Title

Transfer RNA synthesis-coupled translation and DNA replication in a reconstituted transcription/translation system

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Abstract

Transfer RNAs (tRNA) are key molecules involved in translation. *In vitro* synthesis of tRNAs and their coupled translation are important challenges in the construction of a self-regenerative molecular system. Here, we first purified EF-Tu and ribosome components in a reconstituted translation system of *Escherichia coli* to remove residual tRNAs. Next, we expressed 15 types of tRNAs in the repurified translation system and performed translation of the reporter luciferase gene depending on the expression. Furthermore, we demonstrated DNA replication through expression of a tRNA encoded by DNA, mimicking information processing within the cell. Our findings highlight the feasibility of an *in vitro* self-reproductive system in which tRNAs can be synthesized from replicating DNA.

Introduction

Cell-free or *in vitro* synthetic biology aims to reconstitute the biological functions from defined molecules, such as DNA, RNA, and proteins, to reveal the design principles of living systems and also provide new biotechnology[1–14]. To date, various cellular functions have been constructed, such as gene expression[15], energy metabolism[16], DNA replication[17–19], and membrane synthesis[20,21]. Despite much progress in this regard, however, the construction of a self-reproductive system, which requires regeneration of the gene expression system from the gene expression system itself, remains a significant challenge[22–25].

One of the requirements for reconstituting a self-regenerative system is the reproduction of tRNA—a key molecule that links the genetic code to amino acids in a protein. In *Escherichia coli*, a set of tRNAs with 41 different anticodons serves this function[26], although, theoretically, 21 tRNAs are sufficient for encoding the 20 amino acids and formylmethionine. In previous studies, *in vitro* translation using 21 chemically synthesized[27] or *in vitro*-transcribed[28,29] tRNA sets has been demonstrated. However, in

these studies, tRNAs were synthesized and purified before being used for translation. The next important step for realizing a tRNA regeneration system is a one-pot reaction, in which tRNA expression is coupled to translation.

In the present study, we first repurified two components of a reconstituted translation system of *E. coli* (PURE system)[15] to eliminate native tRNA contamination. Using the resultant "tRNA-free" PURE system, we demonstrated the tRNA expression-coupled translation of luciferase gene for each of the 15 tRNAs. In addition, we simultaneously performed all 15 tRNAs expression-coupled translation. Furthermore, we succeeded in the self-replication of DNA encoding $tRNA^{Ala}$ through tRNA expression. Our findings provide a step forward in the realization of a tRNA-reproducible system.

Results

Preparation of 21 *in vitro*-synthesized (IVS) tRNAs

In previous studies[28,29], tRNAs were enzymatically transcribed using T7 RNA polymerase as those with extra 5′ sequences, followed by the removal of the extra sequences using RNaseP. This processing step is essential because T7 RNA polymerase requires guanine as the first transcription site[30,31], and some tRNAs do not start with guanine. In another study, tRNAs were chemically synthesized[27]. In the present study, as a simpler and more economic method, 15 tRNAs with guanine at the 5′-end were prepared through *in vitro* transcription, and the other 6 tRNAs were chemically synthesized (see Supplemental text for detail). To improve the *in vitro* transcription yield of the 15 tRNAs, we modified the composition of the reaction mixture. A composition with increased NTP and magnesium concentration and GMP supplementation enhanced RNA yield by 1.5-fold compared with the manufacture's original composition (Fig. S1). After purification, the 15 *in vitro*-transcribed tRNAs and the 6 chemically synthesized tRNAs were used as 21 IVS tRNAs.

Translation with the 21 IVS tRNAs

We first examined the effect of total concentration of the 21 IVS tRNAs on translation. The mixture of 21 IVS tRNAs used here contained 2-time higher total proportions of $tRNA^{Ile}$, $tRNA^{Pro}$, $tRNA^{Glu}$, and $tRNA^{Asn}$ (IPEN) than the mixture of other 17 tRNAs, because these proportions are known to increase translation in a previous study[28]. As a reporter, we used the firefly luciferase gene, which harbors codons corresponding to the 21 IVS tRNA. We incubated the PCR fragment (1 nM) containing the reporter gene under the T7 promoter in the customized PURE system containing different concentrations of the 21 IVS tRNAs or native *E. coli* tRNAs at 30°C for 16 h and measured luminescence produced by the translated luciferase (Fig. 1). Luminescence was the highest around the total tRNA concentration of 0.5 $\mu g \cdot \mu L^{-1}$ and gradually decreased with increase in the concentration of both native and IVS tRNAs. Comparing the highest points, the translation activity of the 21 IVS tRNAs was approximately 31% of the activity of native tRNAs.

Next, we attempted to couple translation with tRNA expression from DNA. First, we assessed the translation activity in a PURE system without one of the 21 IVS tRNAs ($tRNA^{Cys}$, $tRNA^{His}$, $tRNA^{mMet}$, or $tRNA^{Ser}$). Unexpectedly, the activity was almost the same as that including all 21 tRNAs (Fig. S2), suggesting that the omitted tRNAs were carried over from some components of the PURE system. To confirm this possibility, we subjected the protein components of PURE systems to urea-PAGE and detected substantial tRNA concentration in our laboratory-made PURE system and commercial PURExpress (Fig. 2A). The residual concentrations were 34 and 46 $ng \cdot \mu L^{-1}$ in the laboratory-made PURE system and PURExpress, respectively, corresponding to approximately 10% of the usual tRNA concentration in PURE systems.

