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An Easy Cell-Free Protein Synthesis System Dependent on the Addition of Crude *Escherichia coli* tRNA[1]

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The protein-synthesizing S30 extract of *Escherichia coli* contains tRNA, which limits its applications in cell-free protein synthesis. Here, we show that at least Arg- and Ser-acceptor activities can be removed from a standard S30 extract by treatment with an immobilized RNase A resin. This RNase-treated extract exhibits no protein synthesis activity, but regains it when supplied with crude *E. coli* tRNA and a small amount of human placental RNase inhibitor. The protein synthesis is dependent on the addition of tRNA in the presence of the RNase inhibitor. Chloramphenicol acetyltransferase was synthesized with this system and found to be active.

Key words: cell-free protein synthesis, immobilized RNase A, ribosome, S30, tRNA.

The S30 extract of *Escherichia coli* contains ribosomes, proteins, and tRNAs that are necessary for cell-free protein synthesis. The efficiency of protein synthesis has been improved so that milligrams of proteins can be produced in a 1-ml reaction by means of optimized methods (*1–4*). The cell-free methods of protein synthesis are advantageous over the *in vivo* methods in their flexibility. For example, many unnatural amino acids have been introduced into proteins through externally-added aminoacyl-tRNA molecules, which has facilitated atomic-resolution mutagenesis studies (*5*, *6*), analyses of the mitochondrial protein transport pathway (*7*, *8*), and theoretical studies of electron transfer (*9*). Isotopically-labeled amino acids can also be introduced into specific positions or regions of proteins (*3*, *10*, *11*), which facilitates NMR signal assignment.

The external aminoacyl-tRNA molecules read a stop codon (*3*, *5*) or a four-base codon (*9*, *12–14*) for the insertion of the desired amino acids. These codon-readings compete with the peptide-chain termination in the case of the stop codon-dependent method, or with the insertion of the natural amino acid by the internal aminoacyl-tRNA in the case of the frameshift-suppression method. These competitions reduce the yields of the full-length products. In principle, the competition can be avoided if the intrinsic tRNA can be eliminated from the S30 extract, and the synthesis can be performed only with the tRNA species required for the translation of the mRNA.

On the other hand, several methods based on the fractionation and reconstitution of the cell extract have been developed for analysis of translation kinetics (*15*, *16*). In these systems, translation can be completely dependent on the addition of tRNA molecules, and the peptide-chain elongation rates are as high as those in the *in vivo* systems. Isotope-labeling of a specific region of a protein has also been achieved by means of ingenious fractionation/reconstitution methods (*11*). However, they have not been applied to large-scale protein preparations. This may be partly due to the complexity of the procedure for the fractionation and reconstitution. In the present study, we developed an easy method for the preparation of a cell-free protein synthesis system that is dependent on the addition of crude tRNA.

MATERIALS AND METHODS

Preparation of the S30 Extract of E. coli—The S30 extract of *E. coli* A19 (*17*) was prepared as described (*18*). In short, A19 cells were grown in 2×YT medium until A_{600} reached 4, and then homogenized with an MSK cell homogenizer (B. Braun). The 30,000 ×*g* supernatant of this lysate was incubated and dialyzed as described (*19*).

Treatment of the S30 Extract with an Immobilized RNase A Resin—The S30 extract was diluted with one volume of the S30 buffer (10 mM Tris acetate, pH 8.2, 14 mM magnesium acetate, 60 mM potassium acetate, 1 mM DTT) before it was treated with RNase. The resin from an immobilized RNase A column (Immobilized RNase A F7m from Mo Bi Tec Inc.) containing 1 mg of the enzyme was removed and suspended in 1 ml of water. An aliquot (25 µl) was placed in a 5-µm Ultrafree filtration cartridge (Millipore), washed with a buffer containing 50 mM Tris-HCl, pH 7.5, and 1 M sodium chloride, and then incubated in another buffer (50 mM Tris-HCl, pH 7.5, 1% SDS) at room temperature for 5 min. The buffer was removed by centrifugation, and 200 µl of the diluted S30 extract was added. The mixture was incubated at 37°C for 1 h, with mixing by pipetting every 20 min. After the reaction, the S30 extract was recovered

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Abbreviation: CAT, chloramphenicol acetyltransferase.

by centrifugation, and the resin was rinsed with 100 µl of the S30 buffer. The solution was recovered and mixed with the treated S30 extract. The S30 extract is diluted threefold through this procedure as compared to the original S30 extract.

