The ribosomal binding and peptidyl-tRNA hydrolysis functions of Escherichia coli release factor 2 are linked through residue 246

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The ribosomal binding and peptidyl-tRNA hydrolysis functions of *Escherichia coli* release factor 2 are linked through residue 246

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ABSTRACT

Replacing a cassette of 31 residues from *Escherichia coli* release factor 1 with the equivalent residues in release factor 2 gave a protein active in codon-specific binding to the ribosome but inactive in peptidyl-tRNA hydrolysis. Such a phenotype is also found unexpectedly with release factor 2 when expressed at high concentration in bacteria. Substituting threonine with the release factor 1 equivalent serine at position 246 within the cassette restored the impaired activity of the chimeric protein, and also that of inactive recombinant release factor 2, both in vitro and in vivo. The differences in activity are not due to posttranslational modifications or a lack of it at this residue. Random mutagenesis of codon 246 suggests that this position is pivotal for the function of the release factor, being able to affect differentially both its binding to the ribosome and its peptide release activities. We propose that amino acid 246 is close to a sharp turn (GGQ motif at position 250), and is essential for transmitting the signal from cognate codon recognition by correctly positioning the peptidyl-tRNA hydrolysis domain of the release factor into the peptidyltransferase center.

Keywords: peptidyltransferase; release factors; RF2; ribosome; translational termination

INTRODUCTION

Termination of protein synthesis commences with the arrival of a stop codon in phase at the ribosomal A site. The release of the nascent polypeptide chain from the terminal tRNA, in response to the termination signal, is mediated through protein release factors (RFs). In *Escherichia coli*, two RFs decode three stop signals with partially overlapping specificities, such that RF1 decodes UAG and UAA and RF2 decodes UGA and UAA (Capecchi & Klein, 1969; Caskey et al., 1969).

The decoding RFs were initially proposed to have two functional domains (Moffat et al., 1993): a ribosome-binding domain associated with codon recognition and a peptidyltransferase domain associated with release of the nascent peptide. The functional constraints of RFs, reminiscent of tRNAs, led to the "tRNA analog model," a proposal that the decoding RFs were analogous to tRNAs, spanning the ribosome between the decoding site on the small subunit and the peptidyltransferase center on the large subunit (Moffat & Tate, 1994). Support for this concept came from site-directed crosslinking from the stop codon to one functional domain (Domain I) of the RF (Brown & Tate, 1994) and from the fact that a single cleavage at a putative turn region inactivated peptidyl-tRNA hydrolysis activity of the RF (Moffat & Tate, 1994).

Subsequently, the similarity between the tertiary structures of the EF-Tu ternary complex and EF-G led to the concept of molecular mimicry between translational factors and was proposed to include the termination and initiation phases of translation (reviewed in Nissen et al., 2000). Sequence homology between EF-G and the initiation factors IF1 and IF2 has provided further support for the mimicry concept (Brock et al., 1998). Although there was no obvious homology, a careful comparison of the sequences between eubacterial decoding RFs and EF-G revealed a small number of conserved residues and identified a potential anticodon-like mimicry sequence in the RFs (reviewed in Nakamura & Ito, 1998). An independent study identified a primary sequence motif common between IF1 (which is proposed to bind in the ribosomal A site region; Moazed et al., 1995), and sequences within functional Domain I of both RF1 and RF2 (Wilson et al., 2000). The proposed anticodon-like mimicry region has now been identified by Nakamura and coworkers (Ito et al., 2000), as a tripeptide (pro.ala.thr-RF1 and ser.pro.phe-RF2). A

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number of variant RFs deficient in ribosomal binding have amino acid substitutions near or within this region (reviewed in Wilson et al., 2000).

The functional domain of the RF (Domain II) that interacts with or is close to the ribosomal peptidyltransferase center might be expected to show a high level of conservation between RFs. A candidate for this domain has been a region highly conserved between all known eubacterial RFs (residues 240–270 in *E. coli* RF2). It is significant that no mutations resulting in viable RFs have been mapped to this region. However, gene swapping studies between *E. coli* RF1 and RF2 identified 10 residues located within or near the highly conserved region of RF2 that, when substituted into RF1, abolished peptidyl-tRNA hydrolysis activity but not ribosome binding or codon recognition (Moffat et al., 1993). Interestingly, overexpression of *E. coli* RF2 from a plasmid also produced proteins with ribosome binding activity but without peptidyl-tRNA hydrolysis function (Adamski, 1992) suggesting that at least 1 of the 10 RF2 residues that were substituted in RF1 might be responsible for the inactive RF2 phenotype. Recombinant RF2 from *Salmonella typhimurium* and *Streptomyces coelicolor* does not have reduced activity (Uno et al., 1996). Substituting serially each of the small number of *S. typhimurium* RF2 residues that differ from *E. coli* RF2 into *E. coli* RF2 identified a single residue, threonine 246, as responsible for the inactivity and toxicity associated with overexpression of *E. coli* RF2 (Uno et al., 1996).

After screening RF2 clones randomized at position 246 and measuring factor activity and bacterial growth interference, Nakamura et al. (2000) concluded that the amino acid at 246 needs to be small and relatively hydrophilic. Residue 246 was 1 of the 10 RF2 residues in our studies that led to an inactive variant RF1. Of the 35 known eubacterial RF1 and RF2 sequences, *E. coli* RF2 is unique in being the only factor to have threonine at position 246; all other species have either alanine or serine. Very recently, Song et al. (2000) have reported the first structure of a decoding release factor, eRF1. One of the rare conserved sequence motifs between prokaryotic and eukaryotic factors, a GGQ motif at position 250–252 in *E. coli* RF2 close to 246 is found at position 183–185 in eRF1 (Frolova et al., 1999). Significantly, this motif was found at a turn in a subdomain in the eRF1 structure at the tip believed to be orientated at the peptidyltransferase end of the ribosomal active center (Song et al., 2000).

