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A Bifunctional tRNA for In Vitro Selection

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Summary

In vitro selection is a powerful approach for generating novel aptamers and catalysts. Currently, several methods are being developed to extend this technique to proteins. In principle, selection methods could be applied to any library whose members can be replicated. Here, we describe a bifunctional tRNA that fuses translation products to their mRNAs. The utility of peptide-tRNA-mRNA fusions for in vitro selection was illustrated by the selective enrichment of tagged peptides—together with their mRNAs—by affinity chromatography. Our system can generate libraries larger than 10^{11}. Because library members can be copied and amplified, they provide a means for applying in vitro selection procedures to peptides and proteins. Furthermore, because the system is amenable to translation with misacylated tRNAs, a wide range of unusual monomers could be used to make libraries of nonstandard polymers for selection experiments.

Introduction

In vitro selection is regularly used to search libraries of nucleic acids for rare molecules with desirable functions [see references 1–4 for reviews]. Molecules with specific functions are isolated from libraries with more than 10^{15} sequences through iterative rounds of selection and amplification; molecules that fulfill the selective criteria increase in representation, and amplification increases their number. Thus, with each round, the functional molecules replace less capable members of the initial population. Although nucleic acids are endowed with recognition and catalytic potential, the application of in vitro selection to polymers with greater chemical diversity would be beneficial. Toward this end, several methods have been developed to attach peptides and proteins to their encoding DNAs or mRNAs [5–9]. Such fusions contain the essential elements needed for selection and amplification: a potentially active protein and a corresponding nucleic acid sequence that stores the information needed to make copies of the protein. In phage display [5] and plasmid display [6], fusion proteins that associate with their encoding nucleic acids are expressed in vivo; the cell membrane encapsulates corresponding protein and nucleic acid sequences during complex formation. Ribosome display [7] relies on the integrity of stalled translation complexes to maintain a link between an mRNA and its protein product. With mRNA display [8, 9], covalent protein-mRNA fusions are formed on stalled translation complexes; the 3′ ends of mRNAs are modified in such a way that they stall protein synthesis, enter the A site of the ribosome, and act like an aminoacyl tRNA to become attached to their protein products.

We have been developing an alternative method for generating libraries of mRNA-encoded peptides, which also has the potential to work with other peptide-like polymers. In our system, a modified tRNA ($tRNA^x$) acts as a bifunctional crosslinking agent to attach an mRNA to its translation product. For an in vitro selection experiment to succeed, a molecule with some degree of the desired activity must reside in the initial population, and thus library complexity is critical. Completely in vitro methods like ours, ribosome display [7], and mRNA display [8, 9] have an advantage over methods that require the use of cells because library complexity is not limited by transformation efficiency (bacterial transformation currently limits libraries to $\sim 10^9$ [10]). In our system, as with mRNA display, physically linking a peptide to its mRNA by covalent bonds simplifies the purification of peptide-mRNA fusions and increases the range of selectable properties because the integrity of the peptide-nucleic acid complex is not susceptible to disruption. Optimized bacterial systems are capable of translating about 10% of input mRNAs into protein [11]. Ultimately, this could increase library complexity and simplify selections by decreasing the interference from free mRNA. Other advantages of bacterial systems involve the ability to add and subtract specific components. For example, release factors could be removed and suppressor tRNAs supplied to obviate the need of removing stop codons from mRNAs [12]. Our system, as with ribosome display, uses *E. coli* extracts that could take advantage of these features. Here, we show that the bifunctional tRNA can be used to generate 10^{11} covalent peptide-$tRNA^x$-mRNA fusions (PTM fusions) per milliliter of translation reaction and that the fusions can be used to selectively enrich and amplify peptide libraries.

