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Eukaryotic polypeptide chain release factor eRF3 is an eRF1- and ribosome-dependent guanosine triphosphatase

LYUDMILA FROLOVA,[1,2] XAVIER LE GOFF,[1] GALINA ZHOURAVLEVA,[1,4] ELENA DAVYDOVA,[3] MICHEL PHILIPPE,[1] and LEV KISSELEV[1,2]

[1] Département de Biologie et Génétique du Développement, CNRS URA 256, Université de Rennes 1, 35042 Rennes Cedex, France
[2] Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov str. 32, 117984 Moscow, Russia
[3] Institute of Protein Research, Russian Academy of Sciences, 142292 Pushchino, Moscow Region, Russia

ABSTRACT

Termination of translation in eukaryotes is governed by two polypeptide chain release factors, eRF1 and eRF3 on the ribosome. eRF1 promotes stop-codon-dependent hydrolysis of peptidyl-tRNA, and eRF3 interacts with eRF1 and stimulates eRF1 activity in the presence of GTP. Here, we have demonstrated that eRF3 is a GTP-binding protein endowed with a negligible, if any, intrinsic GTPase activity that is profoundly stimulated by the joint action of eRF1 and the ribosome. Separately, neither eRF1 nor the ribosome display this effect. Thus, eRF3 functions as a GTPase in the quaternary complex with ribosome, eRF1, and GTP. From the in vitro uncoupling of the peptidyl-tRNA and GTP hydrolyses achieved in this work, we conclude that in ribosomes both hydrolytic reactions are mediated by the formation of the ternary eRF1·eRF3·GTP complex. eRF1 and the ribosome form a composite GTPase-activating protein (GAP) as described for other G proteins. A dual role for the revealed GTPase complex is proposed: in "GTP state," it controls the positioning of eRF1 toward stop codon and peptidyl-tRNA, whereas in "GDP state," it promotes release of eRFs from the ribosome. The initiation, elongation, and termination steps of protein synthesis seem to be similar with respect to GTPase cycles.

Keywords: eukaryotes; GTPase; polypeptide chain release factors (RF); protein biosynthesis; termination of translation

INTRODUCTION

For genes encoding proteins, the final chemical step in the expression of genetic information is the termination of translation that proceeds in the ribosomes and is governed by two major components, the stop codon in the messenger RNA at the ribosomal A site, and the polypeptide chain release factor(s) (RF) (reviewed in Caskey, 1980; Craigen et al., 1990; Tate & Brown, 1992; Stansfield & Tuite, 1994). The termination reaction leads to hydrolysis of the last peptidyl-tRNA, generating a free nascent polypeptide chain and a free (uncharged) tRNA, followed by the release of these components from the ribosome.

Reprint requests to: Lev Kisselev, Engelhardt Institute of Molecular Biology, Russian Academy of Sciences, Vavilov str. 32, 117984 Moscow, Russia; e-mail: kissel@imb.imb.ac.ru.

[4] Permanent address: St. Petersburg State University, Department of Genetics and Biotechnology Center of St. Petersburg University, Russia.

In prokaryotes, translation termination is controlled by three RFs. RF1 and RF2 recognize each two of the three stop codons and catalyze the hydrolytic reaction, whereas RF3 is assumed to be a GTP-binding protein, on its own being inactive as a release factor; however, in the in vitro assay, RF3 stimulates RF activity in termination reaction (Grentzmann et al., 1994, 1995; Mikuni et al., 1994). The GTPase activity of the prokaryotic RF3 has not been described so far, although the effect of GTP and GDP on translation termination was noticed long ago (Capecchi & Klein, 1969; Caskey et al., 1969; Milman et al., 1969).

In higher eukaryotes, translational termination is accompanied by the ribosome-dependent GTP hydrolysis (Goldstein et al., 1970; Beaudet & Caskey, 1971; Konecki et al., 1977). It was thought (but not proved) that this GTPase activity was associated with a single eRF partially purified from mammalian cells (Caskey et al., 1974). However, when eRF1 has been identified

(Frolova et al., 1994) and the eRF1 sequences for many species became available, it appeared that this protein had no GTP-binding motifs known to be present in G proteins (reviewed in Bourne et al., 1991). Furthermore, eRF1 was active in the in vitro RF assay even without GTP (Zhouravleva et al., 1995). From these observations, it became evident that eRF1 was unable to confer a GTPase activity to the termination process (Frolova et al., 1994). In fact, it was demonstrated that a newly identified protein, termed eRF3, greatly stimulated the termination reaction in vitro in the presence of GTP and displayed typical GTP-binding motifs in its structure (Zhouravleva et al., 1995). Consequently, eRF3 might be considered as a candidate protein for possessing a GTPase activity.

