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Stabilization and Translation of Immobilized mRNA on Latex Beads for Cell-Free Protein Synthesis System

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Abstract

The stability of immobilized mRNA against ribonucleases was investigated in a cell-free protein synthesis system. The plasmid-encoding protein A with the 20-mer poly(A) tail under the control of T7 promoter was constructed, and the corresponding mRNA was synthesized by T7 RNA polymerase reaction. The resulting mRNA was immobilized on oligo(dT)-immobilized latex beads by hybridization utilizing the poly(A) tail of mRNA at the 3'-terminus. The mRNA was stabilized against three types of nucleases (3'-OH exonuclease, 5'-OH exonuclease, and endonuclease) by immobilization. Translation of immobilized mRNA with a continuous-flow cell-free protein-synthesizing system from *Saccharomyces cerevisiae* was ascertained. Reusability of the immobilized mRNA as genetic information was also examined.

Index Entries: Stabilization of mRNA; immobilized mRNA; cell-free protein synthesis; continuous flow system; *Saccharomyces cerevisiae*; protein A.

Introduction

Since the 1980s, cell-free protein synthesis has progressed remarkably by the development of a flow system by Spirin et al. *(1–3)*. In their system, a feeding buffer solution including amino acids, adenosine 5'-triphosphate

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(ATP), and guanosine 5'-triphosphate (GTP) is supplied to the cell-free reaction mixture containing exogenous mRNA. The produced protein is eluted out through an ultrafiltration membrane. Through the use of this system, proteins have been produced in high yield.

One of the drawbacks of cell-free protein synthesis is the degradation of mRNA by contaminating nucleases. Specific and selective production of a desired protein can be performed when a given mRNA is stabilized and translated continuously to the corresponding protein. The addition of some ribonuclease inhibitors to the reaction mixture is insufficient to protect the mRNA from nucleases. An *Escherichia coli*–coupled transcription-translation system, developed by Zubay *(4)*, is a useful method for protein synthesis since the preparation of labile mRNA is not necessary. This *E. coli*–coupled system has been applied to continuous cell-free protein synthesis systems *(2,5)*. However, its application to the eukaryotic cell-free system appears to be difficult, because addition of 5'-cap structure to mRNA is needed for stabilization of mRNA and efficient translation.

Some researchers have tried to modify mRNA in order to protect free mRNA from nucleases. Ueda et al. *(6)* incorporated phosphorothioate groups into mRNAs, then examined their stability against nucleases and template properties in translational systems. The 3'-terminus of mRNA was modified with trideoxyribonucleotide or its phosphorothioate analog, and its stability against nucleases under the conditions of the formation of the translational initiation complex in vitro was studied *(7)*.

We have immobilized mRNA on a solid-phase matrix for stabilization, and the matrix-bound solid-phase mRNA may be reused as genetic information. Previously, we reported the selective production of silk fibroin in the mRNA-immobilized form by addition of amino acids and energy-source substances *(8)*. The mRNA for fibroin was extracted from the posterior gland of *Bombyx mori* and was immobilized on poly(U)-agarose gel by the poly(A) tail at the 3'-terminus of the mRNA. A number of eukaryotic cell-free systems, such as rabbit reticulocyte lysate *(9)*, wheat germ *(10)*, mouse Krebs II ascites cells *(11)*, and yeast *(12,13)*, are now available for the translation of exogenous mRNA. Among these eukaryotic cell-free systems, yeast (*Saccharomyces cerevisiae*) lysate has several advantages, including easy preparation and a good understanding of gene expression. In our previous study *(14)*, mRNA was immobilized on an oligo (dT)-cellulose matrix by the poly(A) tail at the 3'-terminus of the mRNA with translation done using the yeast cell-free system. The genetic information of the immobilized mRNA was translated to polypeptide, which was confirmed by incorporation of ^{3}H-labeled amino acids into the produced polypeptides.

In the present study, we used latex beads as the matrix for immobilization of the mRNA in order to construct an efficient protein synthesis system. Since the dispersion of the latex beads in the reaction mixture is better than that of the cellulose matrix used in our previous study, improved efficiency in translation of the mRNA would be expected, especially in a continuous-flow system. The stability of immobilized mRNAs against

nucleases was investigated. Furthermore, cell-free translation of immobilized mRNA for protein A, as a model for a functional protein, was examined with a continuous-flow system.

