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An efficient mammalian cell-free translation system supplemented with translation factors

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Abstract

Development of an efficient cell-free translation system from mammalian cells is an important goal. We examined whether supplementation of HeLa cell extracts with any translation initiation factor or translational regulator could enhance protein synthesis. eIF2 (eukaryotic translation initiation factor 2) and eIF2B augmented translation of capped, uncapped and encephalomyocarditis virus-internal ribosome entry site-promoted mRNAs. eIF4E specifically stimulated capped mRNA translation, while p97, a homologue to the C-terminal two-thirds of eIF4G, increased uncapped mRNA translation. When the HeLa cell extract was supplemented with a combination of eIF2, eIF2B, and p97, the capacity to synthesize a protein from an uncapped mRNA became comparable to that from the capped counterpart stimulated with a combination of eIF2, eIF2B, and eIF4E. A dialysis method rendered the HeLa cell extract capable of synthesizing proteins for 36 h, and the yield was augmented when supplemented with initiation factors. In contrast, the productivity of a rabbit reticulocyte lysate was not enhanced by this method. Collectively, the translation factor-supplemented HeLa cell extract should become an important tool for the production of recombinant proteins.

Keywords: Cell-free; Eukaryotic translation initiation factor; HeLa

Translation in eukaryotes is mainly regulated at the initiation step. Among the factors involved in translation initiation, eukaryotic translation initiation factor 4F (eIF4F) and eIF2 play pivotal roles in translational regulation [1,2]. eIF4F is a protein complex, consisting of eIF4E, eIF4A, and eIF4G. eIF4E directly interacts with a cap structure (m^7GpppN, where N is any nucleotide) of the mRNA, and recruits the 40S ribosome to the 5′-end of the mRNA through binding to eIF4G. eIF4A, an RNA-helicase, also binds to eIF4G, and is subsequently thought to facilitate ribosome binding to the mRNA by unwinding the secondary structure near the 5′-end of the mRNA. Association of eIF4G with eIF4E is regulated by 4E-BPs, eIF4E-binding inhibitory proteins. 4E-BPs share an eIF4E-binding motif with eIF4G, thereby evicting eIF4G from eIF4E and consequently inhibiting translation. When 4E-BPs are phosphorylated through the FRAP/mTOR signaling pathway, 4E-BPs become unable to bind to eIF4E, thus allowing eIF4G to associate with eIF4E and increase translation [3]. eIF2 comprises three subunits: α, β, and γ. A ternary complex consisting of eIF2–GTP-methionyl initiator tRNA (Met-tRNAi^{Met}) transfers Met-tRNAi^{Met} to the 40S ribosomal subunit. When the anticodon of Met-tRNAi^{Met} base-pairs with the AUG initiation codon, the eIF2-bound GTP is hydrolyzed to GDP, and eIF2–GDP is subsequently released from the ribosomal complex. For the next round of translation initiation, eIF2–GDP must be converted to eIF2–GTP to

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regenerate the ternary complex, and this reaction is catalyzed by eIF2B, a multiprotein complex consisting of five subunits. When the α-subunit of eIF2 is phosphorylated, the affinity of eIF2 to eIF2B dramatically increases, and eIF2B is thereby sequestered by eIF2 [4]. eIF2B is then unable to regenerate the ternary complex, and translation is consequently attenuated [2]. Phosphorylation of the α-subunit of eIF2 occurs in response to stress conditions such as virus infection, oxidation, deprivation of amino acids, and accumulation of misfolded proteins [5,6].

The use of cell-free translation systems have played a key role in the elucidation of the mechanism of translation. Cell extracts from different sources can faithfully recapitulate translational control observed in vivo, as exemplified by cap/poly(A)-dependent translation in yeast [7] and mammalian cell extracts [8,9], and repression of translation by a sequence specific RNA binding protein in a *Drosophila* embryo extract [10]. In addition, cell-free translation systems are important for production of recombinant proteins. *Escherichia coli* [11] and wheat germ [12]-derived cell-free systems can produce, in large amounts, proteins which are hardly expressed to high levels in vivo, and these in vitro-expressed proteins can be used for functional and structural analyses. Furthermore, productivity of the prokaryotic cell-free system can be improved by addition of translation elongation factors [13]. Cell-free translation systems were also used to produce, in a high-throughput manner, thousands of gene products derived from cDNA libraries, and those products are then easily screened to identify targets of kinases or protienases [14]. An important issue for successful production of recombinant proteins in vitro is whether a highly efficient translation system is readily available to the researcher. Among several mammalian cell-based translation systems, the rabbit reticulocyte lysate (RRL)[1] is the most popular system, being claimed to be more efficient than other mammalian cell-based systems. An obvious advantage of RRL is its cap-independency. Uncapped RNAs can be translated in RRL with a comparable efficiency to capped RNAs, while HeLa and Krebs cell-derived systems require the cap structure for efficient translation [9,15]. This property is beneficial, since in vitro-synthesis of capped RNAs cost more than that of uncapped RNAs, yet the yield of the former is lower than that of the latter. An obvious 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. Here, we improved a HeLa cell-derived translation system by supplementing it with translation factors.

[1] *Abbreviations used:* eIF, eukaryotic translation initiation factor; EMCV, encephalomyocarditis virus; IRES, internal ribosome entry site; RRL, rabbit reticulocyte lysate.

Materials and methods

mRNAs

All mRNAs were synthesized using RiboMAX large scale RNA production system (Promega). When capped mRNAs were synthesized, m^7GpppG was included at a eight-fold molar excess relative to GTP in the transcription reaction. Capped and uncapped luciferase mRNAs (cap-LUC-A and LUC-A, respectively) were transcribed from pSP72-LUC-A [16]. EMCV-IRES-LUC-A mRNA (uncapped) was synthesized from pSP-72-EMCV-LUC-A [9]. Glutathione-*S*-transferase (GST) encoding region from pcDNA3-GST [17] was inserted in the multicloning site of pUC119, together with T7 RNA polymerase promoter and poly (A) sequences to finally construct pUC-T7-GST-STOP-A, from which cap-GST-A RNA was synthesized. The synthesized RNAs were purified by using Chroma-spin-30 (BD Biosciences), and the integrity of each RNA was examined by electrophoresis on formaldehyde–agarose gels.