Here, we demonstrate that eRF3 binds to GTP and exhibits a very low intrinsic GTPase activity on its own. However, in the presence of the ribosomes and eRF1, GTP hydrolysis occurs, generating GDP and P_i, and this reaction is peptidyl-tRNA- and stop-codon-independent in vitro.

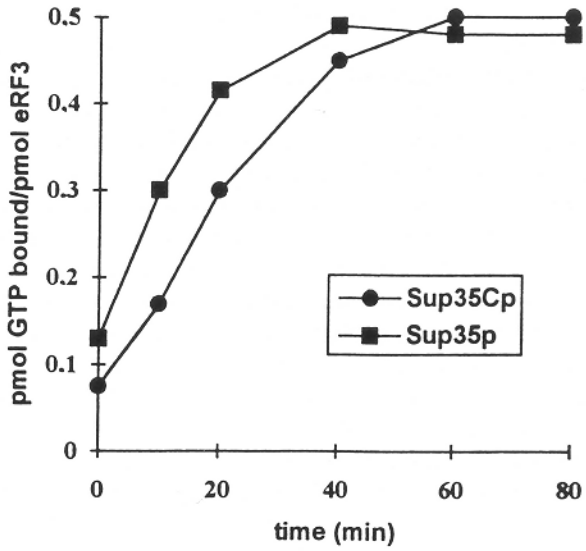


FIGURE 1. Binding of GTP to eRF3. The incubation mixture contained buffer B, 1 mg/mL of BSA, 10 μM [α-^{32}P]GTP (sp. activity 5,000 cpm/pmol), eRF3/Sup35p (25 μg/mL) or eRF3/Sup35Cp (10 μg/mL). After incubation at 25 °C at the indicated time, 12.5-μL aliquots were withdrawn and bound GTP was quantitated as described in the Materials and methods.

RESULTS

eRF3 binds to GTP

eRF3 of higher eukaryotes (Hoshino et al., 1989; Zhouravleva et al., 1995) and the putative yeast eRF3 (Sup35 protein) (Kikuchi et al., 1988; Kushnirov et al., 1988; Wilson & Culbertson, 1988) exhibit typical GTP-binding motifs recognized earlier in a number of G proteins (reviewed in Bourne et al., 1991). In fact, eRF3 binds to GTP in solution, as it is evident from the data presented in Figure 1 and Table 1. The technique applied in these experiments is a standard one utilizing the ability of a protein·GTP complex to be retained on nitrocellulose membrane, whereas free GTP remains in solution. The binding at 25 °C initiates immediately (zero time) and slowly reaches the plateau (Fig. 1). The GTP/eRF3 ratio varies depending on the given protein preparation, time, temperature, and GTP concentration. At optimal conditions, it reaches the value of approximately 0.5 pmol of GTP per pmol of eRF3. This reduced stoichiometry may result from partial occupation of the GTP-binding site by tightly bound GDP (small G proteins are often isolated as protein·GDP complexes) and/or an incomplete retention of the protein·GTP complex on nitrocellulose membrane, as has been shown for another G protein (Kabcenell et al., 1990).

The extended and the truncated forms of the factor, eRF3/Sup35p and eRF3/Sup35Cp, respectively (see the Materials and methods), possess the same binding activity toward GTP (Fig. 1). Therefore, the N-terminal domain of the *Xenopus laevis* eRF3 does not influence the GTP binding. This result is expected because the GTP-binding motifs are located in the C part of the protein and, moreover, the truncated eRF3 stimulates activity of eRF1 (Zhouravleva et al., 1995).

The eRF1 preparation exhibits a very low binding capacity toward GTP, but distinguishable from the background value (Table 1). We believe that this is due to the presence of trace amounts of *Escherichia coli* GTPases not removed during the eRF1 purification. From Table 1 it is evident that eRF1, the ribosomes, and their mixture affect insignificantly the GTP binding to eRF3, whereas the ribosomes without factors bind no GTP. We conclude that GTP-binding capacity of eRF3 is an intrinsic property of this protein that is virtually not influenced by the presence of the other components of termination apparatus.

All components of the termination machinery exhibit negligible GTPase activity

Although the ability of eRF3 to bind to GTP is evident (Fig. 1; Table 1), its GTPase activity is very low (Ta-

TABLE 1. Effect of the eRF1 and the ribosomes on the eRF3 binding to GTP.

