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A human cell-derived *in vitro* coupled transcription/translation system optimized for production of recombinant proteins

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ABSTRACT

The aim of this study was to develop an efficient cell-free protein expression system derived from mammalian cells. We established a HeLa cell-based *in vitro* coupled transcription/translation system with T7 RNA polymerase and a plasmid that harbored a T7 promoter/terminator unit. To enhance protein synthesis in the coupled system, we placed the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) or the hepatitis C virus (HCV) IRES between the T7 promoter and the coding region of the plasmid. Remarkably, we found that these IRES-dependent systems were able to produce large proteins including GCN2 (160 kD), Dicer (200 kD) and mTOR (260 kD) to levels detectable on SDS–PAGE by Comassie Brilliant Blue-staining. We purified the synthesized proteins to near homogeneity, and validated their functionalities in the appropriate biochemical assays. In conclusion, the HeLa cell-based *in vitro* coupled transcription/translation system using the EMCV or HCV IRES is a convenient tool, particularly for the production of large recombinant proteins.

Introduction

Cell-free translation systems are important tools for the production of recombinant proteins. Two systems have been successfully established for practical uses, *Escherichia coli*-[1,2] and wheat germ-[3] derived extracts. These cell-free systems can incorporate labeled amino acids or unnatural amino acids into recombinant proteins, and efficiently produce proteins that are only expressed in limited amounts *in vivo*. The *in vitro*-expressed proteins can be used for functional and structural analyses. Cell-free translation systems have also been used in the high-throughput production of thousands of gene products derived from cDNA libraries to facilitate screening in the identification of kinase or proteinase targets [4,5].

Rabbit reticulocyte lysate (RRL)[1] is one of the most popular mammalian cell-free protein synthesis systems. A drawback of RRL is, however, that commercially available RRLs are expensive with varied activities depending on supplied lots, and preparation of RRL by a researcher's own hands is not an easy task, since this system requires sacrifice of animals. Thus, the development of efficient cell-free translation systems from cultured mammalian cells should benefit many researchers.

We previously improved a HeLa cell-derived translation system by supplementing it with translation factors or kinase regulators [6,7]. This system, combined with a dialysis technique, produced far higher amounts of proteins than the RRL-based system [6]. We also modified the HeLa cell-based cell-free system to efficiently synthesize the infectious Encephalomyocarditis virus (EMCV) from its genome RNA [8].

In cell-free translation systems, mRNA is added either intact from an *in vitro*-synthesized mRNA, or it is synthesized with the addition of a DNA (a plasmid or a PCR product) with a promoter sequence and the corresponding bacteriophage RNA polymerase (T7, SP6, or T3 RNA polymerase). The latter method is called a coupled transcription/translation system, and has been successfully employed in *E. coli* [9,10], wheat germ [11], and RRL [12]-derived systems.

In this paper, we developed a very efficient HeLa cell-based *in vitro* coupled transcription/translation system by utilizing the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) or the hepatitis C virus (HCV) IRES to enhance translation.

Materials and methods

Plasmids

T7 RNA polymerase promoter, the multi-cloning site (MCS), the encephalomyocarditis virus-internal ribosome entry site (EMCV IRES) [7] and the T7 RNA polymerase termination sequences were inserted into pUC119 in order to construct the expression vector pUC-T7-EMCV-MCS-ter. A FLAG- or His-tag sequence was inserted

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[1] *Abbreviation used:* RRL, Rabbit reticulocyte lysate; EMCV, encephalomyocarditis virus; IRES, ribosome entry site; HCV, hepatitis C virus; MCS, multi-cloning site; GST, glutathione *S*-transferase; R-Luc, Renilla luciferase; IPTG, isopropyl-β-D-thiogalactopyranoside.

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between the EMCV IRES and the MCS in order to construct the pUC-T7-EMCV-FLAG-MCS-ter and pUC-T7-EMCV-His-MCS-ter expression vectors. The EMCV IRES was replaced by the HCV (hepatitis type-C virus) IRES [13] to construct the pUC-T7-HCV-MCS-ter, pUC-T7-HCV-FLAG-MCS-ter and pUC-T7-HCV-His-MCS-ter expression vectors.