Cell culture and cell-free extract

HeLa S3 cells were maintained in an incubator (5% CO_2) at 37 °C in minimal essential medium Eagle (SIGMA) supplemented with 10% heat-inactivated fetal calf serum, L-glutamine (2 mM), penicillin (1 U/ml), and streptomycin (0.1 mg/ml). Suspension culture of HeLa cells (1 L) was performed using a spinner flask with a cell culture controller Cellmaster Model 1700 (Wakenyaku, Japan). The control values of temperature, pH, oxygen density, and stirring speed were 37 °C, 7.2, 6.7 ppm, and 50 rpm, respectively. When the cell density reached $0.7–0.8 \times 10^6$ 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 then suspended in 1.0 volume of an extraction buffer (20 mM Hepes–KOH pH 7.5, 135 mM potassium acetate, 30 mM KCl, and 1.65 mM magnesium acetate). The cells (approximately 2.3×10^8 cells/ml) were disrupted by nitrogen pressure (1.0 MPa, 30 min) in the Mini-Bomb cell disruption chamber (KONTES). Cell homogenates were centrifuged at 10,000*g* for 5 min at 4 °C, and the supernatant was passed through a PD-10 desalting column (Amersham) equilibrated with the extraction buffer. The eluted extract (18–22 mg protein/ml) was frozen in liquid nitrogen, and stored at −80 °C.

Cell-free proteins synthesis

HeLa cell extracts

Prior to cell-free protein synthesis, 100 μl of the extract prepared as above was treated with 1 μl of 7500 U/ml nuclease S7 (Roche) and 1 μl of 100 mM $CaCl_2$ for 5 min at 23 °C to degrade endogenous mRNA, and then mixed with 8 μl of 30 mM EGTA to stop the reaction. In the batch system, a translation mixture containing 4.5 μl of the above-treated

extract, 30 μM of each of the 20 amino acids, 27 mM Hepes–KOH (pH 7.5), 1.2 mM ATP, 0.12 mM GTP, 18 mM creatine phosphate, 0.3 mM spermidine, 44–224 mM potassium acetate, 16 mM KCl, 1.2 mM magnesium acetate, 90 μg/ml calf liver tRNA, and 60 μg/ml creatine kinase was incubated with mRNA and (a) translation factor(s) in a total volume of 6 μl for 1 h at 32 °C. After dilution by 40-fold with water, an aliquot (2 μl) was mixed with Luciferase assay reagent (Promega) (30 μl), and the total luminescence for 10 s was measured using a luminometer MiniLumat LB9506 (Perkin Elmer) and the obtained value was taken as luciferase activity. When a protein was radiolabeled, the translation mixture contained 30 μM of each of 19 amino acids (all except methionine) and [^{35}S]methionine. The radiolabeled products were resolved by SDS–PAGE, and visualized with BAS 2000 (Fuji).

In the dialysis system, 120 μl of the translation mixture was dialyzed at 31–32 °C in a dialysis chamber (molecular weight cut-off 50,000, regenerated cellulose) against 5 ml of an external solution containing all components of translation mixture except for creatine kinase, tRNAs, and the cell extracts. At 24 h of incubation, 10 μg creatine kinase was supplied in the reaction mixture, and the external solution was exchanged with new one. Cap-GST-A mRNA was supplied at the time 0 and 24 h.

Rabbit reticulocyte lysate (RRL)

Flexi rabbit reticulocyte lysate (Promega) (3.8 μl) was incubated with mRNA and (a) translation factor(s), and amino acids (20 μM, final) in a total volume of 6 μl for 1 h at 30 °C. For optimal translation of cap-LUC-A, LUC-A and EMCV-IRES-LUC-A in RRL, the concentration of potassium acetate was raised by 120, 60, and 90 mM, respectively, and the concentration of potassium chloride was increased by 20 mM in all cases. The optimal amount of RNA was 1.58 μg/ml in all cases. The final concentration of magnesium was 1.25 mM in all cases, while that of potassium was unknown, since the concentration of potassium in RRL was not provided by the company. Luciferase activity was measured as done with the HeLa cell extract. The dialysis system with RRL was performed as described for the HeLa cell extract.

