

Competition between *trans*-translation and termination or elongation of translation

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ABSTRACT

The effects of tRNA, RF1 and RRF on *trans*-translation by tmRNA were examined using a stalled complex of ribosome prepared using a synthetic mRNA and pure *Escherichia coli* translation factors. No endoribonucleolytic cleavage of mRNA around the A site was found in the stalled ribosome and was required for the tmRNA action. When the A site was occupied by a stop codon, alanyl-tmRNA competed with RF1 with the efficiency of peptidyl-transfer to alanyl-tmRNA for *trans*-translation inversely correlated to the efficiency of translation termination. The competition was not affected by RF3. A sense codon also serves as a target for alanyl-tmRNA with competition of aminoacyl-tRNA. The extent of inhibition was decreased with the length of the 3'-extension of mRNA. RRF, only at a high concentration, slightly affected peptidyl-transfer for *trans*-translation, although it did not affect the canonical elongation. These results indicate that alanyl-tmRNA does not absolutely require the truncation of mRNA around the A site but prefers an mRNA of a short 3'-extension from the A site and that it can operate on either a sense or termination codon at the A site, at which alanyl-tmRNA competes with aminoacyl-tRNA, RF and RRF.

INTRODUCTION

A special RNA called tmRNA or SsrA RNA has been found in a wide variety of eubacteria and in some chloroplasts and mitochondria (1–3). tmRNA having both tRNA and mRNA properties plays an important role in a quality control system during protein synthesis. When a ribosome stalls on a problematic mRNA, presumably at the 3' end of a truncated mRNA

lacking a stop codon, tmRNA charged with alanine enters the A site of the ribosome to act first as an alanyl-tRNA (4,5). Then it acts as an mRNA to direct the addition of a short peptide tail (6). This co-translation, known as *trans*-translation, terminates at a stop codon that follows the tmRNA reading frame, releasing tagged polypeptide and rescuing the stalled ribosome (7–9). The C-terminal tag-peptide is recognized by several ATP-dependent proteases, resulting in degradation of tagged polypeptide (10). In addition, *trans*-translation has been shown to facilitate the degradation of truncated mRNA by releasing a stalled ribosome and thereby allowing 3'- to 5'-exonucleases to access the 3' end of free mRNA (11).

Since tmRNA comprises two functional domains, a tRNA domain partially mimicking tRNA (12) and an mRNA domain including the coding region for tag-peptide surrounded by four characteristic pseudoknot structures (13), an elaborate interplay of the two domains should be required for *trans*-translation. Although the mode of tmRNA action for *trans*-translation has been extensively studied, it has not been clarified how tmRNA finds the ribosome of stalled translation. Initially, the target for the tmRNA system has been assumed to be the ribosome with a stalled translation in which the P site but not A site is occupied by mRNA owing to the lack of its 3'-portion (7). Tandem rare codons on an artificial mRNA can also be a target for *trans*-translation (14). Full-length and C-terminally truncated polypeptides with a tag-sequence at the C-terminus have also been identified as endogenous products of *trans*-translation in the cell (15,16), and *trans*-translation efficiently occurs at an inefficient UGA termination codon preceded by rare arginine codons (17). These *in vivo* data suggest that *trans*-translation can occur on either a sense or nonsense codon at the A site when translation is stalled. However, a recent finding of bacterial toxin that cleaves an mRNA specifically at the A site in the ribosome stalled upon amino acid starvation provides a new concept that mRNA of stalled translation is targeted initially by these A site-specific endoribonucleases and subsequently by alanyl-tmRNA for *trans*-translation (18,19).

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In prokaryotes, either RF1 or RF2 recognizes a stop codon and catalyzes the hydrolysis of peptidyl-tRNA to release the polypeptide from the ribosome (20). After this event, RF3 stimulates the dissociation of RF1 or RF2 from the ribosome (21), and then RRF, with the help of EF-G, allows the ribosome to enter a new round of translation (22,23). In the present study, a stalled complex of the ribosome with a polyphenylalanyl-tRNA at the P site was prepared from ribosomes programmed with synthetic mRNAs and other pure translation factors from *Escherichia coli*. Using this stalled complex of ribosome, the competition between alanyl-tmRNA and RF1 at a nonsense codon was examined. Ivanova *et al.* (24) have recently shown the competition at a nonsense codon, and such a competition was also observed in our system. We further examined the competition between alanyl-tmRNA and valyl-tRNA or RRF at a sense codon with different lengths of the 3'-extension of mRNAs. We found that alanyl-tmRNA can enter an A site for *trans*-translation at either the sense or termination codon with competition among aminoacyl-tRNA, RF1 and RRF, even without cleavage of mRNA at the A site. We also found that alanyl-tmRNA prefers a shorter 3'-extension of mRNA from the A site.

MATERIALS AND METHODS

Preparation of RF1

The gene encoding *E.coli* RF1, *prfA*, ligated into a pKK223-3 plasmid was cloned into *E.coli* strain JM109. Eight grams of cells that overproduced RF1 were suspended in 16 ml of buffer A [50 mM Tris-HCl (pH 7.5) and 10 µg/ml of 4-(2-aminoethyl)benzenesulfonyl fluoride] and lysed by sonication. The debris was removed by centrifugation at 9000 r.p.m. (Hitachi R10A2) for 10 min, 20 000 r.p.m. (Hitachi P65A) for 30 min and 80 000 r.p.m. (Hitachi RP80AT) for 30 min. The supernatant was fractionated by DEAE-Sepharose column chromatography with a linear gradient of KCl from 0 to 200 mM in buffer A. The fractions containing RF1 were pooled and applied to an Ni-NTA column (Pharmacia). RF1 was eluted by a linear gradient of imidazole from 0 to 20 mM. The protein concentration was measured by Bradford's method using BSA as a standard (25). The purity was analyzed by electrophoresis on an SDS polyacrylamide gel (26). The polypeptide-chain releasing activity of RF1 was determined by measuring free formyl-[³H]methionine released from formyl-[³H]methionyl-tRNA_f^{met}•UUCAUGUAA•ribosome complex as described by Grentzmann and Kelly (27).

