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The "eRF" clone corresponds to tryptophanyl-tRNA synthetase, not mammalian release factor

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ABSTRACT **To study the similarity between a putative cloned mammalian release factor (RF) and tryptophanyl-tRNA synthetase (TRS), a recombinant rabbit RF fusion protein was expressed from prokaryotic expression vectors. Purified fractions of the fusion proteins were tested for TRS and RF activities. Addition of the fusion protein to a TRS assay increased the binding of tryptophan to $tRNA^{Trp}$. However, in an assay for RF activity, the addition of the fusion protein resulted in release of only 1–3% of formylmethionine from an fMet-tRNA–AUG–ribosome intermediate that had been provided with UAAA as message. To confirm this result, the coding region of the putative eukaryotic RF clone "eRF" was used for *in vitro* transcription and translation in a rabbit reticulocyte lysate system, resulting in the synthesis of a single 56-kDa protein. The influence of this 56-kDa protein on the termination of translation directed by tobacco mosaic virus was studied. Tobacco mosaic virus RNA produced a major 126-kDa protein and a minor 184-kDa readthrough protein in an *in vitro* translation system. The protein generated from the "eRF" coding region did not inhibit biosynthesis of the 184-kDa readthrough virus protein. Instead, it increased the yield of both viral proteins. This increase was presumably due to its TRS activity. Chromatography of proteins derived from human lymphoblasts separated RF from TRS activity. Thus, our results indicate that the previously cloned "eRF" clone encodes TRS and that rabbit reticulocyte RF activity lies in a different protein.**

Release factors (RFs) are proteins that recognize termination codons and release peptide chains from the ribosome (1). Lee *et al.* (2) have reported the isolation of a putative eukaryotic RF (eRF) (termed herein "eRF") from rabbit reticulocytes by using monoclonal antibodies to screen a cDNA library. The conserved regions of the protein sequence of this "eRF" showed 70% identity with prokaryotic amino acid tRNA synthetases but no homology with prokaryotic RFs. Nevertheless, some RF activity was observed in an *in vitro* assay after expression of the "eRF" protein in bacterial cells (2). Subsequently, tryptophanyl-tRNA synthetase (TRS) genes from bovine (3) and human (4) cells were isolated and sequenced, as was the gene for an interferon-inducible protein in human cells (5–7). The sequences of the "eRF," TRS, and interferon-inducible proteins were found to be virtually identical (up to 90%), and it was speculated that "eRF" and TRS were the same protein with two activities. Recently, the gene encoding a yeast mitochondrial RF was identified by complementation of mutant cells carrying an intron-splicing defect (8) and considerable homology (30–40%) between yeast mitochondrial RF and prokaryotic RF proteins was found, particularly in conserved regions.

This paper describes studies of TRS and RF activities of the previously isolated "eRF" clone, expressed as a fusion protein with maltose-binding protein (MBP). High levels of TRS activity were detected, but little RF activity was observed in an *in vitro* assay. In addition we studied the influence of the "eRF" on the termination of tobacco mosaic virus (TMV) protein synthesis in a reticulocyte lysate system. Instead of inhibiting the production of TMV readthrough protein, newly synthesized "eRF" actually stimulated total viral protein synthesis. We believe that the increase in production of viral proteins was due to the TRS activity of the fusion protein. These data indicate that the primary activity of the "eRF" isolated previously is that of TRS.

METHODS

Generation of Constructs Containing the "eRF" Coding Region. The recombinant "eRF" clone (2) was digested with *Sma* I and *Hin*dIII and a 1922-bp fragment containing the full-length open reading frame region of "eRF" was cloned into *Xmn* I–*Hin*dIII sites of pMAL-c2 or pMAL-p2 (expression vectors for generation of MBP fusion proteins; New England Biolabs). The resulting recombinant plasmids encode residues −22 to 700 of "eRF" inserted downstream from the *malE* gene, which encodes MBP. MBP fusion proteins can be reasonably purified in a single step because of the affinity of MBP for maltose. pMAL-p2 directs secretion of the fusion protein into the bacterial periplasm while pMAL-c2 fusion proteins accumulate in the cytoplasm. The construct containing the "eRF" coding region cloned into pMAL-c2 was called pMAL-c24A; the construct containing the "eRF" coding region cloned into pMAL-p2 was called pMAL-p24A.