Materials and Methods

Materials

Oligotex™-dT30 (oligotex), T7 RNA polymerase and restriction endonucleases were purchased from Takara Shuzo (Shiga, Japan). Tritium-labeled phenylalanine and ^{32}P-CTP were provided from Dupont/NEN (Boston, MA). Phosphodiesterase I (PDE I), phosphodiesterase II (PDE II), and ribonuclease A (RNaseA) were purchased from Funakoshi (Tokyo, Japan), and IgG-Sepharose was purchased from Pharmacia (Uppsala, Sweden).

Preparation of Yeast Cell-Free System

The preparation method of the yeast cell-free translation system was as described previously *(15)*, except cell lysis was done with glass beads *(16)*. *S. cerevisiae* was grown in YEPD medium, centrifuged, resuspended in 1.0 *M* sorbitol, and incubated for 1 h at 20°C with slight agitation. Cells were centrifuged and resuspended in YM5 with $MgSO_4$ and incubated for 90 min at 20°C with slight agitation. The cells were collected by centrifugation, resuspended in 20 m*M* HEPES-KOH buffer solution (pH 7.4) containing 100 m*M* KOAc, 2 m*M* $Mg(OAc)_2$, and dithiothreitol (DTT). Acid-washed glass beads (0.5 mm diameter) were added to the tube, and the cells were agitated vigorously by a high-speed vortex mixer. The cell lysate was centrifuged for 10 min at 30,000*g*, and the supernatant without the lipid layer was centrifuged again for 30 min at 100,000*g*. The supernatant was passed through a Sephadex G-25 column, and void fractions, which were used as the cell-free system, were collected.

Plasmid Construction

The plasmid, which encoded a structural gene for protein A with the poly(A) tail at the 3'-terminus, was constructed as follows. The structural gene for protein A from pMPRA1, which was constructed in our laboratory, was inserted under the T7 promoter of pBluescriptSK+ (TOYOBO, Tokyo, Japan). A synthesized 20-mer DNA fragment including the poly(A) tail was inserted at the 3'-terminus of the gene for protein A. The resulting plasmid, designated pBSPAA3, is schematically drawn in Fig. 1A.

Preparation and Immobilization of mRNA

mRNA of mouse myeloma cells was prepared by following the method of Premkumar et al. *(17)*. Myeloma cells growing at a density of 6×10^5 cells/mL were sedimented, washed, and separated into nuclei and cytoplasm, and the total RNA was isolated from the cytoplasmic fraction by the