Preparation of RF3

The gene encoding *E.coli* RF3, *prfC*, ligated into a pKK223-3 plasmid was cloned into *E.coli* strain JM109. Eight grams of cells that overproduced RF3 were suspended in 16 ml of buffer B [50 mM Tris-HCl (pH 7.5), 5 µM GDP and 10 µg/ml of 4-(2-aminoethyl)benzenesulfonyl fluoride] and were lysed by sonication. The debris was removed by centrifugation three times at 9000 r.p.m. (Hitachi R10A2) for 10 min, 20 000 r.p.m. (Hitachi P65A) for 30 min and 80 000 r.p.m. (Hitachi RP80AT) for 30 min. The supernatant was then applied to DEAE-Sepharose, and the proteins were eluted with a linear gradient of KCl from 25 to 250 mM in buffer B. The fractions containing RF3 were pooled, adjusted to be pH 6.0 with 20 mM

sodium phosphate, and applied to a CM-Sepharose column (Tosoh). RF3 was eluted with a linear gradient of NaCl from 0 to 200 mM. The GTPase activity of RF3 was measured in the presence of ribosome. The hydrolysis of [³²P]GTP into [³²P]GDP was measured by thin-layer chromatography (28).

Preparation of RRF

The gene encoding *E.coli* RRF, *frr*, ligated into a pKK223-3 plasmid was cloned into *E.coli* strain JM109. Eight grams of cells that overproduced RRF were suspended in 16 ml of buffer A and lysed by sonication. The debris was removed by centrifugation at 9000 r.p.m. (Hitachi R10A2) for 10 min, 20 000 r.p.m. (Hitachi P65A) for 30 min and 80 000 r.p.m. (Hitachi RP80AT) for 30 min. The supernatant was then applied to Q-Sepharose Fast Flow (Pharmacia). The flow-through fraction containing RRF was adjusted to be 1.5 M ammonium sulfate and applied to Phenyl Sepharose 6 Fast Flow (Pharmacia). RRF was eluted with a linear gradient of ammonium sulfate from 1.5 to 0.5 M. The activity of RRF was confirmed by monitoring the conversion from polysomes to monosomes in the presence of EF-G by sucrose density gradient centrifugation (23).

Preparation of tmRNA and SmpB

E.coli tmRNA was induced from an overproducing strain by the addition of 1.0 mM Isopropyl-β-D-thiogalactopyranoside (IPTG) and was purified as described previously (29). The nucleic acid fraction was extracted with phenol and then subjected to ethanol precipitation. After performing phenol extraction and ethanol precipitation, the resulting fraction was subjected to differential isopropylalcohol precipitations to roughly remove DNA, followed by incubation with RNase-free DNase I (Pharmacia). tmRNA was purified by electrophoresis on a 5% polyacrylamide gel containing 7M urea.

C-terminally His₆-tagged SmpB was induced from an overproducing strain by the addition of 1.0 mM IPTG and was purified as described previously (30).

Preparation of the stalled complex of ribosome

Ribosomes were prepared from *E.coli* W3110 (28,31). *E.coli* EF-G and EF-Tu with the addition of the sequence of His-tag at the C-terminal were cloned and overproduced in *E.coli*. They were purified using an Ni-NTA column (32,33) and their ribosome-dependent GTPase activities were confirmed by thin-layer chromatography (28).

E.coli tRNA^{Phe} (Sigma) was aminoacylated with [¹⁴C]phenylalanine by *E.coli* phenylalanyl-tRNA synthetase. Polyphenylalanines were synthesized using ribosome programmed with synthetic mRNAs. Translation reaction mixture (50 µl) contained 5 pmol ribosome, 100 pmol mRNA, 100 pmol EF-Tu, 3 pmol EF-G, 10 pmol [¹⁴C]phenylalanyl-tRNA^{Phe}, 2 mM spermidine, 1 mM ATP, 0.2 mM GTP, 80 mM Tris-HCl (pH 7.5), 30 mM ammonium chloride, 5 mM magnesium acetate and 2.5 mM DTT. After different periods of incubation at 37°C, a 10 µl aliquot of the mixture was withdrawn and put in 5 ml of hot 5% trichloroacetic acid, preheated at 90°C for 5 min. Then ¹⁴C-labeled phenylalanines incorporated into a polypeptide, which were in the acid-insoluble fraction, were quantified by using a liquid scintillation counter (LSC-3500; Aloka). The phenylalanine incorporation was

saturated by incubation at 37°C for 10 min, and the solution was then used as a stalled complex of ribosome for further studies.

Trans-translation and translation using the stalled complex of ribosome

tmRNA was aminoacylated with [³H]alanine by alanyl-tRNA synthetase from *E. coli* (4,34). Alanine was incorporated from alanyl-tmRNA into a polyphenylalanine peptide chain by incubating the stalled complex of ribosome with a tmRNA mixture containing 5 pmol [³H]alanyl-tmRNA and 30 pmol SmpB. After different periods of incubation at 37°C, an aliquot of the mixture was withdrawn and put in 5 ml of hot 5% trichloroacetic acid to stop the reaction, and the radioactivity of the acid-insoluble fraction was measured by using a liquid scintillation counter.

