Expression of a cascading genetic network within liposomes

Keitaro Ishikawa[a,1], Kanetomo Sato[a,1], Yasufumi Shima[a], Itaru Urabe[a], Tetsuya Yomo[a,b,c,d,e,*]

[a]Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan
[b]Department of Bioinformatics Engineering, Graduate School of Information Science and Technology, Osaka University, Osaka, Japan
[c]Department of Pure and Applied Sciences, The University of Tokyo, 3-8-1 Komaba, Meguro-ku, Tokyo 153-8902, Japan
[d]Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan
[e]ERATO, ZST, Tokyo, Japan

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Abstract **Liposomes have long been used as possible compartments for artificial cells, and it has been shown that liposomes can sustain various types of biochemical reactions. To elevate the degree of molecular complexity of the system in liposomes, we have constructed a two-stage genetic network encapsulated in liposomes. This two-stage genetic network was constructed with the plasmid pTH, in which the protein product of the first stage (T7 RNA polymerase) is required to drive the protein synthesis of the second stage (GFP). We show that the two-stage genetic network constructed in a cell-free expression system is functional within liposomes.**

Keywords: Liposome; Genetic network; Cell-free protein synthesis; T7 RNA polymerase; GFP; Flow cytometry

1. Introduction

Challenges for synthesizing a minimal cell are expected to provide clues for comprehending the essence of life and its origin [1]. As an experimental approach to the minimal cell, liposomes have long been used as a boundary for compartmentation [1–12], which is crucial to bring forth life and its evolution [1,13]. Various types of biochemical reactions have already been carried out within liposomes [2–11] and one of the most essential of these reactions in living organisms is RNA synthesis. Walde et al. [5] reported the enzymatic polymerization of ADP to poly(A) in vesicles using polynucleotide phosphorylase. As this work developed further, replication of a RNA template by Qβ replicase [6] and mRNA synthesis using a DNA template and T7 RNA polymerase [10] were carried out in vesicles.

Another important reaction is protein synthesis, a more complex reaction catalyzed by ribosomes. Oberholzer et al. reported the ribosomal synthesis of poly(Phe) in liposomes using poly(U) as the RNA messenger. By combining mRNA synthesis and protein synthesis, we can construct a gene-expression system. Furthermore, Yu et al. [9] demonstrated that a GFP (green fluorescent protein) can be synthesized in liposomes by a coupled cell-free transcription and translation system and that the synthesized protein is fluorescent and hence functional. Another GFP was also synthesized by a similar coupled transcription and translation system in giant vesicles [11].

The next step toward a higher degree of genetic complexity is to construct a functional cell-free genetic network [14] in liposomes. Here, we engineered a transcriptional activation cascade in which the protein product of the first stage is the input required to drive the second stage. We selected a T7 RNA polymerase gene for the first stage and a GFP gene, in which expression is controlled by a T7 promoter, for the second stage. We demonstrated that the transcriptional activation cascade constructed in a cell-free expression system is functional within liposomes. This study, hence, provides another step forward in overcoming many challenges in pursuance of synthesizing a minimal cell.

2. Materials and methods

2.1. Materials

Escherichia coli strains used were BL21(DE3) (Novagen), JM109 [16], and DPB267 [17]. The *E. coli* DPB267 was a kind gift from Dr. S.N. Cohen of Stanford Medical School. Plasmids used were pQE-30 (QIAGEN), pET21-GFPmut1-His6 [9], pQE-T7RNAP, pTH, and pTHΔT7RNAP. The plasmid pET21-GFPmut1-His6 contains a gene for GFPmut1 with a C-terminal His-tag under the control of T7 promoter and was constructed as described previously [9]. The plasmids pQE-T7RNAP, pTH, and pTHΔT7RNAP were prepared as described below. T7 RNA polymerase and SP6 RNA polymerase were purchased from TAKARA BIO INC., RNase was from Sigma, and cholesterol was from Nacarai Tesque INC. EggPC (egg yolk phosphatidyl choline) and DSPE-PEG5000 (distearoyl phosphatidyl ethanolamine-poly(ethylene glycol) 5000) were kindly supplied by NOF CORPORATION (Tokyo), and S-30 cell extract was prepared from *E. coli* DPB267 as described by Zubay [18,19].