In this work we show that substituting threonine 246 back to the RF1 residue serine restores activity not only to the RF1/2 chimera but also to *E. coli* RF2 expressed from a plasmid. The loss of activity of overexpressed RF2, and the regained activity of the RF2-T246S mutant is not due to a loss or gain of a posttranslational modification at this residue. Substitution of various residues into position 246 modulates both codon-directed ribosome binding and peptidyltransferase albeit in an independent manner, and affects the growth of the bacterium. Based on these observations we propose a model for mechanism of RF action.

RESULTS

Substitution of a single RF1 residue restores peptidyl-tRNA hydrolysis activity to an overexpressed RF chimera and to exogenously expressed RF2 in vitro and in vivo

A chimera between RF1 and RF2 containing residues 224–255 of RF2 substituted for the equivalent RF1 residues gave a factor active for ribosome binding and codon recognition but not for peptidyl-tRNA hydrolysis. A single residue was responsible for restoring the activity to the original sequence of this RF chimera (RF1/2 chim). Substitution of serine (the equivalent residue in RF1) for threonine at position 246 (RF2 numbering) to produce RF1/2 chim-T246S gave a factor active in all known functions. The same mutation was introduced into the RF2 gene to give the protein $RF2_{exo}$-T246S (Fig. 1). All RF constructs were expressed and the products purified to homogeneity for in vitro functional assays (Materials and Methods).

The peptide release activities of RF1/2 chim and RF1/2 chim-T246S were determined with all three stop codons (Table 1). RF1/2 chim exhibited no peptidyl-tRNA hydrolysis activity unless ethanol was added, whereas codon-dependent release activity was obtained independent of ethanol for RF1/2 chim-T246S. It was equivalent to that observed with RF1. No release activity was detected with either noncognate stop codons, or with the near-cognate UGG codon (data not shown). The in vitro peptide release activities of $RF2_{exo}$ and $RF2_{exo}$-T246S (Table 1) indicated that when RF2

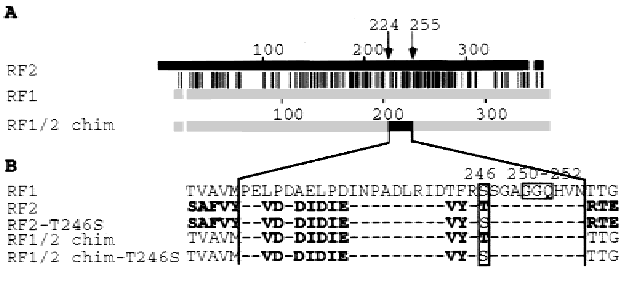

FIGURE 1. Sequence substitutions between RF2, RF1, and RF hybrids. **A**: Sequence homology between *E. coli* RF2 (black) and RF1 (gray) with positions of identity indicated by vertical lines. RF1/2 chim was created by substituting RF2 residues between 224 and 255 into the equivalent position in RF1. **B**: Amino acid sequences of *E. coli* RF2, RF1, and hybrid constructs between positions 219 and 258 (*E. coli* RF2 numbering) encompassing the hybrid region as indicated. RF2 sequence is shown in bold. Position 246 and the universally conserved GGQ (250–252) are boxed.

protein ($RF2_{exo}$) was overexpressed to a high concentration in the bacteria, the factor had no peptide release activity with any of the stop codons unless ethanol (+EtOH) was added together with the codons into the in vitro assay. Nevertheless, the activity of overexpressed RF2 in the presence of ethanol was specific for the cognate codons UGA and UAA. However, the activity in the presence of UAA was lower than endogenously expressed RF2 purified from the bacteria ($RF2_{endo}$). Ethanol alone was not able to activate the exogenously expressed protein when codon was not present (data not shown). In contrast, the $RF2_{exo}$-T246S variant could be expressed to a high concentration in bacteria and isolated without loss of specific activity for the peptidyl-tRNA hydrolysis function. It did not require ethanol for activation, but had consistently higher activity with UGA than endogenous RF2.

The functional activity of RFs can be assessed in vivo with a MalE reporter system containing the RF2 frameshift window inserted upstream of the MalE gene (Poole et al., 1995). Competition between frameshifting and termination at this recoding site has proved to be a useful tool for measuring the efficiency of the termination components in vivo (Poole et al., 1995; Crawford et al., 1999). This regulatory site responds to exogenously expressed RF, and the outcome of these events produces two protein products, easily separated by size, and detected with an antibody to the maltose-binding protein (Fig. 2A). Changes in the termination efficiency due to the exogenously expressed normal or variant RFs in vivo were measured as a function of different termination signals. Because the growth rate of the bacteria expressing the RF1/2 chim was profoundly affected, particularly when it carried an additional test plasmid, we have completed these studies only with the overexpressed normal or variant RF2. A representative western blot is presented in Figure 2B showing results obtained when $RF2_{exo}$ and $RF2_{exo}$-T246S were expressed in conjunction with the MalE reporter system. A vector lacking an RF2 gene (pTG) was used as a control. The results of several experiments are presented as termination efficiency (Fig. 2C).

The termination efficiency was not markedly different between experiments conducted either with a vector control or with the vector allowing overexpression of $RF2_{exo}$. There was only a small gain of termination efficiency at cognate stop codons with $RF2_{exo}$, suggesting the factor was binding into the ribosomal A site but not supporting release of the polypeptide. Interestingly, an extra band (47 kDa) was evident in the experiments