Termination factors	Ribosomes	GTP bound (pmol)
eRF1	–	0.08
eRF1	+	0.25
eRF3	–	1.80
eRF3	+	1.45
eRF3 + eRF1	–	1.94
eRF3 + eRF1	+	2.07
None	+	0

ble 2). The same holds true for each component of the termination process. The ribosomes without RFs are completely inactive in a GTPase reaction. The lack of a GTPase activity of the highly purified ribosomes correlates with its inability to bind GTP (Table 1) and demonstrates that the GTPase activity of the ribosomes mentioned in some publications is due to the presence of GTPase-bearing factors rather than the intrinsic property of the ribosomal particles. eRF1 alone or mixed with the ribosomes exhibits a very low GTPase activity that is probably not associated with this protein and may arise from contaminating *E. coli* GTPases mentioned above. eRF3 on its own or in the presence of the ribosomes manifests a low GTPase activity that is somewhat higher than that of eRF1 (Fig. 2; Table 2). The GTPase activity of eRF3 over the same activity of eRF1 plus the ribosomes might indicate a genuine residual GTPase activity, although it seems obvious that it has no biological meaning due to its negligible value.

From the data shown in Figure 2 and Table 2, we conclude that ribosome-stimulated GTPase activity described for translational termination (Beaudet & Caskey, 1971) is not an intrinsic feature of the ribosomes, eRF1, or eRF3 on their own, although a very low GTPase activity is not ruled out for eRF3.

The GTPase activity is an intrinsic property of the quaternary eRF1·eRF3·GTP·ribosome complex

From the data shown in Figure 2 and Table 2, it becomes evident that the GTPase activity known to be involved in the translation termination should be a complex phenomenon not attributed to any single component of the termination process. As shown in Figure 2, only in the presence of eRF3, eRF1, and the ribosomes does a rapid hydrolysis of GTP takes place. Contrary to the earlier experiments (Beaudet & Caskey, 1971), we fail to observe any stimulatory effect of the stop codon, tetranucleotide UAAA, on the GTPase activity (Table 2). This difference might be somehow related to the fact that, in our experiments, the stimulated level of the GTPase activity is 10–20 times higher than in the incomplete mixture, whereas in the partly purified system, the overall stimulation is 5–10-fold (Beaudet & Caskey, 1971).

About 40% of GTP molecules present in the incubation mixture were hydrolyzed. This incompleteness of the reaction might be caused by several reasons: a partial inactivation of one of the components of the incubation mixture in the course of the reaction, an inhibition of the reaction due to accumulation of the reaction products, or an absence of the guanine nucle-

TABLE 2. Dependence of a GTPase activity on the composition of the incubation mixture.

Components present in the incubation mixture	[γ-^{32}P]GTP hydrolyzed (pmol)
Ribosomes	0.11
eRF1	0.60
eRF1 + ribosomes	1.10
eRF1+ UAAA	0.75
eRF1 + ribosomes + UAAA	1.15
eRF3	0.80
eRF3 + ribosomes	1.50
eRF3 + UAAA	0.85
eRF3 + ribosomes +UAAA	1.35
eRF3 + eRF1	1.50
eRF3 + eRF1 + UAAA	1.45
eRF3 + eRF1 + ribosomes	19.5
eRF3 + eRF1 + ribosomes + UAAA	19.1

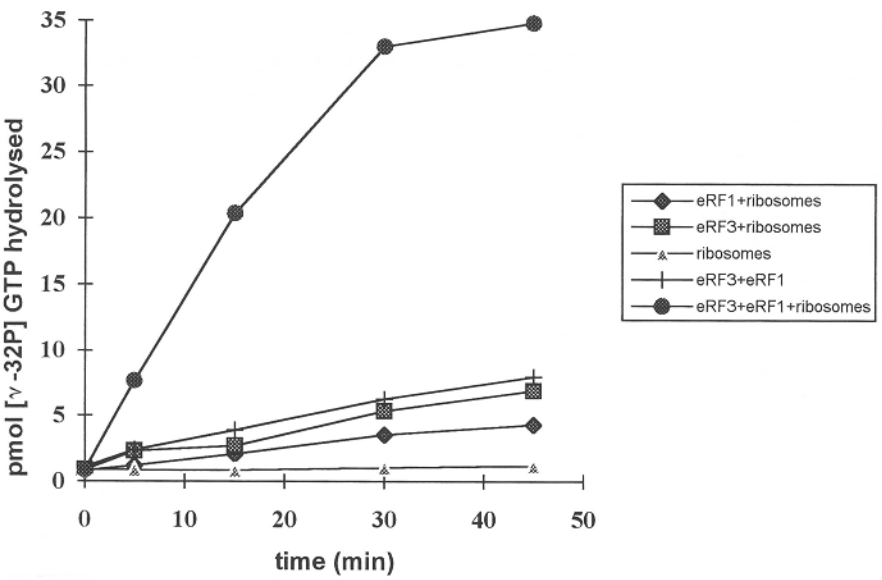


FIGURE 2. Time-course of GTP hydrolysis catalyzed by the components of translational termination machinery. The amount of released [^{32}P]P_i was quantitated by the charcoal technique as described in the Materials and methods.