2.2. Construction of plasmids

Cloning was performed by following routine procedures [20]. The T7 RNA polymerase gene was obtained from the *E. coli* BL21(DE3) genome by PCR (polymerase chain reaction) using the forward primer AGGCACGGATCCAACACGATTAACATCGCTAAG (underlined bases indicate the *Bam*HI site) and the reverse primer TGATTTAAGCTTACGCGAACGCGAAGTCCGACTC (underlined bases indicate the *Hin*dIII site). The amplified gene was inserted into the *Bam*HI and *Hin*dIII sites of plasmid pQE30 to produce the plasmid pQE-T7RNAP. The cloned T7 RNA polymerase gene on the

* Corresponding author. Address: Department of Biotechnology, Graduate School of Engineering, Osaka University, 2-1 Yamadaoka, Suita, Osaka 565-0871, Japan. Fax: +81-6-6879-7428.
E-mail address: yomo@bio.eng.osaka-u.ac.jp (T. Yomo).

[1]These authors contributed equally to this work.

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plasmid was amplified by PCR using the forward primer GGGGGGAGATCT**ATTTAGGTGACACTATAGAA**TTGTGAGC-GGATAACAATTTC (underlined bases indicate the *Bgl*II site and bold bases the SP6 promoter) and the reverse primer CCCCCC-AGATCTGCTAGCTTGGATTCTCACCAATAAAAAAC (underlined bases indicate the *Bgl*II site). The PCR product was inserted into the *Bgl*II site of plasmid pET21-GFPmut1-His6 to produce the plasmid pTH (Fig. 1A). Plasmid pTHΔT7RNAP, shown in Fig. 1B, was prepared by digesting pTH with Eco81I and then self-ligating the resulting fragments, deleting an approximately 180-bp fragment from the T7 RNA polymerase gene.

2.3. Liposome preparation

The lipid mixture (1.2 μmol; molar ratio of eggPC:cholesterol:DSPE-PEG5000 = 1.5:1:0.08) dissolved in dichloromethane/diethyl ether (1:1, v/v) was rotary-evaporated in a pear-shaped flask under vacuum at room temperature to yield a thin lipid film. Added to the lipid film was 100 μl of deionized water at room temperature. After standing for 15 min at room temperature, the lipid film was vortexed to disperse the liposomes, with a final concentration of 12 mM. The liposome dispersion sonicated with an ultrasonic disrupter (TOMY SEIKO Co, Ltd.) was filtered with a 0.4-μm polycarbonate filter (Whatman Inc.). The solution was transferred into an Eppendorf tube and lyophilized (Labconco Corporation). The reaction mixture (10 μl) was added to yield the liposome dispersion as described below.

2.4. Coupled transcription and translation

The reaction conditions were almost the same as those described previously [9]. The reaction mixture (40 μl) contained 88 mM Tris–OAc (pH 8.2), 30 mM NH_4OAc, 90 mM KOAc, 17 mM $Mg(OAc)_2$, 2.5% PEG8000, 7.9 mM ATP, 0.88 mM each of GTP, UTP, and CTP, 0.325 mM each of the 20 types of amino acids, 1.75 mM dithiothreitol, 0.5 mM IPTG (isopropylthiogalactoside), 128 μg/ml folic acid, 0.17 mg/ml tRNA, 28.2 mM phosphoenol pyruvate, 0.65 mM cAMP, 16 nM of template DNA, and 8.0 μl of *E. coli* S-30 cell extract. When reactions were carried out in liposomes, 10 μl of the reaction mixture was added to the lyophilized liposomes described above and mixed by pipetting at 4 °C to disperse the liposomes to give a final lipid concentration of 120 mM. The liposome dispersion kept at 4 °C was diluted with 70 μl of the cold reaction mixture, which is the same as that entrapped inside the liposomes except that it contains 12 μg/ml RNase and no template DNA. The RNase concentration used was confirmed to ensure that protein synthesis would not occur outside the liposomes. To initiate protein expression, the reaction mixture or diluted liposome dispersion prepared as described above was kept at 4 °C and incubated at 37 °C.