The *Sma* I–*Hin*dIII "eRF" fragment was also cloned into the *Pvu* II and *Hin*dIII sites of pRSET-C (Invitrogen). The "eRF" insert (1922-bp region, encoding residues −22 to 700) was positioned downstream and in-frame with the sequence that encodes an N-terminal fusion peptide. This N-terminal sequence encodes, 5′ to 3′ (from N-terminal to C-terminal), an AUG translation initiation codon, a tract of six histidine residues that function as a metal binding domain, and a transcript-stabilizing sequence from gene *10* of phage T7. The resulting construct was called pRRF.

Generation of MBP–"eRF" Fusion Protein. Protein expression in XL1 Blue cells (Stratagene) was induced with 0.1 mM isopropyl β-D-thiogalactoside (IPTG) (Sigma). Cells were collected by centrifugation at 4000 × *g* for 10 min, and the pellet was resuspended in 10 mM Tris·HCl, pH 8.0/200 mM NaCl/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride (for the pMAL-c2 construct) or in 30 mM Tris·HCl, pH 8.0/20% (wt/vol) sucrose/1 mM EDTA/0.1 mM phenylmethylsulfonyl fluoride (for the pMAL-p2 construct). Cells containing the pMAL-c2 construct were frozen overnight at −20°C and then lysed by sonication. The lysate was centrifuged at 9000 × *g* for 20 min at 4°C and the fusion protein was

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Abbreviations: RF, release factor; eRF, eukaryotic RF; IPTG, isopropyl β-D-thiogalactoside; MBP, maltose-binding protein; TMV, tobacco mosaic virus; TRS, tryptophanyl-tRNA synthetase.

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isolated from the supernatant by affinity chromatography (9) with amylose resin (New England Biolabs). Cells containing the pMAL-p2 construct were centrifuged at 8000 × g for 10 min at 4°C and the pellet was resuspended as described above. The fusion protein was purified from the periplasm by affinity chromatography using amylose resin. Protein samples were analyzed by polyacrylamide gel electrophoresis (10) and Western blots (11) were probed with antibodies against MBP (New England Biolabs).

Determination of TRS Activity. tRNA-aminoacylation was performed in a 200-μl reaction mixture containing 50 or 100 ng of purified fusion protein, 100 mM Tris·HCl (pH 8.0), 1 mM EDTA, 15 mM magnesium acetate, bovine serum albumin (0.05 mg/ml), 10 μCi of [5-^{3}H]tryptophan (32 Ci/mmol; 1 Ci = 37 GBq; Amersham), 0.1 mM unlabeled tryptophan, and total yeast tRNA (7 mg/ml) (3). The reaction was initiated by the addition of 10 mM ATP and incubated at room temperature. Aliquots of 20 μl were taken at various times and spotted on GF/A filters (Whatman) for determination of trichloroacetic acid-precipitated radioactivity. In the control experiment, we used the protein fraction (50 ng) that was purified under the same conditions as a fusion protein, but without the addition of IPTG.

***In Vitro* Assay for RF Activity.** The intermediate of [^{3}H]fMet-tRNA–AUG–ribosomes was formed as described (12). RF activity of the fusion protein was studied in a 50-μl reaction mixture containing 4 pmol of [^{3}H]fMet-tRNA–AUG–ribosomes, 0.1 A_{260} unit of UAAA, 50 mM Tris·HCl (pH 7.6), 50 mM NH_4Cl, 20 mM $MgCl_2$, 0.1 mM GTP, and 10 ng of purified fusion protein. The release of [^{3}H]formylmethionine from the intermediate was measured as described (12).