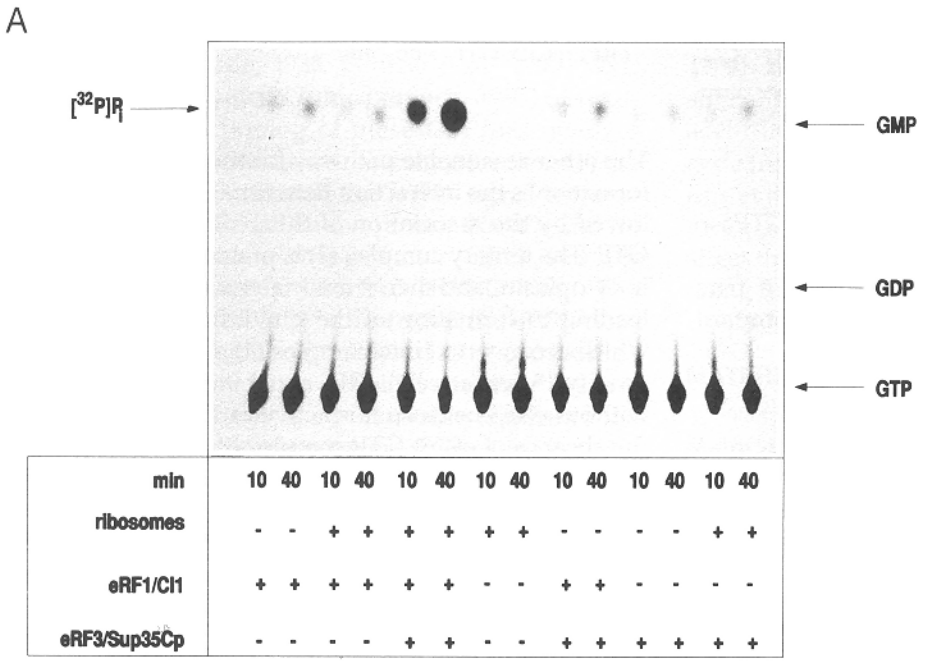
otide exchange protein that may dissociate GDP from the eRF3·GDP complex (see Discussion).

The application of α-^{32}P- and γ-^{32}P-labeled GTP allows one to follow simultaneously the splitting of GTP and the accumulation of the both reaction products, GDP and P_i, in the labeled form. The GTPase activity of the quaternary complex leads to conversion of GTP to GDP and P_i (Fig. 3A,B). The results of the thin-layer chromatography (TLC) experiments shown in Figure 3 are in a full agreement with the data obtained with a charcoal technique (Fig. 2; Table 2).

The turnover rate was calculated to be 14 mmol GTP hydrolyzed·mol^{-1} eRF3·s^{-1} at 25 °C in the complete incubation mixture. For comparison, the same value measured for the EF-Tu GTPase in the presence of ribosomes and kirromycin was found to be 28 mmol GTP hydrolyzed·mol^{-1} EF-Tu·s^{-1} at 37 °C (Fasano et al., 1982).

The ternary complex composed of eRF3, eRF1, and the ribosomes is specific toward the nucleotide substrate because another purine triphosphate, ATP, is not hydrolyzed at all (not shown). The 100-M excess of GMP or ATP over GTP does not influence the rate of GTP hydrolysis in the presence of eRF3, eRF1, and the ribosomes, whereas the nonhydrolyzable analogue of GTP, GTPγS, completely blocks the GTPase activity (Fig. 4). GDP inhibits the GTPase activity incompletely even at 100-M excess (Fig. 4).