Removal of tRNAs from the protein components of the PURE system

We then removed carried over tRNA from the laboratory-made PURE system. First, we performed urea–PAGE analysis of all protein components of the PURE system to identify components containing tRNA. Bands corresponding to tRNA were detected in only two components: EF-Tu and 70S ribosomes (Fig. S3). According to band intensity, the residual tRNA in EF-Tu corresponded to 0.006 tRNA molecule per EF-Tu molecule. We hypothesized that such a low frequency of carry-over could be caused by insufficient washing. Thus, the purified EF-Tu was washed with a high-stringency buffer, which contains 1 M KCl and 1% Triton X-100, and repurified using a nickel affinity column. The resulting repurified EF-Tu contained undetectable tRNA (Fig. 2B, left panel). In 70S ribosome, the residual tRNA corresponded to 0.45 tRNA molecule per ribosome molecule. We hypothesized that this high frequency of carry-over could result from tRNA binding inside the 70S ribosome. We then separated the 70S complex into 30S and 50S subunits and purified each subunit using sucrose-gradient ultracentrifugation. The concentration of residual tRNA in the resultant 30S and 50S subunits was significantly reduced (Fig. 2B, right panel).

Subsequently, using these repurified EF-Tu and ribosomes, we prepared a new PURE system, the protein fraction of which exhibited no detectable tRNA (Fig. 2A, new). We used this “tRNA-free” PURE (tfPURE) system in the following experiments.

To examine the translation activity of the new tfPURE system, we performed luciferase translation reactions with or without the 21 IVS tRNA using the method presented in Fig. 1. Luciferase activity in the experiment without tRNA was at the same level with the control experiment, which lacked all translation proteins of the PURE system (Fig. 2C). Meanwhile, luciferase activity of the new tfPURE system with the 21 IVS tRNAs was comparable to that of the original laboratory-made PURE system.

Translation coupled with tRNA expression

Next, we examined whether translation could be coupled with tRNA expression in the same reaction mixture using the tfPURE system. The reaction scheme is presented in Fig. 3A. Two types of linear DNA templates were used in this reaction: one encoding reporter luciferase and the other encoding each tRNA (tRNA template). In this experiment, we used only one of the 15 tRNAs with G at the 5′-end. The tRNA templates encoded each tRNA sequence directly downstream of the T7 promoter, and the 3′-end was the same as the end of the tRNA. The remaining 20 tRNAs that were not encoded by the tRNA template were added as purified IVS tRNAs to the reaction mixture. If the tRNA encoded by the tRNA template is properly expressed with T7 RNA polymerase, luciferase should be translated. Based on this scheme, we mixed one of the tRNA templates (50 nM), DNA template for luciferase (1 nM), and T7 RNA polymerase (1.67 $U \cdot \mu L^{-1}$) in the tfPURE system and incubated the mixture at 30°C for 16 h. As control, the same experiment was performed without the tRNA template. Luciferase activity with all tRNA templates, except $tRNA^{Glu}$ and $tRNA^{Val}$, was higher than that without the tRNA template (Fig. 3B), indicating that the 13 tRNAs were expressed in their active form by their respective template DNA. For $tRNA^{Cys}$, luciferase activity without the tRNA template was higher than that with the other tRNAs perhaps due to the much smaller number of cysteine residues in luciferase than in other amino acids (only 4 in 550 amino acids, see supplemental text); hence, the remaining undetectable level of residual $tRNA^{Cys}$ may be sufficient for luciferase translation.

For $tRNA^{Glu}$ and $tRNA^{Val}$, no differences were observed with or without the tRNA template (Fig. 3B). We speculate that this may be caused by insufficient tRNA expression, as NTP concentration in this experiment was decreased to approximately $1/10^{th}$ of that in the original PURE system[15]. NTP reduction is aimed at coupling this reaction with DNA replication in the next experiment, because

DNA replication proceeds more efficiently at a low NTP concentration[32]; however, this was not necessary in the present experiment. Hence, we increased NTP concentration from 0.88 to 3.1 mM and magnesium acetate concentration from 7.9 to 14 mM. Consequently, for both $tRNA^{Glu}$ and $tRNA^{Val}$, luciferase activity was significantly higher with the tRNA template than without it (Fig. 3C), indicating that these two tRNAs were expressed in their active form under this condition.

Simultaneous expression of the 15 tRNAs

We tested whether translation proceeded when all 15 tRNAs that started with guanine were simultaneously expressed. The reaction scheme is presented in Fig. 4A, in which 15 tRNAs are expressed from the 15 tRNA templates for translation of the luciferase. As the first trial, all 15 tRNA templates at the same concentration (total 200 nM) and a DNA fragment encoding luciferase gene (1 nM) were incubated in the tfPURE system containing the other 6 IVS tRNAs ($tRNA^{Met}$, $tRNA^{Ile}$, $tRNA^{Trp}$, $tRNA^{Gln}$, $tRNA^{Asn}$, and $tRNA^{Pro}$), T7 RNA polymerase (3.3 $U \cdot \mu L^{-1}$), NTP (5.3 mM), and magnesium acetate (16.2 mM). Under these conditions, luminescence was 30, which was 3.4 times higher than that without tRNA templates (Fig. 4B, Equal, NTP & T7RNAP increase−). Next, we increased T7 RNA polymerase (4.2 $U \cdot \mu L^{-1}$), NTP (9.6 mM), and magnesium acetate (20.6 mM) concentrations and found that luminescence increased to 630, which was 21-fold higher than that before the increase (Fig. 3B, Equal, NTP & T7RNAP increase+), although the activity was still much lower than that with all 21 IVS tRNAs (~10^7; Fig. 3B, IVS tRNA).

To further increase translation with simultaneous tRNA expression, we modified the composition of the 15 tRNA templates based on the required expression level of each tRNA. We hypothesized that the results of individual tRNA expression in Figs. 3B and 3C would roughly represent the required expression level for each tRNA; if translation with a tRNA expression is lower, the required expression

level for the tRNA would be higher. Based on this notion, we divided the 15 tRNAs into four groups (groups A–D in Fig. 3B) in the order of the presumed required level. The concentration of each tRNA template was adjusted to balance the expression level as follows: 4 nM for group A, 8 nM for group B, 20 nM for group C, and 50 nM for group D. With this "balanced" tRNA template mixture, luminescence increased to 3.4×10^5 before the increase in NTP and T7 RNAP concentration and to 5.7×10^6 after the increase (Fig. 4B, balanced). The highest luciferase activity was approximately half of that with all 21 IVS tRNAs in tfPURE system (Fig. 3B, IVS tRNA).

