occurring mRNAs. Our results give physical evidence that RF3 does in fact replace EF-G in ribosome recycling in vitro, allowing mRNA and tRNA release by RRF after translation termination.

On the basis of the data presented in this communication and other published information, we propose a model of the events related to termination and the disassembly of the posttermination complex (Fig. 7). RF3 stabilizes RF2 binding to the termination complex prior to the hydrolysis step (Fig. 7, I and II), because it has been shown to amplify termination by RF2 in vitro and in vivo (Grentzmann et al., 1995) by increasing its affinity to the ribosome (Pel et al., 1998). In our system, such a stabilizing effect by RF3 is not observed for RF1 presumably because of its stronger affinity to complex I (Grentzmann et al., 1995). We show that in the presence of GTP, RF3 amplifies termination at limiting concentrations of RF1 in vitro and suggest that RF3, in the presence of GTP, recycles RF1 (Fig. 7, III). We did not find evidence for recycling of RF2 through RF3 and GTP. To establish the exact conditions under which RF2 is released from the posttranslation complex, experiments using radioactive labeled RF2 might be worth pursuing. Furthermore, we show that RF3, in the presence of RRF and GTP, efficiently releases mRNA from the terminated ribosome (Fig. 7, III). This activity can be observed independent of whether the translation is terminated by RF1 or RF2.

