Cell-free translation reconstituted with purified components

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We have developed a protein-synthesizing system reconstituted from recombinant tagged protein factors purified to homogeneity. The system was able to produce protein at a rate of about 160 μg/ml/h in a batch mode without the need for any supplementary apparatus. The protein products were easily purified within 1 h using affinity chromatography to remove the tagged protein factors. Moreover, omission of a release factor allowed efficient incorporation of an unnatural amino acid using suppressor transfer RNA (tRNA).

The development of cell-free strategies based on purified enzymes is an expanding field in biotechnology. DNA polymerases have made it possible to amplify DNA rapidly and efficiently by PCR without the need for cloning. RNA polymerases are used to synthesize novel RNA molecules through *in vitro* evolution methods. Reverse transcriptases generate complementary DNA (cDNA), which does not exist in natural living systems. The availability of these purified enzymes essential to the "central-dogma" pathway of molecular biology has removed many of the constraints in conventional gene-manipulation techniques that rely on living cells. So far, however, the translation process has eluded efficient reconstitution *in vitro*, in large part because of its complexity. The achievement of this goal would offer advantages in many areas of biotechnology.

Two approaches have guided efforts to achieve cell-free translation. One approach, developed over the past decade, is based on crude cell extract, often derived from *Escherichia coli*, rabbit reticulocytes, or wheat germ[1,2]. But the use of such extracts inevitably encounters two problems: a rapid depletion of energy charge, independent of peptide bond formation[3,4], and degradation of protein products or template nucleic acids by proteases or nucleases. These problems have been partly overcome by a continuous-flow cell-free protein-synthesis system[5,6].

The second approach attempts to reconstitute protein synthesis from purified components of the translation machinery. More than 100 molecules participate in prokaryotic and eukaryotic translation, many of which have been individually purified for biochemical studies of their functions and structures. In 1977, Weissbach's group endeavored to reconstitute a translation system using purified soluble components[7] but did not achieve a satisfactory production of proteins, perhaps because of a complex purification protocol and the absence of release factor 3 (RF3) and ribosome-recycling factor (RRF). A few years later, Ganoza and co-workers reconstructed a cell-free translation system from precharged aminoacyl-tRNAs and purified translation factors[8]. Although this system did not require aminoacyl-tRNA synthetases, the tRNAs were not recycled, resulting in low productivity. More recently, Pavlov and co-workers produced an *in vitro* translation system using a partially purified aminoacyl-tRNA synthetase mixture with purified translation factors[9]. They also constructed a completely purified *in vitro* translation system using short artificial messenger RNA (mRNA)[10]. However, this system lacked a full set of aminoacyl-tRNA synthetases and its productivity was relatively low.

Here we describe a cell-free translation system reconstructed from purified histidine (His)-tagged translation factors and "programmed" by natural mRNA. The system—termed the "protein synthesis using recombinant elements" (PURE) system—contains all necessary translation factors, purified with high specific activity, and allows efficient protein production. We succeeded in producing active dihydrofolic acid reductase (DHFR) with a productivity on the order of submilligrams in a 1 ml reaction volume. The PURE system is also advantageous in terms of product purification. Protein products in the native form (with no His tag) were purified easily and rapidly through ultrafiltration followed by affinity chromatography to remove the His-tagged translation factors. Omission of release factor 1 (RF1) allowed the suppression of an amber codon using chemically synthesized UAG-specific suppressor tRNA with a chemically attached valine (Val–tRNA^{sup}), demonstrating that the PURE system can be used for the efficient synthesis of proteins containing unnatural amino acids.

Results

The PURE system. Protein biosynthesis proceeds in three steps: initiation, elongation, and termination. In *E. coli*, the translation factors responsible for completing these steps are three initiation factors (IF1, IF2, and IF3), three elongation factors (EF-G, EF-Tu, and EF-Ts), and three release factors (RF1, RF2, and RF3), as well as RRF for termination. However, RF2 is not required for the translation of genes terminating with the codons UAG or UAA. The PURE system includes 32 components that we purified individually: IF1, IF2, IF3, EF-G, EF-Tu, EF-Ts, RF1, RF3, RRF, 20 aminoacyl-tRNA synthetases (ARSs), methionyl-tRNA transformylase (MTF), T7 RNA polymerase, and ribosomes. In addition, the system contains 46 tRNAs, NTPs, creatine phosphate, 10-formyl-5,6,7,8-tetrahydrofolic acid, 20 amino acids, creatine kinase, myokinase, nucleoside-diphosphate kinase, and pyrophosphatase.