Transcription and Translation of pRRF in a Reticulocyte Lysate System. A transcription/translation reaction was performed in 50 μl according to the Promega protocol, except that only 1 μg of pRRF plasmid DNA was used. After incubation at 30°C for 1 h, 5 μl of the denatured sample was loaded onto a SDS/6% polyacrylamide gel. After electrophoresis the gel was dried and exposed to X-Omat AR film (Kodak) for 5–10 h at −70°C.

Termination of Biosynthesis of TMV Proteins. pRRF plasmid DNA (0.2 μg) was added to a 10-μl TnT coupled reticulocyte lysate system (Promega). After 1 h of incubation at 30°C for transcription and translation of the "eRF" protein, the reaction mixture was mixed with fresh TnT coupled reticulocyte lysate system, according to the Promega protocol, except that 0.05 μg of TMV RNA was added. The final volume of the reaction was 25 μl and the incubation was continued for 2 h at 30°C. Aliquots of 5 μl from each reaction mixture were mixed with 15 μl of 1× Laemmli sample loading buffer [0.015 M Tris·HCl, pH 6.8/2.5% (vol/vol) glycerol/1.25% (vol/vol) 2-mercaptoethanol/0.5% SDS/0.0125% bromophenol blue]. The proteins were denatured at 100°C for 5 min and centrifuged at 15,000 × g for 5 min, and 10-μl samples were loaded onto a SDS/6% polyacrylamide gel.

Separation of Protein Fractions from Human Lymphoblasts. Cells (strain RJK 853) were grown in RPMI 1640 medium, supplemented with 10% (vol/vol) heat-inactivated fetal calf serum, penicillin, (100 units/ml), and streptomycin (100 μg/ml). Cells were collected by centrifugation at 4000 × g for 10 min, and the resulting supernatant was used for ribosome purification.

Ribosomes were collected by centrifugation at 45,000 × g for 3 h and suspended in 50 mM Tris·HCl, pH 7.4/0.1 M KCl/1 mM dithiothreitol/2 mM $MgCl_2$/15% glycerol. The ribosomes were incubated overnight at 4°C. In a second step, ribosomes were collected by centrifugation as above but were resuspended in 50 mM Tris·HCl, pH 7.4/0.25 M sucrose/2 mM $MgCl_2$/1 mM dithiothreitol.

The supernatant that resulted from ribosome sedimentation was used for isolation of RF and TRS activities. Ammonium sulfate (42.4 g/100 ml) was added to the postsedimentation supernatant. The resulting precipitate was collected by centrifugation at 15,000 × g for 15 min, suspended in buffer A (0.1 M KCl/20 mM Tris·HCl, pH 7.8/1 mM dithiothreitol/0.1 mM EDTA), dialyzed against buffer A overnight, and loaded onto a DEAE-Sephadex A-50 column. Proteins were eluted with a KCl (0.1 M–0.2 M–0.3 M–0.4 M) step gradient in buffer A. Fractions of 1.5 ml were divided in two parts. Each part was equilibrated in the appropriate buffer for RF or TRS, which have been determined as described above.

RESULTS

XL1 Blue cells were transformed with pMAL-c24A or pMAL-p24A plasmid DNA and expression of MBP–"eRF" fusion protein was induced with IPTG. The resulting fusion protein includes MBP sequence in N-terminal and "eRF" full-length open reading frame region (residues −22 to 700). The fusion protein was isolated from crude bacterial extracts for the pMAL-c2 construct or from the periplasm for the pMAL-p2 construct. In these studies, we found the pMAL-p2 vector to be more convenient because the fusion protein was ≈40% of the periplasmic protein. The fusion protein was purified by affinity chromatography using amylose resin. Fig. 1*A* shows the chromatographically purified MBP–"eRF" fusion protein. Two proteins, 92 and 42 kDa, specifically bond to the amylose resin. The expected size for the fusion protein is 92 kDa (42 kDa of MBP and 50 kDa of "eRF"). The 42-kDa protein is predicted to be MBP. The formation of MBP after expression of the fusion "eRF" protein could be due to the preliminary translation termination of the full-length fusion protein. Controls indicated that some amount of MBP was formed also without the addition of IPTG (Fig. 1*A*, lane 2). Fig. 1*B* demonstrates the results of a Western blot illustrating that the fusion protein reacts with anti-MBP antibodies.