cDNAs and baculoviruses

cDNAs for all five subunits of human eIF2B were obtained by reverse transcription followed by PCR (RT-PCR). Poly(A) RNA for RT-PCR was obtained from HeLa cells. DNA primers for RT-PCR were chosen based on the reported sequences (GenBank Accession Nos.: NM_001414 for eIF2B1, NM_014239 for eIF2B2, NM_020365 for eIF2B3, Q9UI10 for eIF2B4, XM_291076 for eIF2B5). eIF2B1, eIF2B2 and eIF2B4 cDNAs were cloned together in pACDB3 (PharMingen) to construct pACDB3–2B1–2B2–2B4. eIF2B3 and eIF2B5 cDNAs were cloned together in pACDB3 to construct pACDB3–2B3–2B5. To facilitate purification and detection of expressed proteins, a FLAG sequence was appended to the C-terminus of eIF2B3 sequence, and a His-tag sequence was added to the N-terminus of eIF2B4. Baculoviruses expressing eIF2B1, 2B2 and 2B4 (Baculo-2B1-2-4) and expressing eIF2B3 and 2B5 (Baculo-2B-3-5) were generated by recombination between a baculovirus DNA BaculoGold (PharMingen) and pACDB3–2B1–2B2–2B4 and pACDB3–2B3–2B5, respectively. p97 cDNA [18] appended with a FLAG sequence at the C-terminus, was cloned in pGEX-6P(Pharmacia) to generate pGEX-6P-p97-FLAG. Mouse eIF4E [19] and eIF4A [20] cDNAs were cloned in pGEX-6P to generate pGEX-6P-eIF4E and pGEX-6P-eIF4A, respectively. For expression of eIF4G and eIF5B in bacteria, modified expression vectors were constructed as follows: the malE region pMAL-p2X (NEB) was replaced by FLAG and His sequences to generate pTac-FLAG and pTac-His, respectively. A cDNA encoding eIF4G (amino acids 45–1560) [16] was appended with a His sequence at the C-terminus, and cloned in pTac-FLAG to generate pTac-FLAG-eIF4G(45–1560)-His. Human eIF5 and eIF5B cDNAs were obtained by RT-PCR as described for eIF2B using primers based on the deposited sequences, HSU49436 for eIF5 and NM_015904 for eIF5B. The eIF5 cDNA was cloned in pET19 (Novagen) to generate pET19-eIF5. The eIF5B cDNA appended with a FLAG sequence at the C-terminus was cloned in pTac-His to generate pTac-His-eIF5B-FLAG. 4E-BP1 cDNA was cloned in pGEX-6P to generate pGEX-6P-4E-BP1. All the PCR-amplified cDNAs were confirmed by sequencing to encode the same amino acid sequences as the deposited ones.

Purification of proteins

eIF3 was purified from Krebs-2 ascites cells as described [21]. eIF2 was purified from HeLa cells as follows: HeLa cells ($1–1.5 \times 10^{10}$) were suspended in an extraction buffer (50 ml) (100 mM KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, one tablet of a cocktail of protease inhibitors [Complete, Roche]), and disrupted in Mini-Bomb cell disruption chamber (KONTES) at the pressure of 3 MPa for 15 min. The disrupted cells were centrifuged at $3.3 \times 10^3 g$ for 10 min. The supernatant was applied onto heparin–Sepharose (10 ml bed volume) column, and washed with the extraction buffer (100 ml) and with buffer A (20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 1 mM EDTA) (40 ml) containing 0.2 M KCl. The bound proteins were eluted out with buffer A (40 ml) containing 0.5 M KCl. The eluate was diluted by 2.5 times with buffer A, and applied to a phosphocellurose column (P11, 2 ml bed volume). After washing with buffer A (50 ml) containing 0.2 M KCl, bound proteins were eluted with buffer A (10 ml) containing 0.6 M KCl. The eluate was concentrated to 2 ml by using Amicon Ultra 30,000 MWCO (MILLIPORE), and then applied on Superdex 200 HiLoad 16/60 (Amersham). Elution (0.3 ml/min, 1 ml/fraction) was performed with buffer A containing 0.5 M KCl, and an aliquot of each fraction was examined by Western blotting for the presence of eIF2. eIF2-

containing fractions were combined, and passed through PD-10 column (Amersham) to reduce the potassium concentration to 0.1 M, and then applied to a Resource S column (Amersham) (1 ml, bed volume). After washing with buffer A containing 0.1 M KCl, bound proteins were eluted by a gradient buffer A containing 0.1–1 M KCl in a total volume of 20 ml. An aliquot of each fraction (0.5 ml) was examined by SDS–PAGE followed by CBB staining: eIF2 was fractionated at the KCl concentration of ~0.55 M.

Recombinant eIF2B was expressed and purified as follows. Hi-5 cells (Invitrogen) (1 L) were grown by Cell-master controller (Waken, Japan) (oxygen concentration, 6.8 ppm; temperature, 27 °C), and were infected at a cell density ($4–8 \times 10^5$/ml) with baculo-2B3–2B5 alone for the 2B3–2B5 complex or with both baculo-2B3–2B5 and baculo-2B1–2B2–2B4 for the five subunit complex. Fifty hours later, cells were recovered, and kept at −20 °C until use. For the eIF2B3–2B5 complex, the cell pellet from 1 L culture was suspended in a buffer (50 ml) (0.1 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 0.5% Triton X-100, one tablet of a cocktail of the protease inhibitors, 2 mM EDTA), and kept at 4 °C for 20 min. After centrifugation at 10,500*g* for 20 min, the supernatant was mixed with anti-FLAG agarose resin (1 ml) (Sigma). Following incubation at 4 °C for 1 h with constant rotation, proteins unbound to the resin were removed by passing the resin through a Poly-Prep chromatography column (Bio-Rad), and then by washing with a buffer (50 ml) (0.1 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, one tablet of a cocktail of the protease inhibitors, 2 mM EDTA). Bound proteins were eluted out with FLAG peptide (Sigma) (5 ml, 100 μg/ml) in the washing buffer. The eluate was applied on Superdex 200 HiLoad 16/60 column. Fractionation was preformed as described for eIF2. The fractions that contained both 2B3 and 2B5 as determined by Western blotting were obtained.

