Chapter 4

Protein Synthesis *in vitro*: Cell-Free Systems Derived from Human Cells

Kodai Machida, Mamiko Masutan and Hiroaki Imataka

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1. Introduction

When researchers wish to obtain recombinant proteins, a primary choice of the method is in most cases the expression in *E. coli*. If this system does not work for the protein of interest, they may turn to insect or mammalian cells. Protein expression *in vitro* may be chosen if these *in vivo* expression systems do not give the protein satisfactorily. There are several reasons why expression of some recombinant proteins in living cells is poor. If the protein to be expressed is toxic to host cells or inhibitory for growth, it should be difficult to express the protein to a high level. The cell-free system is derived from the extract from broken cells, and therefore the above-mentioned problems that occur in the living cells, if not all, are avoidable.

The value of the cell-free protein system seems unlimited. Radioactive amino acids or unnatural amino acids are relatively easily incorporated into proteins, rendering the system very useful for structural analysis of the synthesized proteins (1). The 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. While *E. coli*- (2), wheat germ- (3) and rabbit reticulocyte- (4) derived systems have been widely employed for the above-mentioned purposes for decades, human cells-derived *in vitro*-protein expression systems are now beginning to gain attention.

What is the merit of a human or mammalian cells-derived system compared with other cell-free systems? Firstly, many different cell lines that are derived from various organs or tissues such as neurons, endocrine glands and immunocytes are available from cell banks (eg., ATCC and RIKEN BRC). Since each cell line maintains some properties specific to the originated organs or tissues, one can establish a variety of cell-free systems from different

cell lines. A successful example is a cell-free glycoprotein-synthesis system derived from a monoclonal antibody-producing hybridoma (5). Another merit of the mammalian system is that mammalian cell-derived extracts seem to have greater capacity to synthesize large proteins (6) than other systems. Lastly, mammalian cell-free systems can directly lead to application for medical and pharmaceutical purposes. A remarkable example is the synthesis of RNA virus in a test tube (7), which is impossible by other cell-free systems. The RNA virus is a super-high molecular weight complex consisting of its RNA genome and capsid proteins, and the viral particles are assembled through a complex process. The assembly process of the RNA virus such as picornaviruses can be recapitulated in a human cell-derived *in vitro* protein synthesis system. This system can be used for screening anti-virus chemicals. In the following sections, we discuss these three advantages of the human cell-based in vitro protein synthesis systems.

2. Cell-free synthesis of glycoproteins

Glycosylation is one of the major post-translation modifications of proteins. The polypeptides destined to be localized to the plasma membrane or to be secreted outside of the cell enter the endoplasmic reticulum (ER) while being translated. Immediately after the polypeptide enters the ER, *N*-linked glycosylation starts (8). Whereas glycans linked to proteins are implicated in many biological aspects such as development, differentiation and physiology (9), *N*-glycosylation itself is thought to be necessary for the proper folding of proteins through disulfide bond formation in the ER (8). More than half of eukaryotic proteins are predicted to be glycoproteins(10), and thus, it is an urgent task to establish an efficient system to produce glycoproteins *in vitro*. However, neither the *E. coli* nor wheat germ system can glycosylate proteins. rabbit reticulocyte lysates (RRL) combined with microsomes from dog pancreas have been a popular system for *in vitro N*-glycosylation (11), but commercially available RRL and canine microsomes are expensive, and the activities of the preparations vary depending on the source. Furthermore, preparation of these extracts by a researcher is not an easy task, since these systems entail sacrifice of animals.