The chromatography-purified MBP–"eRF" fusion protein was used to study TRS activity in an *in vitro* assay. Incubation of total yeast tRNA with the fusion protein increased the association between [^{3}H]tryptophan and $tRNA^{Trp}$ by 20 times during 4 min of incubation (Fig. 2). In a control experiment using protein fraction, which was isolated under the same conditions as the fusion MBP–"eRF" from periplasmic fractions of bacteria that had not been induced with IPTG, the amount of radioactivity in the intermediate [^{3}H]Trp-$tRNA^{Trp}$

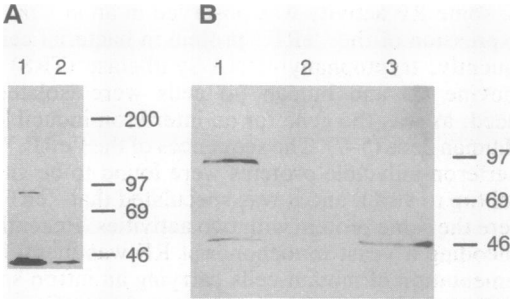

FIG. 1. Purification of fusion RF protein from bacterial cells. (*A*) SDS/polyacrylamide gel stained with Coomassie blue, showing purified fusion protein after column chromatography with amylose resin. Lanes: 1, cells after induction with IPTG; 2, cells incubated without IPTG. Molecular mass markers (kDa) are shown. (*B*) Bacterial periplasm from IPTG-induced (lane 1) and noninduced (lane 2) cells and pure MBP (New England Biolabs) (lane 3) were resolved by SDS/polyacrylamide gel electrophoresis in 6% gels, transferred to a nitrocellulose membrane, and probed with antibodies against MBP. Molecular mass markers (kDa) are shown.

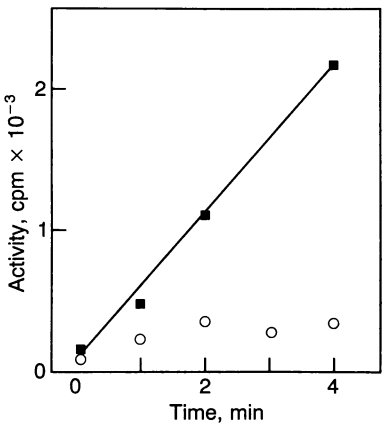

FIG. 2. [³H]Tryptophan aminoacylation activity of purified MBP–"eRF" fusion protein produced after IPTG stimulation (■) and control extracts produced without IPTG stimulation (○).

was close to background. These results show that the fusion protein has significant TRS activity and that MBP does not effect the enzymatic activity of the fusion protein.

In further experiments, we studied the RF activity of the fusion protein. As was reported earlier, RF activity was observed in crude cell extracts after expression of "eRF" in bacterial cells (2). We were unable to measure any significant RF activity in our purified fusion protein by using a standard *in vitro* assay. On average, only 1–3% of [³H]methionine was released after UAAA treatment from the intermediate [³H]fMet-tRNA–AUG–ribosomes (data not shown). To verify this result, we studied the influence of "eRF" on the termination of protein translation in a TMV *in vitro* system.