Purification of translation factors. We overexpressed in *E. coli* and purified IF1, IF2, IF3, EF-G, EF-Tu, EF-Ts, RF1, RF3, RRF, 20 ARSs, MTF, and T7 RNA polymerase. We reconstructed these genes in the

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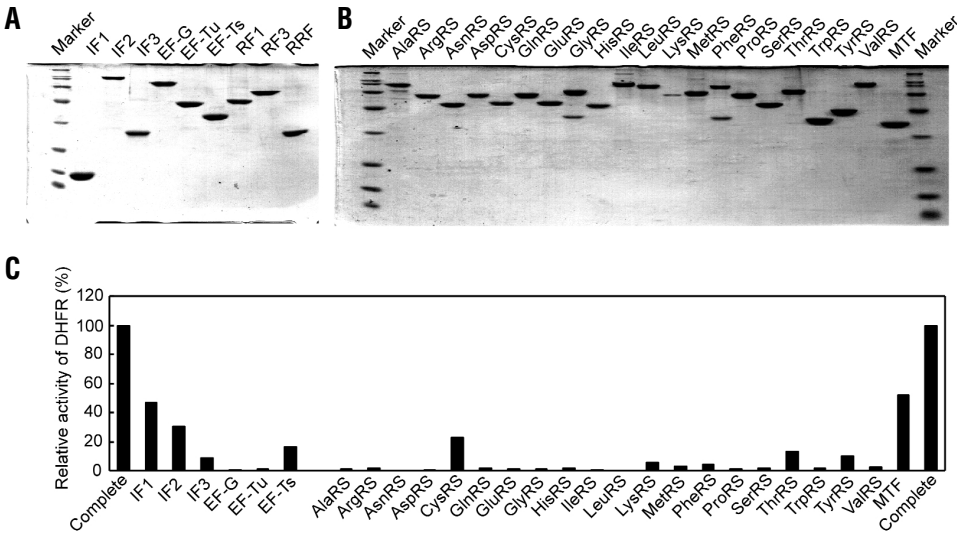

Figure 1. SDS–PAGE gel patterns of purified translation factors (A) and enzymes (B). Marker sizes (top to bottom 200, 116, 97, 66, 45, 31, 21, 14, and 6.5 kDa. The two main bands in glycyl tRNA transferase (GlyRS) and phenylalanyl tRNA transferase (PheRS) result from the subunit structures of these enzymes. (C) The effect of omitting individual components from the PURE system. DHFR template was translated with each factor subtracted in turn from the reaction mixture. Data represent the activities of DHFR relative to that of the complete reaction mixture after 60 min of incubation.

His-tagged fusion form for easy and high-grade protein purification by a Ni^{2+} column. An imidazole gradient strategy was successful in achieving effective purification. Figure 1 shows the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) gel patterns of the purified translation factors (Fig. 1A) and enzymes (Fig. 1B). The electrophoretic pattern indicated that the proteins were purified to homogeneity.

Activities of the purified translation components. We evaluated the activities of the purified translation factors and enzymes except for RF1, RF3, and RRF from the reduction in protein synthesis caused by excluding each component in turn from the PURE system (Fig. 1C). The results indicated that most of the components were indispensable for translation and simultaneously ruled out the possibility of sample contamination by other *E. coli* proteins. The results also suggested that IF1 and MTF were least essential for protein synthesis. These observations are consistent with previous reports showing that deletion of the gene for MTF results in an extreme reduction of the viability but not the lethality of *E. coli*[11], and that formation of the initiation complex can be achieved in the absence of IF1 (ref. 12). However, it is likely that IF1 and MTF contribute to the efficiency and accuracy of protein synthesis.

Table I. Activities of termination factors (RF1, RF3, RRF).