These problems can be solved by using a specific cell line. HeLa cells represent one of the most popular cell lines as a source of mammalian cell-derived *in vitro* translation systems. However, HeLa cell extracts (12) fail to produce a recombinant glycoprotein (5). The major envelope glycoprotein (gp120) of human immunodeficiency virus type-1 (HIV-1) consists of a core polypeptide of ~60 kDa and ~20 *N*-linked glycans which increase the total mass of the molecule to ~120 kDa (13). When mRNA encoding the HIV-1-gp120 region is translated in the HeLa cell extract, a ~60 kDa protein is synthesized as the major product. This indicates that *N*-glycosylation of gp120 is inefficient in the HeLa cell-derived cell-free system. The endoplasmic reticulum (ER), where *N*-glycosylation occurs, may not be well-developed in HeLa cells, because HeLa is not a secretory cell line. A monoclonal antibody-producing hybridoma cell line is now chosen, because the hybridoma cell should possess a

highly developed ER system to secrete large amounts of immunoglobulins, and, from the practical point of view, they can be easily propagated in a suspension culture (5). When programmed with the mRNA encoding HIV-1-gp120, the hybridoma extract prominently synthesizes one product with a molecular mass of ~100 kDa (Figure 1). This 100 kDa product is a glycosylated form of gp120, since treatment with PNGase F changed it into a ~60 kDa product (Figure 1). Other than HIV-1-gp120, biologically active human choriogonadotropin (hCG), a glycoprotein hormone consisting of α and β subunits was successfully synthesized (5).

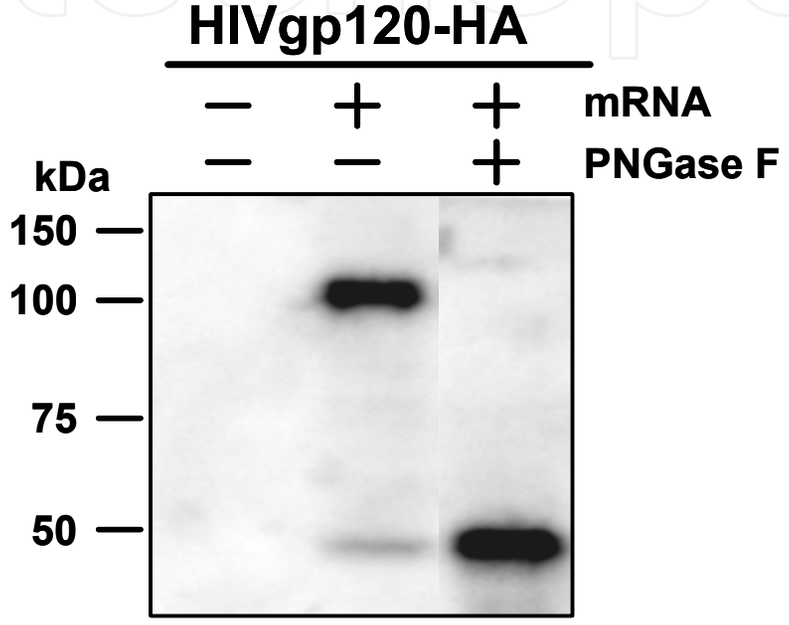

Figure 1. Glycosylation of HIV gp120 in the hybridoma-derived cell-free translation system.
The hybridoma extract was incubated with mRNA encoding gp120-HA. After incubation, samples were treated with or without PNGase F, resolved by SDS-PAGE, and analyzed by western blotting with anti-HA antibody.

3. Cell-free synthesis of large proteins

Many human proteins are very large (>150 kDa), and these large proteins consist of several functional domains. Each domain may be expressed by conventional protein expression systems such as in *E. coli* for the functional and structural analysis. However, it is obviously necessary to examine the structure and function of the whole molecule to gain insight into the real function of the protein, yet many large proteins remain uncovered for the structure and function due to the difficulty in preparation of the full-length form. Since the mammalian cells have many large proteins, it is expected that the mammalian translation machinery basically possesses a high capacity to elongate a long chain of the peptide. Translation initiation is the limiting step in the eukaryotic protein synthesis, and therefore the capacity to elongate a peptide chain of thousands amino acids can be recapitulated *in vitro* if the initiation step is not impaired.