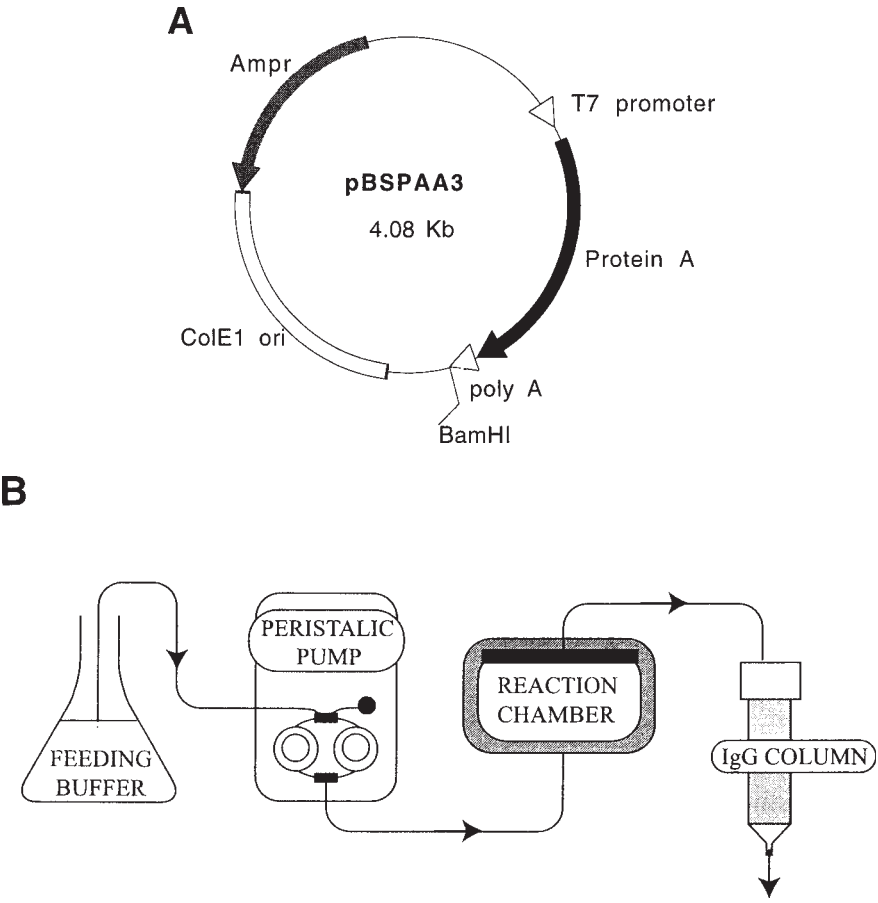

Fig. 1. **(A)** Schematic drawing of plasmid pBSPAA3. The positions of the structural genes coding for protein A and poly(A) are indicated. The T7 promoter, ColE1 ori, and β-lactamase gene (*Ampr*) are also indicated. **(B)** Schematic illustration of the continuous-flow cell-free translation system for protein A production. Immobilized mRNA for protein A is translated in a reaction chamber and the produced protein is specifically trapped in an IgG column.

phenol-chloroform method. After phenol extraction, the RNA was precipitated with 2 vol of ethanol at –20°C. The precipitate was collected by centrifugation and dissolved in a phosphate buffer solution (pH 7.6) containing 0.5 m*M* $MgCl_2$ and 75 m*M* NaCl.

The pBSPAA3 was linearized with *Bam*HI and transcribed by T7 RNA polymerase. The resulting 1.1-kb mRNA including the poly(A) tail of 20-mer was suspended in an elution buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, 0.1% sodium dodecyl sulfate [SDS], pH 7.5). Oligotex is a 30-mer of oligo(dT) immobilized at the 5'-end on latex beads by chemical bonding. Oligotex was added to the mRNA solution and incubated at 65°C for 5 min. Approximately 4 μg of mRNA can be immobilized on 5 mg (50 μL) of oligotex by hybridization. After cooling on ice, 1/10 vol of 5 *M* NaCl was

added to the reaction mixture, which was then incubated at 37°C for 10 min. The mRNA-immobilized beads were centrifuged at 15,000*g* for 3 min and washed with buffer (10 m*M* Tris-HCl, 1 m*M* EDTA, 0.1% SDS, 0.5 *M* NaCl, pH 7.5).

mRNA Stability Against Nuclease

To estimate the stability of mRNA, ^{32}P-CTP was incorporated into mRNA. mRNA was quantified by measuring the absorbance at 260 nm, and 1.5 µg of ^{32}P-labeled mRNA was used for each experiment to estimate stabilization against nucleases. Immobilized mRNA or free mRNA was incubated with PDE I, PDE II, and RNaseA, respectively.

PDE I was used as 3'-OH exonuclease *(18)* and 1.5 µg of free mRNA or immobilized mRNA, labeled with ^{32}P-CTP, was mixed with 10^{-1}, 10^{-2}, and 10^{-3} U of PDE I in Tris buffer (10 m*M* Tris-HCl, pH 7.0). One unit is defined as the amount of enzyme required to hydrolyze 1 m*M* *p*-nitrophenylthymidine-5'-phosphate for 1 min at 25°C. The mixtures were incubated at 37°C for 15 min. Proteinase K (1 mg/mL) and $CaCl_2$ (4 m*M*) were added and incubated at 37°C for 30 min to stop the reaction. In the case of immobilized mRNA, the matrix was recovered by centrifugation (15,000*g*, 3 min), and the mRNA was detached from the matrix by incubation at 65°C for 10 min in diethyl pyrocarbonate–treated water. The recovered mRNA was purified by a phenol/chloroform extraction, then ethanol precipitated, and analyzed by electrophoresis using agarose (1.4%) containing formaldehyde. The radioactivity in full-length mRNA was quantified for each sample by radioactivity of the corresponding bands on a gel using Bioimaging Analyzer BAS 2000 (Fuji Film, Japan).