Factor added	Peptide released (c.p.m.)
RF1, RF3, RRF	4509
RF1, RRF	3254
RF1, RF3	1767
RF1	1646
RF3, RRF	529
none	860

The translation reaction was done according to the method of Pavlov *et al.*[10] with a slight modification using a short synthetic mRNA (mMFL) with the sequence AUGUUCUUGUAA, corresponding to fMet-Phe-Leu-Stop (fMFL). Reaction mixtures (50 µl) containing 50 pmol ^{35}S-labeled formyl methionyl-tRNA^{Met} with each termination factor subtracted in turn were pre-incubated at 37°C for 5 min, followed by the addition of 1 µg MFL mRNA. An aliquot (5 µl) was withdrawn after 8 min of incubation and added to the same volume of 1 N HCl. The tripeptides (fMFL) were extracted by ethyl acetate and the radioactivity was measured.

We evaluated the activities of RF1, RF3, and RRF by determining peptide release from ribosomes according to the method of Pavlov *et al.*[10], using a short synthetic mRNA (mMFL) with the sequence AUGUUCUUGUAA, corresponding to fMet–Phe–Leu–Stop (fMFL). Complete peptide release was dependent on the presence of RF1 (Table 1). The addition of RRF significantly increased the amount of peptide released and gave multiple-turnover protein synthesis. The observation that the turnover rate mediated by RF1 and RRF was enhanced by supplementation with RF3 indicated that RF3 was purified as the active form.

From the results of the two experiments, we concluded that all the purified translation components were active despite the His tags.

Additonal information about the cloning vectors used for expression of the translation factors and enzymes, the yields following purification, the concentrations and specific activities of the purified proteins, and the units used per 50 µl reaction is available on the Web Extras page of *Nature Biotechnology* Online.

Protein synthesis. We next addressed the construction of the transcription/translation-coupled PURE system using the purified translation components and T7 RNA polymerase. The PURE system was able to synthesize DHFR of *E. coli*, λ-lysozyme, green fluorescent protein (GFP), glutathione *S*-transferase (GST), and bacteriophage T7 gene 10 product as full-length polypeptides (Fig. 2). Except for GST, more than 100 µg of each protein was produced in a 1 ml reaction volume after 1 h (Fig. 2). Although we could not explain the low production of GST, we concluded that the PURE system contains all the components essential for translation.

We measured the enzymatic activity of the DHFR, the bacteriolytic ability of the λ-lysozyme, and the GFP fluorescence, and

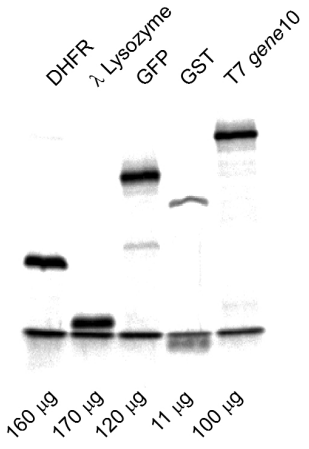

Figure 2. SDS–PAGE gel patterns of the products of the PURE system. After incubation of the translation reaction for 1 h in mixtures containing [^{35}S]methionine and 2.4 µM ribosome, samples were separated by SDS–PAGE and subjected to autoradiography. Below autoradiograph, yields calculated from the radioactivities of the labeled products and the productivity in 1 ml reaction. The bottom row of bands indicates the dye front.

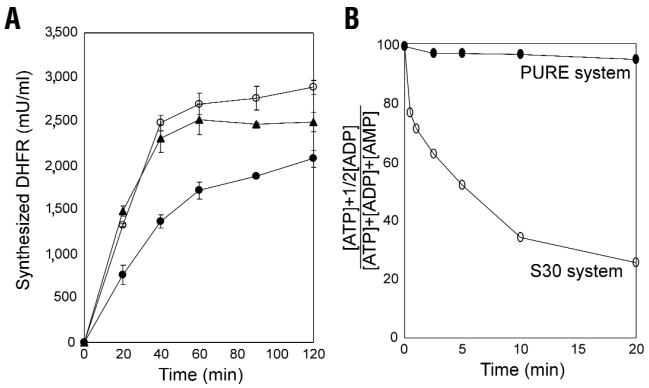

Figure 3. Productivity and energy consumption of the PURE system. (A) Time course of DHFR synthesis. Translation reactions used ribosome concentrations of 1.2 μM (●), 2.4 μM (▲), or 3.6 μM (○). Reaction mixtures contained 0.3 mM amino acids to prevent amino acid "starvation." The concentrations of EF-G, EF-Tu, and EF-Ts are intensified threefold, fivefold (▲), and sevenfold (○). (B) Abortive energy consumption in the PURE and S30 systems. The decrease in the energy charge of each system was monitored.