For the whole complex of eIF2B, the pellet of the infected cells from 1 L culture was suspended in a buffer (100 ml) (0.5 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, one tablet of a cocktail of protease), disrupted and centrifuged as for the eIF2B3–2B5 complex. The supernatant was supplemented with imidazole (20 mM), mixed with Ni–NTA–agarose resin (2 ml, QIAGEN), and incubated at 4 °C for 1 h with constant rotation. Unbound proteins were removed as for the eIF2B3–2B5 complex. Bound proteins were eluted with a buffer (10 ml) (0.25 M imidazole, 0.15 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol). The eluate was supplemented with EDTA (1 mM), and concentrated to 2 ml by using Amicon Ultra 30,000 MWCO, and then applied on Superdex 200 HiLoad 16/60. Fractionation was performed as described for eIF2. The fractions that contained all the eIF2B subunits, as determined by Western blotting or CBB staining, were diluted by three times with a buffer (20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 1 mM EDTA), and applied to anti-FLAG agarose column (0.2 ml). After washing with a buffer (10 ml) (0.1 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM EDTA), the recombinant eIF2B was eluted with the same buffer (700 μl) containing FLAG peptide (50 μg/ml).

eIF4E (non-tagged)

A bacterial strain BL-21 (DE-3) (pLys) was transformed with pGEX-6P-eIF4E, and was grown in Luria broth (LB) (2 L) until optical density at 600 nm (OD_{600}) reached 0.6–1.0. Isopropyl-β-D-thiogalactopyranoside (IPTG) (0.1 mM) was added, and cells were cultured at 30 °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 kept at −20 °C until use. The frozen pellet was suspended in a 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), 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 (0.5 ml) (Amersham), and incubated at 4 °C for 1 h with constant rotation. Unbound proteins were removed by passing the resin through a Poly-Prep column (Bio-Rad), and washing with a buffer (30 ml) (0.1 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, 1 mM EDTA). The resin was incubated in the column with the washing buffer (0.5 ml) containing PreScission Protease (12 unit) (Amersham) at 4 °C for 12–16 h to cleave between GST and eIF4E. Non-tagged eIF4E was drained out from the column, and passed through glutathione Sepharose 4B resin (0.3 ml) to remove possibly contaminating GST or GST-eIF4E. To complete purification of eIF4E, the eluted eIF4E was applied on SP-Sepharose (0.3 ml) (Pharmacia) after pH was adjusted to 7.0. After washing with a buffer (10 ml) (0.1 M KCl, 20 mM Hepes~KOH pH 7.0, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM EDTA), eIF4E was eluted with a buffer (1 ml) (0.3 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM EDTA).

p97 (C-teminally FLAG tagged)

GST-p97-FLAG was expressed as described for GST-eIF4E except that the plasmid pGEX-6P-p97-FLAG was used. The bacterial extract derived from 2 L culture was mixed with heparin Sepharose CL-6B (0.5 ml) (Amersham), and incubated at 4 °C for 1 h with constant rotation. Unbound proteins were removed as described for GST-eIF4E. Bound proteins were eluted with a buffer (0.5 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, 1 mM EDTA), and applied to glutathione Sepharose 4B column (0.5 ml). After washing with a buffer (15 ml) (0.1 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, 1 mM EDTA), cleavage between GST and p97-FLAG was carried out as described for eIF4E. p97-FLAG was further purified by anti-FLAG chromatography as described for eIF2B.

N-terminally His-tagged eIF1 and eIF1A were obtained as described [17]. GST-eIF4A and GST-4E-BP1 were expressed in bacteria, and eIF4A and 4E-BP1 were cleaved out as described for eIF4E. eIF4A was further purified by HiTrap-Blue chromatography (Pharmacia). eIF4B (N-terminally FLAG tagged) was expressed in Sf9 cells by the baculovirus system, and purified by anti-FLAG chromatography.

FLAG-eIF4G(45–1560)-His was purified as follows. BL-21 (DE-3) (pLys) transformed with pTac-FLAG-eIF4G(45–1560)-His was grown and induced as described for eIF4E. The bacterial pellet from 4 L culture was suspended in a buffer (0.1 M KCl, 20 mM Hepes–KOH pH 7.0, 10% glycerol, 10 mM DTT, 5 mM EDTA, 0.1% Triton X-100, two tablets of the cocktail of protease inhibitors) (100 ml). Disruption of cells followed by centrifugation was the same as described for eIF4E. The supernatant (100 ml) was applied on SP-Sepharose column (4 ml), and washed with a buffer (0.1 M KCl, 20 mM Hepes∼KOH pH 7.0, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM EDTA, 0.1% Triton X-100) (100 ml). Bound proteins were eluted with a buffer (0.5 M KCl, 20 mM Hepes–KOH pH 8.0, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1% Triton X-100) (20 ml). The eluate was supplemented with imidazole (20 mM), mixed with Ni–NTA–agarose resin (1 ml), and incubated at 4 °C for 1 h with constant rotation. Unbound proteins were removed as for the eIF2B3–2B5 complex. Bound proteins were eluted with a buffer (5 ml) (0.25 M imidazole, 0.15 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol). The eluate was subjected to the anti-FLAG chromatography.

His-eIF5B–FLAG was expressed and purified as described for eIF4G except that pTac-His-eIF5B-FLAG was used. His-eIF5 was expressed in bacteria as described for eIF4E except that pET19-eIF5 was used. His-eIF5 was purified through the NTA-chromatography followed by HiTrapQ chromatography (Amersham).

All the purified proteins were dialyzed against a buffer (0.1 M KCl, 20 mM Hepes–KOH pH 7.5, 10% glycerol, 5 mM 2-mercaptoethanol, 0.1 mM EDTA) by using a Slide-A-Lyzed Mini Dialysis unit (10,000 MWCO) (PIERCE).

Results

Optimization of HeLa cell extracts for efficient protein synthesis

To find optimal conditions for protein synthesis in a HeLa cell-derived extract (Fig. 1), we first translated capped (cap-LUC-A), uncapped (LUC-A) or encephalomyocarditis virus (EMCV)-internal ribosome entry site (IRES)-directed (EMCV-IRES-LUC-A) luciferase mRNAs with varied concentrations of potassium acetate (44–224 mM; the concentration of potassium chloride was kept at 16 mM), and measured luciferase activity. The optimal concentrations of potassium were determined to be 120 mM for cap-LUC-A, 90 mM for LUC-A, and 180 mM for EMCV-IRES-LUC-A. We then translated various amounts of each mRNA with these concentrations of potassium, and determined that the optimal concentration of mRNA were 40 μg/ml for cap-LUC-A, 80 μg/ml for LUC-A, and 100 μg/ml for EMCV-IRES-LUC-A. These conditions were hereafter employed unless otherwise stated. All three mRNAs were poly(A)-tailed (∼100 A residues), since the poly(A) structure at the 3′-end was found to enhance protein synthesis irrespective of the 5′-end structure by 5- to 50-fold (data not shown), probably because the poly(A) tail enhances both translation initiation [22] and stability of mRNA [23].