K_m for GTP in the quaternary complex determined from the Lineweaver–Burk double reciprocal plot (not shown) is rather small (5 ± 1 mM), reflecting the high affinity of the activated eRF3 toward GTP. This value



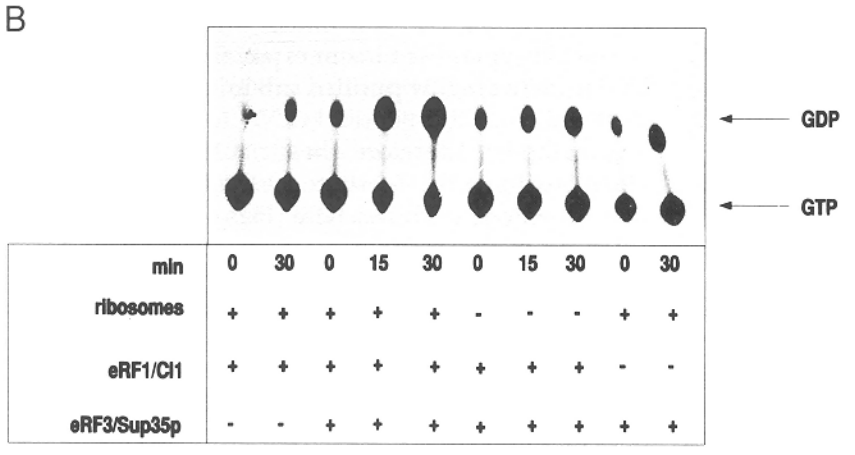


FIGURE 3. GTPase activity of the quaternary complex analyzed by a thin-layer chromatography. Mixtures for measuring GTPase activity contained (**A**) [γ-^{32}P]GTP or (**B**) [α-^{32}P]GTP and were incubated at 25 °C. The reaction products were analyzed as described in the Materials and methods. The positions for GTP, GDP, and GMP markers are shown. The position of [^{32}P]P_i formed after hydrolysis of [γ-^{32}P]GTP is indicated.

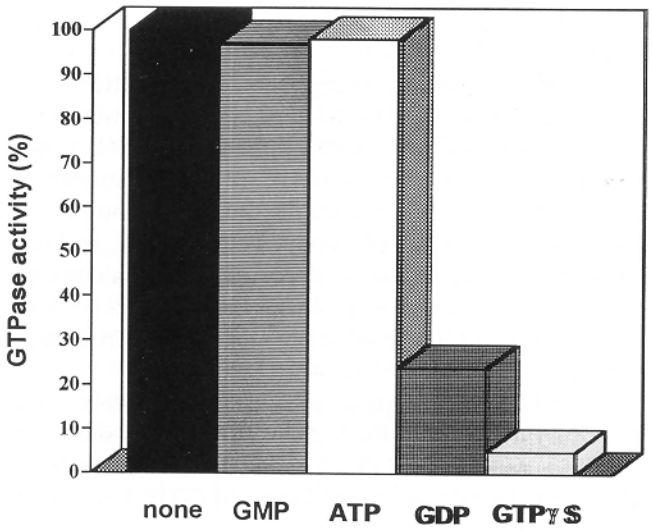


FIGURE 4. Inhibition of GTP hydrolysis in the quaternary complex by various nucleotides. The complete incubation mixture for measuring a GTPase activity (see the Materials and methods) contained eRF1/Cl1, eRF3/Sup35p, ribosomes, 5 μM [γ-^{32}P]GTP, and 500 μM each of the indicated nucleotides. The reaction was stopped after 5 min by adding the charcoal and the amount of released [^{32}P]P_i was measured. One-hundred percent of a GTPase activity corresponds to 22% of the total GTP hydrolyzed during 5 min at 25 °C.

is close to that estimated for the *E. coli* EF-Tu GTPase (Andersen & Wiborg, 1994).

Consequently, we conclude that eRF3 is a true GTPase that is greatly stimulated by the concomitant presence of the ribosomes and eRF1.

DISCUSSION

Here, it has been shown (Fig. 1; Table 1) that eRF3 on its own binds to GTP and this binding is not affected by the presence of the ribosomes and/or eRF1. Nevertheless, in solution, eRF3 exhibits a very low if any GTPase activity (Table 2). Neither eRF1 nor the ribosomes added separately to eRF3 stimulate the GTPase activity (Fig. 2; Table 2). However, when eRF1 and the ribosomes are added to eRF3 at once, a tremendous stimulation of a GTPase activity has been observed (Figs. 2, 3; Table 2). Consequently, these data indicate that (1) the catalytic molecule is eRF3, which binds to GTP and subsequently splits it into GDP and P_i (Fig. 3), and (2) this hydrolytic reaction is ribosome-dependent, as expected from previous observations (Beaudet & Caskey, 1971), but the ternary eRF3·GTP·ribosome complex lacks the GTPase activity: eRF1 has to be present, too.