Among the factors involved in translation initiation, eukaryotic translation initiation factor (eIF) 2 plays a pivotal role in translational regulation (14). 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 regenerate the ternary complex, a reaction catalyzed by eIF2B, a multi-protein complex with 5 subunits. When the α subunit of eIF2 is phosphorylated, the affinity of eIF2 for eIF2B dramatically increases, and eIF2B is thereby sequestered by eIF2. Since eIF2B is then unable to regenerate the ternary complex, translation is consequently attenuated (14). Phosphorylation of the α subunit of eIF2 occurs in response to stress conditions such as viral infection, oxidation, deprivation of amino acids, and accumulation of misfolded proteins (15).

The mammalian cell extract-derived *in vitro* translation system has an intrinsic problem, namely, phosphorylation of the α subunit of eIF2 due to a high concentration of ATP (5). During preparation of the cell extract, eIF2α-kinases seem to aggregate or dimerize, and addition of ATP causes auto--phosphorylation and activation of the kinases. The ATP-induced phosphorylation of eIF2α in the cell-free system is a serious problem, because phosphorylation of eIF2α attenuates translation initiation (14), yet, ATP is indispensable to maintain protein synthesis. This problem is now solved by addition of K3L and GADD (growth arrest and DNA damage gene) 34. K3L is a vaccinia virus-encoded protein, which acts as a pseudosubstrate for eIF2α-kinases because of its structural resemblance to an N-terminal part of eIF2α (16, 17). K3L prevents phosphorylation of eIF2α during the virus infection, thereby counteracting the otherwise repressed translation (18). GADD34 recruits a phosphatase PP1c to eIF2 to dephosphorylate eIF2α (19). As expected, addition of recombinant K3L and / or GADD34 relieves phosphorylation of eIF2α and effectively stimulates protein synthesis in the cell-free system (5, 20).

The cell-free protein expression system supplemented with K3K/GADD34 is further improved by introduction of coupled transcription/translation system and internal ribosome entry site (IRES). In cell-free translation systems, mRNA is added or it is synthesized with the addition of a DNA (a plasmid or a PCR product) and the bacteriophage RNA polymerase (T7, SP6, or T3 RNA polymerase). The latter method, called a coupled transcription/translation system (6), is more convenient than the mRNA-dependent system, because researchers do not need to synthesize and purify RNA. Furthermore, mRNA is continuously supplied to compensate for degradation of the mRNA in the system.

A drawback of the coupled transcription/translation system is that RNA produced by a bacteriophage RNA polymerase is not 5′-capped unless a high concentration of the cap-

analogue is supplied. Uncapped RNAs are less efficient for translation than the capped counterpart in the HeLa cell-derived cell-free system, and the cap-analogue is very expensive. This problem is solved by placing 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. The ribosome binds to IRES and initiates translation without aid of the cap structure.

Collectively, the HeLa cell-based *in vitro* coupled transcription/translation system supplemented with K3L/GADD34 is able to synthesize large proteins such as GCN2 (170 kDa), Dicer (200 kD), eIF4G (220 kDa) and mTOR (260 kD) from the IRES-harboring plasmids that encode for these proteins (Figure 2).