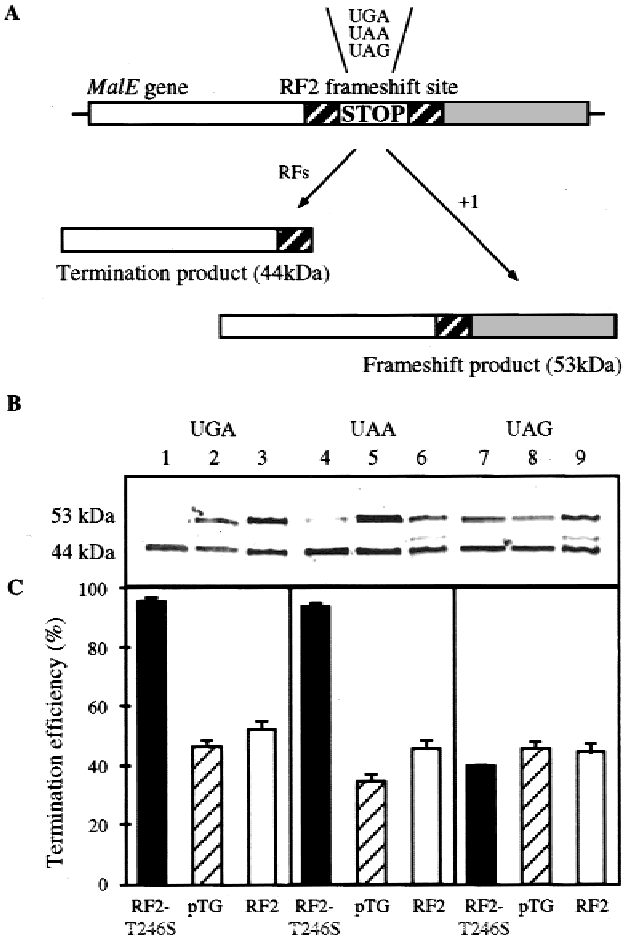

FIGURE 2. An experimental system for investigating the effect of RF2 and RF2-T246S expression on translation termination in vivo. **A**: A schematic diagram of the protein products produced in the translational frameshift assay system (Poole et al., 1995). **B**: A western blot probed with an antibody to the MBP, demonstrating the relative intensities of the frameshift (53 kDa) and termination (44 kDa) protein products at UGA (lanes 1–3), UAA (lanes 4–6), and UAG (lanes 7–9), in the presence of RF2-T246S (lanes 1, 4, and 7), RF2 (lanes 2, 5, and 8), or pTG vector only (lanes 3, 6, and 9). **C**: The intensities of the protein bands shown in **B** were quantitated using laser densitometry, and the termination efficiency is expressed as a percentage of the total alternative events competing at the stop signal (termination, frameshifting, and readthrough). RF2-T246S: filled bar; RF2: open bar; and pTG: hatched bar. The experiments were performed in triplicate with at least two separate isolates. The mean and standard errors of these experiments are shown.

TABLE 1. Codon-dependent peptidyl-tRNA hydrolysis activities with different RF variants.

Factors	UGA	UAG	UAA
RF1/2 chim	0 ± 34[a]	0 ± 61	0 ± 43
RF1/2 chim (+EtOH)	0 ± 72	180 ± 54	200 ± 34
RF1/2 chim-T246S	0 ± 53	1,080 ± 134	700 ± 49
$RF1_{endo}$	0 ± 42	995 ± 89	1,008 ± 104
$RF2_{exo}$	0 ± 45	0 ± 30	0 ± 10
$RF2_{exo}$(+EtOH)	745 ± 103	0 ± 57	350 ± 47
$RF2_{exo}$-T246S	1,120 ± 130	0 ± 84	700 ± 62
$RF2_{endo}$	645 ± 54	0 ± 27	608 ± 59

[a]Peptide release activities are presented as specific activity, where each RF unit is defined by the amount of fMet hydrolyzed (fmol) per μg of RF protein (Donly et al., 1990a). Release assays were performed at UGA, UAG, and UAA. RF2 and RF1/2 chim were assayed in the presence or absence of ethanol as indicated. The mean of three experiments (plus standard deviations) is shown.

with $RF2_{exo}$ (Fig. 2B). The size of this product was consistent with a readthrough event occurring at the RF2 recoding site and suggested that the presence of the inactive factor affected ribosomal fidelity to enable suppression of the stop codon by near-cognate tRNAs. In contrast, overexpression of $RF2_{exo}$-T246S, measured by western analysis as being at a 5-fold higher concentration than that of the endogenous factor alone, markedly increased the termination efficiency at cognate stop codons UGA and UAA, but not at noncognate UAG. The competing events are shown at this increased concentration of the factor (Fig. 2B,C; UGA, UAA).

The loss of activity of overexpressed RF2 and the restored activity of RF2-T246S are not due to a posttranslational modification at position 246

It has been known for some time that expression of *E. coli* RF2 from a plasmid resulted in a protein with lower specific activity for peptidyl-tRNA hydrolysis. Moreover, the specific activity decreased with the concentration of the expressed RF2 in the bacterial cell (a function of the expression system used). For example, an expression system giving a 20-fold increase in RF2 concentration had a 10-fold lower specific activity (data not shown). The pET expression vector used in this study gave much higher concentrations and produced a factor with no activity for peptidyl-tRNA hydrolysis. The lack of a posttranslational modification at residue 246 might have explained why the $RF2_{exo}$-specific activity for peptide release was inversely proportional to its expression. In vivo phosphorylation studies and the use of antibodies against threonine or serine phosphates did not support the contention that native RF2 was modified with a phosphate at threonine 246, or that RF1 had a modified serine at the equivalent residue. Electrospray ionization mass spectroscopy was used to determine the molecular mass of overexpressed RF2 and RF2-T246S (Materials and Methods). Analyses of the RF protein samples did not support a modification as large as a phosphate on one of the exogenously expressed proteins that could explain the differential activity, although there was a small difference between the predicted and experimental values for unmodified proteins (10–24 Da; data not shown). Tryptic digests of each sample were analyzed and the fragments containing residue 246 in $RF2_{exo}$ and $RF2_{exo}$-T246S were identified (Fig. 3A,B). Both fragments had a mass corresponding to the amino acid being in an unmodified state. The difference of seven between the mass/charge values for the [M + 2H] ion of the tryptic fragment containing position 246 for both RF2 and RF2-T246S was in concordance with the threonine-to-serine substitution. Although pure endogenous RF2 has not been obtained at a sufficiently high concentration for such detailed analysis, the exogenous-expressed factor with the threonine to serine substitution did show a minor peak where unmodified threonine was expected that may reflect the small fraction of endogenous protein in this preparation.