Results

Design and Synthesis of the Bifunctional tRNA

We designed a tRNA that can be used to form a stable linkage between polypeptides and the mRNA that encodes them. Within the ribosome, the growing peptide chain is normally linked to a tRNA by a labile ester bond. In turn, the tRNA is transiently linked to the mRNA that encodes the peptide by the base pairs of the codon-anticodon interaction. If the 3′-terminal adenosine of an aminoacyl tRNA is replaced by 3′-amino-3′-deoxyadenosine, the labile ester bond is replaced by a stable amide

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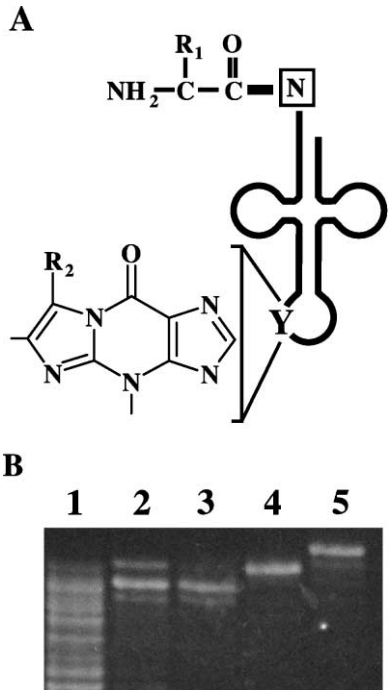

Figure 1. Design and Synthesis of tRNA^x

(A) Schematic highlighting the important components of tRNA^x. The nitrogen that replaces the 3′-terminal oxygen of yeast tRNA^{phe} is boxed. The amide bond between the tRNA and phenylalanine is shown as a thick line, and the naturally occurring wybutine base at position 37 of yeast tRNA^{phe} is labeled (Y).

(B) Synthesis of tRNA^x. RNAs were resolved on a denaturing 6% polyacrylamide gel (PAGE) and visualized with SYBR-gold: lane 1, total yeast tRNA; lane 2, yeast tRNA^{phe}; lane 3, purified ΔA-yeast tRNA^{phe}; lane 4, ΔA-yeast tRNA^{phe} repaired with 3′-amino-3′-deoxyadenosine triphosphate; lane 5, repaired yeast tRNA^{phe} aminoacylated with phenylalanine (tRNA^x). From top to bottom, the three bands in lane 2 correspond to the full-length tRNA and truncated forms missing the 3′-terminal A and CA.

bond [13]. Similarly, crosslinking the wybutine base (Y base) of yeast tRNA^{phe} to the mRNA within the ribosome complex covalently joins the tRNA and mRNA [14]. Thus, a tRNA containing both an amide linkage to its amino acid and a Y base (Figure 1A, "tRNA^x") could be useful for crosslinking proteins to their encoding mRNAs. Yeast tRNA^{phe}, which contains the Y base, was purified by benzoylated DEAE cellulose chromatography, and tRNA missing its 3′-terminal adenosine was repaired with tRNA nucleotidyl transferase and 3′-amino-3′-deoxyadenosine triphosphate (Figure 1B, lanes 1–4). Several methods exist for removing the 3′-terminal adenosine of a tRNA, but with yeast tRNA^{phe} this is generally unnecessary because the nucleotide is lost during purification [15]. Once repaired, the modified tRNA contained an intact acceptor stem with a 3′-terminal amine, and it contained the naturally occurring Y base in the anticodon loop. When the tRNA is aminoacylated with phenylalanine, the amino acid migrates to the 3′-amine forming the desired amide bond [13]. The tRNA purification, repair, and aminoacylation steps were all efficient (Figure 1B), and it was not necessary to purify the intermediates or product.

Synthesis of PTM Fusions

PTM fusions were made by translating synthetic mRNAs (Table 1) in the presence of tRNA^x and subsequently irradiating the translation products with UV light. In the translation mix, protein synthesis proceeded normally until a phenylalanine codon (Phe codon) in the mRNA reached the A site of the ribosome. At this point, either tRNA^x or *E. coli* tRNA^{phe} could be incorporated. If tRNA^x was selected, it was attached to the translated peptide chain (formyl-[^{35}S]-MKDYKDDDDK) (Figure 2). No fusion products were formed if the mRNA did not code for tRNA^x (Figure 2, lane 1). If normal tRNA^{phe} was selected at a Phe codon, translation continued, as shown by the production of multiple peptide-tRNA fusions (PT fusions) with an mRNA that contained multiple Phe codons (Figure 2, lane 2). Because the number of PT fusions produced was always equal to the number of in-frame Phe codons in the mRNA, the process was likely the result of normal translation (Figure 2, lanes 2–5). Furthermore, the mobilities of PT fusions in acrylamide gels were consistent with the size and charge of the polypeptide encoded by the translated mRNA (Figure 2, lanes 2–5).