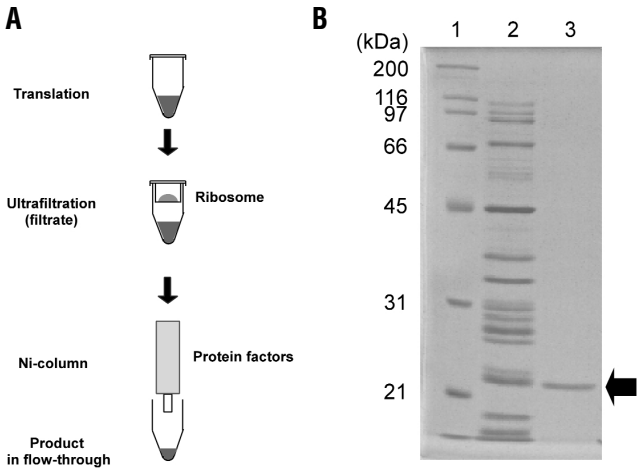

Figure 4. Rapid purification of the translation product. (A) Product purification procedures. Ribosome was first eliminated by ultrafiltration using a membrane with a cutoff of 100 kDa. Subsequently, all His-tagged components were eliminated by passage through a Ni^{2+} column. (B) Purity of purified translation product. The DHFR template was translated for 1 h at 37°C in reaction mixtures containing 2.4 μM ribosome; products were separated by 12% SDS–PAGE and visualized by staining with Coomassie blue. Lane 1, marker; lane 2, reaction mixture; lane 3, purified DHFR. Arrow, DHFR band.

found that these proteins were synthesized in their active forms. The specific activity of the synthesized DHFR was 16.7 ± 1.1 U/mg, which is similar to the specific activity value of DHFR that we overexpressed in cells and purified (16.0 ± 0.80 U/mg). From these data we concluded that the components of the PURE system are sufficient to produce active proteins.

Monitoring of the time course of DHFR synthesis at different ribosome concentrations showed that in each case the reaction rate decreased after 1 h (Fig. 3A). To clarify the cause of this deceleration, we simultaneously monitored the biochemical energy level, or energy charge,—defined by Atkinson[13] as ([ATP] + 1/2[ADP])/([ATP] + [ADP] + [AMP])—in the reaction mixture. Despite supplementation of the energy-recycling system consisting of creatine kinase, myokinase, and nucleoside diphosphate kinase, the energy charge decreased to <30% after 1 h. Thus, it is likely that reaction quenching resulted mainly from a deprivation of chemical energy. The PURE system showed no abortive energy consumption, whereas in a crude cell extract system most nucleotide triphosphates were hydrolyzed regardless of protein synthesis (Fig. 3B). The PURE system is therefore very efficient in terms of energy conversion.

Product purification. Taking advantage of the fact that most components of the PURE system exist in the His-tagged form, we designed the purification process shown in Figure 4A. After the protein-synthesis reaction was terminated, ribosomes were removed by ultrafiltration using a membrane with a cutoff of 100 kDa. Next, His-tagged components were eliminated by passage through a Ni^{2+} column. We analyzed the purity of the product by SDS–PAGE and found that we had obtained highly purified DHFR (Fig. 4B). The purification steps in the PURE system can be completed within a few hours, including the protein-synthesis reaction. Moreover, the target protein is expressed and purified in its native form without any tag.

Incorporation of unnatural amino acids. One of the great advantages of cell-free translation is the ease of incorporating unnatural amino acids[14]. We attempted to develop the PURE system in this respect by omitting the release factor RF1. Suppression of an amber codon using Val–$tRNA^{sup}$ was employed as a model for unnatural-amino-acid incorporation. The template for the experiment was a mutant DHFR mRNA in which the codon UAG was substituted for the Asn37 codon, terminated by the RF2-specific codon UGA. In this case, RF1 cannot be used as a termination factor and we prepared RF2 to substitute for RF1. Although the PURE system in the presence of RF1 produced a truncated protein terminating at UAG, omission of RF1 resulted in an extremely faint band corresponding to the truncated protein, which is likely to have resulted from single-turnover synthesis (Fig. 5, lanes 2 and 3). Addition of Val–$tRNA^{sup}$ produced a full-length protein, with complete disappearance of the truncated protein. This finding suggested highly efficient read-through of the UAG codon by suppressor tRNA. Thus, the PURE system is able to insert an amino acid corresponding to a particular termination codon, indicating that it can be used to synthesize proteins containing unnatural amino acids.