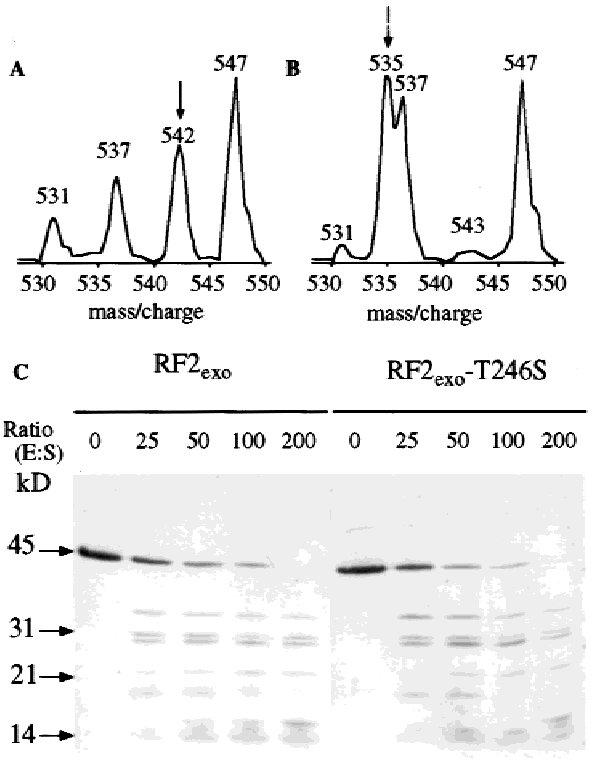

FIGURE 3. Electrospray ionization mass spectroscopy of tryptic digests of overexpressed RF2 (**A**) and RF2-T246S (**B**). The mass/charge value of the [M + 2H] ion of the tryptic fragment containing position 246 of RF2 (542) and RF2-T246S (535) are indicated (arrows). **C**: Limited proteolysis of overexpressed RF2 and RF2-T246S with chymotrypsin. SDS-PAGE of RF2 and RF2-T246S digested with chymotrypsin using different ratios of enzyme to substrate (E:S) are indicated. Positions of molecular weight markers are indicated by arrows.

Does substitution of T246S in RF2 result in major changes in conformation?

Chymotrypsin cleavage of RF2 gives two initial products before subsequent degradation (Moffat & Tate, 1994). Limited proteolysis was used to determine whether there was a difference in sensitivity of the $RF2_{exo}$ and T246S variants to either trypsin or chymotrypsin. Identical cleavage rates and patterns were obtained for RF2 and RF2-T246S with different ratios of chymotrypsin to substrate (Fig. 3C). This suggested that the gross conformation of the two proteins was not significantly different despite their different activities. Similar cleavage patterns were also obtained with both trypsin and chymotrypsin for a variety of different RF2 variants as described for Figure 5 (data not shown).

Substitutions at position 246 of RF2 affect growth rate and viability

Random mutagenesis was used to generate a pool of RF variants modified only at amino acid position 246. The growth rate of transformants was monitored before and after induction by measuring the extinction at 600 nm (Fig. 4). Differences between growth rates provided the first indication of toxicity for some of the variant RFs. The growth rates were assigned to four main classes (labeled A–D). An example of each is shown in Figure 4. The growth of BL21(DE3) pLysS is shown for comparison (labeled BL21). Group A, with the least affected growth rate, consisted of $RF2_{exo}$ variants with alanine, serine, or valine at position 246. Interestingly, these factor variants were either fully active functionally or completely inactive. Those with alanine or serine, naturally occurring amino acids at position 246 among RFs from different bacteria and mitochondria, were characterized as fully active, whereas those with valine at position 246 were essentially inactive. Groups B and C represented the majority of the isolated RF variants; all have reduced ribosome binding and peptide release efficiencies. The more severely affected RF2 variants, such as those with glutamine or phenylalanine at position 246, corresponded to the recombinant strains with the lower growth rate (group C). Group D contained RF2 variants with lysine at position 246. Expression of these were lethal to the cell and emphasized the functional significance of this position in the RF2 structure. An initial puzzle was that one isolate with lysine at position 246 retained some functional activity. However, it was determined that there was a truncation of the 94 C-terminal amino acids that allowed cell viability (data not shown).

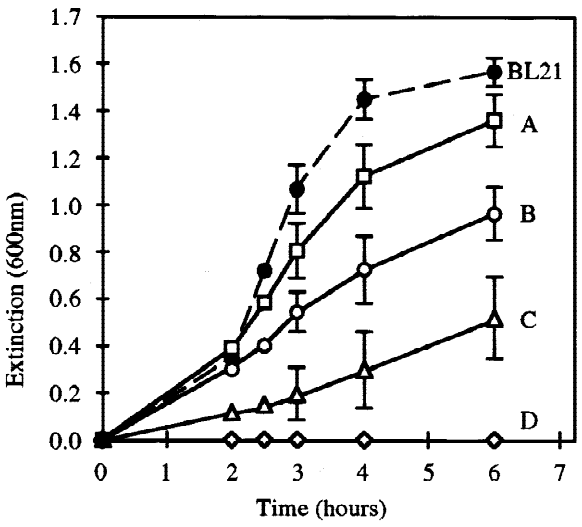

FIGURE 4. The effect of mutations at position 246 of *E. coli* RF2 on growth rate. The host strain BL21(DE3) pLysS (closed circles) was included to compare growth rates with RF2 expression strains. Mutations at position 246 were observed to exhibit four distinct rates of growth; serine, alanine, and valine (A = squares), threonine, proline, and leucine (B = open circles), phenylalanine, glutamine (C = triangles), and lysine (D = diamonds). Growth rate was monitored by measuring the Extinction at 600 nm over a 6-h period. Expression was induced with IPTG (1 mM final concentration) after 2.5 h. The standard error was calculated from two separate experiments with duplicates of two different clones.