Once linked to the peptide chain, tRNA^x stalls protein synthesis because the ribosome cannot break the amide bond that connects it to its amino acid [13]. The stalled ribosomal complexes were stable on sucrose gradients (data not shown). Therefore, high-salt sucrose cushions were used to purify ribosomal complexes from free mRNA and ribonucleases. The ribosome-bound PT fusions were then linked to their mRNAs by UV irradiation (Figure 3A), which crosslinks the wybutine base in tRNA^x to the 5′-U of the Phe codon [16] (Figure 3B). As expected from a process that links a peptide to its mRNA, the product detected when the peptide was labeled comigrated with the product detected when mRNA was labeled (Figure 3A, lanes 4 and 5, respectively). Controls showed that crosslinking required Phe codons in the mRNA to recruit tRNA^x (Figure 3A, lanes 1 and 8), inclusion of tRNA^x in the translation mix (Figure 3A, lanes 2 and 7), and exposure to UV light (Figure 3A, lanes 3 and 6). Quantitation of the PT and PTM fusion bands (Figure 3A, lane 4) indicated that about 0.2% of the mRNA was decoded by tRNA^x and that 1% of the PT fusions formed PTM fusions. Although the bulk of input mRNA is degraded by contaminating ribonucleases, 10^{11} PTM fusions were made in a 1 ml translation reaction. These results open the prospect of using tRNA^x to make complex pools of mRNA-encoded polypeptides.

Enrichment of Mixed Populations by Peptide Selection

Mock in vitro selections were performed to show that PTM fusions could be used to enrich RNA sequences that encode peptides with specified properties. In the first experiment, a synthetic mRNA coding for a Cys-containing peptide and an mRNA coding for six consecutive histidines (poly-His) were mixed at a ratio of 1:10. The mRNA mixture was added to a translation reaction that contained tRNA^x, and translated peptides were crosslinked to their encoding mRNAs. PTM fusions were then removed from the ribosome and partially purified by urea-LiCl precipitation. To selectively isolate PTM

Table 1. Sequences of the mRNAs Used to Direct In Vitro Protein Synthesis

mRNA	Sequence
1	GGAUCCUAGGAAGCUUGAAGGAGAUAUACC**AUG** AAA GAC UAC AAG GAC GAC GAC GAC AAG UAU AAA GUU . . .
2	GGAUCCUAGGAAGCUUGAAGGAGAUAUACC**AUG** AAA GAC UAC AAG GAC GAC GAC GAC AAG **UUU UUU UUU** . . .
3	GGAUCCUAGGAAGCUUGAAGGAGAUAUACC**AUG** AAA GAC UAC AAG GAC GAC GAC GAC AAG **UUU** AAA GUU . . .
4	GGAUCCUAGGAAGCUUGAAGGAGAUAUACC**AUG** AAA GAC UAC AAG GAC GAC GAC GAC AAG UAU **UUU** GUU . . .
5	GGAUCCUAGGAAGCUUGAAGGAGAUAUACC**AUG** AAA GAC UAC AAG GAC GAC GAC GAC AAG UAU AAA **UUU** . . .
6	GGGUUAACUUUAGAAGGAGGUAAAAAAA**AUG** AAA CGU GAA AAG ACA **UUU UUU UUU**
7	GGGUUAACUUUAGAAGGAGGUAAAAAAA**AUG** AAA CGU GAA AAG ACA GAA CGU ACA
8	GGGUUAACUUUAGAAGGAGGUAAAAAAA**AUG** AAA CAC CAU CAC CAC CAU CAC GGA AAU CGU **UUU UUC UUU** **UUC UUU UUC** CGC UAG CGU CAG GGC UAU UCA CCA UUA ACC CAC UAG GGC GUU
9	GGGUUAACUUUAGAAGGAGGUAAAAAAA**AUG** CGU UGC GAU CAC GGA AAU CGU UUU UUC UUU UUC UUU UUC CGC UAG CGU CAG GGC UAU UCA CCA UUA ACC CAC UAG GGC GUU
10	GGGUUAACUUUAGAAGGAGGUAAAAAAA**AUG** **UUU** AAA GAA AAG **UUU** GAA CGU ACA