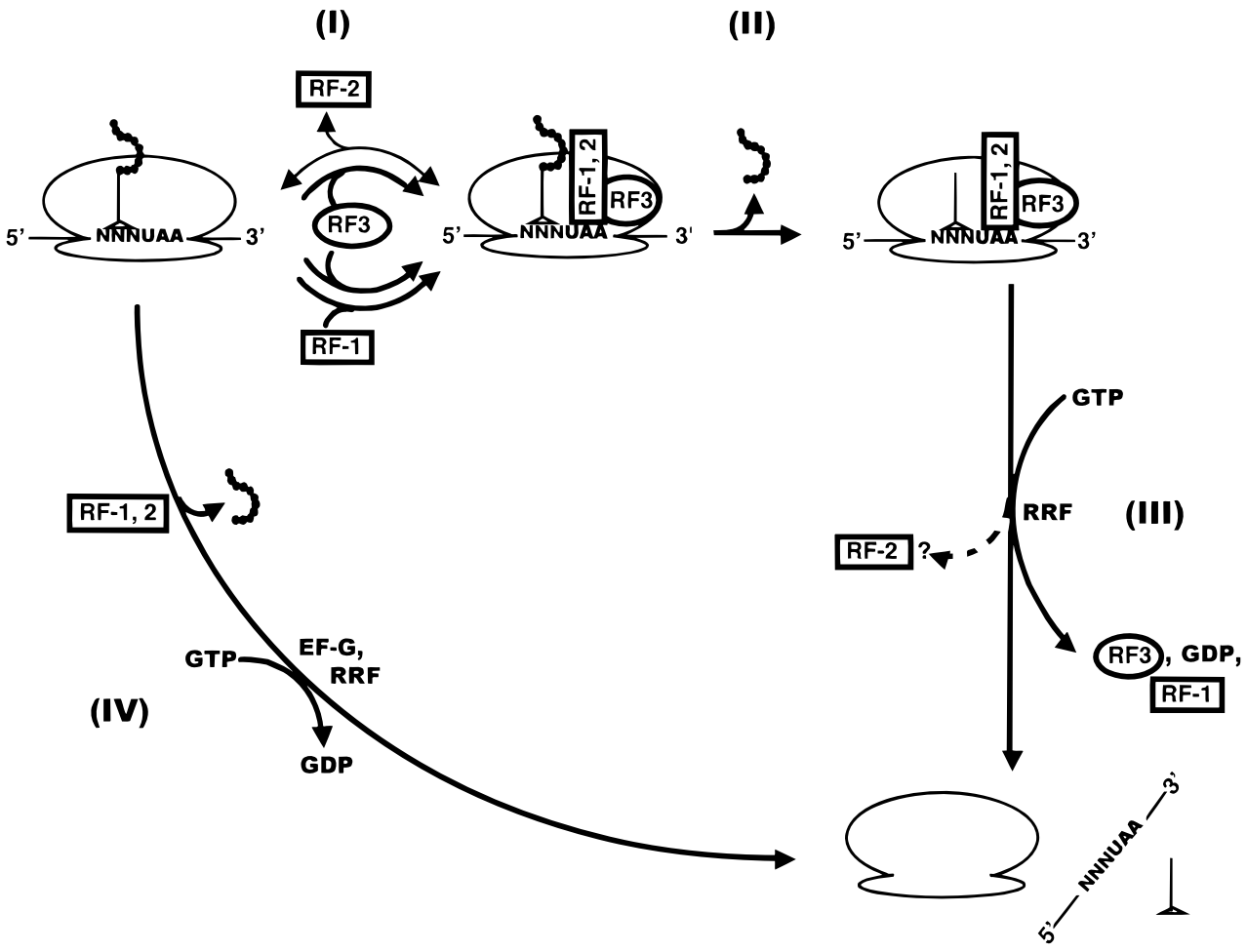

FIGURE 7. Model for RF3 function in translational termination. I, complex formation RF1 binds stronger to the termination complex than RF2 (Goldstein et al., 1970b; Grentzmann et al., 1995), the thin double-headed arrow indicates lower binding affinity for RF2; II, terminal peptidyl-tRNA hydrolysis; III, ribosome recycling; IV, termination and ribosome recycling in absence of RF3 (Janosi et al., 1996).

RF3 is not essential (Grentzmann et al., 1994; Mikuni et al., 1994) and termination occurs in the absence of RF3 (Caskey et al., 1971). EF-G has been shown to catalyze ribosome recycling in vitro, in the presence of RRF and GTP (Ogawa & Kaji, 1975; Janosi et al., 1996) (Fig. 7, IV). At present, we do not know whether EF-G, RF3, or both factors function for disassembly of the termination complex by RRF in vivo. It is possible that EF-G can replace RF3 in this function in vivo in an $RF3^-$ mutant or in organisms like *Mycoplasma genitalium*, which do not contain an RF3 gene (Fraser et al., 1995). On the other hand, it is also possible that the function of RF3 is limited to the termination stop and the ribosome recycling step may as well be catalyzed by EF-G and RRF, as proposed previously (Janosi et al., 1996).

MATERIALS AND METHODS

Purified elements for in vitro reactions

Release factors RF1 and RF2 were purified from wild-type strains (Tate & Caskey, 1990) or from release factor overexpressing strains (Adamski et al., 1994). RF3 was purified as described (Grentzmann et al., 1994) by HPLC on DEAE (Waters). Contamination by elongation factors was efficiently eliminated by a supplementary step on CM-Sephadex (Pharmacia) (Caskey et al., 1969; Grentzmann et al., 1994). Release factor fractions were checked for GTP contamination using electrospray mass spectrometry (Crain, 1990; Straub & Voyksner, 1993). Concentrations of purified release factor fractions in terms of amount of protein per unit of volume was not constant compared to the number of molecules of active protein per unit of volume. For example, overexpression of release factors has been shown to result in loss of specific activity (Adamski et al., 1994) and a possible site for post-translational modification for RF2 has been reported (Uno et al., 1996). We therefore estimated the quantities of active release factors in units of activity as described previously (Caskey et al., 1971; Grentzmann et al., 1994).

tRNAfmet (Subriden) was charged and purified according to Tate and Caskey (1990). Tight couple ribosomes were purified as described (Spedding, 1990). UUC AUG UAA, UUC AUG UAG, and UUC AUG UGA RNA oligonucleotides were synthesized on an ABI synthesizer, deprotected, and purified on Sephadex G-25 (Pharmacia). Sequences were verified by mass spectroscopy (Limbach et al., 1995). RRF was purified from an overexpressing strain (Ichikawa & Kaji, 1989) as described (Hirashima & Kaji, 1972). EF-G was purified and its activity was tested as described (Kaziro et al., 1972).