Mutations at codon position 246 of the RF2 gene differentially affect ribosome binding and peptide release activity of RF2

A selection of RF2 variants at position 246 were assayed for codon-dependent ribosome binding and peptidyl-tRNA hydrolysis activities at cognate stop codons UAA (Fig. 5A) and UGA (Fig. 5B) as well as at the noncognate UAG stop codon (data not shown). The results demonstrated that substitutions at position 246 affected not only the peptide release activity of RFs,

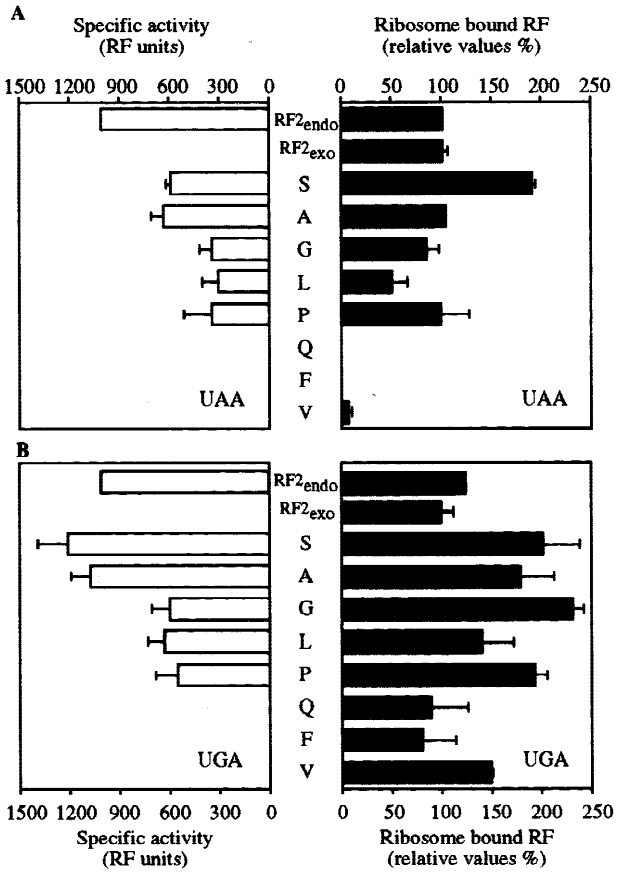

FIGURE 5. In vitro peptide release and ribosomal binding activities of RF1, RF2, and RF2 variants at UAA (**A**) and UGA stop codons (**B**). The RF2 variants are represented by the single letter code for the amino acid substituted into RF2 at position 246. Peptide release activities (open bars) are presented as specific activity in RF units (fMet hydrolyzed (fmol) per μg of RF). The ribosomal binding activity (filled bars) of each RF is presented relative to the binding activity of overexpressed RF2, which is arbitrarily assigned to be 100% at the respective stop codons. The mean of three experiments (plus standard errors) is shown.

but also affected the affinity of the RFs for the ribosome. There were quite dramatic differences in relative binding and release activities among the RF2 variants. Three had lost RF2 specificity with UAA (Q, F, V, Fig. 5A) but maintained an RF2-type ribosomal binding specificity with UGA (Fig. 5B). However, they could not catalyze peptidyl-tRNA hydrolysis, unlike those with T246S or A or G or P or L, which retained both activities. No RF2 variants either bound to the ribosome or exhibited peptidyl-tRNA hydrolysis activity in the presence of the noncognate stop codon UAG or the sense codon UGG. Surprisingly, with UGA as the stop codon, the ribosome-binding ability of a number of the RF2 variants (S, A, G, and P) was greater than that of both $RF2_{endo}$ and $RF2_{exo}$. This may account for the higher peptide release activities of some of these variants with the UGA codon. Recent work using a more "physiological" in vitro translation assay demonstrated that a $RF2_{exo}$-T246A mutant had a significantly higher affinity for the ribosome than overexpressed RF2 (Pavlov et al., 1998).

DISCUSSION

The overexpression of *E. coli* RF2 produced a protein inactive for peptidyl-tRNA hydrolysis. This situation is unique to *E. coli* RF2, as overexpression of *E. coli* RF1 (Donly et al., 1990b) and RF2 from other bacterial species (Uno et al., 1996) produced active protein. RF2 from *E. coli* and *S. typhimurium* differ by only a few residues, and substitution of threonine at position 246 in *E. coli* RF2 with alanine, the equivalent residue in *S. typhimurium*, was critical for restoring activity to the *E. coli* protein (Uno et al., 1996).

The results presented here show that position 246 alone is responsible for the loss of peptide release activity associated with an RF1/2 chimera that had only 10 RF2 residues in a region inferred from a range of published data to be important for peptidyl-tRNA hydrolysis. Restoring the RF1 residue, serine, to this position reactivated the protein. Furthermore, making an identical threonine-to-serine substitution into $RF2_{exo}$ produced a variant factor that retained activity when overexpressed, similar to the RF2-T246A variant documented by Uno et al. (1996).