For each mRNA, the codons are underlined and the initiator methionine codon (AUG) and Phe codons (UUU or UUC) are in bold. For mRNAs 1–5, part of the sequence is not shown (. . .). The remaining sequence is free of phenylalanine codons and is the same as nucleotides 217–444 of β-lactamase.

fusions that contained Cys, the purified translation reaction was subjected to thiol-affinity chromatography (Figure 4A). Quantitation of the band intensities from the initial (Figure 4A, lane 1) and selected populations (Figure 4A, lane 3) indicated that the initially underrepresented Cys fusion was enriched about 15-fold. To control for inadvertent skewing of the makeup of the mixed population by mechanisms other than peptide selection, we performed the inverse experiment; the mRNA ratio was switched and used to generate a second population of PTM fusions which were subjected to metal-affinity chromatography. Again, the initially underrepresented fusion—in this case the one containing poly-His—was enriched, but by about 5-fold (Figure 4A, lanes 4 and 6).

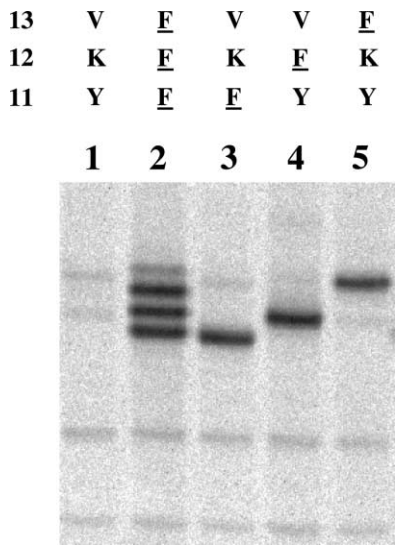

Figure 2. Attachment of tRNA^x to Growing Peptide Chains

Denaturing 6% PAGE was used to separate the products of in vitro translation reactions that contained [^{35}S]-methionine and tRNA^x. Amino acids and peptides bound to normal tRNAs were removed by base treatment and phenol extraction. Five different mRNAs were used to direct translation in an S30 extract (mRNAs 1–5, lanes 1–5, respectively). Each of the mRNAs coded for the same leader peptide (MKDYKDDDDK). Only amino acids specified at codons 11, 12, and 13 differed, as indicated above each lane.

To show that peptide selection was reflected at the genetic level, the mRNAs contained in the initial and enriched populations were subjected to RT-PCR and compared (Figure 4B). When thiol-affinity chromatography was used as the selective step, the PCR product encoding Cys was enriched (Figure 4B, lanes 1–3), whereas when metal-affinity chromatography was used, the PCR product encoding poly-His was enriched (Figure 4B, lanes 4–6). Thus, the intended selective step drives the evolution of the nucleic acid sequences that encode PTM fusions; if another mechanism were dominant—such as preferential RT-PCR amplification of a specific template—the same species would have overtaken both populations. Because RT-PCR products could be used to produce a new population of fusions, selection and amplification could be repeated to provide exponential enrichment of target molecules.

Discussion

By fusing a peptide to its mRNA, tRNA^x linked corresponding functional and replicable sequences in a single molecule. Two simple libraries of mRNAs were translated and fused to their peptide products, and the mRNAs coding for the selected peptide were amplified. These results indicate that the system can be used for the in vitro selection of peptides and proteins from complex libraries.