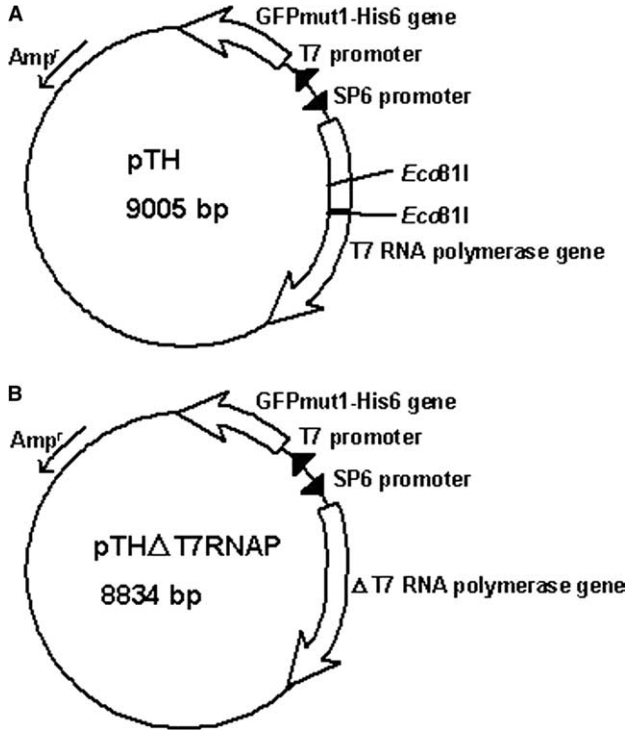

Fig. 1. Structures of plasmids pTH (A) and pTHΔT7RNAP (B). The abbreviation Amp^r represents the ampicillin-resistance-coding region.

2.5. SDS–PAGE analysis of proteins produced by coupled transcription-translation

Proteins were synthesized in the cell-free expression system (40 μl) as described above, with the addition of 1.1 mCi/μl L-[^{35}S] Methionine (Amersham Pharmacia Biotech). After 1-h incubation at 37 °C, the solution was added to 320 μl of ice-cold acetone and kept on ice for 15 min to precipitate the translation products. The precipitate was collected by centrifugation, dried under vacuum at room temperature, and analyzed by SDS–PAGE using a 12% gel. Following electrophoresis, the gel was soaked in a fixing buffer (isopropanol:acetic acid:-water = 5:2:13, v/v) for 30 min and then in Amplify (Amersham Bioscience K.K.) for 30 min. After drying by heating under vacuum, the gel was exposed to Hyperfilm™ MP (Amersham Pharmacia Biotech) at −70 °C for 8 h. The film was developed with a film processor (FPM100, Fuji Medical Systems Ltd., Tokyo, Japan).

2.6. Flow cytometric analysis of protein synthesis in liposomes

Liposomes encapsulating the cell-free expression system were prepared at 4 °C as described above. The liposome dispersion was incubated at 37 °C for 1 h, after which the reaction was terminated by immediately placing the tube on ice. The production of GFPmut1-His6 in liposomes was analyzed with an EPICS® ALTRA™ HyPerSort flow cytometer (Beckman Coulter, Miami, FL, USA) equipped with a 488-nm water-cooled argon ion laser (Coherent Japan, Inc.) at 20 mW. The analytical conditions were almost the same as those described previously [9]. The Flow-check™ Fluorospheres 10-μm bead (Beckman Coulter, Inc.) was introduced prior to measuring samples for alignment of the 488-nm argon ion laser source to set half-peak coefficients of variation (CVs) to less than 1.5%. In order to calibrate the daily variance, fluorescence-labeled latex beads of 0.5, 1, 2, 3, and 6 μm (fluorescent flow cytometer calibration grade-size range kit, Polyscience Inc., Warrington, PA, USA) were used. A discriminator of 60 channels out of 1024 was set on forward scatter (FS). Fluorescence signals were then measured successively using a 488-nm band block filter, a 550-nm dichroic lens, and a bandpass filter at 525 ± 10 nm. Fluorescence intensity and FS data were collected with a logarithmic amplifier, and GFPmut1-His6 synthesis in 2.5×10^5 liposomes of each sample was analyzed.

3. Results and discussion

A two-stage cascading genetic network was constructed with the plasmid pTH, which contains the T7 RNA polymerase gene under the control of the SP6 promoter and the GFPmut1-His6 gene under the T7 promoter (Fig. 1A). The cascading network was designed to work in a manner that the SP6 RNA polymerase input drives the production of T7 RNA polymerase, which in turn induces the expression of GFPmut1-His6. GFPmut1 is a mutant of GFP exhibiting fluorescence 35 times as intense as that of the wild type [15]. The cascading network was characterized in a test tube using an *E. coli* cell-free expression system described in Section 2. SDS–PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) analysis of the proteins labeled by the incorporation of [^{35}S]Met during the cell-free protein synthesis revealed that both GFPmut1-His6, with an expected molecular weight of about 28 kDa, and T7 RNA polymerase, with an expected molecular weight of about 100 kDa, were produced irrespective of whether SP6 RNA polymerase was added (Fig. 2, lanes 4 and 6). The unexpected expression of the T7 RNA polymerase gene without the addition of SP6 RNA polymerase may be due to the possibilities that RNA polymerase in the *E. coli* S-30 cell extract recognizes the SP6 promoter and/or unknown pro-