Ethanol is known to increase the affinity of the RF for the ribosome and to allow factors from one bacterial species to bind to the ribosomes of another, suggesting it "loosens" determinants needed to position the factor correctly for function (W.P. Tate, unpubl.). Ethanol is important for stable ribosomal binding of RFs in a filter assay (Tate & Caskey, 1990) and even allowed activity of RFs on ribosomes when the binding site for the factor was altered (for example, the substitution of *Bacillus stearothermophilus* L11 for *E. coli* L11; W.P. Tate, unpubl.). Evidence has also been presented that the affinity of the RF for the ribosome was directly affected by the length of the growing peptide on the peptidyl-tRNA (Pavlov et al., 1998). Overexpressed native RF2 was as efficient as the variant RF2-T246A mutant if there was a tri- or tetrapeptidyl-tRNA in the P site, but not when the substrate was a dipeptidyl-tRNA. These elegant experiments were interpreted to suggest that in the case of overexpressed native RF2 the ribosomal affinity was too weak. The conclusions are compatible with our experiments using the triplet assay (Caskey et al., 1971), where ethanol was necessary to observe peptide release with $RF2_{exo}$. On the other hand, previous in vivo studies (Uno et al., 1996) and those presented here (Fig. 2C) indicate that normal mRNAs encoding longer polypeptides were also not translated well by recombinant RF2. Overexpression of RF2 in vivo has been shown to be toxic to the cell (Mikuni et al., 1991; Adamski, 1992). Our results indicate there is toxicity with even modest overexpression of RF2 (approximately fivefold). Overexpressed RF2 did not compete with frameshifting at the RF2 frameshift site to increase termination efficiency like the RF2-T246S variant. Furthermore, an additional protein product appeared, the size of which was consistent with a readthrough protein resulting from a suppression event. The overexpressed RF2 is apparently affecting the fidelity of the ribosome, enabling near-cognate tRNAs to be more competitive in decoding the stop codon as sense. The ability of RF2-T246S to enhance termination efficiency at the frameshift site to almost 100% with modest overexpression and the lack of a readthrough product is a dramatic demonstration of how residue 246 is solely responsible for the difference in phenotype.

How might a change from threonine to serine, normally a conservative substitution, influence RF2 activity? One possibility we considered was a posttranslational modification, such as a phosphorylation, at position 246. This was not supported by analysis with mass spectrometry, by determination of in vivo phosphorylation with radiolabeled phosphate, or by western analysis with antibodies against threonine or serine phosphates. Further, the mass spectrometry analysis did not give evidence for an alternative modification, suggesting that the activity restored by the serine mutation was more likely to be due to a conformational change. Limited proteolysis was not able to detect a difference between the active variant and the inactive exogenously-expressed wild-type protein. This was puzzling, but the recently published structure of eRF1 might provide an explanation (Song et al., 2000). If position 246 was within an autonomous minidomain at the "aminoacyl end" of the structure, as is seen in the structure of eRF1 (Song et al., 2000), then no major structural changes might result from substitutions at this position.

The functional sensitivity of RF2 to the presence of a variety of different amino acids at position 246 emphasizes the pivotal nature of this region (Nakamura et al., 2000; this study). The location of position 246 and

the ability to differentially affect ribosome binding and peptidyl-tRNA hydrolysis activities suggests that position 246 may act to place the two domains at the relevant ribosomal active sites (Fig. 6). Interestingly, in our laboratory, unrelated research that introduced cysteine substitutions in 20–30 residues upstream and downstream of position 246 have not identified any variants (except 246) with activities that deviated from the overexpressed RF2 phenotype. This suggests that position 246 may represent the sole residue able to switch the RF between active and inactive forms. Residue 246 is close to a turn-forming GGQ motif, and because some yeast mitochondrial RF1s have large insertions within this region, it would support the inference that 246 is positioned within a putative "loop" region (Pel et al., 1992). Consistent with this proposal, proteolytic studies indicated the most sensitive cleavage site of RF1 and RF2 was close to residue 246 (Moffat & Tate, 1994). Chymotrypsin cleaved RF2 between the neighboring residues Tyr^{244} and Arg^{245} and produced a "nicked" RF2 molecule that was deficient in promoting peptidyl-tRNA hydrolysis but had enhanced ribosome binding (Moffat & Tate, 1994).

A recent analysis of the primary sequences of all known RFs from eukaryotes and prokaryotes identified that the GGQ motif was universally conserved (Frolova et al., 1999). In eubacteria, this motif is four residues downstream of position 246 and is located within the most highly conserved region of eubacterial RFs. Mutations to either of the glycyl residues completely abolished the release activity of human eRF1, but did not affect its ability to form a complex with the ribosome and stimulate the GTPase activity of human eRF3 (Frolova et al., 1999). An examination of the PDB database identified numerous protein structures (for example, the HIV proteases) with a GGQ motif. In almost all cases the consecutive glycyl residues provide the flexibility necessary for a very sharp turn. It is interesting to note that there are two other consecutive glycyl residues that are strictly conserved between all known eubacterial RF1 and RF2s (*E. coli* RF2: residues 137–138).

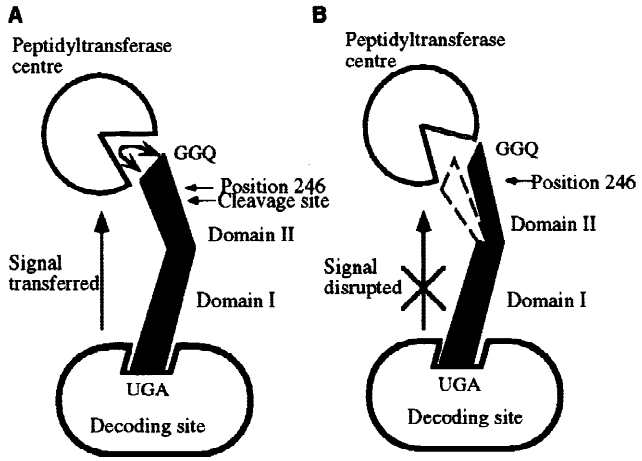

FIGURE 6. A model for RF-dependent signal transfer during translational termination. **A**: In the active state, such as endogenous RF2 or overexpressed RF2-T246S, correct codon recognition in the decoding site is signaled from domain I to domain II, enabling efficient peptidyl-tRNA hydrolysis. Position 246 is involved in orientation of the GGQ turn motif, which is important for efficient interaction of domain II with the peptidyltransferase center of the ribosome. **B**: In the inactive state, such as when native RF2 is overexpressed or contains certain substitutions at position 246, the correct alignment of domain II (hatched) is disturbed (filled) to prevent efficient peptidyl-tRNA hydrolysis. Disturbed codon recognition may also prevent transfer of the signal to domain II such that the orientation of the GGQ motif is misaligned. This, in turn, does not allow a correct interaction between domain II of the RF and the peptidyltransferase center of the ribosome, and, thus, peptidyl-tRNA hydrolysis activity is diminished.