E. coli tRNA^{Val} (Sigma) was aminoacylated with [³H]valine by valyl-tRNA synthetase from *E. coli* (35). Valine incorporation into polypeptide was determined by mixing the stalled complex of ribosome with a valyl-tRNA^{Val} mixture containing 1 pmol [³H]valyl-tRNA^{Val}, 0.1 pmol EF-G and 20 pmol EF-Tuz. After incubation at 37°C for 30 s, an aliquot of the mixture was withdrawn and put in 5 ml of hot 5% trichloroacetic acid to stop the reaction and the radioactivity of the acid-insoluble fraction was measured by using a liquid scintillation counter.

RESULTS

***In vitro* translation and peptidyl-transfer to alanyl-tmRNA for trans-translation**

Using pure translation factors, [¹⁴C]phenylalanine was incorporated into polyphenylalanine from (UUC)₁₀ or its derivatives having a varying sequence of 3'-extension. After incubation of the mixture at 37°C for 10 min, the phenylalanine incorporation was saturated (data not shown). The levels of phenylalanine incorporation at 10 min from six kinds of mRNAs were similar as shown in Figure 1A, and the solution was used as a stalled complex of ribosome for further studies.

The stalled complex of ribosome was then incubated with ³H-labeled alanyl-tmRNA mixture. Alanine was gradually incorporated only in the presence of SmpB, a tmRNA binding protein (30,36), and the alanine incorporation was almost saturated after 10 min of incubation (Figure 1B). During this period, the amount of phenylalanines incorporated did not change (Figure 1B, dotted lines). At a saturated time point, the ratio of alanines to phenylalanines incorporated was ~0.09. The alanine incorporation at 30 s from six kinds of mRNAs is shown in Figure 1C. When mRNA having the longest 3'-extension, (UUC)₁₀GUA(A)₁₂, was used, alanine incorporation was significantly less than the alanine incorporations using the other kinds of mRNAs.

Competition between RF1 and alanyl-tmRNA

The efficiency of termination depends on the stop codon and its 3'-adjacent (+4) nucleotide. UAAU and UAAG serve as strong stop signals, while UGAC and UAGC give poor termination efficiency (37). In the present study, two different contexts of stop codons UAAU and UAGC that are strong and weak stop signals for RF1, respectively,

were used as mRNAs to examine the competition between RF1 and alanyl-tmRNA. Various amounts of purified RF1 were added to the tmRNA mixture with a fixed amount of ³H-labeled alanyl-tmRNA. Each of the tmRNA mixtures containing various amounts of RF1 was incubated with the stalled complex of ribosome at 37°C for 1 min. During this process, phenylalanine incorporation was not affected by RF1. By using mRNA without a stop signal sequence, RF1 had no influence on the alanine incorporation by alanyl-tmRNA (Figure 2). When using (UUC)₁₀UAAU and (UUC)₁₀UAGC as mRNAs containing a stop signal for RF1, however, alanine incorporation was significantly affected by RF1, the effect being much greater when (UUC)₁₀UAAU was used. When using the same amounts of tmRNA and RF1 in the reaction, the percentages remaining of alanine incorporation from the stalled complex of ribosome programmed with (UUC)₁₀UAAU and (UUC)₁₀UAGC were 66 and 78%, respectively. Apparently, the efficiency of peptidyl-transfer for *trans*-translation in the presence of RF1 depended on the preference of the stop signal for RF1. These results indicate that alanyl-tmRNA competes with RF1 at a termination codon with the efficiency of *trans*-translation inversely correlated to the efficiency of translation termination.

RF3 did not affect the inhibition of RF1 on peptidyl-transfer for trans-translation at a termination codon

RF3 has been shown to stimulate the dissociation of RF1 from the ribosome after peptidyl-tRNA hydrolysis (21). It accentuates the strength of the stop signal specified by the 4 nt stop signal. Therefore, RF3 was added to the reaction mixture. However, no significant difference was found between alanine incorporation or between phenylalanine incorporation in the presence of RF3 and absence of RF3 (Figure 3). This result indicates that RF3 neither enhances nor inhibits the step of peptidyl-transfer for *trans*-translation at a termination codon.

Competition between RRF and alanyl-tmRNA

The effect of RRF on alanine incorporation by tmRNA was examined by the addition of RRF to the [³H]alanyl-tmRNA mixture. After incubation of this mixture for 30 s with the stalled complex of ribosome programmed with six kinds of mRNAs, alanine incorporation was measured. Alanine incorporation was decreased when the reaction mixture contained 0.5 and 5.0 μM of RRF, which were 10 and 100 times greater than the amount of tmRNA, respectively (Figure 4). In the case of a smaller amount of RRF, no reduction of alanine incorporation was detected (data not shown). The patterns of inhibition by RRF for all kinds of mRNAs were similar. Since phenylalanine incorporation was not decreased by the addition of RRF, the observed inhibition by RRF was not due to the decrease in the stalled complex of ribosome. These results indicate that alanyl-tmRNA competes with RRF to enter the A site of the stalled ribosome either in the presence or absence of mRNA in the A site.

Effect of alanyl-tmRNA on valine incorporation

To study the competition between tRNA and tmRNA at a sense codon, the effect of alanyl-tmRNA on the incorporation of valine using mRNAs containing a GUA codon were observed. After a saturating level of polyphenylalanine was synthesized