To further enhance the protein synthesis, we examined whether supplementation with any translation initiation factor or translational regulator would stimulate translation (Fig. 1). Recombinant eIF1, eIF1A, eIF4A, eIF4E, eIF4B, eIF4G, p97, eIF5, eIF5B and eIF2B were expressed in bacteria or insect cells, and purified to near homogeneity (Fig. 2) (see Materials and methods for details), while native eIF2 and eIF3 were purified form HeLa and Krebs-2 ascites cells, respectively (Fig. 2). All the purified factors were confirmed to be active by reconstituted translation initiation systems (H. Imataka, unpublished data) [17]. We supplemented the HeLa cell extract with each of the purified factors for translation of cap-LUC-A (Fig. 3A), LUC-A (Fig. 3B), or EMCV-IRES-LUC-A (Fig. 3C). Translation of all three mRNAs was increased by 1.4- to 2.5-fold compared to their respective controls by the

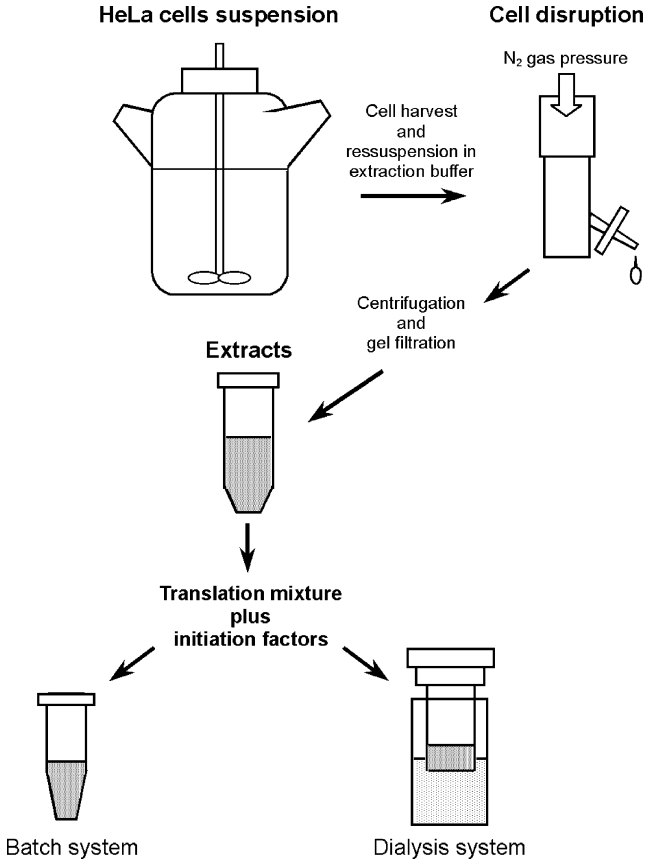

Fig. 1. Cartoon depicting preparation of cell-extracts and translation.

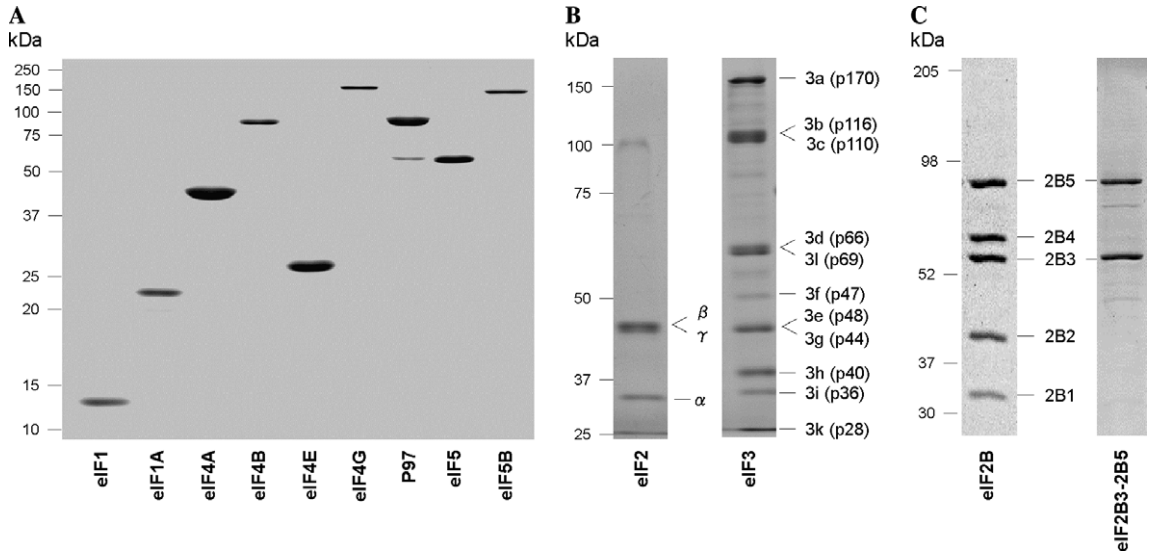

Fig. 2. Purification of translation factors. Recombinant eIF1, eIF1A, eIF4A, eIF4B, eIF4E, eIF4G, p97, eIF5, and eIF5B (1.8 μg each), (A), native eIF2 and eIF3 complex (1.5 and 3.5 μg, respectively) (B), and recombinant eIF2B and eIF2B3–2B5 complex (1.0 μg each) (C) were resolved on SDS–PAGE, and stained with Coomassie brilliant blue.