Discussion

We have demonstrated here that the PURE system for reconstituting translation from purified translation factors is capable of producing active proteins from template nucleic acids in considerable amounts. Because it uses only highly purified components, the system is free of inhibitory substances such as nucleases, proteases, and enzymes that hydrolyze nucleoside triphosphates. ATPase activity in the PURE system was observed to be low, in contrast to the rapid energy depletion seen when the conventional S30 extract of *E. coli* (Fig. 3B) was used. Although the productivity of the PURE system was generally higher than that of the S30 systems we examined, it has not yet surpassed that reported by Kim *et al.*, in which protein was produced at 400 μg/ml in batch culture[15]. However, that system contained fivefold more creatine phosphate (50 mM) than ours, and its reaction conditions—such as the addition of polyethylene glycol[15]—also differed considerably. Because energy conversion in the PURE system is highly efficient, we estimate that its protein productivity in relation to the concentration of the energy source is high compared with that of S30 systems. Moreover, optimization of the PURE system is not yet complete; further study of the reaction conditions should allow us to improve its productivity.

We consider the PURE system as a tool not only for producing natural proteins but also for synthesizing unnatural proteins with new functions. The finding that omitting particular factors from the reaction mixture largely stopped the reaction (Fig. 1C and Table 1) suggests various strategies for artificially controlling protein synthe-

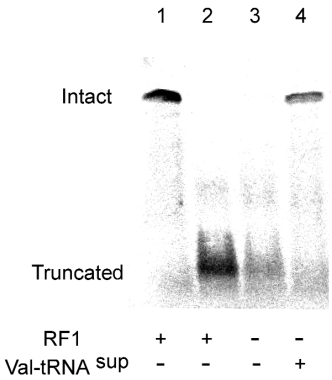

Fig. 5. Suppression of an amber codon using Val–tRNA^{sup} in the PURE system. The *in vitro* suppression reaction included 20 µl of RF1-deprived PURE system, as well as 0.2 A_{260} unit suppressor tRNA-Val. The DHFR template (lane 1) and the N37Amber DHFR template (lanes 2–4) were translated in the PURE system containing RF2 substituted for RF1. A truncated peptide terminating at UAG is produced in the PURE system containing RF1 (lane 2), whereas subtraction of RF1 produces an extremely faint band corresponding to the truncated peptide (lane 3), which is likely to be the result of synthesis for a single turnover. Addition of Val–tRNA^{sup} produces a full-length peptide, whereas the truncated peptide completely disappears (lane 4).

sis—strategies that would not be possible in crude-cell-extract systems. Subtraction of RF1 from the PURE system enabled complete conversion of the termination codon to a sense codon (Fig. 5). With a crude cell extract, competition between suppressor tRNA and termination factors inevitably causes inefficient incorporation of the amino acid corresponding to the termination codon. In the PURE system, this problem is overcome simply by omitting a termination factor. A similar approach might be adopted with other components, such as particular aminoacyl-tRNA synthetases. Substitution of native aminoacyl-tRNA synthetases with mutant synthetases having new specificities should enable the construction of a translation system founded on a noncanonical genetic code.

In other experiments, we succeeded in producing proteins using an mRNA-directed PURE system (data not shown), but found variable productivity depending on the template mRNAs used. This variability may arise from mRNA secondary structure. Because the PURE system lacks RNA helicases, it may be more susceptible to this problem than a crude-cell-extract system. However, this problem could be addressed by the addition of RNA helicases. We are evaluating the influence of mRNA secondary structure on translation efficiency using the mRNA-directed PURE system. The PURE system will also allow us to examine many factors that interact with the translation apparatus, including chaperones and several factors thought to stimulate translation efficiency[8,16–18].