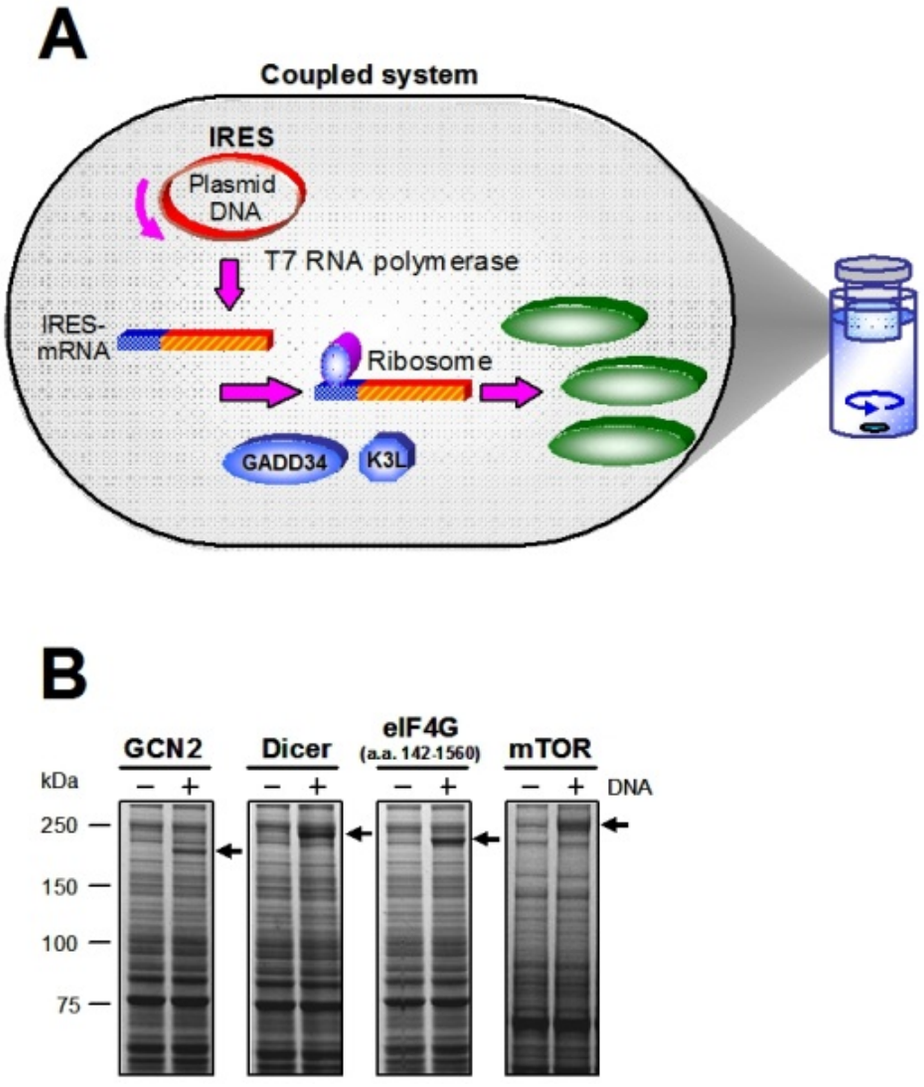

Figure 2. The HeLa cell-based in vitro coupled transcription/translation system.
(A) Cartoon depicting the HeLa cell-based in vitro coupled transcription/translation system that utilizes IRES and is supplemented with K3L and GADD34. (B) Large proteins were synthesized using the system depicted in (A). Samples were separated by SDS-PAGE and stained with CBB. Arrows indicate synthesized proteins.

4. Cell-free synthesis of RNA virus

Cell-free synthesis of an infectious virus is an ideal tool for elucidating the mechanism of viral replication and for screening anti-viral drugs. *Encephalomyocarditis virus* (EMCV) is a *Cardiovirus* in the family *Picornaviridae*. The genome of EMCV is a single-stranded positive-sense RNA of 7.9 kb. Upon infection by EMCV, the genomic RNA is translated into a single polyprotein, which is subsequently processed via a series of proteolytic events into structural (capsid) and nonstructural proteins (21). The RNA-dependent RNA polymerase (RdRp), one of the viral nonstructural proteins, replicates the genomic RNA, which is incorporated into a viral capsid intermediate structure to constitute an infectious virion (22) (23). EMCV can be synthesized from its RNA in the HeLa extract-derived cell-free protein synthesis systems (24, 25) (Figure 3). Since the synthesized virus is proved to be infectious (24) (Figure 4), the *in vitro* system is a choice of the method to obtain virus particles.