From our results, it becomes obvious that two eRFs rather than a single eRF are responsible for the GTPase activity. In early experiments, eRF1 and eRF3 were not separated from each other, and, being added to the ribosomes together, manifested the GTPase activity.

eRF1 and eRF3 form a complex in vitro both in the presence (Zhouravleva et al., 1995) or in the absence (Stansfield et al., 1995) of GTP. This complex, as shown here, is inactive as a GTPase and should be associated with the ribosome to acquire the GTPase activity. Therefore, the GTPase activity is a complex phenomenon that involves the quaternary complex formation (eRF1·eRF3·GTP·ribosome). Presumably, the stop codons are not implicated in the activation process because they do not affect the GTPase activity in vitro (Table 2).

We may consider the following chain of the reactions leading to GTP hydrolysis:

$$\text{eRF3} + \text{GTP} \rightarrow \text{eRF3}\cdot\text{GTP} \quad (1)$$

$$\text{eRF3}\cdot\text{GTP} + \text{eRF1} \rightarrow \text{eRF1}\cdot\text{eRF3}\cdot\text{GTP} \quad (2)$$

$$\text{eRF1}\cdot\text{eRF3}\cdot\text{GTP} + \text{ribosome} \rightarrow \text{eRF1}\cdot\text{eRF3}\cdot\text{GTP}\cdot\text{ribosome} \quad (3)$$

$$\text{eRF1}\cdot\text{eRF3}\cdot\text{GTP}\cdot\text{ribosome} \rightarrow \text{eRF1}\cdot\text{eRF3}\cdot\text{GDP}\cdot\text{ribosome} + P_i. \quad (4)$$

The other reasonable pathway for the ternary complex formation is the interaction between eRF1 and eRF3 followed by the association of this binary complex with GTP. The ternary complex (2) is presumably assembled in cytoplasm, and then it associates with the ribosome, leading to formation of the quaternary complex (3), which is converted into complex (4) due to the GTPase activity. Alternatively, eRF1 may bind to the ribosome without eRF3 because it is active as a release factor in the absence of eRF3·GTP complex (Zhouravleva et al., 1995). Subsequently, the eRF1·ribosome complex may associate with the eRF3·GTP complex (1) forming the same quaternary complex (3). Perhaps the eRF1·ribosome complex is rather unstable, and, for this reason, this alternative pathway of the quaternary complex formation seems to be less efficient than that described above. In spite of the fact that the order of these partial reactions has not yet been defined, the quaternary complex formation, whatever formed, is the main prerequisite for the manifestation of the GTPase activity.

The ribosomes used in our experiments are reassembled from the highly purified subunits and completely depleted from the peptidyl-tRNA (see the Materials and methods). Therefore, these ribosomes are incapable of providing the substrate for the termination reaction. However, as shown here (Figs. 2, 3; Table 2), the GTPase activity of the quaternary complex is high, although the concomitant hydrolysis of the peptidyl-tRNA does not proceed. Consequently, we have successfully dissociated the termination process into two distinct stages: eRF1 is capable of stimulating the splitting of peptidyl(formylmethionyl)-tRNA in the absence of eRF3 and GTP (Zhouravleva et al., 1995) and, at the

same time, it stimulates the GTPase activity without peptidyl-tRNA hydrolysis (this work). The complete uncoupling of these reactions in the in vitro system is achieved for the first time. From the in vitro modeling of translational termination process, one may conclude that the energy of GTP hydrolysis is not an absolute requirement for promoting peptidyl-tRNA cleavage. Furthermore, the GTPase activity is not dependent on the presence of peptidyl-tRNA at the ribosomal P site at least in vitro. Thus, the coupling between the peptidyl-tRNA and GTP hydrolyses should be mediated by the ternary complex formation.

Structurally, eRF3 belongs to the family of small G proteins that bind to GTP and possess a GTPase activity. Among these G proteins are those involved in protein synthesis, such as elongation factors EF-Tu and EF-G in prokaryotes and EF-1α and EF-2 in eukaryotes, and initiation factors IF-2 and eIF-2 in prokaryotes and eukaryotes, respectively. These G proteins are molecular switchers that are able to alternate between the active "ON" and inactive "OFF" states. A general model (reviewed in Allende, 1988; Bourne et al., 1990, 1991; Riis et al., 1990; Boguski & McCormick, 1993; Sprinzl, 1994) for the functioning of these GTPases involves interaction with at least three macromolecules: (1) the effector molecule, to which the "ON" signal is transmitted; (2) a GTPase-activating protein (GAP), and (3) a guanine nucleotide exchange factor (GEF), and it is applicable to the newly described eRF3 GTPase. The results (Figs. 2, 3; Table 2) indicate that the eRF1·ribosome complex should be considered as a "composite" GAP. The synergistic action of eRF1 and the ribosome on the GTPase activity is a remarkable feature of the eukaryotic termination apparatus. In prokaryotes, the role of the composite GAP for EF-Tu is fulfilled by the aminoacyl-tRNA and the ribosome (reviewed in Sprinzl, 1994; Weijland & Parmeggiani, 1994). It is noteworthy that eRF1 plays a dual role: it operates as a part of the GAP toward eRF3, as has been already mentioned, and, at the same time, it serves as an effector molecule activated in ribosomes by eRF3·GTP complex, whereas the peptidyl-tRNA is a target molecule, the hydrolysis of which is induced by the activated form of eRF1.