Experimental protocol

Construction of plasmids for overexpression of components of the PURE system. The genes encoding ARS, MTF, T7 RNA polymerase, nucleoside-diphosphate kinase, IF1, IF2, IF3, EF-G, EF-Tu, EF-Ts, RF1, RF2, RF3, and RRF were amplified by PCR from the *E. coli* genome and cloned into vector pET21a (Novagen, Madison, WI), pQE30, or pQE60 (Qiagen, Valencia, CA) to generate each His-tagged factor plasmid. The plasmids obtained were transformed into *E. coli* BL21/pREP4 (pQE series) or BL21/DE3 (pET series) strains.

Preparation of PURE system components. Tightly coupled ribosomes were purified from *E. coli* A19 cells by sucrose-density-gradient centrifugation according to the method of Spedding[19]. His-tagged protein was purified as follows: BL21 cells with each vector were grown to an OD_{600} of 0.5–0.9 in 2–6 L Luria–Bertani (LB) broth. Isopropyl-β-D-thiogalactoside (IPTG) was added to a final concentration of 0.1 mM, and the cells were grown for an additional 4 h at 37°C. Collected cells were resuspended in a buffer (50 mM *N*-[2-hydroxyethyl]piperazine-*N*'-[2-ethanesulfonic acid] (HEPES)–KOH, pH 7.6, 1 M NH_4Cl, 10 mM $MgCl_2$, 0.3 mg/ml lysozyme, 0.1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 7 mM β-mercaptoethanol) and were lysed by sonication. Cell debris was removed by centrifugation at 100,000 *g* for 1 h at 4°C and the supernatant was applied to a Ni^{2+} precharged 10-ml Hi-Trap chelating column (Amersham Pharmacia Biotech, Arlington Heights, IL) and was washed with 100 ml HT buffer (50 mM HEPES–KOH, pH 7.6, 1 M NH_4Cl, 10 mM $MgCl_2$, and 7 mM β-mercaptoethanol) containing 10 mM imidazole. Factors were eluted with a linear gradient from 10 mM to 400 mM imidazole in HT buffer. Fractions containing His-tagged protein were dialyzed against a stock buffer (50 mM HEPES–KOH, pH 7.6, 100 mM KCl, 10 mM $MgCl_2$, 30% glycerol, and 7 mM β-mercaptoethanol) and were frozen in small aliquots at -80°C.

Construction of template plasmids used for translation. The gene used for the translation experiment was cloned into vector pET17b, pET29a (Novagen), pUC18 (Takara, Shuzo, Japan), or pGEMEX-2 (Promega, Madison, WI). The gene contained a T7 promoter upstream of a ribosome-binding site with the "epsilon sequence" originating from bacteriophage T7 gene 10 (ref. 20) followed by a Shine–Dalgarno sequence. The plasmid encoding the N37Amber DHFR mutant was constructed using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The plasmid encoding the gene for mMFL was transcribed according to the method of Sampson and Uhlenbeck[21] with a slight modification.

Translation reaction. Unless otherwise specified, the standard translation mixtures (50 µl) were prepared as follows: reaction mixtures were generally prepared in polymix buffer as described before[22,23] with a slight modification. Reaction mixtures contained 9 mM magnesium acetate, 5 mM potassium phosphate, pH 7.3, 95 mM potassium glutamate, 5 mM ammonium chloride, 0.5 mM calcium chloride, 1 mM spermidine, 8 mM putrescine, 1 mM dithiothreitol (DTT), 2 mM each ATP and GTP, 1 mM each of CTP and UTP, 10 mM creatine phosphate, 2.8 A_{260} units tRNA mix (Roche, Mannheim, Germany), 0.5 µg 10-formyl-5, 6, 7, 8-tetrahydrofolic acid, 0.1 mM each of amino acids, and factor mix. The factor mix contained 12 pmol ribosome, 1 µg IF1, 2 µg IF2, 0.75 µg IF3, 1 µg EF-G, 2 µg EF-Tu, 1 µg EF-Ts, 0.5 µg RF1, 0.5 µg RF3, 0.5 µg RRF, 30–300 units of each ARS and MTF, 0.2 µg creatine kinase (Roche), 0.15 µg myokinase (Sigma, St. Louis, MO), 0.054 µg nucleoside-diphosphate kinase, 0.1 units pyrophosphatase (Sigma), and 0.5 µg T7 RNA polymerase. The final concentrations of these components, except for the ARSs, were determined by dose-response translation experiments. Activities of the ARSs conformed to aminoacylation activities in S100 cell extract. After the reaction mixture was incubated at 37°C for 5 min, the template plasmid was added and the reaction was incubated at 37°C. The reaction was carried out for one hour and terminated on ice.