RF3 GTPase activity

RF3-mediated GTP hydrolysis was monitored according to Kolakofsky et al. (1968) with the modifications described below, in 80 mM Tris, pH 7.2, 100 mM KCl, and 8 mM $Mg(OAc)_2$. The incubation mixture contained 2 pmol of 70S ribosomes, UUC AUG UAA·ribosome complex, or fmet-$tRNA_f^{met}$· UUC AUG UAA·ribosome complex, and RF1, RF2, RF3 as indicated, in a total volume of 10 μL. Reactions were performed for 15 min at 37 °C and then stopped by the addition of 0.1 mL of 1 M perchloric acid and 1 mL of 1 mM KH_2PO_4. The mixtures were treated as described by Beaudry et al. (1979), and hydrolyzed (^{32}P) phosphate was counted in 5 mL of Quicksafe A (Zinsser Analytic). Values were corrected for background GTP hydrolysis in buffer alone.

Translational termination by f[35S]met release

f[^{35}S]met-$tRNA_f^{met}$·messenger·ribosome complex was prepared as described (Grentzmann & Kelly, 1997), incubating 50 pmol of 70S ribosomes with 50 pmol charged tRNA in the presence of 250 pmol RNA oligonucleotide for 20 min in 20 mM Tris, pH 7.2, 150 mM NH_4Cl, and 30 mM $Mg(OAc)_2$ at 30 °C in a final volume of 50 μL. Termination kinetics were run in 80 mM Tris, pH 7.2, 100 mM KCl, and 8 mM $Mg(OAc)_2$ as described at 24 °C or 30 °C (Caskey et al., 1971). Ribosome complex was added at 0.5 pmol per 10 μL reaction volume. GTP, when added, was 160 μM. RRF in pmol, RF1 and RF2 in picounits (Caskey et al., 1971), RF3 in units (Grentzmann et al., 1994) were as indicated. Increase of the GTP concentration due to contamination of RF fractions by GTP was less than 0.2 μM. Hydrolyzed formyl-methionine was quantified by ethyl acetate extraction at pH 1. Maximal release ranged between 50% and 70% of total [^{35}S]f-met. Assays were repeated two to six times.

Release of [32P]mRNA from ribosomes

f[^{35}S]met-$tRNA_f^{met}$·[^{32}P]messenger·ribosome complex was prepared by incubating 300 pmol of 70S ribosomes with 300 pmol charged tRNA in presence of 500 pmol 5′[^{32}P]RNA-oligonucleotide (Sambrook et al., 1989) for 20 min at 20 mM Tris, pH 7.2, 150 mM NH_4Cl, and 30 mM $Mg(OAc)_2$, at 30 °C in a final volume of 50 μL. Complex was purified from unbound message and tRNA by Sephacryl S-300 spun columns (Pharmacia) at 4 °C. Reactions were run in 8 mM Tris, pH 7.2, 40 mM NH_4Cl, and 8 mM $Mg(OAc)_2$ (Ogawa & Kaji, 1975) at 30 °C. RF-1, -2 and -3, EF-G, RRF, and 160 μM GTP were added as indicated. When establishing conditions for this assay, we found that addition of 0.2 mM phosphoenolpyruvate and 3 μg of pyruvate kinase, which significantly enhances ribosome recycling in the presence of EF-G, was not essential when using RF3 (data not shown). To minimize the risk of mRNA release due to contamination of RF3 fractions by EF-G, we performed our RF3 experiments without the above-mentioned GTP-generating system. Hydrolyzed formyl-methionine was counted after ethyl acetate extraction. Released mRNA was counted after filtration through Microcon 10 filters (Amicon) at 4 °C. Released nonhydrolyzed fmet-$tRNA^{fmet}$ was estimated by counting the ^{35}S difference after repurification of reaction mixtures on S-300 spun columns and subtracting values for hydrolyzed formyl-methionine. Assays were repeated two to four times with independent complex preparations.

ACKNOWLEDGMENTS

RF1- and RF2-overexpression plasmids were a kind gift from Dr. W.P. Tate. We thank Dr. Y. Kaziro for providing EF-G and

Dr. Chad C. Nelson for mass spectrometry analysis. First steps of the RF3 purification were realized in Dr. R.H. Buckingham's laboratory in Paris. We thank him for his generosity. Major parts of the presented work were performed in the laboratory of Drs. Ray F. Gesteland and John F. Atkins in Salt Lake City. We extend our sincere thanks to them for their support. Thanks to Drs. Douglas J. Bucklin, Herman J. Pell, and Waren P. Tate for critical reading of the manuscript.