The cell-free synthesis of EMCV is enhanced by employing a dialysis system (Figure 3). A batch system (a closed test tube system) does not allow for sustained synthesis of proteins over a period of several hours due to amino acid and ATP deficiencies, and to the 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 HeLa cell extracts to maintain protein synthesis for up to one day. To investigate the means by which the dialysis system enhances virus synthesis, the efficiencies of translation and processing steps were monitored by labeling with radio-labeled leucine during a 10-h incubation. Neither the processing pattern of the viral proteins nor the intensity of each product varied substantially when HeLa cell extracts were incubated with the viral RNA by the batch or dialysis system (24). In contrast, the capacity of the HeLa cell extract to synthesize EMCV RNA was increased seven-fold by employing the dialysis system compared with the batch system (24). Thus, replication of the RNA, rather than translation or processing of the viral proteins, is enhanced by the dialysis system (Figure 5).

A ribozyme technology provides opportunities for mutational analyses of EMCV *in vitro* and for production of EMCV particles (24, 26) (Figure 3). Efficient RNA synthesis by the virus-encoded RdRp requires a precise sequence at the 5′-end of the template RNA. Synthetic RNAs produced by T7, SP6, and T3 RNA polymerases have a guanine nucleotide at the 5′-end, which hampers the plus stranded RNA synthesis of EMCV. No detectable virus was generated from a synthetic EMCV RNA that possessed extra nucleotides (GG) at the 5′-end (24). If genomic RNA purified from EMCV particles were the only available template, the usefulness of the cell-free system would be limited, because mutational studies could not be done. Thus, a hammerhead ribozyme sequence was introduced at the 5′-end of the RNA to catalyze removal of the extra nucleotides; introduction of an appropriately designed hammerhead ribozyme sequence at the 5′-end of the RNA yields an RNA with the same nucleotide sequence at the 5′-end as the viral genomic RNA (24). Synthetic EMCV

RNAs were translated with comparable efficiencies in HeLa cell extracts by the dialysis method irrespective of the presence of the ribozyme at the 5′ end. However, while replication of synthetic EMCV RNA without the ribozyme was not appreciable, the EMCV RNA with the ribozyme replicated at 25% the efficiency of the genomic EMCV RNA (24) (Figure 6).