Library production requires the translation of mRNA pools that contain randomized coding regions. Phe codons within the randomized region could recruit tRNA^x early, which would produce truncated peptides. However, selection of a normal tRNA^{phe} at a Phe codon allows translation to proceed. Thus, by adding tRNA^x at a low effective concentration and placing a large number of Phe codons after the randomized region, most fusions will be formed near the end of an mRNA. Another concern is the presence of stop codons in the randomized region. Of the existing methods for dealing with this problem, perhaps the easiest is to use translation mixes that contain suppressor tRNAs but no release factors [17, 18]. Suppressor tRNAs that are chemically misacylated would have the added advantage of allowing the

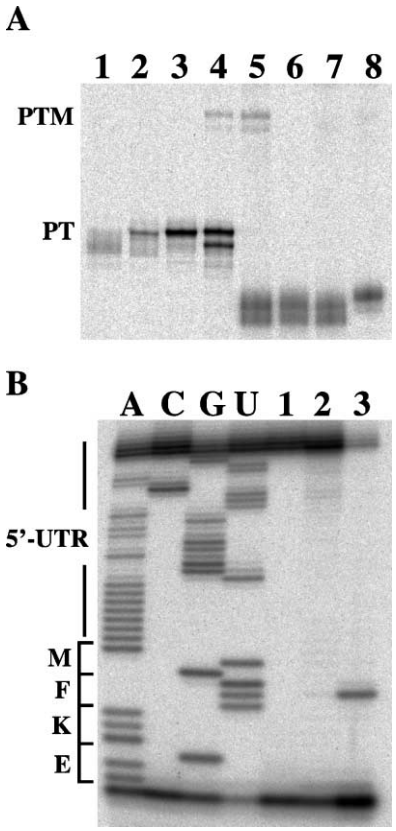

Figure 3. PTM Fusions

(A) Denaturing 4% PAGE of the products of in vitro translation reactions. Translation reactions contained [^{35}S]-methionine (lanes 1–4) or ^{32}P-labeled mRNA (lanes 5–8). In lanes 1 and 8, translation was directed by an mRNA that contained no Phe codons (mRNA 7), whereas the mRNA in lanes 2–7 contained Phe codons at positions 7, 8, and 9 (mRNA 6). Transfer RNA^x was not added to lanes 2 and 7. Lanes 3 and 6 were not exposed to UV. The mobilities of peptide-tRNA^x (PT) and peptide-tRNA^x-mRNA (PTM) fusions are indicated. The band below the PT fusion in lane 4 probably resulted from UV-dependent degradation of the Y base [29]. In the absence of translation, mRNA 7 ran as two separate bands which could also account for the two PTM fusion products seen in lanes 4 and 5.

(B) Denaturing 6% PAGE of the primer-extension stops for reverse transcriptase on mRNA 10 that was crosslinked to tRNA^x. A, C, G, and U are dideoxy-sequencing lanes using mRNA as the template. Lane 1, mRNA; lane 2, crosslinking reaction; lane 3, gel-purified PTM fusions from the crosslinking reaction. The 5′-UTR of the mRNA and codons 1–4 (MFKE) are indicated.

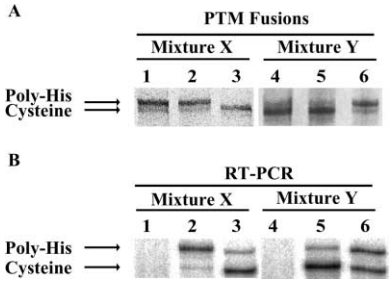

Figure 4. In Vitro Selection of mRNA-Encoded Peptides

(A) Denaturing 4% PAGE of peptide-tRNA^x-mRNA fusions before and after in vitro selection. Two mixtures of mRNA 8 (Cys mRNA) and mRNA 9 (poly-His mRNA) were translated in the presence of tRNA^x. In mixture X (lanes 1–3), the Cys:poly-His mRNA ratio was 1:10. In mixture Y (lanes 4–6), the mRNA ratio was inverted. Following translation, PTM fusions were formed and partially purified. The products of mixture X were selected by thiol-affinity chromatography and the products of mixture Y by metal-affinity chromatography. Lanes 1 and 4, PTM fusions from 1 μl of translation; lanes 2 and 5, column wash from 1 μl of translation; lanes 3 and 6, column eluant from 10 μl of translation.