To measure the concentration of synthesized tRNA, the reaction mixtures were subjected to urea–PAGE analysis, followed by SYBR Green II staining (Fig. S5). The intensities of tRNA bands were increased for samples with tRNA templates (lanes 2–5) compared to those for samples without tRNA templates (lane 1). In particular, high-molecular-weight tRNA bands (indicated with white arrowheads, corresponding to $tRNA^{Leu}$, $tRNA^{Tyr}$, and $tRNA^{Ser}$) were not detected in samples without tRNA templates but detected in samples with tRNA templates. Moreover, the tRNA concentration estimated from band intensity (shown under the panel) was higher for samples with tRNA templates (0.87–1.2 $\mu g\cdot\mu L^{-1}$) than for those without tRNA templates (0.38 $\mu g\cdot\mu L^{-1}$), and that the difference (i.e., 0.49–0.82 $\mu g\cdot\mu L^{-1}$) was attributed to newly synthesized tRNAs from the tRNA templates. The concentration of newly synthesized tRNA was equivalent to the total tRNA concentration that allows the highest translation, as shown in Fig. 1 (0.6 $\mu g\cdot\mu L^{-1}$), indicating that a sufficient amount of total tRNA could be synthesized in this reaction.

Replication of the tRNA gene coupled with its own expression

To establish an artificial self-regenerating system, one of the possible future directions is coupling tRNA expression with the replication of the tRNA gene. A major hurdle is processing of the 3′-end of

tRNA. In the above experiments, we used linear tRNA templates, whose 3′-ends matched the 3′-ends of the tRNA; thus, 3′-end processing was unnecessary. However, for coupling with DNA replication, 3′-end processing is required, because some DNA replication schemes require a circular DNA[33,34]. Moreover, even in the linear DNA replication scheme[35], tRNA genes that are not at the 3′-end of the DNA require 3′ processing. In this experiment, we employed a previously established artificial circular DNA replication scheme[18], in which a gene encoded in circular DNA is replicated through a rolling circle replication scheme with phi29 DNA polymerase. The problem was how to express tRNA with a defined 3' end from a circular DNA template.

To solve this problem, we inserted a nick in only one strand of circular DNA while keeping the other strand circular for rolling circle replication. Nicking the strand allowed the expression of tRNA with the correct 3′-end. The overall reaction scheme is presented in Fig. 5A. The $tRNA^{Ala}$ gene was encoded in circular DNA under the T7 promoter, and the Nt.BspQI recognition site was placed downstream of the tRNA 3′-end. After treating the circular DNA with Nt.BspQI to introduce a nick at the 3′-end of the tRNA gene, the circular DNA (5 nM) was incubated in the tfPURE system containing 20 IVS tRNAs other than $tRNA^{Ala}$, mRNA encoding phi29 DNA polymerase (200 nM), and T7 RNA polymerase (1.67 $U \cdot \mu L^{-1}$). T7 RNA polymerase transcribed the nicked circular DNA to synthesize $tRNA^{Ala}$, which was then used for translation by phi29 DNA polymerase. The DNA polymerase proceeded with rolling-circle replication using circular DNA as the template to synthesize long linear single-stranded DNA with repeated sequences. DNA polymerase also synthesized a complementary strand, resulting in the replication of a long linear double-stranded DNA harboring multiple $tRNA^{Ala}$ genes.

The reaction was performed at 30°C for 16 h. Before and after incubation, aliquots of the reaction

mixtures were treated with DpnI to selectively degrade the original, methylated circular DNA. The concentration of tRNA genes in the newly synthesized DNA was measured using quantitative PCR (Fig. 5B). As control experiments, we performed reactions without any translation proteins (Translation proteins -), and with a nicked circular DNA that does not encode tRNA ($tRNA^{Ala}$ gene -). In all the control experiments, the DNA concentration was similar before (0 h) and after (16 h) incubation (Fig. 5B). In the presence of translation proteins and tRNA genes, the DNA concentration significantly increased after 16 h of incubation, indicating that the tRNA gene was replicated depending on tRNA expression and translation. The replicated DNA concentration was approximately 0.3 nM, corresponding to 6% the initial concentration of the circular DNA. Although there is room for improvement, this result suggests that tRNA genes can indeed be replicated through their own expression.

Discussion

In the present study, we established repurification methods to remove contaminated tRNAs from the conventional PURE system. Using the resultant highly purified PURE system, we successfully demonstrated luciferase translation coupled with tRNA expression for a single (Fig. 3) or all of (Fig. 4) the 15 tRNAs with guanine at their 5′-end. We also highlighted the importance of balancing the tRNA template concentration and increasing the NTP, magnesium acetate, and T7 RNA polymerase concentrations for efficient tRNA expression. Our results support the feasibility of *in vitro* translation coupled with tRNA expression. We further demonstrated the replication of the $tRNA^{Ala}$ gene through its own expression using half-nicked circular DNA, demonstrating that DNA replication can also be coupled with tRNA expression. These findings provide a step forward to a self-regenerative artificial system in which tRNAs are produced from replicating DNA.

The chemical group at the 5′-end of IVS tRNAs used in the present experiment was different from that of the native *E. coli* tRNAs. While the 5′-ends of native tRNAs are monophosphates[36], the 5′-ends of our six chemically synthesized tRNAs were hydroxylated, and those of our 15 tRNAs synthesized by T7 RNA polymerase were a mixture of triphosphates and monophosphates because we performed *in vitro* transcription in the presence of GMP, which increases monophosphates at 5′-ends[37]. Although sufficient level of translation proceeded with such mixed state of the 5′-end as demonstrated in this study, end correction may further improve translation.