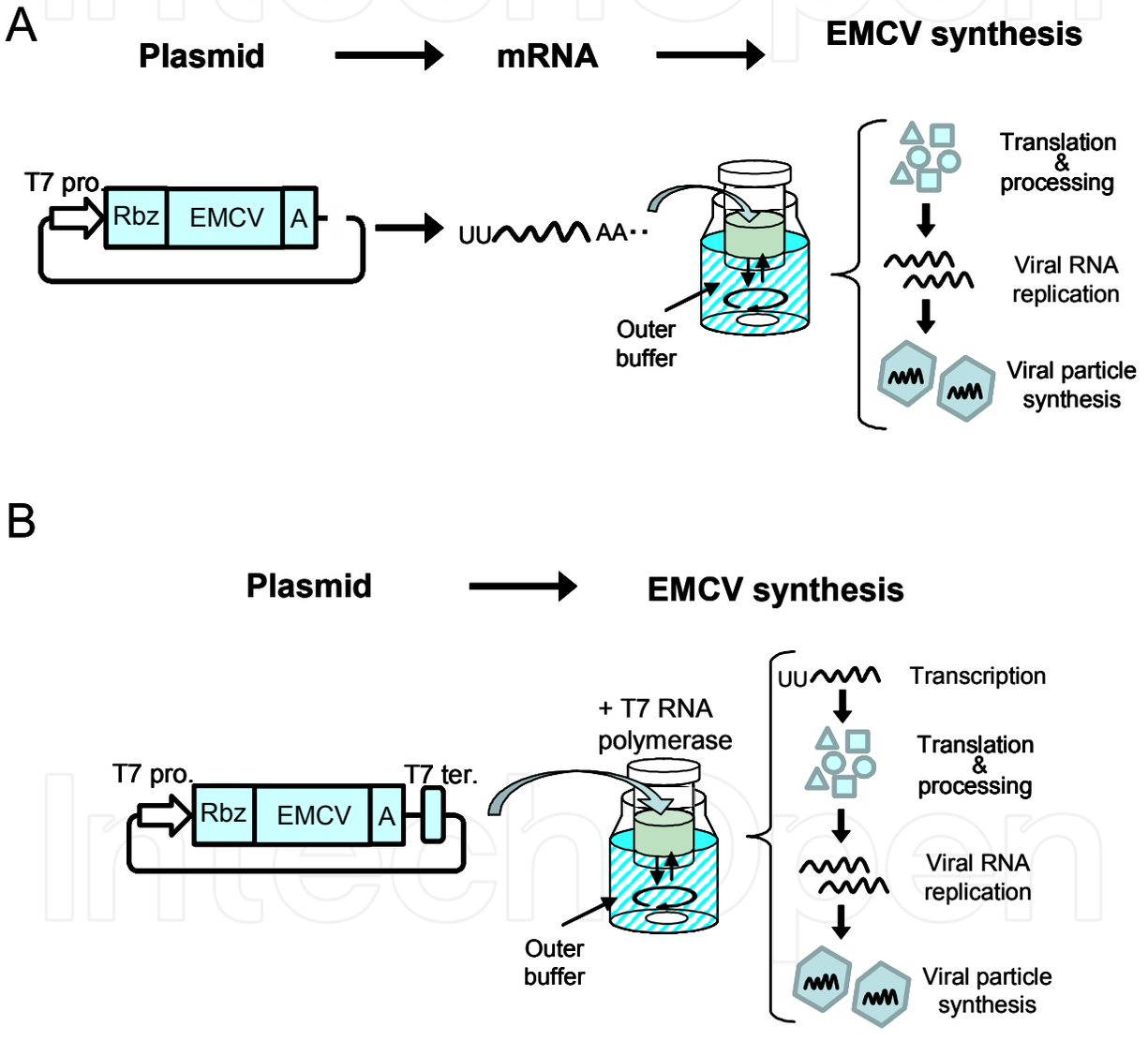

Figure 3. RNA-dependent and DNA-dependent cell-free systems for EMCV synthesis
(A) mRNA dependent system. EMCV RNA is synthesized in vitro and purified. The purified EMCV RNA is incubated with the HeLa cell-derived cell-free protein synthesis system. (B) DNA-dependent system. The plasmid encoding the EMCV RNA is directly incubated with the HeLa cell-derived cell-free protein synthesis system supplemented with T7 RNA polymerase.

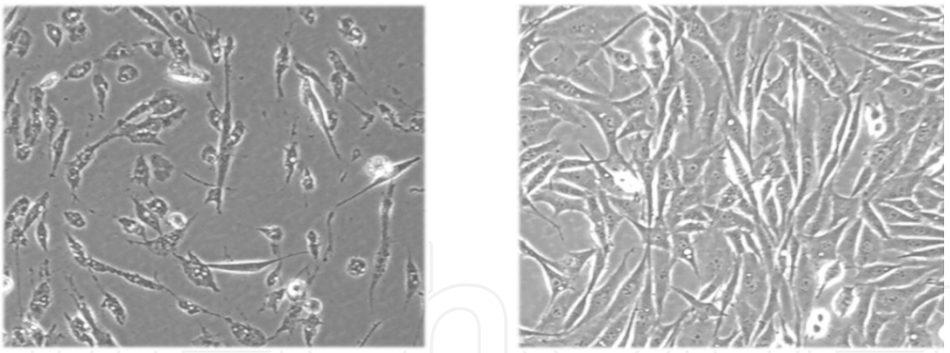

Figure 4. Infection of BHK-21 cells with EMCV synthesized by the cell-free system.
BHK-21 cells were incubated with RNase-treated HeLa cell extract programmed with (left panel) or without (right panel) EMCV RNA. Twenty hours later, cells were observed by microscopy.