At least two GEFs are known to be involved in elongation: EF-Ts for EF-Tu and eEF1$\beta\gamma$ for eEF1α (reviewed in Riis et al., 1990). For EF-G, it is suggested (Bourne et al., 1991; Ævarsson et al., 1994; Czworkowski et al., 1994) that a certain domain in EF-G may fulfill the function of an "internal" GEF. Because at initiation and elongation steps GEFs are required for GTPase recycling, we have to admit the involvement in the termination process of the GEF activity that might be an intrinsic property of eRF3, as suggested for EF-G, or a distinct, yet unidentified protein exists that could be provisionally termed eRF3B. Further experiments are needed to solve this dilemma.

It follows from our observations that, in the quaternary complex, both eRF1 and eRF3 have to undergo structural rearrangements that have not yet been observed experimentally. It was proved by X-ray analysis that $p21^{ras}$ (Pai et al, 1990; Wittinghofer & Pai, 1991), EF-Tu (Berchtold et al., 1993; Kjeldgaard et al., 1993), and EF-G (Ævarsson et al., 1994; Czworkowski et al., 1994), bound to GTP or GDP, exhibited different conformation. It is now generally accepted that the conformational differences between GTP- and GDP-bound proteins are implicated in the signal amplification and transduction. To apply this general scheme to the quaternary termination complex, one may assume that the main role of the "GTP state" is to control the positioning of eRF1 against two essential ribosomal regions, the stop codon in the mRNA at the A site, and the ester linkage between peptidyl and tRNA moieties at the peptidyltransferase center. It is shown for prokaryotic RF2 that it is composed of two domains, the stop-codon-oriented and the peptidyl-tRNA-oriented (Tate et al., 1990; Brown & Tate, 1994; Moffat & Tate, 1994). This proposed role for eRF3·GTP appears to be similar to what has already been suggested for aminoacyl-tRNA positioning at the A site in the elongation cycle (reviewed in Thompson, 1988; Bourne et al., 1989; Riis et al., 1990; Yarus, 1992a, 1992b; Weijland & Parmeggiani, 1994). The GTPase·GDP complex seems to affect the recycling of translational machinery by facilitating the dissociation of the factors from the ribosome.

MATERIALS AND METHODS

Materials

[α-^{32}P]GTP, [γ-^{32}P]GTP, and [γ-^{32}P]ATP were purchased from ICN; L-[^{35}S]methionine from Amersham; unlabeled nucleotides, bovine serum albumin (BSA), $tRNA^{fmet}$, and activated charcoal from Sigma; nitrocellulose filters from Whatman; pET21b plasmid from Novagen; oligodeoxyribonucleotide primers for PCR from Bioprobe; and polyethyleneimine-cellulose (PEI) plates from Schleicher & Schull. UAAA and AUG were synthesized by A. Veniaminova and M. Ryabkova (Novosibirsk State University, Russia).

The expression and purification of eRF1 and eRF3 proteins

The expression and purification of *X. laevis* eRF1/Cl1 protein were performed as described by Frolova et al. (1994). The eRF3 amino acid sequence deduced from *X. laevis* cDNA was established by Zhouravleva et al. (1995). The expression of the C domain of *X. laevis eRF3/SUP35* cDNA starting from Met 116 (Sup35Cp) was performed as described by Zhouravleva et al. (1995). For expression of the *X. laevis eRF3/SUP35* cDNA starting from Met 42 (Sup35p), the cDNA was inserted into the Bluescript vector and amplified by PCR using the following oligos: the forward primer *Nhe* I, 5′-CCCGGCTAGCACGCGAG-3′, and the reverse primer *Xho* I,