A wealth of evidence is available that indicates the importance of correct positioning of the RF on the ribosome for efficient peptidyl-tRNA hydrolysis (reviewed in Wilson et al., 2000). In particular, the orientation of the stop codon has been shown to play a major role in the binding of the RF to the ribosome and that "correct" binding is necessary for efficient peptide release (McCaughan et al., 1998). Inefficient peptide release could result from an incorrectly oriented RF being unable to physically interact with the peptidyltransferase site and activate it or, alternatively, the RF may not receive the appropriate signal from the decoding site necessary to activate a conformational switch.

Residue 246 may affect peptidyl-tRNA hydrolysis activity by a subtle disturbance of a domain involving the GGQ turn motif. Whether this motif directly interacts with the ribosome peptidyltransferase center or is involved in positioning of the functional domain (domain II, Fig. 6) will become clearer when the structure of the prokaryotic RF is elucidated. It is interesting that a GGQ motif is located on a turn connecting two elements of structure which forms the tip of the eRF1 molecule and "creates a self-autonomous GGQ domain" (Song et al., 2000). The unique ability of position 246 to influence both ribosome binding and peptide release activities may reflect a highly restrictive environment for this residue, a posttranslational modification at another position nearby, or a physiological switch requiring correct codon recognition and involving residue 246 itself to activate peptide release. A posttranslational modification at a nearby residue would simply mean there is saturation of the posttranslational event when the protein is expressed at high levels, whereas the "switch mechanism" implies that RF2 with 246T could exist in two forms and, perhaps, when it is at a high concentration in the bacterial cell, nonphysiological interactions trap it in a nonfunctional state. The similarity in the cleavage patterns between RF2 variants suggests that the changes in activity do not result from gross structural disturbances and is consistent with a posttranslational modification. Certainly at most there must be only relatively subtle structural move-

ments of the functional domains after correct codon recognition.

The high sensitivity of RF activity to perturbations of ribosomal structure, or factor structure as revealed in this study, could reflect the position of the water molecule for nucleophilic attack on the peptidyl-tRNA. Song et al. (2000) propose that the Q in the GGQ motif participates in the coordination of a water molecule responsible for hydrolyzing the peptidyl-tRNA ester bond at the peptidyltransferase center. A ribosomal mutation, C1052 of the 16SrRNA, was characterized using an in vitro termination system, which demonstrated that this mutation was responsible for defects in RF2-dependent peptidyl-tRNA hydrolysis (Arkov et al., 1998). The authors propose that helix 34 within the small ribosomal subunit and the GTPase-associated center on the large subunit may be part of a signal transduction network with RF2 providing the link. Our evidence would be consistent with this model, with residue 246 in *E. coli* RF2 playing a major role in the transduction of the signal (Fig. 6).

This model may hold for both bacterial decoding RFs. The ability to disrupt and restore RF1 activity by changing residue 246 as evidenced by the chimera studies supports this conclusion. Furthermore, a number of rRNA mutations within helix 34 have been shown to cause defects in both RF1- and RF2-dependent binding of stop codons to the ribosome (Brown et al., 1993). This suggests that the ribosomal sites involved in transmission of the termination signal in the presence of RF1 may be similar to those for RF2 (Arkov et al., 1998).

MATERIALS AND METHODS

The RF2 expression plasmid pTGRF2 was a kind gift from J. Mansell (Mansell, 1999). pTGRF2-T246S was constructed as described by Wilson (1999). Briefly, the PCR products previously used for cloning RF2-T246S into the pET vector were end filled and first blunt cloned into the *Sma*I site of pSP65. The clones were checked for orientation. The appropriate clones were digested with *Eco*RI/*Pst*I and subcloned into pTG so that the RF gene open reading frame started at the *Eco*RI site and terminated at the *Pst*I site. This puts the gene under the control of the tryptophan promoter in pTG. The RF1/2 chimera was supplied by J. Moffat (Moffat et al., 1993) and the pMal clones containing the RF2 frameshift window were constructed by E. Poole (Poole et al., 1995). Anti-MBP was purchased from New England Biolabs. Plasmids were purified using a QIAgen miniprep kit and were electroporated into bacteria using an Electro Cell Manipulator® 600 (BTX). Trypsin was supplied by Boehringer Mannheim.

Growth media and bacterial strains

In vivo termination studies were carried out in *E. coli* strain FJU_{112} [_(*lac-pro*) *gyr*A *ara rec*A*56*^Tn*10*, F′ *lacI*^{Q1}]. This strain has wild-type ribosomes and no suppressor tRNAs that could compete with the termination or frameshifting events. Expression of the RF protein utilized the *E. coli* strain, BL21(DE3) pLysS [*hsdS gal* (_c*Its* 857 *ind*1 *Sam*7 *nin*5 *lac*UV5-T7 gene *1*) pLysS (*cam*^{R})], which was a kind gift from M. Berry. Cultures were grown in LB, containing ampicillin (100 μg/mL) and chloramphenicol (50 μg/mL), at 37 °C overnight with shaking. The overnight cultures were used to inoculate (5% v/v) fresh media containing antibiotics and grown at 37 °C with vigorous shaking. The growth rate was monitored by measuring the Extinction at 600 nm. Plasmid expression was induced with isopropyl-D-thiogalactopyranoside (IPTG) (1 mM final concentration) when growth had reached an Extinction of 0.6–0.8 (~2.5 h). Cultures were grown for a further 4 h.