PDE II was used as 5'-OH exonuclease *(19)*. Free or immobilized mRNAs were mixed with 10^{-1}, 10^{-2}, and 10^{-3} U of PDE II. One unit is defined as the amount of enzyme required to increase A_{260} of acid-soluble total nucleotide in 0.2 for 30 min at 37°C. The procedure was the same as described for PDE I.

RNaseA was used as endonuclease *(20)*. Free or immobilized mRNAs were mixed with 10^{-6}, 10^{-7}, and 10^{-8} U of RNaseA. One unit is defined as the amount of enzyme required to increase A_{260} of acid-soluble total nucleotide in 1.0 for 4 min at 37°C. The procedure for this experiment was the same as described for PDE I.

Cell-Free Translation of Immobilized mRNA with Continuous-Flow System

The 0.5-mL reaction mixture for cell-free translation contained 25 m*M* HEPES-KOH (pH 7.4), 150 m*M* potassium acetate, 3.0 m*M* magnesium acetate, 0.5 m*M* ATP, 0.1 m*M* GTP, 25 m*M* creatine phosphate, 80 µg/mL of creatine phosphokinase, 2.1 m*M* DTT, 6% glycerol, 50 µ*M* each of 20 essential amino acids, and 165 µL of the yeast cell-free system. The feeding buffer contained HEPES-KOH (pH 7.4), 3.0 m*M* magnesium

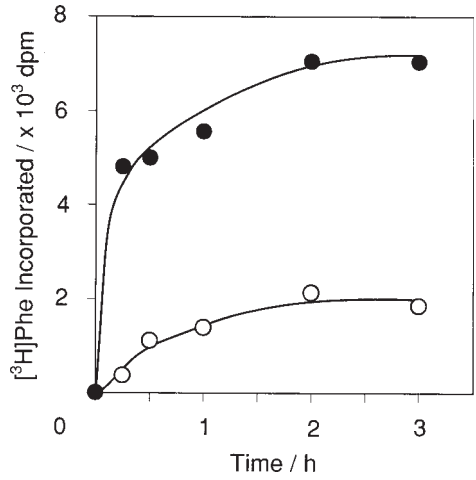

Fig. 2. Translation of immobilized mRNA. mRNA extracted from mouse myeloma cells was immobilized on oligotex beads and incubated with yeast cell-free translation system. Oligotex with mRNA (●) and without mRNA (○) were tested in this system. After incubation for varying time periods, 5 µL of each reaction mixture was spotted on a filter paper. Incorporation of ^{3}H-labeled phenylalanine in proteins as acid-insoluble product was measured by a liquid scintillation counter.

acetate, 1 m*M* ATP, 0.2 m*M* GTP, 25 µ*M* each of 20 amino acids, and 0.5 m*M* phenylmethylsulfonylfluoride. The reaction mixture was set in a reaction chamber (Molcut UFG1TGC24, mol wt 10,000; Millipore, Tokyo, Japan) and the feeding buffer was fed into the chamber. After the reaction chamber, IgG-Sepharose was used to trap the protein A produced. Figure 1B schematically illustrates the flow system.

Results and Discussion

Translation of Immobilized mRNA

To confirm the translational activity of immobilized mRNA, mRNA extracted from mouse myeloma cells was immobilized on oligotex and incubated with the *S. cerevisiae* cell-free translation system. To stabilize the hydrogen-bonding interaction between mRNA and oligotex beads, 500 m*M* NaCl was included in the reaction mixture. Approximately 1 mg of mRNA-immobilized oligotex was suspended in a 50-µL solution for cell-free protein synthesis (*see* Materials and Methods) and incubated at 22°C. Figure 2 shows the time course of ^{3}H-labeled phenylalanine incorporation into the protein product with and without mRNA-immobilized matrix. In the cell-free system without immobilized mRNA, some incorporation of [^{3}H]Phe was observed, which may be owing to yeast endogenous mRNA slightly contaminating in the lysate. However, more incorporation of [^{3}H]Phe was observed by adding immobilized mRNA. The translational activity of immobilized mRNA was about 20% of that of free mRNA (data