The construct pRRF, which contains the full-length open reading frame of "eRF" in the pRSET-C vector, was used as a template in our transcription/translation reticulocyte lysate system. Because pRSET-C vector contains the T7 promoter, the transcription/translation of the fusion protein can be

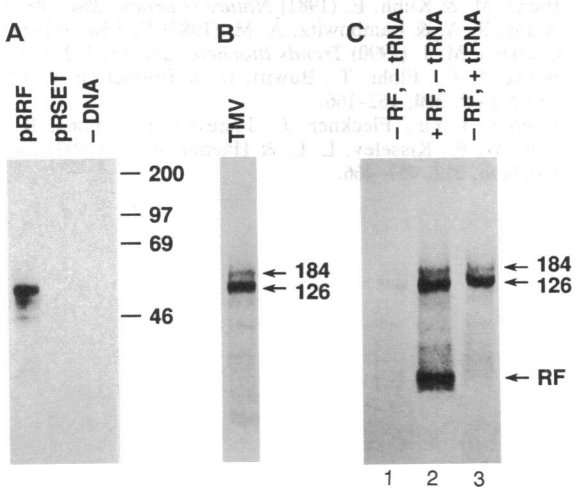

FIG. 3. Effect of "eRF" fusion protein on the termination of TMV readthrough protein. (*A*) SDS/polyacrylamide gel electrophoresis of an *in vitro* transcription/translation of pRRF recombinant DNA, pRSET vector DNA, or no DNA. Molecular mass markers (kDa) are shown. (*B*) SDS/polyacrylamide gel electrophoresis of *in vitro* translation products of TMV RNA. The position of 126- and 184-kDa viral proteins are shown by arrows. (*C*) Effect of "eRF" fusion protein on the termination of TMV readthrough protein. From left to right, the reactions contain the following material. Lanes: 1, 50 ng of TMV RNA only; 2, 50 ng of TMV RNA with 200 ng of recombinant pRRF plasmid DNA; 3, 50 ng of TMV RNA with 0.04 A_{260} unit of calf liver tRNA. The positions of the 126- and 184-kDa viral proteins and recombinant "eRF" are shown by arrows.

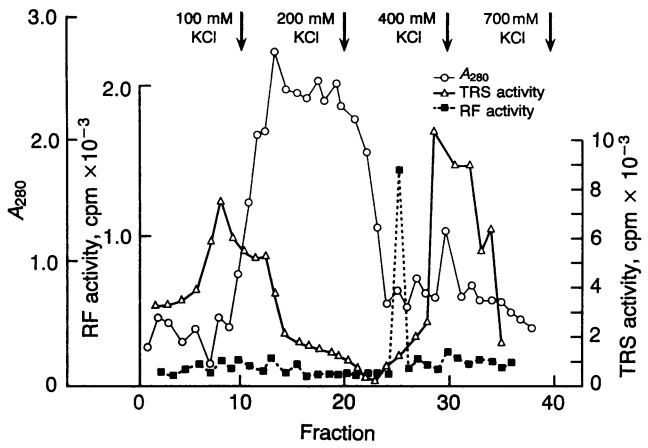

FIG. 4. Column chromatography of the eRF and TRS activities in human lymphoblasts. TRS and RF activities were determined. ○, A_{280}; △, tryptophanylation; ■, formyl[³H]methionine released.

stimulated in the presence of T7 polymerase. Fig. 3*A* shows that pRRF directs the transcription and translation of one 56-kDa fusion protein containing 6 kDa of protein from gene *10* of phage T7 and 50 kDa from "eRF." The effect of this fusion protein on TMV protein termination was tested.