Received September 29, 1997; returned for revision October 27, 1997; revised manuscript received May 1, 1998

REFERENCES

Adamski FM, McCaughan KK, Jorgensen F, Kurland CG, Tate WP. 1994. The concentration of polypeptide chain release factors 1 and 2 at different growth rates of *Escherichia coli*. *J Mol Biol 238*:302–308.

Beaudry P, Sander G, Grunberg-Manago M, Douzou P. 1979. Cation-induced regulatory mechanism of GTPase activity dependent on polypeptide initiation factor 2. *Biochemistry 18*:202–207.

Brot N, Tate WP, Caskey CT, Weissbach H. 1974. The requirement for ribosomal proteins L7/L12 in peptide chain termination. *Proc Natl Acad of Sci USA 71*:89–92.

Buckingham RH, Grentzmann G, Kisselev L. 1997. Polypeptide chain release factors. *Mol Microbiol 24*:449–456.

Capecchi MR. 1967. Polypeptide chain termination in vitro: Isolation of a release factor. *Proc Natl Acad Sci USA 58*:1144–1151.

Capecchi MR, Klein HA. 1969. Characterization of three proteins involved in polypeptide chain termination. *Cold Spring Harbor Symp Quant Biol 34*:469–477.

Caskey CT. 1977. Peptide chain termination. In: Weissbach H, Pestka S, eds. *Molecular mechanisms of protein biosynthesis*. New York: Academic Press. pp 443–465.

Caskey CT, Scolnick E, Tompkins R, Goldstein J, Milman G. 1969. Peptide chain termination, codon, protein factor and ribosomal requirements. *Cold Spring Harbor Symp Quant Biol 34*:479–488.

Caskey CT, Scolnick E, Tompkins R, Milman G, Goldstein J. 1971. Release factors: In vitro assay and purification. *Methods Enzymol 20*:367–375.

Caskey CT, Tompkins R, Scolnick E, Caryk T, Nierenberg M. 1968. Sequential translation of trinucleotide codons for initiation and termination of protein synthesis. *Science 162*:135–138.

Conway TW, Lipmann F. 1964. Characterization of a ribosome-linked guanosine triphosphatase in *Escherichia coli* extracts. *Proc Natl Acad Sci USA 52*:1462–1469.

Crain PF. 1990. Mass spectrometric techniques in nucleic acid research. *Mass Spectrom Rev 9*:505–554.

Fraser CM, Gocayne JD, White O, Adams MD, Clayton RA, Fleischmann RD, Bult CJ, Kerlavage AR, Sutton G, Kelley JM, Fritchman JL, Weidman JF, Small KV, Sandusky M, Fuhrmann J, Nguyen D, Utterback TR, Saudek DM, Phillips CA, Merrick JM, Tomb JF, Dougherty BA, Bott KF, Hu PC, Lucier TS, Peterson SN, Smith HO, Hutchison CA III, Venter JC. 1995. The minimal gene complement of *Mycoplasma genitalium*. *Science 270*:397–403.

Freistroffer DV, Pavlov MY, McDougall J, Buckingham RH, Ehrenberg M. 1997. Release factor RF3 in *E. coli* accelerates the dissociation of release factors RF1 and RF2 from the ribosome in a GTP-dependent manner. *EMBO 16*:4126–4133.

Frolova L, Le Goff X, Rasmussen HH, Cheperegin S, Drugeon G, Kress M, Arman I, Haenni AL, Celis JE, Philippe M, Justesen J, Kisselev L. 1994. A highly conserved eukaryotic protein family possessing properties of polypeptide chain release factor. *Nature 372*:701–703.

Frolova L, LeGoff X, Zhouravleva G, Davydova E, Philippe M, Kisselev L. 1996. Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase. *RNA 2*:334–341.

Ganoza MC. 1966. Polypeptide chain termination in cell-free extracts of *E. coli*. *Cold Spring Harbor Symp Quant Biol 31*:263–268.

Ganoza MC, Nakamoto T. 1966. Studies on the mechanism of polypeptide chain termination in cell-free extracts of *E. coli*. *Proc Natl Acad Sci USA 55*:162–169.