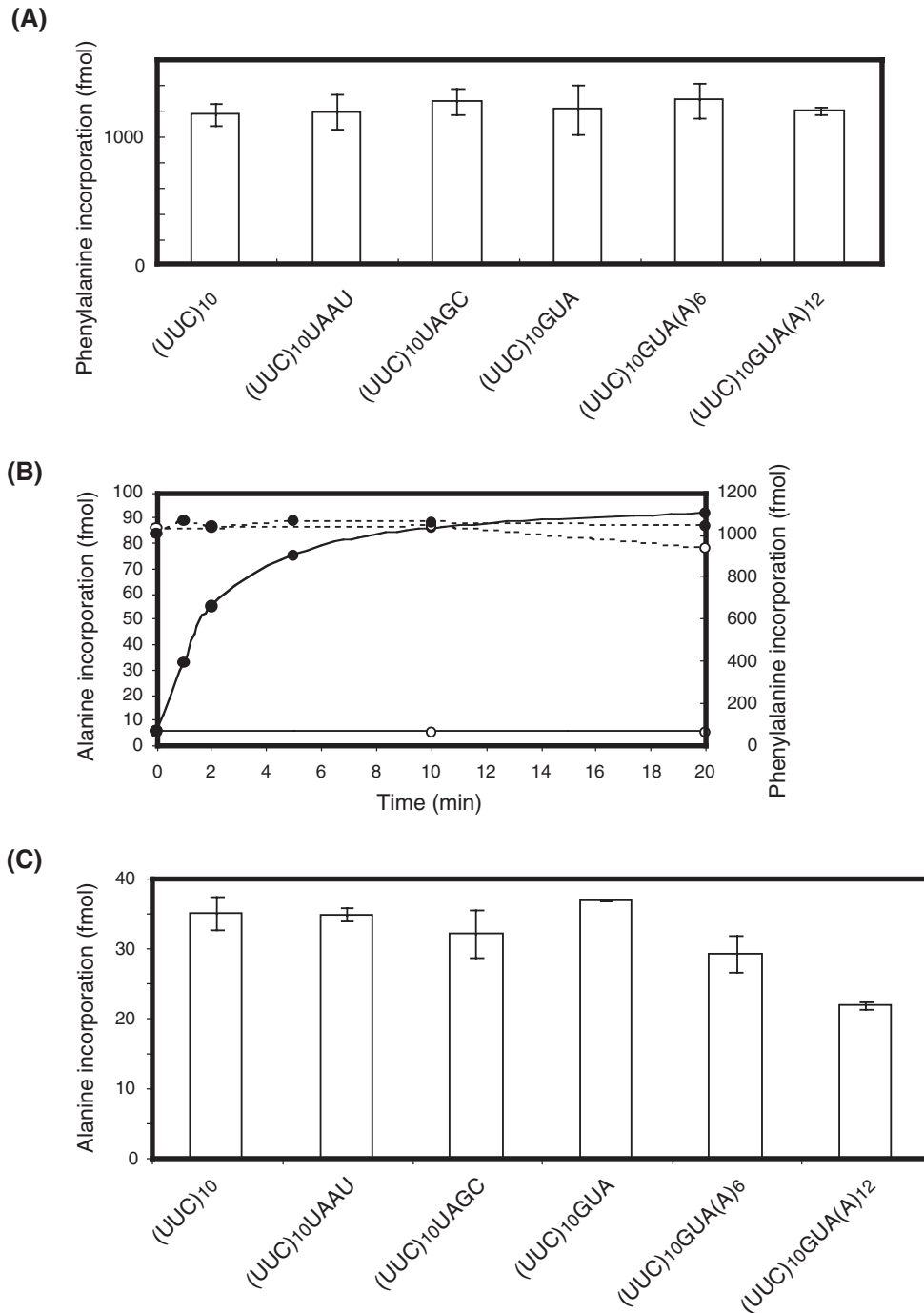


Figure 1. *In vitro* polyphenylalanine synthesis directed by (UUC)₁₀-based mRNAs and alanine incorporation by tmRNA from the stalled complex of ribosome. (A) Fifty microliters of translation reaction mixture contained 5 pmol ribosome, 100 pmol mRNA, 200 pmol EF-Tu, 3 pmol EF-G, 10 pmol [¹⁴C]phenylalanyl-tRNA^{Phe}, 2 mM spermidine, 1 mM ATP, 0.2 mM GTP, 80 mM Tris-HCl (pH 7.5), 30 mM ammonium chloride, 5 mM magnesium acetate and 2.5 mM DTT. After incubation at 37°C for 10 min, a 10 μl aliquot was withdrawn from the reaction mixture and put in 5 ml of hot 5% trichloroacetic acid. (B) Sixty microliters of stalled complex of ribosomes programmed with (UUC)₁₀GUA prepared in (A) was incubated at 37°C with 20 μl of tmRNA mixture containing 4 pmol [³H]alanyl-tmRNA with (solid circle) or without (open circle) 60 pmol SmpB. After incubation at the indicated time point, a 10 μl aliquot was withdrawn and put in 5 ml of hot 5% trichloroacetic acid. The incorporated alanines (solid line) and phenylalanines (dotted line) were quantified. (C) Fifteen microliters of stalled complex of ribosome programmed with each kind of mRNA was incubated with 5 μl of tmRNA mixture containing 1 pmol pre-charged [³H]alanyl-tmRNA and 15 pmol SmpB. After incubation at 37°C for 30 s, a 15 μl aliquot was withdrawn and put in 5 ml of hot 5% trichloroacetic acid. The incorporated alanines were quantified.

as explained above, the [³H]valyl-tRNA^{Val} mixture was added. After incubation at 37°C for 30 s, valine incorporation was measured. As expected, [³H]valine was significantly incorporated when using (UUC)₁₀GUA, (UUC)₁₀GUA(A)₆ or

(UUC)₁₀GUA(A)₁₂ as an mRNA, while valine incorporation was not observed when using (UUC)₁₀UAGC lacking a valine codon (Figure 5A). Similar levels of valine incorporation were observed from three mRNAs having a GUA codon with