In the present study, we demonstrated translation coupled with the expression of 15 tRNAs. The next important challenge is the expression of all 21 tRNAs. For this challenge, the six tRNAs starting with non-guanine nucleotides must be expressed through *in vitro* transcription. A possible method is to use RNase P, which cuts a certain position of RNA[36,38]. Another possibility is the addition of self-cleaving ribozymes, such as hammerhead ribozymes, at the 5′-end of tRNAs[29,39]. Further modifications of the sequence and experimental conditions would be required for these methods to be used in the tfPURE system.

Another relevant problem is 3′-end processing, which is important for coupling tRNA expression with DNA replication. In the present study, we inserted a nick in a one strand of circular DNA, such that tRNA with the correct 3′-end was synthesized while retaining the ability for rolling-circle replication using the other circular strand as the template (Fig. 5). This half-nicking strategy was successful and can be applied to other tRNAs. However, the replication efficiency was not high (approximately 6% replication) under the current conditions. A possible method for improving replication efficiency is *in vitro* evolution. Recently, we found that the replication efficiency of circular DNA spontaneously

improves through long-term replication due to Darwinian evolution[18]. A similar long-term replication would allow the evolution of highly replicable template DNA encoding tRNA. Another advantage of *in vitro* evolution is the improvement of tRNA sequences. The native tRNA were chemically modified and their sequences were adapted to the condition in the cell. Under the present *in vitro* conditions, in which tRNAs were not chemically modified and the condition significantly differed from that within the cell, the optimum tRNA sequences may be different. For the evolution of tRNAs that can adapt to *in vitro* conditions, tRNA gene replication must be coupled with tRNA expression, as demonstrated in the present study. Our work paves the way for an evolutionary method to obtain tRNAs suitable for *in vitro* conditions.

Materials and Methods

DNA preparation

For luciferase expression experiments, the luciferase DNA was prepared through PCR amplification using a plasmid (pUC-T7p-Fluc_21tRNA) as the template with primers 1 (GGCGATTAAGTTGGGTAACGCCAG) and 2 (CCGGCTCGTATGTTGTGTGG). The plasmid was constructed through artificial synthesis of the firefly luciferase gene, which harbored only the codon set for the 21 tRNAs. The plasmid (pUC-T7p_DNAP_21tRNA) used for the synthesis of RNA encoding phi29 DNA polymerase was constructed by artificially synthesizing the phi29 DNA gene, which harbored only the codon set for the 21 tRNAs. The circular DNA used in Fig. 5 was prepared by treating the plasmid (pUC-tRNA-Ala-GGC_NtBspQI) with Nt.BspQI (NEB) at 50°C for 2 h, followed by purification using the QIAquick column (QIAGEN). The plasmid (pUC-tRNA-Ala-GGC_NtBspQI) was constructed by artificially synthesizing the $tRNA^{Ala}$ sequence and inserting it into the pUC19 vector together with the Nt.BspQI site. The original pUC19 vector contained another Nt.BspQI site, which was deleted before inserting the tRNA sequence. The entire sequence of the

plasmids is provided in the supplemental text.

Preparation of IVS tRNAs

Sequences of the 21 IVS tRNAs were based on a previous study[28]. Fifteen tRNAs with G at the 5′-end (Ala, Arg, Asp, Cys, Gly, Glu, His, Leu, Lys, mMet, Phe, Ser, Thr, Tyr, and Val) were prepared through *in vitro* transcription. The remaining six tRNAs were chemically synthesized (Invitrogen). The template DNAs for *in vitro* transcription was prepared using PCR with a plasmid encoding each of the tRNAs[28] as the template and primers listed in supplemental text. Each reverse primer contained a 2′-O-methylation site at the second nucleotide from the 5′-end to prevent nucleotide addition by T7 RNA polymerase[40]. The reaction mixture for *in vitro* transcription contained 5 mM DTT; 1 $U \cdot \mu L^{-1}$ T7 RNA polymerase (Takara, Japan); 6 mM each of ATP, GTP, CTP, and UTP; 3 mM GMP; 40 mM Tris–HCl (pH 8.0), 18 mM magnesium chloride; 2 mM spermidine; 5 $\mu g \cdot \mu L^{-1}$ PCR products; 2 $U \cdot mL^{-1}$ inorganic pyrophosphatase (NEB); and 0.8 $U \cdot \mu L^{-1}$ RNasin (Promega). Reactions were performed at 37°C overnight in 400 μL for tRNAs with 5′-GG tRNAs and in 550 μL for those with 5′-G. The transcribed tRNAs were purified using the PureLink RNA Mini Kit (Invitrogen). tRNAs were dissolved in water and stocked at -80°C until use. tRNA and DNA concentrations were quantified based on A260. The RNA encoding phi29 DNA polymerase was prepared using the same protocol as described above, except that the template DNA was prepared through PCR amplification using plasmid (pUC-T7p_DNAP_21tRNA) as the template and primers 1 and 2 mentioned above.

Preparation and repurification of protein components for the tfPURE system

All components of the laboratory-made PURE system were prepared as previously described[18]. EF-Tu was purified using a nickel column. For EF-Tu repurification, purified EF-Tu was 10-fold diluted with a stringent buffer [50 mM HEPES–KOH (pH 7.6), 1 M KCl, 10 mM $MgCl_2$, 15% glycerol, 1

mM DTT, and 1% Triton X-100] and allowed to stand on ice for 1 h. The solution was injected into to the HisTrap column (Thermo Fisher Scientific) and washed with the same buffer, omitting Triton X-100. EF-Tu was eluted using a 0–250 mM imidazole gradient. This purification process was repeated once. Protein-containing fractions were collected and stored following buffer exchange with the stock buffer [50 mM HEPES–KOH (pH 7.6), 100 M KCl, 10 mM MgCl2, 30% glycerol, and 7 mM 2-mercaptoethanol) using a 30 kDa Amicon Ultra centrifugal filter. For ribosome repurification, 30S and 50S were separated by three rounds of 10–30% sucrose gradient centrifugation according to a previous method[41].