Goldstein JL, Beaudet AL, Caskey CT. 1970a. Peptide chain termination with mammalian release factor. *Proc Natl Acad Sci USA 67*:99–106.

Goldstein JL, Caskey CT. 1970. Peptide chain termination: Effect of protein S on ribosomal binding of release factors. *Proc Natl Acad Sci USA 67*:537–543.

Goldstein J, Milman G, Scolnick E, Caskey T. 1970b. Peptide chain termination. VI. Purification and site of action of S. *Proc Natl Acad Sci USA 65*:430–437.

Grentzmann G. 1994. RF-3, facteur de terminaison de la traduction chez *Escherichia coli* [dissertation]. Paris: Université Paris 6.

Grentzmann G, Brechemier-Baey D, Heurgue-Hamard V, Buckingham RH. 1995. Function of polypeptide chain release factor RF-3 in *Escherichia coli*. RF-3 action in termination is predominantly at UGA-containing stop signals. *J Biol Chem 260*:10595–10600.

Grentzmann G, Brechemier-Baey D, Heurgue V, Mora L, Buckingham RH. 1994. Localization and characterization of the gene encoding release factor RF3 in *Escherichia coli*. *Proc Natl Acad Sci USA 91*:5848–5852.

Grentzmann G, Kelly PJ. 1997. Ribosomal binding site of release factors RF1 and RF2. A new translational termination assay in vitro. *J Biol Chem 272*:12300–12304.

Heurgué-Hamard V, Mora L, MacDougall J, Leboeuf C, Grentzmann G, Ehrenberg M, Buckingham RH. 1998. Ribosome release factor RF4 and termination factor RF3 are involved in dissociation of peptidyl-tRNA from the ribosome. *EMBO J* 17:808–816.

Hirashima A, Kaji A. 1972. Purification and properties of ribosome-releasing factor. *Biochemistry 11*:4037–4044.

Ichikawa S, Kaji A. 1989. Molecular cloning and expression of ribosome releasing factor. *J Biol Chem 264*:20054–20059.

Janosi L, Hara H, Zhang S, Kaji A. 1996. Ribosome recycling by ribosome recycling factor (RRF)—An important but overlooked step of protein biosynthesis. *Adv Biophys 32*:121–201.

Janosi L, Mottagui-Tabar S, Isaksson, LA, Sekine Y, Ohtsubo E, Zhang S, Goon S, Nelken S, Shuda M, Kaji A. 1998. Evidence for in vivo ribosome recycling, the fourth step in protein biosynthesis. *EMBO J 17*:1141–1151.

Janosi L, Shimizu I, Kaji A. 1994. Ribosome recycling factor (ribosome releasing factor) is essential for bacterial growth. *Proc Natl Acad Sci USA 91*:4249–4253.

Kaziro Y, Inoue-Yokosawa N, Kawakita M. 1972. Studies on polypeptide elongation factor from *E. coli*. I. Crystalline factor G. *J Biochem 72*:853–863.

Kolakofsky D, Dewey KF, Hershey JW, Thach RE. 1968. Guanosine 5′-triphosphatase activity of initiation factor f2. *Proc Natl Acad Sci USA 61*:1066–1070.

Laalami S, Grentzmann G, Bremaud L, Cenatiempo Y. 1996. Messenger RNA translation in prokaryotes: GTPase centers associated with translational factors. *Biochimie 78*:577–589.

Limbach PA, Crain PF, McCloskey JA. 1995. Characterization of oligonucleotides and nucleic acids by mass spectrometry. *Curr Opin Biotechnol 6*:96–102.

McCaughan KK, Ward CD, Trotman CNA, Tate WP. 1984. The ribosomal binding domain for the bacterial release factors RF-1, RF-2 and RF-3. *FEBS Lett 175*:90–94.

Menninger JR. 1979. Accumulation of peptidyl tRNA is lethal to *Escherichia coli*. *J Bacteriol 137*:694–696.

Mikuni O, Ito K, Moffat J, Matsumura K, McCaughan K, Nobukuni T, Tate W, Nakamura Y. 1994. Identification of the *prfC* gene, which encodes peptide-chain-release factor 3 of *Escherichia coli*. *Proc Natl Acad Sci USA 9*:5798–5802.