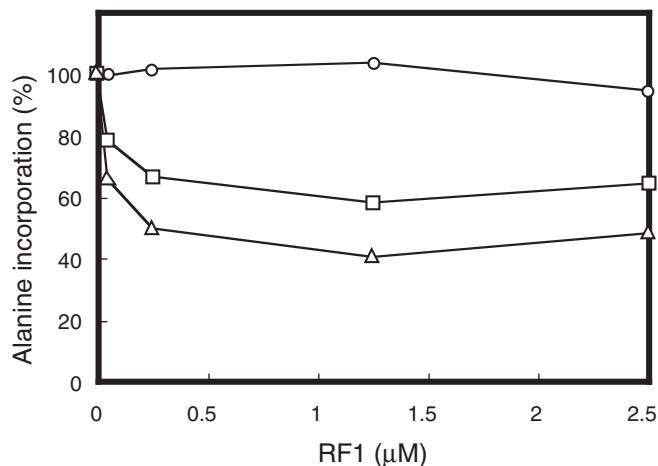


Figure 2. Effect of RF1 on alanine incorporation by tmRNA. Stalled complex of ribosomes (30 μl) programmed with (UUC)₁₀ (circle), (UUC)₁₀UAGC (rectangle) or (UUC)₁₀UAAU (triangle) mRNA was incubated in 10 μl of tmRNA mixture containing 2 pmol pre-charged [³H]alanyl-tmRNA, 30 pmol SmpB and various concentrations of RF1. After incubation at 37°C for 1 min, a 10 μl aliquot was withdrawn and put in 5 ml of hot 5% trichloroacetic acid. Alanine incorporation in the reaction without RF1 is regarded as 100%. The experiments showed a good reproducibility and the error was within 10%.

different lengths of the 3'-extension from the GUA codon. This result indicates that aminoacyl-tRNA, unlike alanyl-tmRNA (Figure 1C), enters the A site of a translating ribosome regardless of the length of the 3'-extension of mRNA from the A site.

Various concentrations of alanyl-tmRNA with 15 pmol SmpB were added to the [³H]valyl-tRNA^{Val} mixtures. These mixtures were then combined with the stalled complex of ribosome. After incubation at 37°C for 30 s, valine incorporations were measured (Figure 5B). At the same concentrations of valyl-tRNA^{Val} and alanyl-tmRNA (50 nM), valine incorporation was reduced by 10–20%. When the concentration of alanyl-tmRNA was increased to 400 nM, 8 times higher than that of valyl-tRNA^{Val}, valine incorporation was decreased more. Alanyl-tmRNA had the smallest inhibitory effect when mRNA having the longest (12 nt) extension from the GUA codon was used. These results indicate that the length of 3'-extension of mRNA from the A site for alanyl-tmRNA is critical for entrance into the stalled complex of ribosome more than that for aminoacyl-tRNA.

RRF did not affect valine incorporation

Various concentrations of RRF were added to the [³H]valyl-tRNA^{Val} mixtures. These mixtures were then mixed with the stalled complex of ribosome. After incubation at 37°C for 30 s, incorporation of [³H]valine was measured. No significant change in valine incorporation from any type of mRNA used was observed by the addition of 50 μM RRF, a 1000-fold higher concentration than that of valyl-tRNA^{Val} (Figure 6). This result indicates that RRF neither enhances nor inhibits substantially the entrance of aminoacyl-tRNA into the A site of the translating ribosome.

Cleavage of mRNAs was not found from the stalled complex of ribosome

mRNA labeled with ³²P at the 5' end was used for synthesizing the stalled complex of ribosome. Then mRNA in the stalled