Product analysis. Synthesized proteins containing [^{35}S]methionine were separated by 12% SDS–PAGE. The radioactivity of each product was measured and the yield was calculated. The activity of DHFR was measured according to the method of Poe *et al.*[24], with a slight modification. The λ-lysozyme assay was done according to the method of Schleif *et al*[25]. Fluorescence spectra of GFP were measured with a spectrofluorometer (type 850; Hitachi, Tokyo, Japan).

Measurement of energy charge. Translation reaction mixtures containing [α-^{32}P]ATP and 1.2 µM ribosome without GTP, UTP, CTP, amino acids, template DNA, and components of the energy-recycling system were incubated at 37°C. Aliquots (2 µl) were withdrawn at different incubation times and added to 150 µl 10% formic acid. An aliquot (0.5 µl) of this was then spotted onto a polyethyleneimine thin-layer chromatography (TLC) plate and the reaction products were separated in 0.75 M potassium phosphate buffer, pH 3.75; then the TLC plates were dried for autoradiography. The S30 extract was purchased from Promega (Madison, WI).

Purification of product. The DHFR template was translated for 1 h at 37°C in reaction mixtures containing 2.4 µM ribosome. After termination of the translation, the reaction mixtures were loaded onto a Centricon 100 device (Millipore, Bedford, MA) and centrifuged, and the flowthrough was recovered. Aliquots obtained were then loaded onto Ni-NTA resin (Qiagen) and the flowthrough was recovered.

Preparation of valyl-tRNA^{sup}. Suppressor tRNA lacking the 3′-terminal –CA sequence was transcribed using T7 RNA polymerase[21] from the plasmid encoding the yeast suppressor tRNA^{Phe}CUA. The products were purified by denaturing 12% PAGE, chemically misacylated with *N*-(4-pentenoyl)valyl-pdCpA, and "de-protected" according to the method of Short *et al*[26].

Note: Supplementary information can be found on the Nature Biotechnology *website in Web Extras (http://biotech.nature.com/web_extras)*