(B) Nondenaturing 8% PAGE analysis of the RT-PCR products produced from mixture X (lanes 1–3) or Y (lanes 4–6) before and after in vitro selection by thiol affinity or metal affinity chromatography, respectively. Lanes 1 and 4, starting mRNA mixtures without reverse transcriptase; lanes 2 and 5, starting mRNA mixtures; lanes 3 and 6, translated, fused, and selected mRNA mixtures. In both panels, the mobilities of the Cys- and poly-His-containing PTM fusions or their RT-PCR products are indicated.

introduction of unnatural amino acids and other monomers [19, 20].

In a selection experiment, a large population is critical because it increases the likelihood that desirable molecules are represented. Currently, we can make 10^{11} fusions in a 1 ml translation reaction, which already surpasses the complexity achieved by in vivo methods. Furthermore, up to a 1000-fold improvement in fusion efficiency might be possible; if fully realized, a 1 ml reaction would yield 10^{14} PTM fusions. The bulk of this anticipated increase comes from improving crosslinking efficiency and increased utilization of the mRNA. For example, only 0.2 percent of the mRNA was translated and decoded by tRNA^x in our experiments, whereas in experiments using more highly purified bacterial translation systems, over 10 percent of the mRNA is utilized for protein synthesis, perhaps because purified systems have less ribonuclease contamination [11]. If still larger libraries are desired, bacterial translation extracts can be scaled up without undue expense.

In conjunction with a more highly purified translation system, our method might offer advantages for constructing libraries synthesized from unusual monomers. Although all of the systems have the potential to incorporate unnatural amino acids by nonsense suppression, incorporating unusual monomers at sense codons is difficult in most other systems. The cognate tRNAs would need to be specifically eliminated, perhaps by use of antisense oligonucleotides, and these tRNAs would need to be replaced with "orthogonal" tRNAs that are designed to avoid editing or charging by aminoacyl-tRNA synthetases [20]. With our system, such measures would not be necessary or would be more easily accomplished because bacterial translation systems are more readily customized. For example, fusions bearing nonstandard polymers have been generated in translation mixes that use misacylated tRNA, EFG, and EFTu rather than total tRNA and S150 (data not shown). In principle, ribosome display and mRNA display could use similar translation systems. However, mRNA display has not been shown to work with bacterial ribosomes, and ribosome display requires the translation of much longer peptides, as over 40 residues must be translated before the peptide begins to emerge from the exit channel of the ribosome [7]. With PTM fusions, even short open reading frames can satisfy the requirements of complexity and accessibility. Thus, because the ribosome can

use hundreds of monomers [e.g., 21–24], it could be possible to build low-molecular-weight libraries that have desirable properties such as protease resistance, permeability, and conformational rigidity. In conjunction with in vitro selection methods, such libraries could open the door to a vast array of useful molecules that could serve as leads for the development of therapeutics and other useful reagents.

Significance

We anticipate that the flexibility of our system with respect to the types of polymers that could be produced will broaden the number of applications to which in vitro selection can be applied. In principle, fusion libraries that are larger than 10^{11} could be constructed from any combination of monomers that the ribosome can polymerize. This large complexity has the potential to generate rare-functional molecules, while the wide range of acceptable monomers could be used to adjust the overall physical and chemical properties of a library. Thus, the method could be useful for evolving nonbiological polymers whose properties depart from those accessible to peptides and proteins.