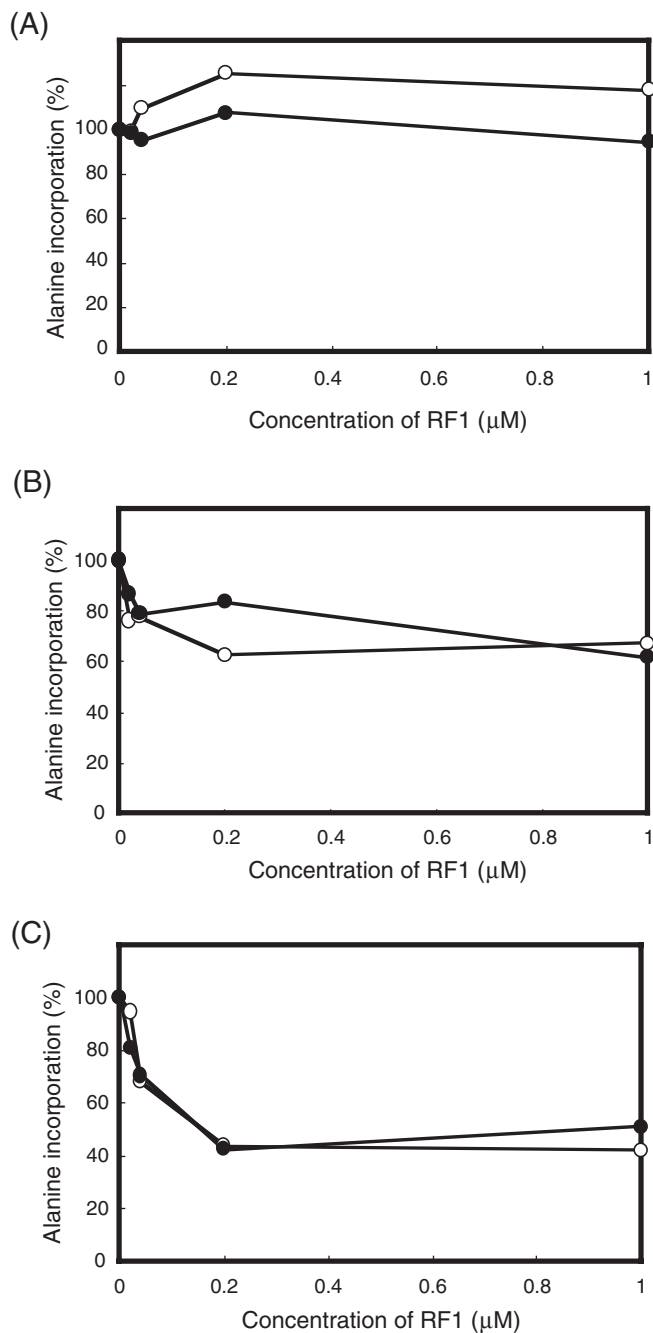


Figure 3. Effect of RF1 on alanine incorporation by tmRNA in the presence of RF3. Stalled complex of ribosomes (30 μl) programmed with a (A) (UUC)₁₀-based mRNA (used as control), (B) (UUC)₁₀UAGC or (C) (UUC)₁₀UAAU mRNAs was incubated with 10 μl of tmRNA mixture containing 2 pmol [³H]alanyl-tmRNA, 30 pmol SmpB, various concentrations of RF1 and 0 (solid circle) or 20 pmol RF3 (open circle). After incubation at 37°C for 1 min a 10 μl aliquot was withdrawn and put in 5 ml of hot 5% trichloroacetic acid. Alanine incorporation in the reaction without release factors is regarded as 100%. The experiments showed a good reproducibility and the error was within 10%.

complex of ribosome was examined by PAGE. To increase the population of mRNA involved in the stalled complex of ribosome, the concentrations of mRNA and ribosome in the mixture of the stalled complex formation were decreased and increased, respectively (Figure 7). In this system, >10%

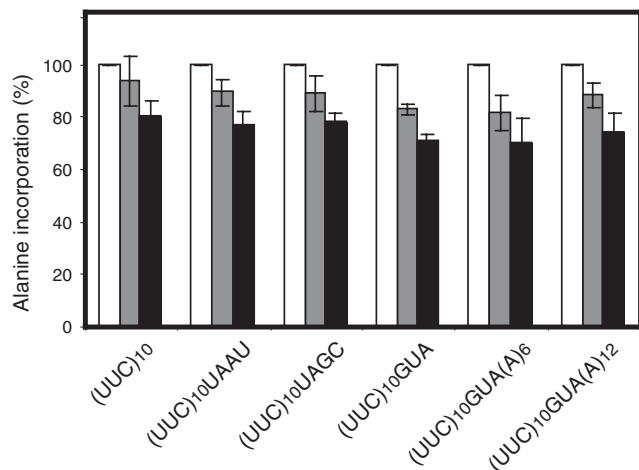


Figure 4. Effect of RRF on alanine incorporation by tmRNA. Stalled complex of ribosome (15 μ l) was incubated with 5 μ l of tmRNA mixture containing 1 pmol pre-charged [3 H]alanyl-tmRNA, 15 pmol SmpB and 0 (white), 0.5 (gray) or 5.0 (black) μ M of RRF. After incubation at 37°C for 30 s, a 15 μ l aliquot was withdrawn and put in 5 ml of hot 5% trichloroacetic acid. The incorporation of alanine in the reaction without RRF is regarded as 100%.

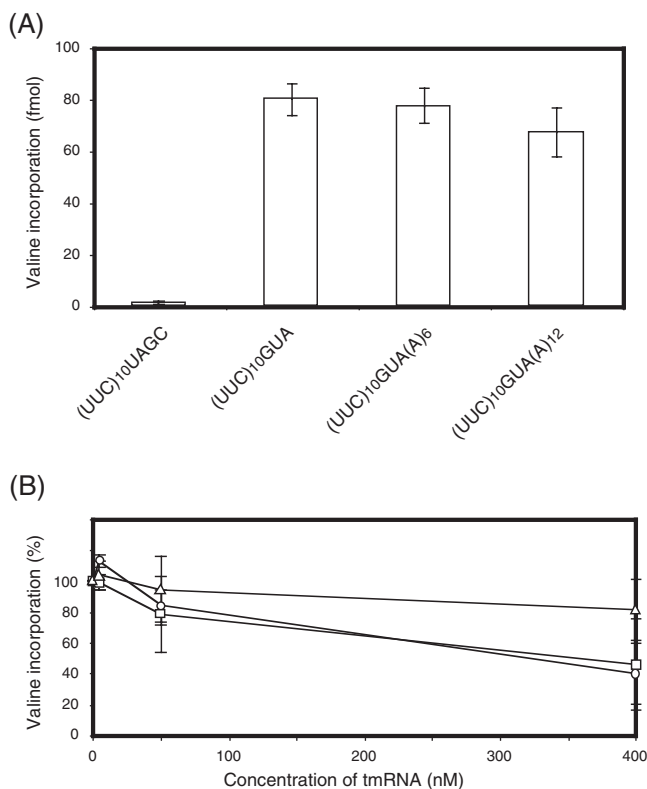


Figure 5. *In vitro* valine incorporation from (UUC)₁₀-dependent polyphenylalanine-ribosome complex and effect of tmRNA on valine incorporation. (A) Stalled complex of ribosome (15 μ l) programmed with a (UUC)₁₀GUA-based mRNA or (UUC)₁₀UAGC (used as control) was incubated with 5 μ l of the mixture containing 1 pmol pre-charged [3 H]valyl-tRNA^{Val}, 0.1 pmol EF-G and 20 pmol EF-Tu. (B) Stalled complex of ribosome (15 μ l) programmed with (UUC)₁₀GUA (circle), (UUC)₁₀GUA(A)₆ (rectangle) or (UUC)₁₀GUA(A)₁₂ (triangle) was incubated with 5 μ l of the mixture containing 1 pmol pre-charged [3 H]valyl-tRNA^{Val}, 0.1 pmol EF-G, 20 pmol EF-Tu, 15 pmol SmpB and various concentrations of alanyl-tmRNA. After incubation at 37°C for 30 s, a 15 μ l aliquot was withdrawn and put in 5 ml of hot 5% trichloroacetic acid. Valine incorporation in the reaction without alanyl-tmRNA is regarded as 100% (B).