addition of either eIF2 or eIF2B. When eIF2 and eIF2B were simultaneously added, translation was further augmented (1.7- to 3.2-fold relative to the respective controls) (Figs. 3A–C). These results are in agreement with the findings that eIF2 and eIF2B are among the principal regulators of eukaryotic translation, and that eIF2B is one of the least abundant translation factors [24]. As expected, eIF4E specifically stimulated translation of cap-LUC-A mRNA (~1.8-fold) (Fig. 3A), supporting an earlier finding that eIF4E was one of the limiting factors in eukaryotic translation [25]; eIF4E may not always be limited in the amount, e.g., in RRL [26], but the presence of the eIF4E inhibitors 4E-BPs renders eIF4E a functionally limiting factor [27]. When eIF4E, eIF2 and eIF2B were added together, translation of cap-LUC-A mRNA was increased by three-fold compared to the control (Fig. 3A). Translation of LUC-A was moderately (1.7-fold) stimulated by p97, a homologue of the C-terminal two-thirds of eIF4G, which binds eIF4A and eIF3, but not eIF4E [18] (Fig. 3B). When p97, eIF2 and eIF2B were added together, translation of the uncapped RNA was increased by five-fold relative to the control (Fig. 3B). No factor other than eIF2 and eIF2B augmented translation of EMCV-IRES-LUC-A mRNA, and the greatest effect (1.7-fold relative to the control) on the translation was observed with the combination of eIF2 and eIF2B (Fig. 3C). The synthesis of luciferase from LUC-A mRNA stimulated by p97 + eIF2 + eIF2B reached a level comparable to that from cap-LUC-A stimulated by eIF4E + eIF2 + eIF2B (Fig. 3D).

Along with the experiments using the HeLa cell extract, we carried out translation of the same mRNAs with a rabbit reticulocyte lysate (RRL) (Fig. 3D). Optimum concentrations of potassium and amounts of mRNA for translation in RRL were determined for each mRNA (see Materials and methods). eIF4E, p97, eIF2 and eIF2B were then tested individually or in combination for their capacity to further stimulate translation in RRL. Since commercially available lots of RRL are variable with respect to the translational activity, we tested several lots, and show the results obtained with the lot that exhibited the best activity (Fig. 3D). eIF2 seemed slightly effective in raising translational activity of RRL for LUC-A and EMCV-IRES-LUC-A, and eIF4E + eIF2 appeared to be moderately effective in enhancing translation of cap-LUC-A (Fig. 3D). Addition of other translation factor(s) or any other combination of the factors had no effect on the translation of any of the three mRNAs in RRL (data not shown). Thus, supplementation of RRL with a translation initiation factor was not as effective for enhancing translation as observed with the HeLa cell extract.

Continued protein synthesis by a dialysis system

The batch system, which was employed in the experiments presented above (Fig. 3), does not allow for the sustained synthesis of proteins (longer than a few hours) because of amino acids and ATP deficiency, and because of accumulation of waste products. In contrast, a dialysis system, which continuously supplies the substrates and energy source for protein synthesis and removes waste products through a dialysis membrane, has enabled *E. coli* and wheat germ extracts to maintain protein synthesis up to several days [11,12,28]. We hence examined whether the dialysis method could further enhance protein synthesis in the HeLa cell extract supplemented with translation factors. To effectively operate the dialysis system, a relatively large volume of the extract (~100 μl, compared to 6–12 μl in the batch method) is required, and increased amounts of the translation factors (eIF2, eIF2B, eIF4E, or p97) are also required accordingly. We thus decided to omit eIF2 from