We inserted FLAG-eIF4G (amino acids 142–1560)-His [14] DNA into pUC-T7-EMCV-MCS-ter and pUC-T7-HCV-MCS-ter to construct the pUC-T7-EMCV-FLAG-eIF4G-His and pUC-T7-HCV-FLAG-eIF4G-His, respectively. Complementary DNAs for mTOR, Raptor and GCN2 cDNAs (kind gifts from Dr. Sonenberg) were ligated with a His or FLAG sequence at the C-terminus and then were inserted into pUC-T7-EMCV-FLAG-MCS-ter, pUC-T7-EMCV-His-MCS-ter, pUC-T7-HCV-FLAG-MCS-ter or pUC-T7-HCV-His-MCS-ter for expression of both N- and C-terminally tagged proteins. Complementary DNAs for the human genes, Dicer, Ago2, TARBP-2, and GβL were obtained by reverse transcription of human placenta poly (A) RNA followed by PCR (RT-PCR). DNA primers for RT-PCR were chosen based on the reported sequences (GenBank Accession No. NM_030621 for Dicer, NM_012154 for Ago2, BC005860 for TARBP-2, and NM_022372 for GβL). Dicer and Ago2 cDNAs were cloned into the EMCV IRES- and HCV IRES-harboring plasmids for expression in the cell-free system. The TARBP-2 and GβL cDNAs were cloned into the glutathione *S*-transferase (GST)-fusion vector pGEX-6P (GE Healthcare) to generate pGEX-6P-TARBP-2 and pGEX-6P-GβL expression vectors, respectively.

The Renilla luciferase (R-Luc) coding region (Promega) with a myc C-terminal tag sequence was inserted into the MCS of pUC-T7-EMCV-MCS-ter to produce the pUC-T7-EMCV-R-Luc-myc reporter plasmid. HCV IRES, Poliovirus-IRES [13], plautia stali intestine virus (PSIV)-IRES [15], and a CAA repeat sequence (10 repeats) [16] were inserted in place of the EMCV IRES in the pUC-T7-EMCV-R-Luc-myc plasmid to construct the pUC-T7-HCV-R-Luc-myc, pUC-T7-polio-R-Luc-myc, pUC-T7-PSIV-R-Luc-myc and pUC-T7-CAA-R-Luc-myc reporter plasmids, respectively. The plasmid pUC-T7-R-Luc-myc was used as the construct for testing expression in the absence of any IRES (minus-IRES): the length between the T7 RNA promoter start site and the R-Luc translation start site of this plasmid is 20 nucleotides.

Proteins

T7 RNA polymerase was expressed and purified by the following procedure: RNA purified from vTF7-3 vaccinia virus [17]-infected HeLa cells was used in an RT-PCR reaction to produce a cDNA for T7 RNA polymerase. The cDNA was cloned into the pGEX-6P (GE Healthcare) plasmid to construct the pGEX-6P-T7 RNA polymerase expression vector. A bacterial strain BL-21 (DE-3) (pLys) was transformed with pGEX-6P-T7 RNA polymerase, and was grown in Luria broth (2 l) until optical density at 600 nm reached 0.6–1.0. Next, isopropyl-β-D-thiogalactopyranoside (IPTG; 0.1 mM) was added, and cells were cultured at 25 °C for 12–16 h. After washing with a buffer (80 ml; 20 mM Tris–HCl, pH 7.5, 0.15 M NaCl), the bacterial pellet was stored at −20 °C until use. The frozen pellet was resuspended in a lysis buffer (50 ml; 0.1 M KCl, 20 mM Hepes-KOH, pH 7.5, 10% glycerol, 10 mM DTT, 5 mM EDTA, 0.1% Triton X-100, one tablet of the cocktail of protease inhibitors (Roche)), and lysed by sonication, and centrifuged at 30,000 rpm for 1 h in the Ti-70 rotor (Beckman). The supernatant was mixed with Glutathione Sepharose 4B resin (1.0 ml; GE Healthcare), and unbound proteins were removed with a washing buffer (30 ml; 0.1 M KCl, 20 mM Hepes-KOH, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% Triton X-100, 1 mM EDTA). Another washing buffer (1 ml) containing PreScission Protease (13 units; GE Healthcare) was added to cleave the T7 RNA polymerase from GST and the column was incubated at 4 °C for 12–16 h. The T7 RNA polymerase was then eluted from the column, and passed through Glutathione Sepharose 4B resin (0.3 ml) to remove any contaminating GST or GST-T7 RNA polymerase. The purified sample was then applied to a Q-sepharose (1 ml; GE Healthcare) column equilibrated with the washing buffer. After washing with a buffer (30 ml; 0.1 M KCl, 20 mM Hepes-KOH, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 1 mM EDTA), the T7 RNA polymerase was eluted with the elution buffer (2.7 ml; 0.6 M KCl, 20 mM Hepes-KOH, pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 1 mM EDTA), and was dialyzed against a buffer (500 ml; 0.1 M KCl, 50 mM Hepes-KOH, pH 7.5, 10% glycerol, 1 mM DTT, 1 mM EDTA) overnight. The sample was then mixed with an equal volume of glycerol and stored at −20 °C at a final concentration of 0.27 μg/ml.