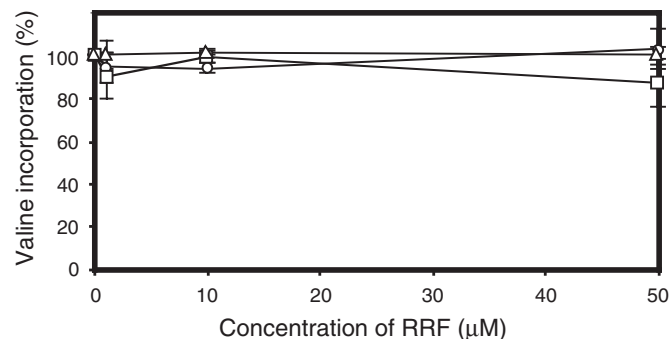


Figure 6. Effect of RRF on valine incorporation. Stalled complex of ribosome (15 μ l) programmed with (UUC)₁₀GUA (circle), (UUC)₁₀GUA(A)₆ (rectangle) or (UUC)₁₀GUA(A)₁₂ (triangle) was incubated with 5 μ l of the mixture containing 1 pmol [3 H]valyl-tRNA^{Val}, 0.1 pmol EF-G, 20 pmol EF-Tu and various concentrations of RRF. After incubation at 37°C for 30 s, a 15 μ l aliquot was withdrawn and put in 5 ml of hot 5% trichloroacetic acid. Valine incorporation in the reaction without RRF is regarded as 100%.

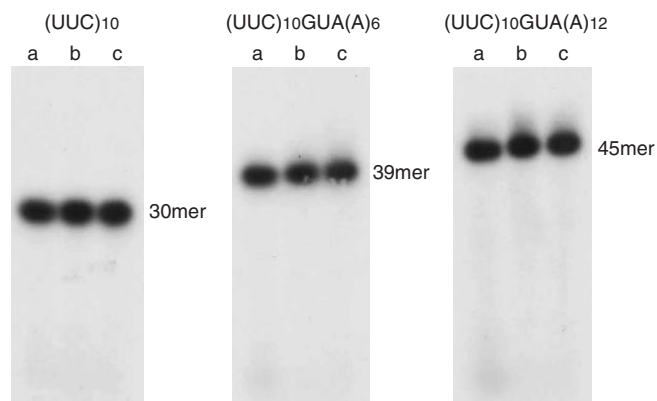


Figure 7. Detection of the degradation of mRNA in the stalled complex of ribosome. The 5' end of synthetic mRNA was labeled with γ -[32 P]ATP by T4 polynucleotide kinase (TaKaRa). The amount of mRNAs was photometrically quantified at a wavelength of 260 nm. The stalled complex of ribosome was formed in a 20 μ l of reaction mixture containing 0 pmol (lane a) or 30 pmol ribosome (lanes b and c), 3 pmol 32 P-labeled-mRNA, 80 pmol EF-Tu, 3 pmol EF-G, 10 pmol [14 C]phenylalanyl-tRNA^{Phe}, 0 pmol (lanes a and b) or 5 pmol [3 H]alanyl-tmRNA (lane c), 2 mM spermidine, 1 mM ATP, 0.2 mM GTP, 80 mM Tris-HCl (pH 7.5), 30 mM ammonium chloride, 5 mM magnesium acetate and 2.5 mM DTT. After incubation at 37°C for 10 min, mRNA was electrophoresed on 12% polyacrylamide gel and visualized on an X-ray film.

of mRNA was involved in the stalled complex of ribosome, judging from the amount of alanine incorporated. If (UUC)₁₀GUA(A)₆ or (UUC)₁₀GUA(A)₁₂ were cleaved at the A site of the stalled complex of ribosome, a band of 30–33mer should appear. However, no band was detected around this position (Figure 7, lane b). Besides, no significant change in the band pattern was observed when the ribosome was removed from the reaction mixture (Figure 7, lane a). We also confirmed that the addition of alanyl-tmRNA did not change the band pattern (Figure 7, lane c). Even after 10-fold longer exposure, no band of cleavage was detected in any lane of Figure 7. These results indicate that no endoribonucleolytic cleavage of mRNA occurs around the A site in the stalled ribosome in our *in vitro* system and that alanyl-tmRNA can accept peptide from peptidyl-tRNA for *trans*-translation without any cleavage of mRNA around the A site in the stalled ribosome.

DISCUSSION

Poly(U)-dependent *in vitro trans*-translation systems using the *E. coli* S30 fraction (8) and using pure translation factors (38) have already been developed. In the present study, we developed an *in vitro trans*-translation system coupled with translation directed by a (UUC)₁₀-based synthetic mRNA using pure translation factors. Compared with poly(U), (UUC)₁₀ has an advantage for specifying the reading frame. In our system, peptidyl-transfer to alanyl-tmRNA for *trans*-translation efficiently occurred in the presence of mRNA, ribosome, aminoacyl-tRNA, EF-Tu, EF-G, alanyl-tmRNA and SmpB. Using this system, we studied the competitions at a codon in the A site of the stalled ribosome between alanyl-tmRNA and RF, between alanyl-tmRNA and aminoacyl-tRNA and between alanyl-tmRNA and RRF. We found that *trans*-translation by tmRNA can operate on sense and termination codons at the A site at which alanyl-tmRNA competes with aminoacyl-tRNA and RF, respectively, and that the peptidyl-transfer step of *trans*-translation does not absolutely require an mRNA truncated at the A site but prefers an mRNA of a shorter 3'-extension from the A site. The addition of RF3 had almost no effect on the competition between RF1 and alanyl-tmRNA at a nonsense codon.