Experimental Procedures

Purification of Ribosomes and S150 Enzyme Fraction

For ribosomes, 5 g of an *E. coli* ribonuclease-deficient strain (A19) was washed with 300 ml of buffer A (10 mM Tris-HCl [pH 7.5], 10 mM Mg-acetate, 22 mM NH_4Cl, and 1 mM DTT), pelleted in a Sorvall SLA-3000 rotor (4600 g), and suspended in buffer A in a final volume of 20 ml. The suspension was lysed in a BeadBeater mixer (Biospec) according to the manufacturer's directions with 80 ml of 0.1 mm zirconia/silica beads. The beads were washed several times with buffer A and the supernatants combined and transferred to 13 ml centrifuge tubes. The lysate was cleared by repeated 20 min centrifugations in a Sorvall SS-34 rotor (17,000 g). Ribosomes were isolated by layering 13 ml of the clarified supernatant on 13 ml of 32% sucrose in buffer A and centrifuging for 13 hr in a Beckman 70Ti rotor (120,000 g). Pellets were dissolved in a small volume of buffer A, and the concentration was adjusted to 45 μM before storage at −80°C in 5–25 μl aliquots. For the S150 enzyme fraction, 4 g of cells was washed in 38 ml of buffer B (10 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 30 mM NH_4Cl, and 6 mM BME) and lysed as before but with buffer B. The lysate was clarified twice by centrifugation for 20 min in a Sorvall SS-34 rotor (30,000 g) and once for 30 min in a Beckman VTi50 rotor (150,000 g). The clarified supernatant was loaded on a 40 ml DEAE sepharose column (Pharmacia) that had been equilibrated with buffer B, and the column was washed with 1 liter of buffer B. The S150 enzyme fraction was eluted with buffer B plus 220 mM NH_4Cl. Fractions were examined by eye, and the dark brown ones were pooled and stored at −80°C in 20–100 μl aliquots.

Synthesis of the Bifunctional $tRNA^x$

Yeast $tRNA^{phe}$ was purified by benzoylated DEAE cellulose chromatography [25]. 3′-Amino-3′-deoxyadenosine triphosphate was prepared by published protocols [26, 27]. The construct pQECCA, expressing *E. coli* tRNA nucleotidyl transferase as a fusion protein with a poly-His tag, was a generous gift from U. RajBhandary (MIT). Yeast $tRNA^{phe}$ missing its 3′-terminal adenosine was repaired by incubating 500 μM tRNA, 2 mM 3′-amino-3′-deoxyadenosine triphosphate, and tRNA nucleotidyl transferase in buffer (50 mM Tris-HCl [pH 8.0], 10 mM $MgCl_2$, 30 mM KCl, 5 mM DTT, and 0.5 mg/ml BSA) for 10 min at 37°C. Protein was removed by phenol extraction, and the tRNA was ethanol precipitated. The intermediate (3′-amino-$tRNA^{phe}$) was charged by incubating 25 μM 3′-amino-$tRNA^{phe}$, 100 μM phenylalanine, and 4 mM dATP with 1/5 (vol/vol) S150 enzyme fraction for 30 min at 37°C in 30 mM Tris-HCl (pH 7.5), 15 mM $MgCl_2$, 25 mM KCl, and 5 mM DTT. The final product ($tRNA^x$) was purified by phenol extraction and ethanol precipitated.

Synthesis of mRNA

DNA templates for T7 in vitro transcription were generated by PCR, using appropriate templates and primers. PCR products were ethanol precipitated and transcribed in half their original volume (40 mM Tris-HCl [pH 7.9], 26 mM $MgCl_2$, 2.5 mM spermidine, 0.01% Triton X-100, 5 mM ATP, 5 mM CTP, 8 mM GTP, 2 mM UTP, and T7 RNA polymerase). All mRNAs (Table 1) were gel purified.

Synthesis of PT Fusions

Three micrograms of mRNA was translated in 40 μl of an *E. coli* S30 extract (Promega) according to the manufacturer's protocol. In addition to exogenous mRNA, reactions contained [^{35}S]-methionine and 2 μM $tRNA^x$. After incubating for 30 min at 37°C, the reactions were terminated by phenol extraction and ethanol precipitated. Amino acids and peptides bound to normal tRNAs were removed by incubation in 0.5 M Ches-KOH (pH 9.5) for 1 hr at 37°C, followed by phenol extraction and ethanol precipitation.