TMV RNA directs the biosynthesis of two nonstructural proteins with molecular masses of 126 and 184 kDa (13). The 184-kDa protein is a larger version of the 126-kDa protein, resulting from readthrough translation of a protein chain-termination UAG codon. Fig. 3*B* shows the synthesis of the 126- and 184-kDa proteins from TMV RNA in a reticulocyte lysate system. It has been shown (13) that readthrough of the TMV termination codon occurs in the rabbit reticulocyte lysate system. Both amber suppressor tRNAs and wild-type tyrosine tRNAs can promote the readthrough of TMV translation (14). A protein with RF activity should compete with suppressor tRNA for recognition of the termination codon and the amount of readthrough protein would, therefore, be expected to decrease (7). The "eRF" protein generated from pRRF increased synthesis of both viral proteins (Fig. 3*C*, lane 2). Instead of observing the decrease in synthesis of the larger TMV protein that would be expected from a protein with RF activity, the effect from "eRF" of increasing the synthesis of both viral proteins resembled the result seen when total tRNA was added to the reaction mixture (Fig. 3*C*, lane 3). It is possible that the TRS activity of "eRF" increases protein synthesis in the reticulocyte lysate system by enhancing protein elongation. It is also possible that TRS activity may be influencing the initiation of translation, since aminoacyl tRNA synthetases are multifunctional proteins (15, 16).

Fig. 4 shows RF and TRS activities in protein fractions of human cells after ion-exchange chromatography on DEAE-Sephadex A-50. Two TRS peaks and one RF peak were observed. Because eRF activity was eluted in one fraction, all procedures with the separation of eRF and TRS activities were repeated. On repeated analysis, the RF activity was observed in the same fraction. Since the RF peak and one of the TRS peaks lies so close together, this result may indicate that the two activities reside in the same protein or that two proteins have been copurified. It is also possible that a common protein cofactor regulates both RF and TRS activities.

DISCUSSION

The cloning and sequencing of a putative rabbit reticulocyte "eRF" clone have been reported (2). After induction with IPTG and fractionation on a Sephadex G-75 gel column (2),

RF activity was tested in a bacterial extract. Some RF activity was observed in a standard *in vitro* assay in the presence of 10% ethanol (2). Bovine (3) and human (4) TRS and interferon-inducible proteins with high homology to TRS and our "eRF" clone were subsequently cloned and sequenced (5–7). TRS activity was observed in preparations of bovine TRS (3) and interferon-inducible proteins γ2 (5) and 56αγ (6). Both RF (7) and TRS (17) activities were found in the interferon-inducible protein IFP53. Given these results, it was important to clarify whether the protein that was originally isolated (2) was actually TRS instead of eRF or whether the two activities do indeed reside in the same protein.

Because the original study (2) did not include a determination of TRS activity, we prepared purified fusion proteins containing the previously cloned "eRF" and tested for both RF and TRS activities. We have shown TRS activity in these extracts but have been unable to demonstrate RF activity using two assays. This is not a fault of the assay because the reticulocyte ribosomes were active with puromycin. The incubation of the intermediate of [^{3}H]fMet-tRNA–AUG–ribosomes in the presence of puromycin (0.3 mM) released 90% of formyl[^{3}H]methionine from the intermediate during a 5-min incubation (data not shown). Other possible explanations include the effect of additional sequence in the fusion proteins on RF activity or the loss of RF activity during protein purification. The fact that neither the MBP–"eRF" fusion protein nor protein translated from the pRRF construct showed RF activity argues against the additional sequence having an effect. Failure to detect RF activity in newly formed "eRF" from a rabbit reticulocyte lysate system (Fig. 3) indicates that the fault does not lie with a protein purification effect.

Our evidence suggests that RF and TRS activities reside in different proteins. We therefore investigated the possibility that the two activities could be separated. After fractionation of protein from human lymphoblasts, separate peaks for RF and TRS activities were observed, although the peaks lie close together. We believe that this is evidence that RF and TRS activities are in different proteins. Indeed, a recent report shows (18) that eukaryotic TRS and eRF have different immunochemical, biochemical, and physicochemical properties. Thus, these data show that TRS and eRF are two different proteins.

In our previous studies, RF activity was determined in crude extracts and in the presence of 10% ethanol. It is possible that under these conditions, ribosomal-bound f[^{3}H]-Met-tRNA can hydrolyze independently from codon-specific RF recognition. Given these possible explanations for the low level of RF activity in the expressed protein reported previously, we feel that this was an artifact. Our current studies associate the earlier reported "eRF" clone with TRS activity and suggest that the mammalian eRF has yet to be found.

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