Classically, tmRNA has been assumed to target a ribosome with a stalled translation in which the P site but not A site is occupied by mRNA owing to the truncation of its 3'-portion. *Trans*-translation can also occur at tandem rare codons on an artificial mRNA (14). Full-length and C-terminally truncated polypeptides with a tag sequence at the C-terminus have also been identified as endogenous products of *trans*-translation in the cell (15,16). These *in vivo* data suggest that *trans*-translation can occur when translation elongation or termination is stalled, although whether these tagged-polypeptides are produced from an intact or truncated mRNA in the cell is unknown. It has been shown recently that bacterial toxins such as RelE or ChpAK/MazF cleave an mRNA specifically at the A site in the stalled ribosome, providing a new concept that mRNA of stalled translation is targeted initially by these A site-specific endoribonucleases and subsequently by tmRNA for *trans*-translation (18,19). More recently, cleavage of mRNA at the A site has been detected even in cells lacking these bacterial toxins and some cellular ribonucleases, raising the possibility that the ribosome itself is directly involved in the cleavage of mRNA at the A site (39–41). The present *in vitro* results clearly showed that cleavage of mRNA around the A site did not occur in the stalled ribosome and that, notwithstanding this, peptidyl-transfer for *trans*-translation efficiently occurred. It might be true that mRNA in the A site of the stalled ribosome in the cell is also a target for some kinds of nucleases, and indeed RelE has been shown to greatly enhance the efficiency of *trans*-translation (24). At the A site codon of the intact mRNA in the stalled ribosome in the cell, aminoacyl-tRNAs, release factors, alanyl-tmRNA and A site-specific nucleases would always compete with one another, and consequently alanyl-tmRNA would enter the ribosome both before and after the cleavage of mRNA around the A site. The competition should significantly be influenced by the activities or levels of these molecules in the cell, which fluctuate with change in the physiological conditions; bacterial toxins are activated upon starvation of amino acids (18,19), while tmRNA is induced under some stressful conditions (42).

The present study showed the competition between tmRNA and a release factor at a termination codon, which has been suggested by recent *in vivo* (43) and *in vitro* (41) studies. We also showed that the efficiency of peptidyl-transfer for *trans*-translation depends on the context of the stop signal. Furthermore, the competition with aminoacyl-tRNA at a sense codon was also shown. Apparently tmRNA prefers a shorter 3'-extension as shown in Figure 1C. Besides, the preference for the length of the 3'-extension of mRNA from the A site was different between alanyl-tmRNA and cognate aminoacyl-tRNA. The entrance of alanyl-tmRNA into the A site may be more critical to the length of the 3'-extension of mRNA than that of aminoacyl-tRNA.

A crystal structure of the ribosome in complex with an mRNA and tRNAs has shown that the downstream tunnel of the small subunit covers the downstream of mRNA encompassing positions +7 to +15 (44). Thus, the downstream tunnel is covered almost entirely by (UUC)₁₀GUA(A)₁₂, partially by (UUC)₁₀GUA(A)₆ and not by (UUC)₁₀GUA. mRNA with a short 3'-extension may be transiently detached from the A site owing to a weak interaction with the downstream tunnel, whereas mRNA with a long 3'-extension can be fixed to the A site owing to a sufficient interaction with the downstream tunnel. Consequently, the present observation described above can be rationalized, assuming that the region at the A site or its proximity in the small subunit required for interaction with alanyl-tmRNA is sequestered extensively by mRNA with a long 3'-extension but only weakly by mRNA with a short 3'-extension. Interaction between tmRNA and the proximity of the A site for *trans*-translation has been suggested (45,46), although such an interaction has not been found in a complex of tmRNA/SmpB/ribosome/EF-Tu/GTP/kirromycin, presumably reflecting the initial binding of the tmRNA/SmpB/EF-Tu/GTP complex with the ribosome (47).

RRF together with EF-G facilitates ribosome recycling after the release of polypeptides by promoting the dissociation of deacylated tRNA in the P site and/or the dissociation of the 50S subunit from the posttermination complex (22,48). Several structural studies have shown that RRF, unlike tRNA, locates across the A site of the 50S subunit, although it is a structural mimic of tRNA (49–51). Apparently, the sites of RRF, aminoacyl-tRNA and alanyl-tmRNA on the ribosome overlap each other. The empty A site of the posttermination complex in which the P site is occupied by a deacyl-tRNA is a preferable substrate for RRF (52). Our results obtained by using a stalled complex of ribosome with a GUA codon at the A site suggest that RRF does not substantially interfere with the binding of cognate aminoacyl-tRNA to the A site during the canonical elongation processes. This is consistent with an earlier finding that the affinity of the cognate EF-Tu/GTP/aminoacyl-tRNA ternary complex to the A site is much higher than that of RRF (53). In the present study, we further demonstrated that RRF inhibits alanine incorporation by alanyl-tmRNA, although only at very high concentration. It is consistent with a previous finding that RRF binds, albeit with very low affinity, to the ribosome in which the P site is occupied by a peptidyl-tRNA (53). The efficiency of peptidyl-transfer for *trans*-translation (Figure 1C) was dependent on the length of the 3'-extension of mRNA on the stalled ribosome, whereas the inhibition of peptidyl-transfer for *trans*-translation by RRF was not (Figure 4). It has been shown

in vivo that drop-off of mRNA from the stalled ribosome is enhanced by RRF in combination with peptidyl-tRNA hydrolase (54–56). The recognition of the stalled ribosomes with different lengths of 3'-extension of mRNA by RRF has yet to be investigated.

The present study demonstrates *in vitro* that the *trans*-translation system can efficiently operate on a stalled ribosome having an mRNA with a short 3'-extension with competition of elongation or termination of translation. Several other factors, such as A site-specific nucleases or peptidyl-tRNA hydrolase, should also be involved in releasing a stalled ribosome having an intact mRNA with a long 3'-extension *in vivo*.

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