Construction of pETRF expression plasmids

PCR was used to introduce restriction sites into the 5′ and 3′ ends of genes encoding the RF of interest. The 5′ primers for RF1 (5′-AGATATACATATGAAGCCTTCTATCGTTCCC) and RF2 (5′-AGATATACATATGTTTGAAATTAATCCGG) introduced an *Nde*I site (underlined) at the start of the gene. The 3′ primers for RF1 (5′-GAGGATCCATTATTCCTGCTC) and RF2 (5′-TTGGATCCTCATAACCCTGC) introduced a *Bam*HI (underlined) restriction site after the translational stop signal. The PCR cycle parameters were as follows: denaturing at 95 °C for 30 s (the first cycle uses a longer denaturing step of 3 min), annealing at 60 °C for 1 min, extension at 72 °C for 2 min. These conditions were cycled 30 times with a final extension time of 10 min. The products were then cloned *Nde*I/*Bam*HI into pET3a.

Site-directed and random mutagenesis

Mutations were constructed using PCR mutagenesis. Substitution of threonine for serine at position 246 of RF1/2 chim and RF2 involved a first round PCR using a mutagenic primer (5′-GACGTTTATCGCTCGTCCGGCGCG) and the appropriate 3′ pETRF primer (as described above). The product of this reaction was then used as a primer, in conjunction with the appropriate 5′ pETRF primer, to generate the full-length product that was cloned *Nde*I/*Bam*HI into pET3a. The random mutagenesis utilized the same approach, but substituted the mutagenic primer with a primer redundant at position 246 (5′-GACGTTTATCGCNNNTCCGGCGCGGGC). Position 246 is underlined in both oligonucleotides. PCR using megaprimers utilized an initial extension step in the absence of the pETRF cloning primer. This consisted of a cycle of denaturing at 95 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 2 min. The appropriate pETRF cloning primer was added and a 30-cycle PCR using the pETRF cycle parameters as described for the pETRF cloning above.

Release factor purification

Purification of RF protein was essentially as described by Tate and Caskey (1990), with the exception that grinding with alumina was omitted and replaced with a simple freeze-thaw step. This was possible because of the efficient cell lysis obtained due to high levels of lysozyme resulting from pLysS expression.

In vivo analysis of termination efficiency using pTG and pMal plasmids

Expression from pTG plasmids is repressed by tryptophan, so all experiments utilizing this vector were carried out in M9 media. Overnight cultures of *E. coli* strain FJU_{112} containing pTG, pTGRF2, or pTGRF2-T246S and pMal (supplemented with 100 μg/mL ampicillin, 10 μg/mL gentamicin, and 20 μg/mL tryptophan) were inoculated into 5 mL of the same media. Bacteria were grown to mid-log phase (OD_{600} of 0.5), then IPTG was added (1 mM final concentration) to induce the expression of the MBP fusion proteins and 3-indoleacrylic acid to induce RF expression. Bacteria were harvested and cellular proteins were analyzed by SDS-PAGE and immunodetection following western transfer.

Electrospray ionization mass spectroscopy

For tryptic digestion, 20 μg of each RF sample was redissolved in 100 μL 10% acetonitrile, 5 μL 0.5M NH_4HCO_3, and 1.25 μL 2 mg/mL trypsin. The samples were incubated at 37 °C overnight, lyophilized to remove NH_4HCO_3, and resuspended in 20 μL of 0.1% acetic acid and 50% acetonitrile prior to injection. The probe was charged to +3,000 and the source maintained at 60 °C. The mass range 200–1600 m/z was scanned every 3.5 s and a cone voltage ramp of 53 V was applied over this range. A resolution setting of 15/15 was used. Data were acquired and processed using Mass-Lynx software and transformed using Maximum Entropy (Max-Ent) software supplied with the instrument. Molecular mass calculations are based on weights of C = 12.011, H = 1.00794, N = 14.00674, O = 15.9994, P = 30.97376, and S = 32.006.

In vitro assays

Codon-directed fMet-tRNA hydrolysis and ribosome binding assays were performed as described by Tate and Caskey (1990). The fMet-tRNA hydrolysis assay measures the RF-mediated release of f[^{3}H]Met from the P site tRNA. The substrate for this assay, f[^{3}H]Met-$tRNA^{fMet}$•AUG•ribosome complex, was formed by incubating 50 pmol of ribosomes with 100,000 cpm f[^{3}H]Met-tRNA, 2.5 nmol AUG in a buffer containing 10 mM $Mg(OAc)_2$, 20 mM Tris-HCl, pH 7.4, and 0.15M NH_4Cl at 30 °C for 20 min. Samples were assayed for termination activity by incubating with codon (or ethanol) and 5 μL substrate in a 50-μL final volume of 1× RF buffer (30 mM $Mg(OAc)_2$, 50 mM Tris-HCl, pH 7.2, 75 mM NH_4Cl) at room temperature for 20 min. The reaction was stopped by the addition of 250 μL 0.1 M HCl, and free f[^{3}H]Met was extracted with 1 mL of ethyl acetate. The top organic phase was counted in a scintillation counter using 6 mL of Starscint™ scintillant. The ribosome-RF binding was measured by adding [^{35}S]-radiolabeled RF to a 200-μL reaction containing 50 pmol 70S ribosomes, 500 pmol stop codon in 1× binding buffer (200 mM $Mg(OAc)_2$, 50 mM Tris-HCl, pH 7.2, 0.1 M NH_4Cl) 10% (v/v) ethanol, or by using ^{32}P-labeled codon instead of radiolabeled factor. After at least 20 min at 4 °C, ribosomes containing radioactive factor were pelleted by centrifuging at 30 psi (95,000 rpm) for 15 min at 4 °C (Beckman airfuge). Ribosome-associated RF was determined by measuring the radioactivity in both pellet and supernatant fractions by counting in 1 mL Starscint™ scintillant. In the experiment with radioactive codon, the complex was trapped on a Millipore filter and the radioactivity measured.

Limited proteolysis

Pure RF2 protein was incubated with trypsin (1:50) and chymotrypsin (1:220) at 30 °C for varying lengths of time as described by Moffat and Tate (1994). Dilutions from a stock of chymotrypsin were created and equal volumes were added to 1 μg of RF protein and incubated for 30 min at 30 °C. The samples were analyzed by Coomassie staining following SDS-PAGE.

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