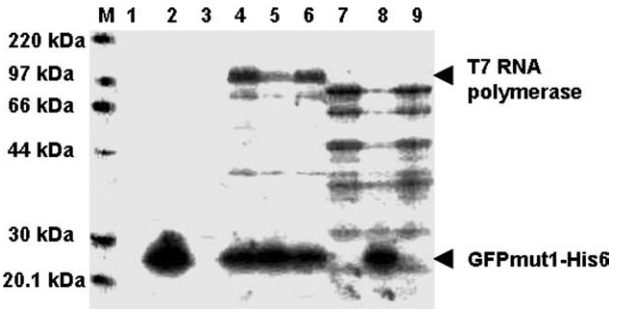

Fig. 2. Expression of RNA polymerases and GFPmut1-His6. Proteins synthesized in the cell-free expression system containing [^{35}S]Met were analyzed by SDS–PAGE and visualized by autoradiography, as described in Section 2. The template DNAs used were: pET21-GFPmut1-His6 for lanes 1–3; pTH for lanes 4–6; and pTHΔT7RNAP for lanes 7–9. To the reaction mixtures for lanes 2, 5, and 8, T7 RNA polymerase was added (0.5 Units/μl), to those for lanes 3, 6, and 9, SP6 RNA polymerase was added (0.5 Units/μl), and to those for lanes 1, 4, and 7, no RNA polymerase was added. Lane M: marker proteins (Rainbow™ [^{14}C]methylated protein molecular-weight markers, 148 kBq/ml).

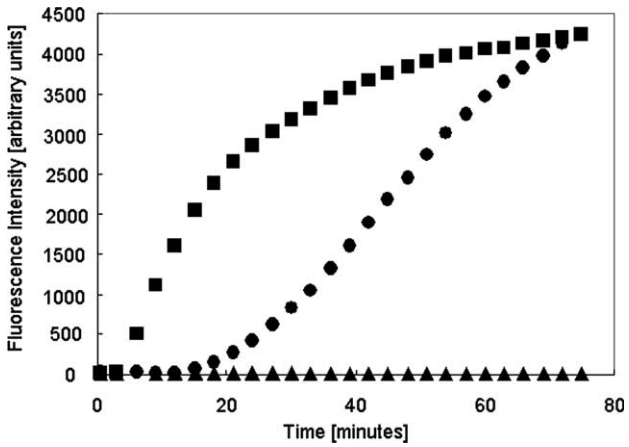

Fig. 3. Kinetics of GEPmut1-His6 production in cell-free expression system. Time courses of GFPmut1-His6 expression in the coupled transcription-translation system described in Section 2 were monitored at 37 °C by the increase in the fluorescence emission at 545 nm (excitation at 488 nm) of the reaction mixture using an ABI PRISM 7700 Sequence Detector (PE Applied Biosystems). The template DNAs used were pTH (filled circle) and pTHΔT7RNAP (filled square, filled triangle). To the reaction mixture for a filled square, T7 RNA polymerase was added.

moter-like sequences upstream of the T7 RNA polymerase gene, although the activity of the SP6 promoter for *E. coli* RNA polymerase was reported to be very weak [21]. However, the unexpected expression of the T7 RNA polymerase gene does not alter the essential system of the two-stage genetic network constructed with the plasmid pTH; it only simplifies the system by omitting the addition of the SP6 RNA polymerase.

To confirm that the expression of the T7 RNA polymerase gene on the plasmid pTH is necessary to express the GFP gene on the same plasmid, the same experiment was conducted replacing pTH with pTHΔT7RNAP, which contains the gene for an inactive deletion mutant of T7 RNA polymerase instead of the wild-type polymerase gene of pTH (Fig. 1B). The results revealed that while the deleted mutant, with an expected molecular weight of about 94 kDa, was produced regardless of the addition of SP6 RNA polymerase (Fig. 2, lanes 7 and 9), GFPmut1-His6 was produced from the plasmid pTHΔT7RNAP only by adding T7 RNA polymerase exogenously (Fig. 2, lanes 7 and 8). The above results indicate that the T7 RNA polymerase gene on the plasmid pTH is expressed without the addition of SP6 RNA polymerase in the cell-free expression system, and that active T7 RNA polymerase is required for the expression of the GFP gene on the plasmid. In these experiments, it was found that the exogenous T7 RNA polymerase depressed the expression of the wild type or the mutant T7 RNA polymerase gene (Fig. 2 lanes 5 and 8). Such a result indicates that competition may exist between the expressions of T7 RNA polymerase (or its mutant) and GFPmut1-His6 genes on the same plasmid at the transcriptional and/or translational levels. Based on the results so far described, we concluded that the two-stage genetic network using pTH operates as a transcriptional activation cascade in the cell-free expression system without the addition of SP6 RNA polymerase.