mRNAs and tRNA—An mRNA encoding a model peptide was prepared by transcription of a *Bgl*I-digest of pART-23GGC3AGA, as described (*18*, *20*). The coding sequence is composed of 49 codons, including an AGA arginine codon. The mRNA for chloramphenicol acetyltransferase (CAT) was prepared as follows. The CAT gene region of pBR328 (*21*) was amplified by polymerase chain reaction (PCR) primed by 5'-TTA-ATA-CGA-CTC-ACT-ATA-GGC-ACG-TAA-GAG-GTT-CCA-ACT-TTC-3' and 5'-AAA-AAA-ACT-ACG-CCT-TTC-GGC-GTA-GCA-CCA-GGC-GTT-TAA-GGG-3' with *Tth* DNA polymerase (Toyobo, Osaka). The former primer contains the promoter for T7 RNA polymerase. This template DNA was purified by polyacrylamide gel electrophoresis and then transcribed. The transcription product was separated on polyacrylamide gels and recovered by electroelution using a Biotrap™ apparatus (Schleicher and Schuell). *E. coli* unfractionated tRNA was prepared by a standard method (*22*).

Cell-Free Protein Synthesis—The cell-free protein synthesis reaction was performed essentially as described (*20*). The mixture (50 µl) contained 55 mM HEPES-KOH, pH 7.5, 1.7 mM DTT, 275 µM GTP, 26 mM phosphoenolpyruvate, potassium salt, 1.2 mM ATP, 1.9% (w/v) polyethyleneglycol 8000, 34 µg/ml folinic acid, calcium salt, 6.9 mM ammonium acetate, 1 mM spermidine, 7.5 mM magnesium acetate, 210 mM potassium glutamate, 40 µg/ml mRNA, and either a one-sixth volume of the original S30 extract or a half volume of the RNase-treated S30 extract. For synthesis of the model peptide, 0.37 mM each of Val, Thr, Ala, Gly, Tyr, Met, Gln, and Lys, and 44 kBq/ml [^{14}C] Arg were also included. For the synthesis of CAT, all 20 standard amino acids were used. Unless mentioned, the translation reaction with the original extract and that with the RNase-treated extract were different in the magnesium ion concentration, since the S30 buffer contains 14 mM magnesium acetate. This difference was eliminated, in some experiments, by changing the amount of the magnesium acetate added for the translation reactions.

Aminoacylation—Aminoacylation was performed as in the previous study (*20*) using the *E. coli* S100 protein fraction passed through a DEAE-cellulose column (*23*).

CAT Assay—CAT enzyme activity was measured using a FAST CAT Yellow Deoxychloramphenicol Acetyltransferase Expression Kit (Molecular Probes). The substrate is a fluorescent deoxychloramphenicol analog that only gives a single spot of an acetylated product on thin-layer chromatography (Silica gel 60 from Merck, developed with methanol:chloroform = 15:85).

RESULTS AND DISCUSSION

Treatment of the S30 Extract with an Immobilized RNase A Resin—We initially planned to reconstitute protein synthesis by mixing the protein fraction (without ribosomes), ribosomes, and tRNAs. Although it has been established that the protein fraction can be obtained by ultracentrifugation followed by DEAE-cellulose treatment (*23*), we examined another method that would be more convenient if it worked. We treated the S30 extract with an immobilized RNase A, expecting that an RNA-deficient protein fraction without RNase A activity could be obtained easily. As the original S30 was too viscous to pass through the RNase cartridge, it was diluted before treatment with the RNase resin, and then passed through a filter after the reaction.

We first performed a protein synthesis assay using the model-peptide mRNA (Fig. 1A). We found that the protein synthesis activity was completely lost after the RNase treatment (filled triangles), but it was restored when human placental RNase inhibitor and crude *E. coli* tRNA were added (filled diamonds). This shows that this RNase-treated S30 could drive protein synthesis without the supplementation of ribosomes, although a small amount of the RNase A was released into it. When the RNase inhibitor was added but the tRNA was not, protein synthesis activity was not observed (open squares). Thus, the protein synthe-