Urea–PAGE

Electrophoresis was performed using 8% (acrylamide:bis = 19:1) polyacrylamide gel containing 8 M urea, 0.1% ammonium persulfate, and 0.1% N,N,N',N'-tetramethylethylene-diamine in Tris–borate EDTA buffer. The samples were prepared by mixing with stripping buffer containing 50 mM EDTA, 90% formamide, and 0.025% bromophenol blue. RNA was stained with SYBR Green II (Takara, Japan).

Translation assays with the 21 IVS tRNAs

The reaction mixture containing the indicated concentrations of tRNAs, luciferase DNA (1 nM), T7 RNA polymerase (0.42 $U \cdot \mu L^{-1}$, Takara, Japan), NTP (0.88 mM), magnesium acetate (7.9 mM), and laboratory-made PURE system omitting tRNA was incubated at 30°C for 16 h. An aliquot (1 μL) was added to 30 μL of luciferase assay reagent (Promega), and luminescence was measured with the GloMax Luminometer (Promega).

tRNA synthesis-coupled translation with the PURE system

The reaction mixture containing luciferase DNA (1 nM), tRNA template DNA, T7 RNA polymerase, and the tfPURE system with repurified EF-Tu and ribosomes was incubated at 30°C for 16 h. In Fig. 3B, the reaction mixture contained 50 nM tRNA template, 1.67 $U \cdot \mu L^{-1}$ T7 RNA polymerase, and 20 tRNAs (100 $ng \cdot \mu L^{-1}$ each for IPEN and 11.7 $ng \cdot \mu L^{-1}$ each for others) except for the tRNA to be expressed. In Fig. 3C, the NTP and magnesium acetate concentrations in the reaction mixture were increased from 0.88 to 3.1 mM and from 7.9 to 14 mM, respectively. In Fig. 4B, the reaction mixture containing luciferase DNA (1 nM), 15 tRNA templates (200 nM in total), and 6 IVS tRNAs (100 $ng \cdot \mu L^{-1}$ each for IPEN and 11.7 $ng \cdot \mu L^{-1}$ each for others) except the tRNA to be expressed was incubated at 30°C for 16 h. Under the "Equal" condition (Fig. 5B), all 15 tRNA templates were added at the equal concentration. Under the "Balanced" condition, the tRNA templates were added at following concentrations: 4 nM for group A, 8 nM for group B, 20 nM for group C, and 50 nM for group D. Under the "NTP & T7 RNAP addition+ " condition, T7 RNA polymerase, NTP, and magnesium acetate concentrations were increased from 3.3 to 4.2 $U \cdot \mu L^{-1}$, from 5.3 to 9.6 mM, and from 16.2 to 20.6 mM, respectively. After incubation, an aliquot (1 μl) was added to 30 μL of luciferase assay reagent (Promega), and luminescence was measured using the GloMax Luminometer (Promega).

tRNA synthesis-coupled DNA replication

The reaction mixture contained circular DNA with a nick (5 nM), T7 RNA polymerase (1.7 $U \cdot \mu L^{-1}$), 20 IVS tRNAs (100 $ng \cdot \mu L^{-1}$ each for IPEN and 11.7 $ng \cdot \mu L^{-1}$ each for others) except for $tRNA^{Ala}$, and RNA encoding phi29 DNA polymerase (200 nM) in the tfPURE system. The concentrations of NTP and magnesium acetate were changed to 3.1 and 14 mM, respectively. The reaction was incubated at 30°C for 16 h. Before and after the incubation, aliquots of the reaction mixtures were 10-fold diluted with a buffer [33 mM Tris–acetate (pH7.9), 10 mM magnesium acetate, 0.5 mM DTT, and 66 mM potassium acetate] and treated with DpnI to degrade the original circular DNA. The reaction mixture

was further diluted 100-fold with 1 mM EDTA (pH 8), and the DNA concentration was measured using quantitative PCR (TB Green Premix Ex TaqII, Takara) with primers 1 and 2 mentioned above. For the control experiments, all translation proteins were omitted, or pUC19 plasmid, which does not contain tRNA gene yet contain a single Nt.BspQI site, was used as a circular DNA after treating with Nt.BspQI as described above.

Conflict of Interest

The authors declare no conflict of interest associated with this manuscript.

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Supporting Information

Supporting Information includes Figures S1-5, Supplemental text (DNA sequences used in this study).

Author contribution

RM and NI plan the experiments and wrote the manuscript. YS prepared the materials and wrote the manuscript. RM performed the experiments.

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Figures

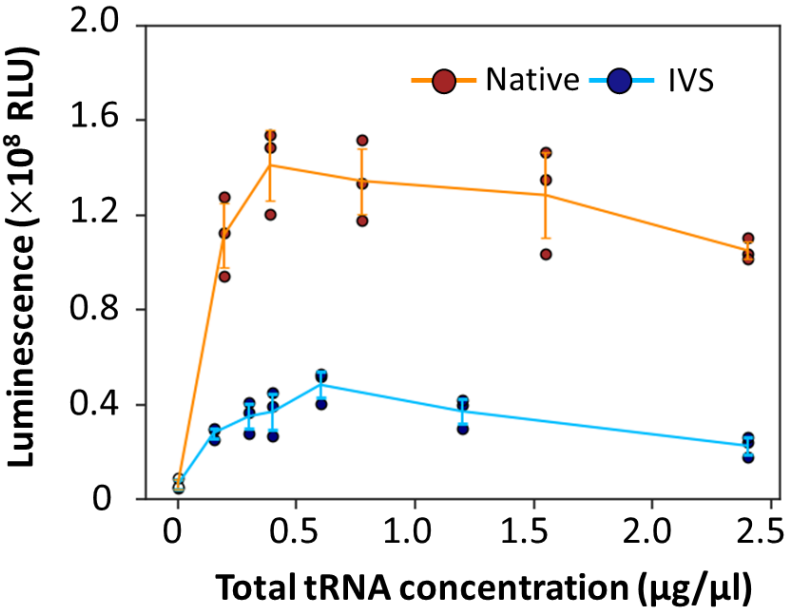

Figure 1. Effect of native and in vitro-synthesized (IVS) tRNAs on luciferase translation

Escherichia coli native tRNAs and 21 IVS tRNAs were mixed in the laboratory-made PURE system. The total proportion of IPEN tRNAs was two times higher than that of other tRNAs. The reaction mixture was incubated at 30°C for 16 h with DNA containing the luciferase gene (1 nM) and T7 RNA polymerase (0.42 U·μL^{-1}), and luminescence was measured. Each dot represents three independent experiments. The error bars represent standard deviation.