5′-CTTCTCGAGGTCCTTTTCTGG-3′. The amplified product was digested with *Nhe* I and *Xho* I and inserted into *Nhe* I/*Xho* I sites of the expression vector pET21b. A hexa-His tail was present at the C terminus of the expressed eRF3/Sup35p. The purification of the expressed eRF3/Sup35p from *E. coli* was performed on NiNTA resin (Qiagen) as described for eRF3/Sup35Cp (Zhouravleva et al., 1995). In our previous work (Zhouravleva et al., 1995), the *X. laevis* eRF3/Sup35Cp starting from Met 116 was shown to possess the GTP-dependent eRF1-stimulating activity in the presence of low concentration of the stop codon in the in vitro RF assay. The *X. laevis* eRF3/Sup35p starting from Met 42 (see Zhouravleva et al. [1995]) behaves similarly to eRF3/Sup35Cp in the in vitro RF assay. Therefore, the N-terminal part of eRF3 has no influence on the stimulation of formylmethionyl-tRNA hydrolysis catalyzed by eRF1 in the ribosome. For this reason, in the experiments described in this paper, both the "extended" and "truncated" forms of eRF3 are used.

Purification of the ribosomal subunits

Rabbit reticulocyte ribosomal subunits were isolated and purified as described by Cox and Hirst (1976). The ribosomes were stripped from endogenous peptidyl-tRNA with puromycin, dissociated, and resolved by zonal centrifugation into 40S and 60S subunits. Before addition to the incubation mixture, the ribosomal subunits were combined in equal molar ratio. One A_{260} unit was assumed to correspond to 25 pmol of the 80S ribosomes.

In vitro RF assay

eRF1 activity was measured in the in vitro RF assay according to Tate and Caskey (1990) with modifications described by Frolova et al. (1994). eRF3 activity was measured as described by Zhouravleva et al. (1995).

Binding of GTP to proteins in solution

Binding of GTP to proteins was detected by a rapid filtration technique. Reaction mixture (25 μL) contained buffer B (20 mM Tris-HCl, pH 7.5, 30 mM NH_4Cl, 15 mM $MgCl_2$), 1 mg/mL of BSA, 8 μM [α-^{32}P]GTP (sp. activity 1,000 cpm/pmol), where indicated, 0.2 μM ribosomes, eRF3/Sup35p (0.25 μg), and eRF1/Cl1 (0.25 μg). After incubation at 30 °C for 20 min (Table 1), or at 25 °C (Fig. 1), the samples were diluted into 2 mL of the cold buffer B just prior to filtration through 25-mm nitrocellulose filters (0.45 μm pore diameter) at a flow rate of 1 mL/min and washed twice with 2 mL of the iced buffer B, dried, immersed in scintillation fluid, and counted.

Assay for GTPase activity

A GTPase activity was followed by accumulation of [^{32}P]P_i using a modified charcoal precipitation assay described by Kabcenell et al. (1990), or by TLC on polyethyleneimine-cellulose coated plates (Richter et al., 1995).

Charcoal assay

Incubation mixture (12.5 μL) contained 2 μM [γ-^{32}P]GTP (sp. activity 10,000 cpm/pmol) (Table 2 only) or 5 μM [γ-^{32}P]GTP (sp. activity 4,000-5,000 cpm/pmol) (all other experiments), buffer B, and, where indicated, 0.1 μM ribosomes, 60 μM UAAA, eRF1/Cl1 (0.1 μg), eRF3/Sup35p, or eRF3/Sup35Cp (0.1 μg each). The reaction was run at 25 °C (for 30 min, Table 2), stopped by adding 750 μL of 5% charcoal in 50 mM NaH_2PO_4 on ice. The mixture was vortexed and centrifuged at 10,000 rpm for 10 min at 4 °C, and the [^{32}P]P_i released into 500 μL of supernatant was quantitated by liquid scintillation counting. Protein-independent release of [^{32}P]P_i due to the decomposition of the labeled GTP in solution was measured simultaneously and this value (approximately 2% of that observed in the presence of all components of the incubation mixture) was subtracted for all samples. At zero time, less than 0.05% of the input radioactivity was a free [^{32}P]P_i.

TLC on PEI-cellulose

Incubation mixture (12.5 μL) for measuring a GTPase activity was the same as described for charcoal assay and contained the release factors and ribosomes where indicated. In addition to [γ-^{32}P]GTP, 5 μM [α-^{32}P]GTP (sp. activity 5,000 cpm/pmol) was used as a substrate. The reaction was stopped by addition of 1 μL of the solution containing 20 mM EDTA and 5% SDS together with 0.5 μL of a reference mixture (GTP, GDP, and GMP, 30 mM each) to enable UV detection (254 nm). One-microliter aliquots were spotted onto PEI-cellulose plates and resolved in 1 M acetic acid and 1 M LiCl during 1.5 h. The plates were dried and exposed to X-ray film (Dupont).

Assay for ATPase activity

An ATPase assay was the same as the GTPase charcoal assay. [γ-^{32}P]ATP was used instead of labeled GTP.

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