The kinetics of the production of GFPmut1-His6 in the cell-free expression system was monitored by the increase in the fluorescence (Fig. 3). In comparison to the production of GFPmut1-His6 by the single-stage system using pTHΔT7RNAP and added T7 RNA polymerase, a delay of about 20 min was observed for the production by the two-stage cascading network using pTH, where T7 RNA polymerase is newly synthesized in the expression system (Fig. 3). This delay seems to be the time needed to accumulate T7 RNA polymerase, which transcribes the GFP gene, and hence, is a characteristic property of a network working as an activation cascade [14].

The reaction mixture for the cell-free production of GFPmut1-His6 by the two-stage cascading genetic network described above was encapsulated in liposomes at 4 °C. After encapsulation, RNase was added to inhibit protein synthesis outside the liposomes resulting from any non-entrapped reaction components. The reaction was then initiated by elevating the temperature to 37 °C. After 1 h, the liposomes were examined by flow cytometry (Fig. 4), with the basal background being determined by running samples in which liposomes were dispersed by the reaction mixture without the plasmid DNA (Fig. 4B). A dot with fluorescence detected beyond the basal background indicates the liposome in which functional GFPmut1-His6 was synthesized. As Fig. 4A shows, it is evident that functional GFP was synthesized within the liposomes encapsulating the reaction mixture for the expression of the two-stage cascading genetic network, as indicated by the significant amount of liposomes fluorescing above the background level. When 12 μg/ml RNase was included both in the reaction mixture entrapped inside the liposome and in the solution outside the liposome, the result shown in Fig. 4C occurred, which was almost the same as that for the basal background (Fig. 4B). Such a result indicates that the presence of RNase inhibits the GFP synthesis almost completely. Therefore, the addition of RNase after the reaction mixture was encapsulated in the liposomes ascertained that the fluorescence detected above the background level could be attributed mostly to GFPmut1-His6 synthesized inside the liposome and not that outside it.

In our previous work, we demonstrated the synthesis of a functional protein, the fluorescent GFPmut1-His6, within liposomes [9]. The present results elevate the degree of

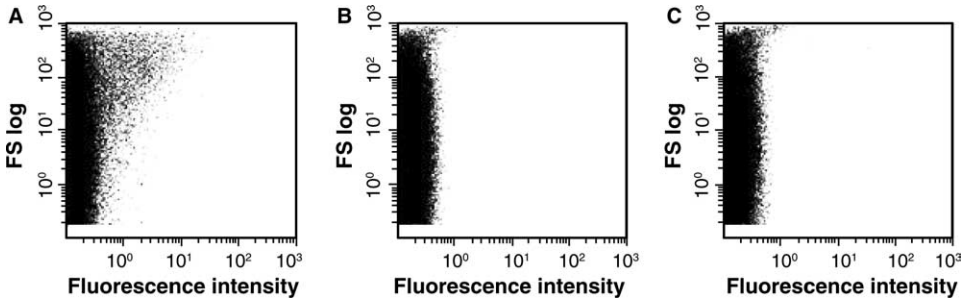

Fig. 4. Flow cytometric analysis of GFPmut1-His6 production by the two-stage cascading network in liposomes. Liposomes encapsulating the cell-free expression system with (A, C) or without (B) the pTH template DNA were analyzed with a flow cytometer after incubation at 37 °C for 1 h, as described in Section 2. (A, B) 12 μg/ml RNase was included in the solution outside the liposome. (C) 12 μg/ml RNase was included both in the solution inside the liposome and outside the liposome. Each dot represents one liposome.

molecular complexity of the system to a level showing that the two-stage genetic network encapsulated in liposomes is functional and works as a transcriptional activation cascade. Although there is still room for improvement to the system, and quantitative analysis is needed for further characterization of it, the fact that a functional genetic network, which is a simple element of a much more complex network in a living cell, can be constructed within liposomes is an important step forward to overcoming many challenges in pursuance of synthesizing a minimal cell.

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