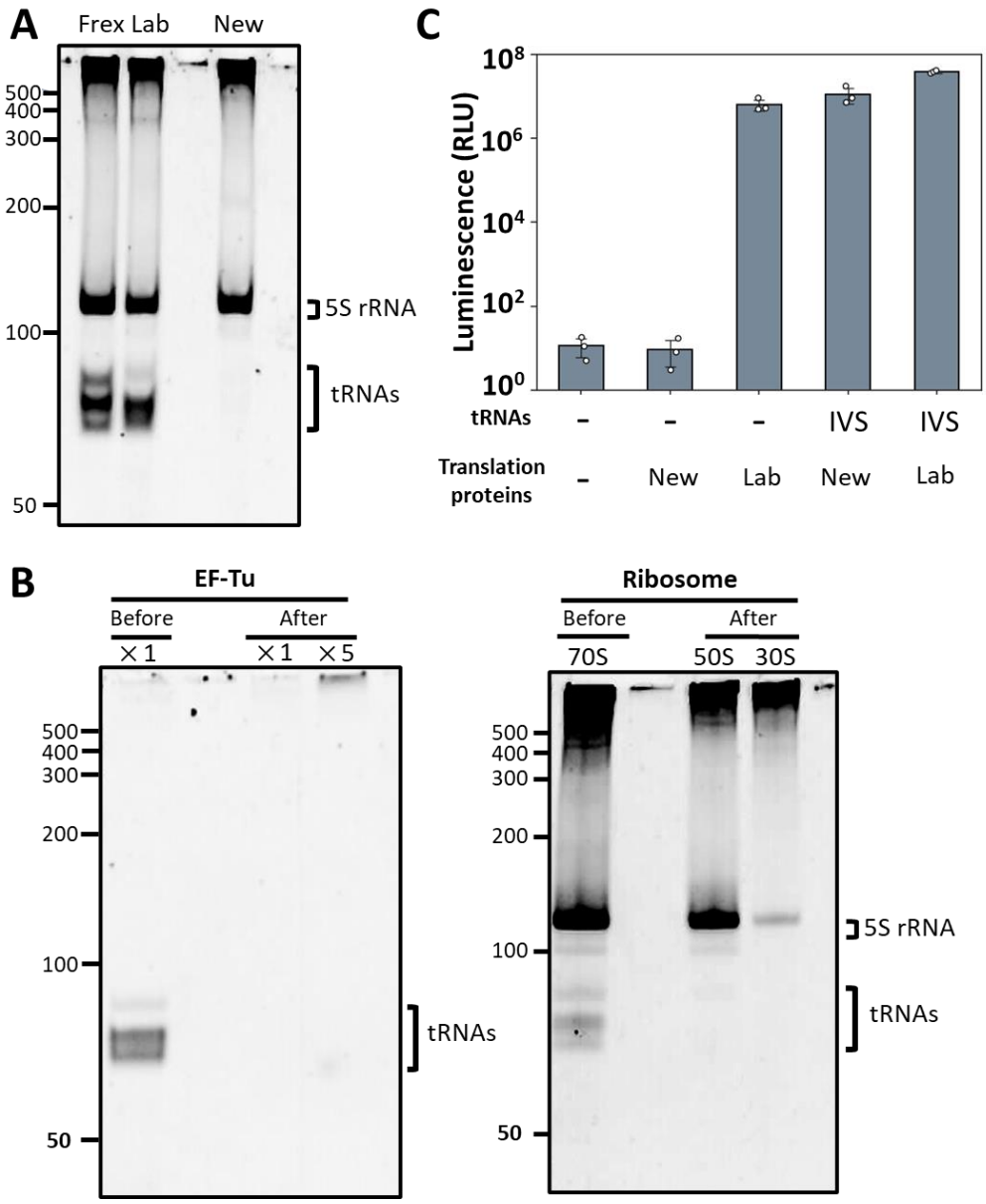

Figure 2. Removal of contaminated tRNA in the PURE system

A) Urea–PAGE analysis of the protein components of different PURE systems. Each lane contains 1 μl equivalent of sol. II and sol. III of PURE frex ver.2 (Frex), the laboratory-made PURE system (Lab), and the new "tRNA-free" PURE (tfPURE) system (New), in which EF-Tu and ribosome were further purified. B) Purified EF-Tu and ribosomes used in the laboratory-made PURE system were subjected to urea–PAGE before and after repurification. In addition to the ×1 amount, ×5 amount was applied for EF-Tu. For ribosome, the original purified 70S and repurified 30S and 50S ribosomes

corresponding to 1 μl PURE system were applied. RNA was stained with SYBR Green II. The whole gel images of A and B are shown in Fig. S4. C) Translation activity of luciferase with the original laboratory-made PURE system (Lab) or new tfPURE system (New). DNA fragment containing the luciferase gene (1 nM) and T7 RNA polymerase (0.42 $U \cdot \mu L^{-1}$) was incubated at 30°C for 16 h in each PURE system, and luminescence was measured. Each dot represents three independent experiments. The error bars represent standard deviation.