Milman G, Goldstein J, Scolnick E, Caskey T. 1969. Peptide chain termination 3. Stimulation of in vitro termination. *Proc Natl Acad Sci USA 63*:183–190.

Nakamura Y, Ito K, Isaksson LA. 1996. Emerging understanding of translation termination. *Cell 87*:147–150.

Nomura M, Gourse R, Baughman G. 1984. Regulation of the synthesis of ribosomes and ribosomal components. *Annu Rev Biochem 53*:75–117.

Ogawa K, Kaji A. 1975. Requirement for ribosome-releasing factor for the release of ribosomes at the termination codon. *Eur J Biochem 58*:411–419.

Oppenheim DS, Yanofsky C. 1980. Translational coupling during expression of the tryptophane operon of *Escherichia coli*. *Genetics 95*:785–795.

Pavlov MY, Freistroffer DV, McDougall J, Buckingham RH, Ehrenberg M. 1997. Fast recycling of *Escherichia coli* ribosomes requires both ribosome recycling factor (RRF) and release factor RF3. *EMBO 16*:4134–4141.

Pel HJ, Moffat JG, Ito K, Nakamura Y, Tate WP. 1998. *Escherichia coli* release factor 3: Resolving the paradox of a typical G protein structure and atypical function with guanine nucleotides. *RNA 4*:47–54.

Remes B, Elseviers D. 1980. Adenosine 5′-triphosphate leakage does not cause abortive infection of bacteriophage T7 in male *Escherichia coli*. *J Bacteriol 143*:1054–1056.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: A laboratory manual, 2nd ed*. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Scolnick E, Tompkins R, Caskey T, Nirenberg M. 1968. Release factors differing in specificity for terminator codons. *Proc Natl Acad Sci USA 61*:768–774.

Spedding G. 1990. *Ribosomes and protein synthesis: A practical approach*. Oxford: IRL.

Straub RF, Voyksner RD. 1993. Negative ion formation in electrospray mass spectrometry. *J Am Soc Mass Spectrom 4*:578–587.

Tate WP, Brown CM. 1992. Translational termination: "Stop" for protein synthesis or "pause" for regulation of gene expression. *Biochemistry 31*:2443–2450.

Tate WP, Caskey CT. 1990. Termination in protein synthesis. In: Spedding G, ed. *Ribosomes and protein synthesis: A practical approach*. Oxford: IRL. pp 81–100.

Tate WP, Dognin MJ, Noah M, Stöffler-Meilicke M, Stöffler G. 1984. The NH2-terminal domain of *Escherichia coli* ribosomal protein L11. Its three-dimensional location and its role in the binding of release factors 1 and 2. *J Biol Chem 259*:7317–7324.

Tate WP, McCaughan KK, Ward CD, Sumpter VG, Trotman CNA, Stöffler-Meilicke M, Maly P, Brimacombe R. 1986. The ribosomal binding domain of the *Escherichia coli* release factors. Modification of tyrosine in the N-terminal domain of ribosomal protein L11 affects release factors 1 and 2 differentially. *J Biol Chem 261*:2289–2293.

Tate WP, Schulze H, Nierhaus KH. 1983. The *Escherichia coli* ribosomal protein L11 suppresses release factor 2 but promotes the release factor 1 activities in peptide chain termination. *J Biol Chem 258*:12810–12815.

Uno M, Ito K, Nakamura Y. 1996. Functional specificity of amino acid at position 246 in the tRNA mimicry domain of bacterial release factor 2. *Biochimie 78*:935–943.

Yanofsky C, Horn V, Nakamura Y. 1996. Loss of overproduction of polypeptide release factor 3 influences expression of the tryptophanase operon of *Escherichia coli*. *J Bacteriol 178*:3755–3762.

Zhouravleva G, Frolova L, Le Goff X, Le Guellec R, Inge-Vechtomov S, Kisselev L, Philippe M. 1995. Termination of translation in eukaryotes is governed by two interacting polypeptide chain release factors, eRF1 and eRF3. *EMBO J 14*:4065–4072.