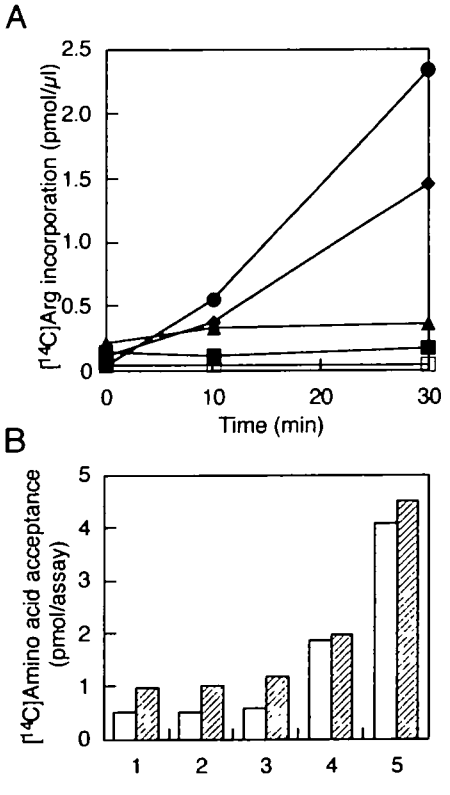

Fig. 1. **Protein synthesis (A) and aminoacylation (B) activities of the RNase-treated and untreated S30 extracts.** (A) A cell-free protein synthesis reaction directed by the model-peptide mRNA (40 µg/ml) was performed with the untreated S30 (without added tRNA) (●), the treated S30 and 34 µM tRNA without RNase inhibitor (▲), the treated S30 and 0.8 unit/µl RNase inhibitor without added tRNA (□), or the treated S30, 34 µM tRNA, and 0.8 unit/µl RNase inhibitor (◆). A negative control reaction was performed with the untreated S30 without mRNA (■). (B) Nucleic acids were phenol-extracted from the RNase-treated or original S30 extract, precipitated with ethanol, and then dissolved in water (50 µl). An aliquot was aminoacylated with the DEAE-cellulose–treated S100 enzyme fraction from *E. coli*. Open bars, Arg acceptance; hatched bars, Ser acceptance. 1, negative control (without added tRNA); 2 and 3, 2- and 5-µl aliquots, respectively, of a sample of the RNase-treated extract; 4 and 5, 2- and 5-µl aliquots, respectively, of a sample of the original extract.

sis was dependent on the crude tRNA. We further measured the amino acid-acceptor activities. Total RNA was phenol-extracted from the RNase-treated S30 and the original S30, and then the Ser- and Arg-acceptor activities were measured (Fig. 1B). The RNA from the treated S30 did not accept these amino acids, while that from the original S30 exhibited significant activities.

The released RNase can partially inhibit the protein synthesis by degrading the mRNA. Thus, we examined the dependence on the amount of added RNase inhibitor (Fig. 2). Cell-free protein synthesis directed by the model-peptide mRNA was performed with the treated S30, in the presence of 0 unit/μl (filled squares), 0.8 unit/μl (filled circles), or more RNase inhibitor. It was found that the protein synthesis activity was constant when more than 0.8 unit/μl of the inhibitor was added (Fig. 2). Thus, the released RNase may be completely inhibited with 0.8 unit/μl RNase inhibitor during the translation reaction. We further examined if the addition of protease inhibitors during the RNase treatment can improve the protein synthesis activity, as a protease originating from the S30 extract may have released the RNase activity from the resin. Phenylmethylsulfonyl fluoride (100 μM), bacitracin (100 μM), and benzamidine (10 μg/ml) had no effects on the translation activity (data not shown). Therefore, it is practical to add the RNase inhibitor during the translation reaction.

tRNA-Dependent Protein Synthesis—The productivity of the S30-based cell-free protein synthesis systems is generally dependent on the concentration of the externally added tRNA. Thus, we measured the dependence of the protein synthesis activity on the added tRNA (Fig. 3). Cell-free translation of the model-peptide RNA was performed with various concentrations of added tRNA. As the total Mg^{2+} concentration affects the protein synthesis rate (*19*), all of the reactions were performed with 10 mM Mg^{2+}, including that within the S30 extract solutions. For the original S30, peptide synthesis was positive at 0 μM tRNA. For the treated S30, peptide synthesis did not proceed at 0 μM but increased as the added tRNA increased. The RNase treatment seems to have damaged the ribosomes slightly, since the productivity of the RNase-treated S30 is lower than that of the original S30 at all of the tested tRNA concentrations. We also analyzed the ribosomal RNAs from the treated S30 and found that they were partially degraded (data not shown). It may be possible to minimize the ribosome damage by optimizing the ratio of the RNase resin and S30 extract, and the incubation conditions.

Synthesis of an Active Enzyme—We examined whether or not the tRNA-dependent translation system can produce an active enzyme. The CAT gene was amplified by PCR with two primers: one of them hybridizes upstream of the coding region and introduces the T7 promoter, and the other introduces a hairpin structure followed by a stretch of T's at the downstream terminus. The PCR product was transcribed, and a purified 807-base mRNA was obtained.

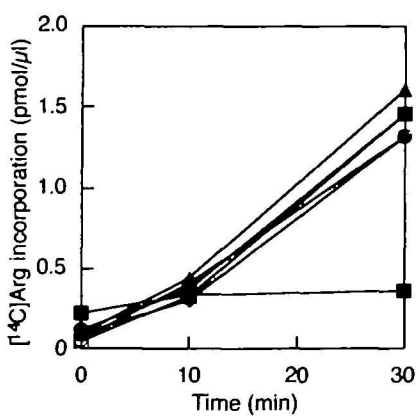

Fig. 2. **Inhibition of the released RNase A by human placental RNase inhibitor.** A cell-free protein synthesis reaction directed by the model-peptide mRNA (40 μg/ml) was performed with the RNase-treated S30 supplemented with 34 μM crude *E. coli* tRNA in the presence of 0 (■), 0.8 (●), 1.6 (▲), 2.4 (◆), 3.2 (□), or 4.0 (○) unit/μl RNase inhibitor.