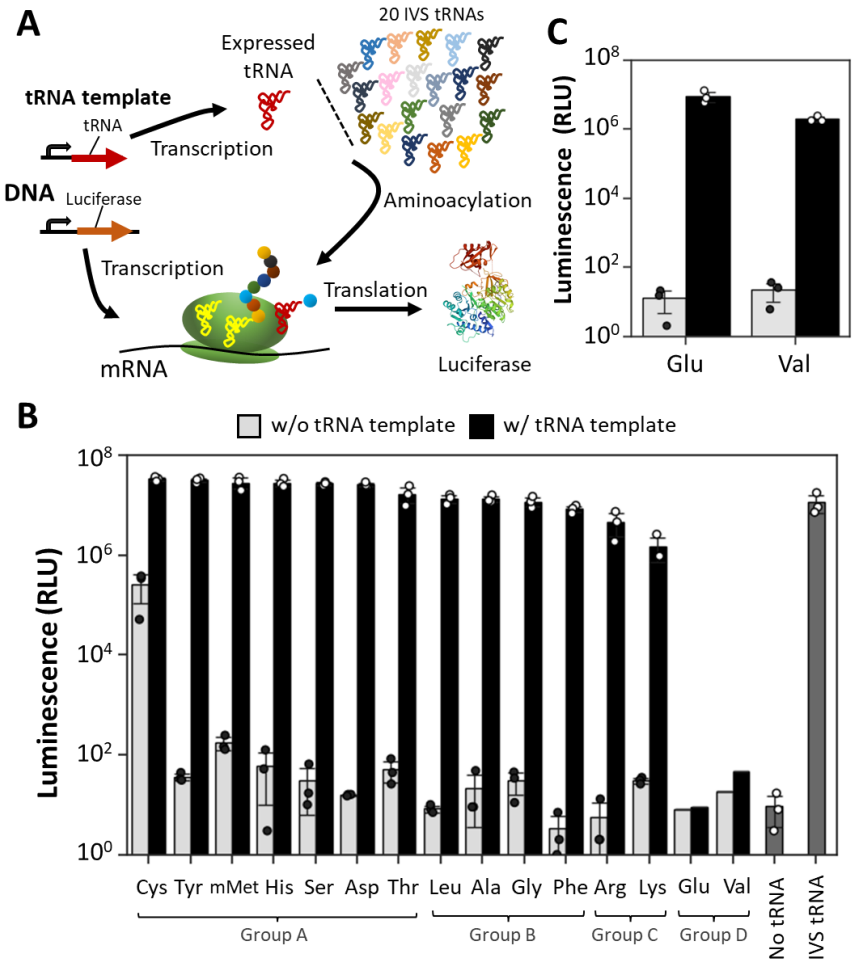

Figure 3. Luciferase translation coupled with one of the 15 tRNA expressions

A) Reaction scheme. One of the 21 tRNA was expressed from the tRNA templates to induce translation of the luciferase gene in the presence of the remaining 20 tRNAs. Luciferase structure is obtained from PDBID:1LCI. B) Luciferase activity after reaction at the original NTP (0.88 mM) and magnesium acetate concentrations (7.9 mM). The reaction mixture comprised a DNA fragment containing one of the 15 tRNAs (tRNA template, 50 nM), a DNA fragment encoding the luciferase gene (1 nM), T7 RNA polymerase (1.7 $U \cdot \mu L^{-1}$), 20 IVS tRNAs, except for the tRNA to be expressed, and the tfPURE system. This reaction mixture was incubated at 30°C for 16 h, and luminescence was measured. The results without (gray bars) and with the tRNA template (black bars) are shown. Results without tRNA (no tRNA) and with 21 IVS tRNAs (IVS tRNA) are also shown for comparison. C) $tRNA^{Glu}$ and

$tRNA^{Val}$ results at increasing NTP and magnesium acetate concentrations. NTP and magnesium acetate were increased to 3.1 and 14 mM, respectively. Each dot represents three independent results. The error bars represent standard deviation.

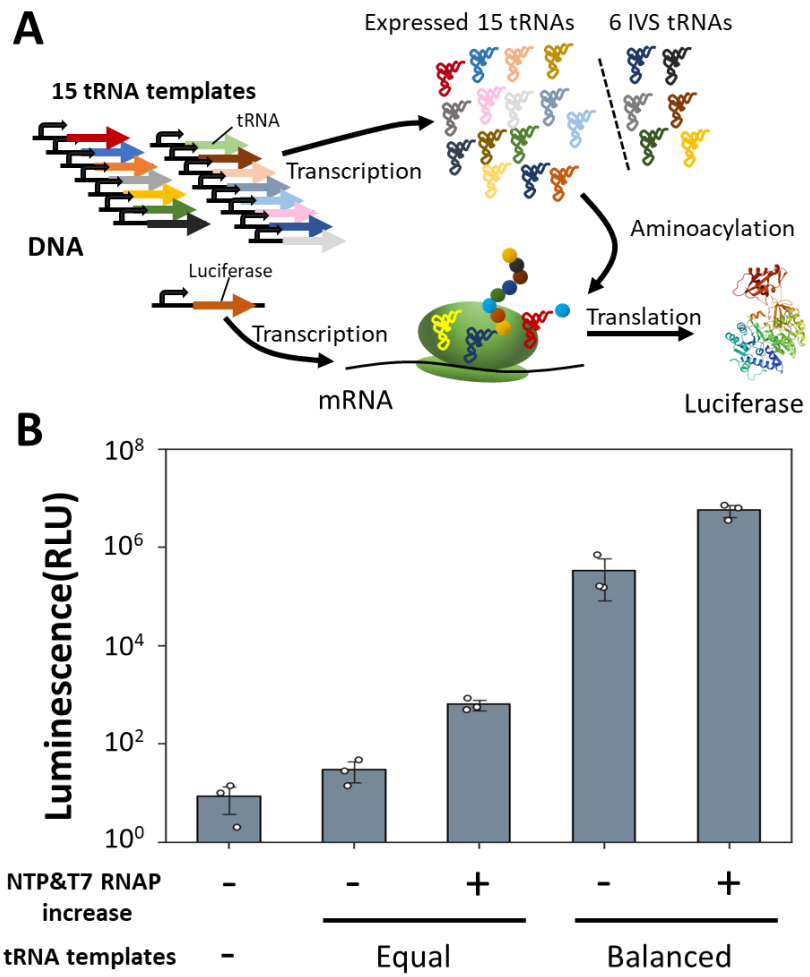

Figure 4. Luciferase translation coupled with the simultaneous expression of 15 tRNAs