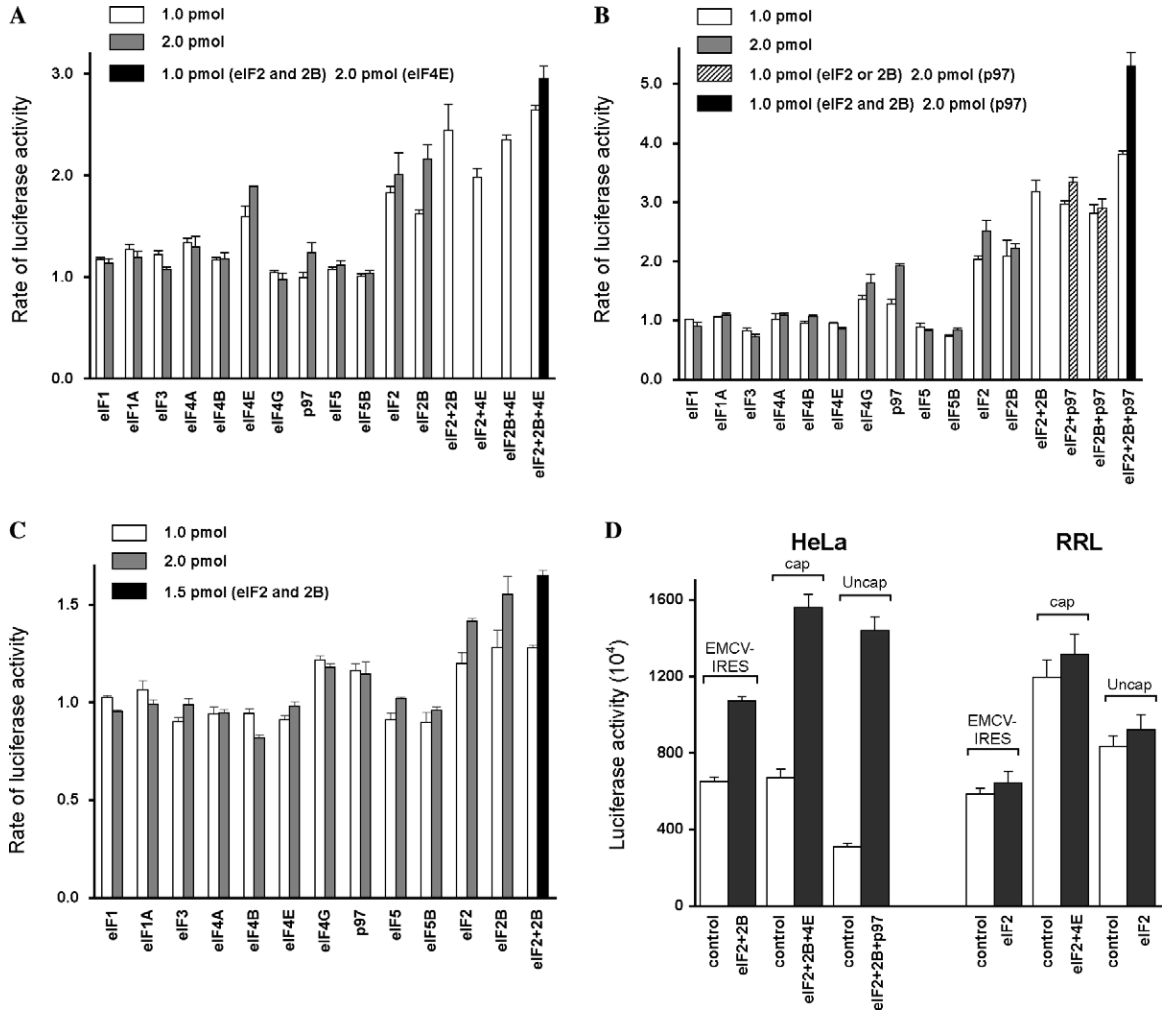

Fig. 3. Effects of translation factors on protein synthesis in HeLa cell extracts and rabbit reticulocyte lysates. (A) Cap-LUC-A mRNA (40 μg/ml), (B) LUC-A mRNA (80 μg/ml), and (C) EMCV-IRES-LUC-A mRNA (100 μg/ml) were translated with HeLa cell extracts (6 μl, total volume) supplemented with eIF1, eIF1A, eIF2, eIF2B, eIF3, eIF4A, eIF4B, eIF4E, eIF4G, p97, eIF5, or eIF5B (1.0, 1.5, or 2.0 pmol). Following incubation for 1 h at 32 °C, an aliquot was used for luciferase assay. The luciferase activity of the reaction in the absence of an exogenous factor was set at 1.0. The data represent the average of three experiments with the standard deviation from the mean. White and gray bars represent 1.0 and 2.0 pmol each factor in a reaction, respectively. Black and slash bars represent a combination of factors as indicated in (A–C). (D) Luciferase activities obtained from translation of each mRNA in HeLa cell extracts or rabbit reticulocyte lysates (RRL) not supplemented (white bar) or supplemented (black bar) with effective factors which were the same combinations as indicated by the black bars in (A–C) for the HeLa cell extract. For RRL, eIF2 (2.0 pmol) and eIF4E (3.0 pmol) were added for translation of cap-LUC-A, while only eIF2 (2.0 pmol) was supplied for that of LUC-A and EMCV-IRES-LUC-A. Data are the average of three experiments with the standard deviation from the mean.

the supplements, since the yield of eIF2 purified from HeLa cells is limited (~15 μg from 1 litter cell culture), and no recombinant form of eIF2 was successfully obtained (Imataka, unpublished). Concerning eIF2B, it was reported that eIF2B3 and eIF2B5 formed a sub-complex catalyzing guanine-nucleotide exchange on eIF2 [29]. We thus prepared a recombinant eIF2B3–eIF2B5 complex (Fig. 2) and tested whether it could enhance translation in the HeLa cell extract. This complex was as effective as the whole complex for enhancing protein synthesis in the dialysis method (data not shown); the eIF2B3–eIF2B5 complex was less active than the whole complex in the batch method for an unknown reason. We therefore used the eIF2B3–eIF2B5 complex in place of the five subunit-eIF2B complex in the dialysis method. The HeLa cell extract was incubated in the dialysis system (see Materials and methods for details) with cap-GST-A mRNA, which was added at the time 0 and 24 h of incubation. An aliquot of the mixture was removed after different times of incubation to monitor the GST protein synthesis by Western blotting (Fig. 4). Protein synthesis in the HeLa cell extract was sustainable for 36 h, and the yield was further enhanced (2.6- to 3.6-fold) by eIF4E + eIF2B3–2B5 (Fig. 4, left). The yield of GST protein from the factor-supplemented extract after 36 h was ~50 μg/ml extract. Similar experiments were carried out with EMCV-IRERS-GST-A and uncapped-GST-A mRNAs. Translation was maintained for 36 h in both cases, and could be stimulated with eIF2B3–2B5 for EMCV-IRES-GST-A and p97 plus eIF2B3–2B5 for uncapped-GST-A. The yield of the GST protein in neither case