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1. Stiege, W. & Erdmann, V.A. The potentials of the *in vitro* protein biosynthesis system. *J. Biotechnol.* **41**, 81–90 (1995).
2. Jermutus, L., Ryabova, L.A. & Pluckthun, A. Recent advances in producing and selecting functional proteins by using cell-free translation. *Curr. Opin. Biotechnol.* **9**, 534–548 (1998).
3. Kitaoka, Y., Nishimura, N. & Niwano, M. Cooperativity of stabilized mRNA and enhanced translation activity in the cell-free system. *J. Biotechnol.* **48**, 1–8 (1996).
4. Matveev, S.V. *et al.* Effect of the ATP level on the overall protein biosynthesis rate in a wheat germ cell-free system. *Biochim. Biophys. Acta* **1293**, 207–212 (1996).
5. Spirin, A.S., Baranov, V.I., Ryabova, L.A., Ovodov, S.Y. & Alakhov, Y.B. A continuous cell-free translation system capable of producing polypeptides in high yield. *Science* **242**, 1162–1164 (1988).
6. Kim, D.M. & Choi, C.Y. A semicontinuous prokaryotic coupled transcription/translation system using a dialysis membrane. *Biotechnol. Prog.* **12**, 645–649 (1996).
7. Kung, H.F. *et al.* DNA-directed *in vitro* synthesis of beta-galactosidase. Studies with purified factors. *J. Biol. Chem.* **252**, 6889–6894 (1977).
8. Ganoza, M.C., Cunningham, C. & Green, R.M. Isolation and point of action of a factor from *Escherichia coli* required to reconstruct translation. *Proc. Natl. Acad. Sci. USA* **82**, 1648–1652 (1985).
9. Pavlov, M.Y. & Ehrenberg, M. Rate of translation of natural mRNAs in an optimized *in vitro* system. *Arch. Biochem. Biophys.* **328**, 9–16 (1996).
10. Pavlov, M.Y., Freistroffer, D.V., Heurgue-Hamard, V., Buckingham, R.H. & Ehrenberg, M. Release factor RF3 abolishes competition between release factor RF1 and ribosome recycling factor (RRF) for a ribosome binding site. *J. Mol. Biol.* **273**, 389–401 (1997).
11. Guillon, J.M., Mechulam, Y., Schmitter, J.M., Blanquet, S. & Fayat, G. Disruption of the gene for Met-tRNA(fMet) formyltransferase severely impairs growth of *Escherichia coli*. *J. Bacteriol.* **174**, 4294–4301 (1992).
12. Benne, R., Arentzen, R. & Voorma, H.O. The mechanism of action of inititation factor F1 from *Escherichia coli*. *Biochim. Biophys. Acta* **269**, 304–310 (1972).
13. Atkinson, D.E. The energy charge of the adenylate pool as a regulatory parameter. Interaction with feedback modifiers. *Biochemistry* **7**, 4030–4034 (1968).
14. Noren, C.J., Anthony-Cahill, S.J., Griffith, M.C. & Schultz, P.G. A general method for site-specific incorporation of unnatural amino acids into proteins. *Science* **244**, 182–188 (1989).
15. Kim, D.M., Kigawa, T., Choi, C.Y. & Yokoyama, S. A highly efficient cell-free protein synthesis system from *Escherichia coli*. *Eur. J. Biochem.* **239**, 881–886 (1996).
16. Glick, B.R. & Ganoza, M.C. Identification of a soluble protein that stimulates peptide bond synthesis. *Proc. Natl. Acad. Sci. USA* **72**, 4257–4260 (1975).
17. Kossel, H. Purification and properties of peptidyl-tRNA hydrolase from *Escherichia coli*. *Biochim. Biophys. Acta* **204**, 191–202 (1970).
18. Lu, J., Aoki, H. & Ganoza, M.C. Molecular characterization of a prokaryotic translation factor homologous to the eukaryotic initiation factor eIF4A. *Int. J. Biochem. Cell. Biol.* **31**, 215–229 (1999).
19. Spedding, G. Isolation and analysis of ribosomes from prokaryotes, eukaryotes, and organelles. In *Ribosomes and protein synthesis: a practical approach*. (ed. Spedding, G.) 1–29 (IRL Press at Oxford University Press, New York, NY; 1990).
20. Olins, P.O. & Rangwala, S.H. A novel sequence element derived from bacteriophage T7 mRNA acts as an enhancer of translation of the *lac*Z gene in *Escherichia coli*. *J. Biol. Chem.* **264**, 16973–16976 (1989).
21. Sampson, J.R. & Uhlenbeck, O.C. Biochemical and physical characterization of an unmodified yeast phenylalanine transfer RNA transcribed *in vitro*. *Proc. Natl. Acad. Sci. USA* **85**, 1033–1037 (1988).
22. Jelenc, P.C. & Kurland, C.G. Nucleoside triphosphate regeneration decreases the frequency of translation errors. *Proc. Natl. Acad. Sci. USA* **76**, 3174–3178 (1979).
23. Wagner, E.G., Jelenc, P.C., Ehrenberg, M. & Kurland, C.G. Rate of elongation of polyphenylalanine *in vitro*. *Eur. J. Biochem.* **122**, 193–197 (1982).
24. Poe, M., Greenfield, N.J., Hirshfield, J.M., Williams, M.N. & Hoogsteen, K. Dihydrofolate reductase. Purification and characterization of the enzyme from an amethopterin-resistant mutant of *Escherichia coli*. *Biochemistry* **11**, 1023–1030 (1972).
25. Schleif, R.F. L-arabinose operon messenger of *Escherichia coli*. Its inducibility and translation efficiency relative to lactose operon messenger. *J. Mol. Biol.* **61**, 275–279 (1971).
26. Short, G.F., Golovine, S.Y. & Hecht, S.M. Effect of release factor 1 on *in vitro* protein translation and the elaboration of protein containing unnatural amino acids. *Biochemistry* **38**, 8808–8819 (1999).