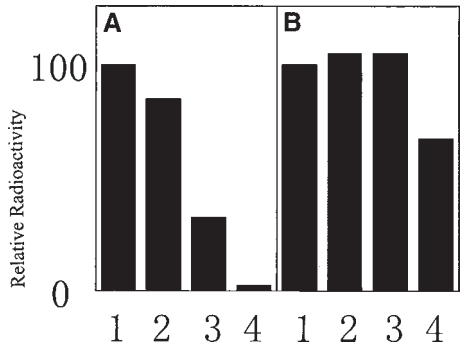

Fig. 3. Stability of mRNA against PDE I. The remaining amounts of full-length mRNA after treatment with PDE I were determined by the radioactivity of the corresponding band on a gel. The relative radioactivities of free mRNAs **(A)** and immobilized mRNAs **(B)** are shown. Lane 1 (100%), mRNA incubated without PDE I as the control; lane 2, 10^{-3} U PDE I; lane 3, 10^{-2} U PDE I; lane 4, 10^{-1} U PDE I.

not shown). Such a drop in translational activity may be caused by steric interference of the ribosome molecules to the bulky immobilized mRNA. Another possibility is that the sodium ions in the reaction mixture affected translation. Although the translational activity of immobilized mRNA was lower than that of free mRNA, the exogenous mRNA was translated in an immobilized form.

Stability of Immobilized mRNA Against Nucleases

The stability of immobilized mRNA was investigated using three types of nuclease as described in Materials and Methods.

PDE I was used as 3'-OH exonuclease and the results are shown in Fig. 3. When the mRNAs were immobilized on oligotex (Fig. 3B), mRNAs were protected from PDE I attack, compared with free mRNA (Fig. 3A). Immobilized mRNAs were not degraded at all when 10 m*M* PDE I was added. When 100 m*M* PDE I was added, approx 70% of immobilized mRNAs remained whereas almost all free mRNAs were degraded.

Figure 4 shows the stability of mRNAs against PDE II as 5'-OH nuclease. Free mRNAs were degraded in a concentration-dependent manner (Fig. 4A). However, immobilized mRNA was stable against PDE II up to 10 mU (Fig. 4B).

In the case of RnaseA, which was used as endonuclease, almost all mRNAs were degraded in their free form (Fig. 5A). However, immobilized mRNAs exposed to RNaseA at 10^{-6}, 10^{-7}, and 10^{-8} U showed very little degradation (Fig. 5B).

The stabilization of mRNA was improved against 3'-OH exonuclease (PDE I), 5'-OH exonuclease (PDE II), and endonuclease (RNaseA) activity. These results might be caused by steric obstruction of the nucleases to the bulky immobilized mRNA. Another reason for the stabilization is the drop of the enzymatic activities owing to the high salt concentration used to form

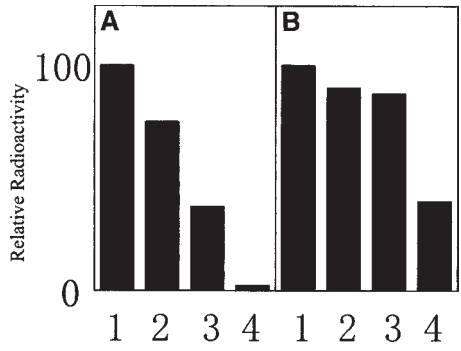

Fig. 4. Stability of mRNA against PDE II. The amount of full-length mRNA remaining after PDE II treatment was determined by the radioactivity of the corresponding band on a gel. The relative radioactivities of free mRNAs **(A)** and immobilized mRNAs **(B)** are shown. Lane 1, mRNA incubated without PDE II as the control; lane 2, 10^{-3} U PDE II; lane 3, 10^{-2} U PDE II; lane 4, 10^{-1} U PDE II.

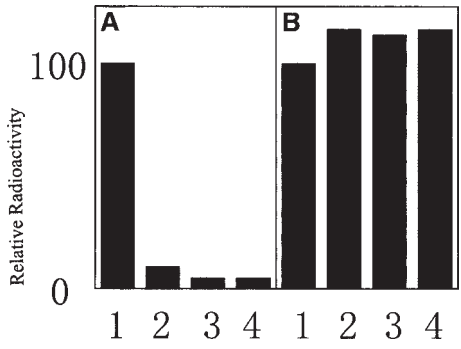

Fig. 5. Stability of mRNA against RNAseA. The remaining amounts of full-length mRNA after treating with RNaseA were determined by the radioactivity of corresponding band on a gel. The relative radioactivities of free mRNAs **(A)** and immobilized mRNAs **(B)** are shown. Lane 1, mRNA incubated without RNaseA as a control; lane 2, 10^{-6} U RNaseA; lane 3, 10^{-7} U RnaseA; lane 4, 10^{-8} U RNaseA.

stable hydrogen bonds between the mRNA and the oligotex beads. Actually, nuclease activities to free mRNAs in the presence of 500 m*M* NaCl were less than those in the absence of NaCl (data not shown).