Synthesis of PTM Fusions

For each reaction, translation premix contained 2.5 μl of 10× translation buffer (500 mM Tris-acetate [pH 8.0], 110 mM Mg-acetate, 1 M NH_4Cl, and 10 mM DTT); 2.5 μl of 100 mM phosphoenol pyruvate; 0.5 μl of 100 mM ATP:10 mM GTP; 0.5 μl of 25 μg/μl total *E. coli* tRNA; 2.5 μl of 1 mM amino acids; 0.5 μl of 1 μg/μl pyruvate kinase; 3 μl of 18 μM ribosomes; and 6 μl of S150 enzyme fraction. Premix was incubated for 10 min at 37°C, after which 2.5 μl of 200 μM $tRNA^x$ was added when appropriate. Aliquots (16 μl) of the premix were distributed among tubes that contained 2 μl of [^{35}S]-methionine-labeled fMet-$tRNA^{Met}$ and 2 μl of 50 μM mRNA. When the mRNA rather than the peptide was labeled, the tubes contained 2 μl of fMet-$tRNA^{Met}$ and 2 μl of 50 μM [^{32}P]-cordycepin-labeled mRNA. Incubation was continued for another 10 min and samples layered onto 500 μl sucrose cushions (32% sucrose, 50 mM HEPES [pH 7.8], 20 mM $MgCl_2$, 500 mM NH_4Cl, and 6 mM BME). Stalled ribosomal complexes were pelleted by centrifugation for 45 min at 4°C in a Beckman TLA 100.2 rotor (360,000 g). Pellets were dissolved in 25 μl of buffer (40 mM HEPES [pH 7.8], 20 mM $MgCl_2$, 80 mM NH_4Cl, and 6 mM BME), spotted on a polystyrene petri dish, covered with the supplied lid, and exposed to UV for 20 min using a 450 watt Hanovia bulb with water jacket (Ace Glass). As before, phenol extraction and base treatment were used to remove undesired translation products.

In Vitro Peptide Selection

PTM fusions were synthesized as described above with minor modifications. In brief, [^{35}S]-methionine-labeled fMet-$tRNA^{Met}$ was generated in situ by adding 1/10 (vol/vol) deprotected 5,10-methenyltetrahydrofolate [28] and 1/10 (vol/vol) [^{35}S]-methionine; the amino acid mix did not contain methionine. To make room for the additional reagents, half as much amino acid mix was added, concentrated stocks of nucleotide mix and ribosomes were used, and the final concentration of $tRNA^x$ was reduced to 10 μM. Rather than individual mRNAs, mixtures were used to direct translation; mRNA encoding the target peptide was at 0.4 μM and mRNA encoding the background peptide was at 4 μM. After crosslinking, ribosomal complexes were mixed 1:1 with lithium buffer (8 M urea, 4 M LiCl, 10 mM EDTA [pH 8.0], and 6 mM BME) and precipitated overnight at 4°C. Pellets were dissolved in 20 mM Tris-HCl (pH 7.5), 2 mM $MgCl_2$, 60 mM KCl, and 6 mM BME and stored at −20°C. Before selection, aliquots were precipitated from ethanol and dried by aspiration to remove BME, then dissolved in 10 μl of water. Poly-His-containing fusions from a 90 μl translation reaction were bound to Talon metal-affinity resin (Clontech) by adding 9 μl of the concentrated product to a resin slurry (100 μl resin: 100 μl 50 mM Tris-HCl [pH 7.5], 300 mM NH_4Cl, and 0.25 mg/ml BSA) and rotating for 16 hr at 4°C. Unbound fusions were removed by washing the resin five times with 100 μl aliquots of wash buffer (50 mM Tris-HCl [pH 7.5] and 1 M NH_4Cl). Bound fusions were eluted by washing the resin three times with 100 μl of wash buffer plus 10 mM EDTA (pH 8.0) and 8.3 μg

of total *E. coli* tRNA. Cys-containing fusions were immobilized in a similar manner with 500 μl of activated Thiol-Sepharose 4B (Pharmacia) in 5 ml of buffer (25 mM Tris-HCl [pH 7.5], 300 mM NaCl, and 7 M urea). After binding, the resin was poured into a column and washed with 100 ml of the same buffer. The resin was then removed and eluted three times with 1 ml aliquots of buffer that also contained 50 mM DTT and 25 μg/ml total *E. coli* tRNA. Column washes and eluants were ethanol precipitated and dissolved in 90 μl or 9 μl of water, respectively. For both selections, before RT-PCR, unfused mRNA was removed from the enriched population of PTM fusions by gel purification; a liberal section of the gel was excised to ensure that gel purification did not influence the ratio of the two PTM fusions.

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