TARBP-2 and GβL were expressed and purified as follows: The BL-21 (DE-3) (pLys) bacteria were transformed with pGEX-6P-TARBP-2 and pGEX-6P-GβL for expression of GST-TARBP-2 and GST-GβL, respectively. TARBP-2 and GβL were cleaved from the GST-tag as described for T7 RNA polymerase.

GADD34 and K3L, two proteins used to lower phosphorylation of eIF2α in mammalian cell-free systems were prepared as previously described [7]. In addition, 4E-BP1 was obtained as previously described [6]. Recombinant eIF2B (five subunits) was expressed by a baculovirus-dependent system and purified as previously described [6].

Cell culture and cell-free extracts

HeLa S3 cells (RIKEN BRC) were cultured as previously described [6] with a few modifications. A suspension culture of HeLa cells (6 l) was grown in a spinner flask regulated with a Cellmaster Model 1700 cell culture controller (Wakenyaku, Japan) in Eagle's Minimal Essential Medium (Sigma) supplemented with 10% heat-inactivated calf serum, penicillin (1 unit/ml), streptomycin (0.1 mg/ml), and GlutaMAX (2 mM; Invitrogen) instead of L-glutamine. The incubation conditions were 37 °C, pH 7.2, 6.7 ppm oxygen density, and stirring at 50 rpm. When the cell density reached 0.8–1.0 × 10^6 cells/ml, cells were harvested and washed three times with a buffer (35 mM Hepes–KOH, pH 7.5, 140 mM NaCl, and 11 mM glucose) and once with an extraction buffer (20 mM Hepes–KOH, pH 7.5, 135 mM potassium acetate, 30 mM KCl, 1.65 mM magnesium acetate, and 1 mM DTT). The cell pellet was then resuspended in an equal volume of the extraction buffer (approximately 3.0 × 10^8 cells/ml) and was disrupted by nitrogen pressure (1.0 MPa, 30 min) in the Mini-Bomb cell disruption chamber (KONTES). Cell homogenates were centrifuged twice at 10,000 g for 5 min at 4 °C, and the supernatant (8–10 ml) was dialyzed against the extraction buffer (500 ml) using a dialysis membrane (molecular weight cut-off 50,000, regenerated cellulose) (SPECTRUM) twice at 4 °C for 1.5 h each. The extract (24–28 mg protein/ml) was divided into aliquots frozen in liquid nitrogen, and stored at −80 °C. The protein concentration was determined by Bradford method using bovine serum albumin as the standard throughout this paper.

In vitro coupled transcription/translation system

Two cell-free methods were used for coupled transcription and translation: the batch method and the dialysis method.

The batch method: The final incubation mixture (18 μl) consisted of the HeLa cell extract (7.5 μl), mixture-1 (6.5 μl), mixture-2 (1.2 μl), mixture-3 (1.8 μl), and plasmid (1.0 μl to make 15 ng/μl final concentration).

Mixture-1 consisted of 5.6 μl of magnesium acetate (100 mM), 35.4 μl of potassium acetate (0, 125, 328 or 532 mM), 8.25 μl of DTT (100 mM), and 15.75 μl of Hepes–KOH (400 mM), pH 7.5.