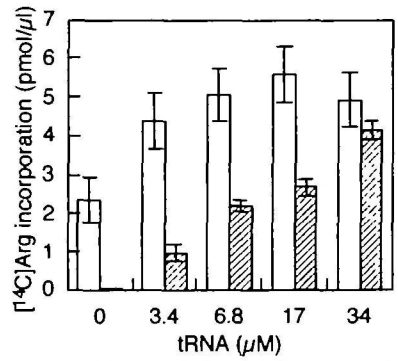

Fig. 3. **Dependence of the model-peptide synthesis on the concentration of added crude *E. coli* tRNA.** A cell-free protein synthesis reaction directed by the model-peptide mRNA (40 μg/ml) was performed for 30 min with various concentrations of added crude *E. coli* tRNA. The total Mg^{2+} concentration was adjusted to 10 mM. The bars show the average Arg incorporation in three experiments with the use of the same preparation of the RNase-treated or original S30 extract. The error bars show the standard deviation. Hatched bars, the RNase-treated S30 supplemented with 0.8 unit/μl RNase inhibitor; open bars, the untreated S30. The Arg incorporation with the RNase-treated S30 without added tRNA was (0.058 ± 0.006) pmol/μl.

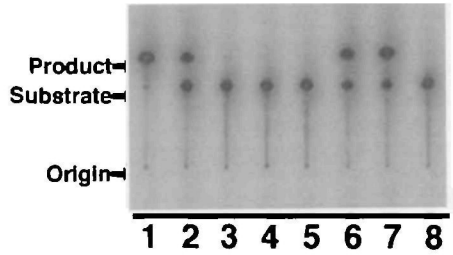

Fig. 4. **CAT enzyme assay.** The result of thin-layer chromatography for separating the substrate chloramphenical analog (lower spots) and the acetylated analog (upper spots) is shown. Lane 1 shows the position of the acetylated chloramphenicol analog. The control CAT enzyme (0.22 ng) provided in the kit (lane 2), 8.4 μl of the RNase-treated S30 extract (lane 3), 25 μl of the original extract (lane 4), the product of the translation reaction with the RNase-treated S30 extract supplemented with 0.8 unit/μl RNase inhibitor (lane 5), the product of the translation reaction with the RNase-treated extract supplemented with the RNase inhibitor and 34 μM crude tRNA (lane 6), the product of the translation reaction with the original S30 extract (lane 7), and the product of the translation reaction with the original S30 without the CAT mRNA (lane 8) were assayed with the use of the commercial kit.

This was translated with the RNase-treated or original S30 (50 µl), and then subjected to the CAT enzyme assay (Fig. 4). While the RNase-treated S30 extract has no CAT activity (lane 3) and cannot synthesize CAT without tRNA (lane 5), it can produce the active enzyme if supplied with tRNA (lane 6). These results indicate that active CAT was synthesized, depending on the addition of tRNA.

Applications of the tRNA-Dependent Cell-Free Protein Synthesis System—The cell-free protein synthesis system driven by the present RNase-treated S30 is totally dependent on the addition of crude tRNA. The present method is more convenient than the reconstitution methods, since the preparation of the protein synthesizing mixture is easier. The RNase-treated S30 is likely to contain all of the molecular chaperones and the other protein factors that the original S30 contains. This may also be an advantage of the present method over the reconstitution methods, because some proteins may not fold correctly during cell-free protein synthesis without molecular chaperones (*24*), and because some unknown protein factor may be involved in translation. On the other hand, RNA factors that are essential for translation, such as 4.5S RNA (*25–27*) and tmRNA (*28–30*), should be supplied externally.

We are now developing a method for the preparation of crude *E. coli* tRNA deficient in the AGU/C-specific serine tRNA isoacceptor. If this mixture can be used with the present RNase-treated S30, it will be possible, in principle, to use these sense codons for the introduction of unnatural/labeled amino acids. By the use of the stop codons or four-base codons, it is difficult to introduce a tRNA-charged amino acid at more than two positions on a single polypeptide, because of the competition mentioned above. The sense codon-based method may be useful for multi-site amino acid introductions. The relationships between the tRNA chemical structure and its coding specificity and efficiency are not fully understood. The present cell-free protein synthesis methods may also be applied to the analysis of some tRNA coding properties.

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