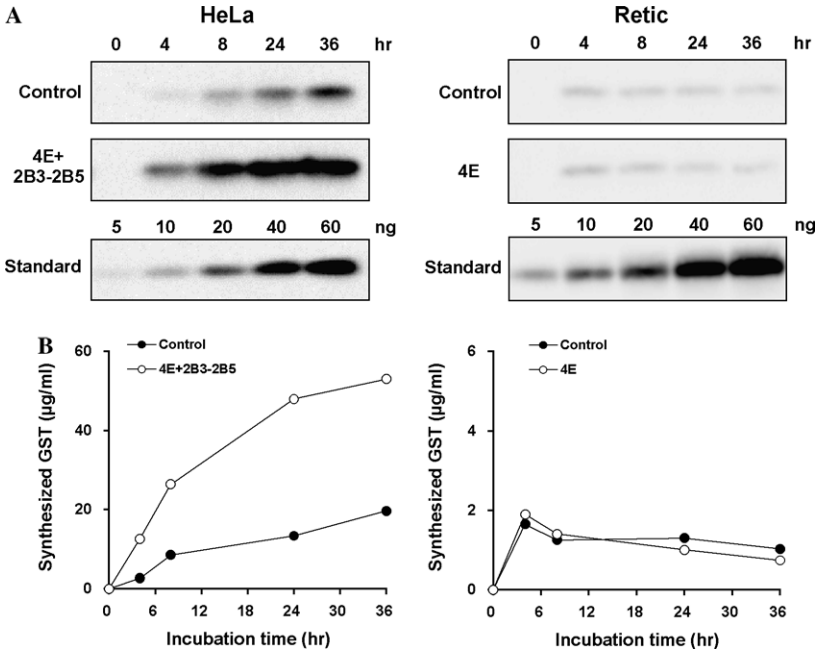

Fig. 4. Protein synthesis by a dialysis system. (A) Cap-GST-A mRNA (6.4 μg) was translated with HeLa extracts (left panels) (120 μl, total volume) not supplemented (upper panel) or supplemented (middle panel) with 45 pmol of eIF4E and 21 pmol of eIF2B3–2B5 in a dialysis system for 36 h at 32 °C (see Materials and methods for details). The same mRNA (2.4 μg) was additionally supplied at 24 h of incubation. At 4, 8, 24, and 36 h of incubation, an aliquot of the sample was removed for SDS–PAGE followed by Western blotting with anti-GST-antibody, and the protein band was visualized by chemiluminescence. A standard GST proteins (0–60 ng) mixed with the translation mixture without mRNA was analyzed as described above (lower panel). RRL (right panels) was incubated in the dialysis system as was the HeLa cell extract except that the supplement was eIF4E. (B) The signal of the GST band detected in (A) was quantified by a phosphoimager, and the amount of the synthesized GST in each sample was calculated by comparing with the signal of the standard GST (left: HeLa cells; right: RRL).

exceeded that obtained with cap-GST-A (data not shown). No enhancement of the protein synthesis by the dialysis system was observed with RRL (Fig. 4). As stated above, we did not use eIF2 as a supplement in the dialysis system, since the amount of the purified eIF2 is limited, and it is not practical therefore to use eIF2 in this system, which requires far more amounts of supplements than does the batch system.

Discussion

We have successfully improved the efficiency of a HeLa cell-derived cell-free translation system by supplementing it with translation initiation factors (eIF2, eIF2B or eIF4E) or a translational regulator (p97). Other initiation factors (eIF1, eIF1A, eIF3, eIF4A, eIF4B, eIF4G eIF5, and eIF5B) failed to stimulate translation of any form of mRNAs (capped, uncapped or EMCV-IRES-directed) tested. It is likely that these factors, which are essential in translation initiation [30–32], already exist in sufficient amounts in HeLa cells, and their addition was of no consequence. eIF2B is, in contrast, one of the least abundant translation initiation factors [24,33], hence effectively enhancing protein synthesis when exogenously supplied. eIF2B catalytically regenerates eIF2–GTP from eIF2–GDP [2], explaining that addition of both eIF2 and eIF2B further enhanced translation. eIF2 is 5–10 times more abundant than eIF2B [24,33] in eukaryotic cells, yet addition of eIF2 alone stimulated protein synthesis. While eIF2 and eIF2B were effective for all forms of RNA tested, eIF4E specifically activated translation of the capped RNA, since eIF4E specifically recognizes the cap structure [34]. While the molar ratio of eIF4E to the ribosome in the HeLa cell was reported to be 0.26 [25], that in the reticulocyte lysate [26] and in yeast [24] was estimated to be 1–2, suggesting that the amount of eIF4E itself is not apparently limited in some cells. However, a significant fraction of eIF4E is being sequestered by 4E-BPs, which were reported to exist at a molar ratio of ~1:1 against eIF4E [26]. Addition of eIF4E to the translation extract should hence increase the amount of eIF4E which is not associated with 4E-BP. In this regard, it is noteworthy that eIF4E has been repeatedly shown to cause or promote malignant transformation of cells when over-expressed [35–37], and that the malignancy of cells due to eIF4E-overexpression was counteracted by overexpression of 4E-BPs [38].

p97 was previously characterized to be an inhibitor of cap-dependent translation in vivo [18]. The results presented above, however, suggest that p97 serves as a translational enhancer in vitro (Fig. 3B). Our unpublished data show that p97 relieved repression of cap-dependent translation caused by 4E-BP. The mechanism by which p97 stimulates translation will be published elsewhere.

The HeLa cell extract is here demonstrated to sustain protein synthesis for longer than 24 h if the extract is being dialyzed. In agreement with this, translation factors

including eIF4G and large subunits of eIF3 were still intact after 24 h of incubation as observed by Western blotting, and the 18S and 28S ribosomal RNAs were not degraded at that time as examined by gel analysis (Mikami et al., unpublished). Of note is the observation that the protein synthesis continued to increase between 8 and 24 h of incubation, although the mRNA (cap-GST-A) was added only at the beginning of the incubation, suggesting that the capped/poly(A)-tailed mRNA is stable and translationally active in the HeLa extracts for many hours. In contrast, when we used an uncapped/poly(A)-tailed mRNA (GST-A) in the dialysis method, the mRNA had to be replenished at every 4 h to achieve a continued synthesis of the protein (data not shown), since an uncapped RNA is generally more unstable than the capped counterpart [39,40]. Importantly, unlike with the HeLa cell extract, the protein synthesis with RRL was not increased by the dialysis method, probably because RRL contains enormous amounts of globin proteins (~200 mg/ml), and thereby traffic of small molecules through the dialysis membrane is rendered inefficient. In conclusion, the translation factor-supplemented HeLa cell extracts which we have optimized here should become a useful tool to produce recombinant proteins.

Acknowledgments

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