A) Reaction scheme. All 15 tRNAs were expressed using the tRNA template to induce the translation of the luciferase gene together with the other six IVS tRNAs. B) Luciferase activity measured after reaction. The reaction mixture contained 15 tRNA template (total 200 nM), DNA encoding luciferase (1 nM), T7 RNA polymerase (3.3 $U \cdot \mu L^{-1}$), the other six IVS tRNAs, and the tfPURE system. The reaction mixture was incubated at 30°C for 16 h, and luminescence was measured. Reaction was performed under the original [5.3 mM NTP, 16.2 mM magnesium acetate, and 3.3 $U \cdot \mu L^{-1}$ T7 RNA polymerase] (NTP & T7RNAP increase −) or NTP & T7 RNA polymerase increased conditions [9.6 mM NTP, 20.6 mM magnesium acetate, and 4.2 $U \cdot \mu L^{-1}$ T7 RNA] (NTP & T7RNAP increase +).

Experiments were performed without the tRNA template (tRNA template−), with 15 different tRNA templates at equal concentration (Equal), and with 15 different tRNA templates at required concentrations (Balanced). Each dot represents three independent results. The error bars represent standard deviation.

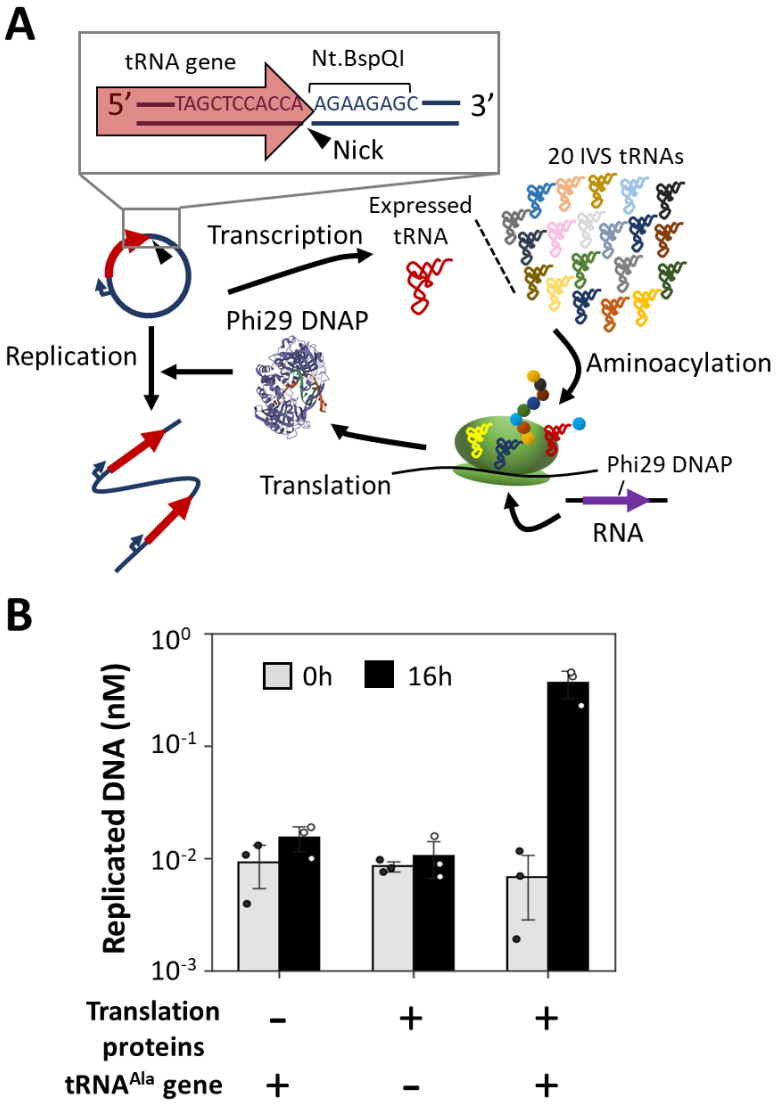

Figure 5. Replication of the tRNA gene coupled with tRNA expression

A) Scheme of tRNA replication. The circular DNA encodes $tRNA^{Ala}$ and harbors a nick at the 3′ termini of the tRNA introduced by Nt.BspQI. The transcription of this nicked circular DNA is expected to produce $tRNA^{Ala}$ with the correct 3′-end. If $tRNA^{Ala}$ is expressed and functions correctly, phi29 DNA polymerase is translated, which starts rolling-circle replication using circular DNA as the template, and linear DNA with repeated sequences is replicated. The structure of phi29 DNA polymerase is from PDBID:2PZS. B) DNA concentration before and after the reaction. The reaction

mixture contained circular DNA (5 nM), T7 RNA polymerase (1.7 $U \cdot \mu L^{-1}$), 20 IVS tRNAs except $tRNA^{Ala}$, mRNA encoding phi29 DNA polymerase (200 nM), and the tfPURE system (3.1 mM NTP and 14 mM magnesium acetate). The mixture was incubated at 30°C for 16 h. Before (0 h) or after (16 h) the reaction, the methylated template circular DNA was selectively degraded with DpnI and the remaining DNA was measured using quantitative PCR with specific primers. As control experiments, reactions without all translation proteins (Translation proteins -) or using a nicked circular DNA that does not encode tRNA ($tRNA^{Ala}$ gene -) were also performed. Each dot represents three independent results. The error bars represent standard deviation.