Translation of Immobilized mRNA Encoding Protein A

Protein A, which binds specifically to the Fc region of IgG, was selected as the functional protein for translation because it is easily detected by immunochemical methods such as Western blotting. The protein A mRNA containing a poly(A) tail was immobilized on oligotex beads and then set in a reaction chamber with the yeast cell-free system. The feeding solution was supplied continuously through a reaction chamber with a constant

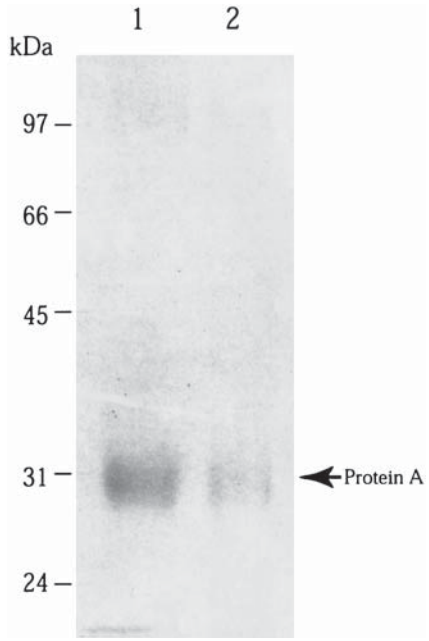

Fig. 6. SDS-PAGE analysis of synthesized protein A with flow system. An eluted fraction from IgG-Sepharose was analyzed by 15% SDS-PAGE. The proteins were stained with Coomassie Brilliant Blue R-250. Lane 1, produced protein from the immobilized mRNA; lane 2, produced protein from the recovered immobilized mRNA.

flow rate (2 mL/h). After 20 h, produced proteins that bound to IgG-Sepharose were eluted with 0.5 *M* acetate buffer (pH 3.4) and analyzed by 15% SDS-polyacrylamide gel electrophoresis (PAGE) *(21)*. Figure 6 shows the result of the SDS-PAGE analysis for eluted protein. A protein of 30 kDa, which is the molecular mass corresponding to protein A, was observed in lane 1. The protein was transferred on a nitrocellulose membrane and reacted with mouse IgG in Western blotting (data not shown). The immobilized mRNA was correctly translated, and active protein A was produced with the yeast cell-free system.

To confirm the stability and reusability of immobilized mRNAs in the cell-free protein synthesis, oligotex beads with immobilized mRNA were isolated from the reaction mixture by centrifugation and translational activity was investigated again. The recovered immobilized mRNA and the reaction mixture were added to a fresh cell-free system in the reaction chamber, and the reaction was continued for another 20 h. The produced proteins were analyzed as described previously and are shown in Fig. 6 (lane 2). The band is observed at the same location as the first reaction in lane 1 of Fig. 6, although its intensity is decreased. These results indicate that mRNA stabilized by immobilization retain translational activity and can be reused for translation.

Conclusion

A major drawback of cell-free protein synthesis is the short lifetime of mRNA as a source of genetic information. In the cell-free system, mRNAs are translated only a few times, whereas mRNAs in living cells are repeatedly translated. Therefore, improvement of the stability and the reusability of mRNAs is necessary for an efficient cell-free protein synthesis system.

In this study, mRNA including a poly(A) tail was immobilized on latex beads by hybridization. Since the mRNA was immobilized at its 3'-terminus, the reading from the 5'-terminus by a ribosome system seemed to be initiated without problems, although the translational activity was less than that of free mRNA. To stabilize the hydrogen bonds between the mRNA and the matrix, a relatively high concentration of sodium chloride was included in the reaction mixture. The stability of mRNA against nucleases could be improved by both immobilization and salt concentration. mRNA was protected and the immobilized mRNA functioned as the source of genetic information after long-term reaction by a continuous-flow system, and functional protein A with IgG-binding activity could be produced.

Since the intrinsically labile mRNA was used as a stable source of genetic information by immobilization, this method is expected to be a useful technique for the construction of efficient cell-free protein synthesis systems.

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