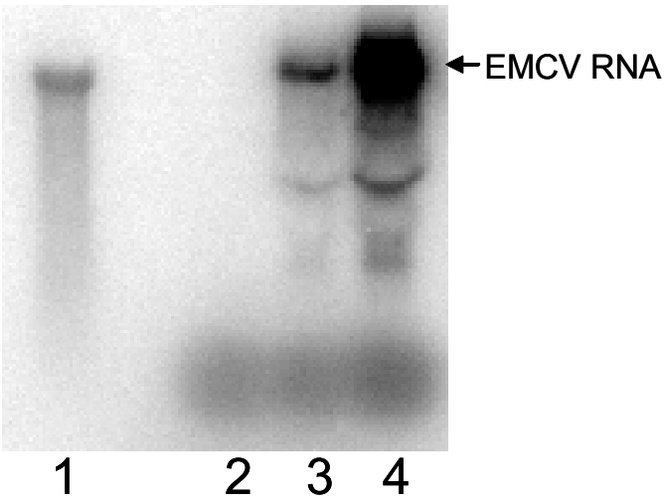

Figure 5. Dialysis enhances replication of EMCV RNA.
EMCV RNA was incubated in the HeLa cell extract with 32P CTP by the batch system (lane 3) or the dialysis system (lane 4).
Lane2: no RNA was Incubated in the batch system. After incubation, RNA was purified, resolved by gel, and detected by autoradiography. Lane 1: in vitro-synthesized EMCV RNA

Furthermore, synthesis of EMCV from DNA templates *in vitro* is now possible (Figure 3). When a plasmid or a PCR product harboring the full-length cDNA of EMCV in the T7 promoter /terminator unit is incubated in the HeLa extract-derived cell-free protein synthesis system supplemented with T7 RNA polymerase, EMCV is progressively produced, thereby circumventing the handling the easily degradable viral RNA (7). This coupled system for the EMCV synthesis provides an opportunity to study the selectiveness of the RNA into the viral particle. Two forms of EMCV RNA are synthesized in the DNA-programmed system: the RNA transcribed from the plasmid by T7 RNA polymerase and the RNA amplified by the viral RNA-dependent RNA polymerase. It is thus imperative to determine which RNA form is incorporated into the EMCV particles. To this end, RNA was purified from EMCV particles from the incubated mixture, and sequencing of the RNA

revealed that the EMCV RNA transcribed by the virally encoded RNA-dependent RNA polymerase was predominantly incorporated into the EMCV particle even in the presence of a larger amount of the EMCV RNA transcribed by T7 RNA polymerase from the plasmid (7). This work is a good example that shows the usefulness of the cell-free system for the study of the RNA virus.

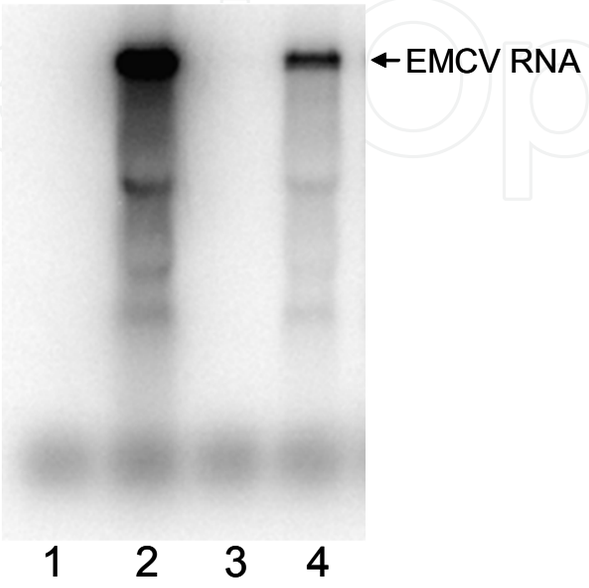

Figure 6. A ribozyme enables replication of synthetic EMCV RNA.
Genomic EMCV RNA (lane 2), synthetic EMCV RNA without (lane 3) or with (lane 4) a ribozyme sequence at the 5′ end was incubated in the HeLa cell extract as in Figure 5 by the dialysis system. Lane 1: no RNA was Incubated. After incubation, RNA was purified, resolved by gel, and detected by autoradiography.

5. Concluding remarks

As discussed in this chapter, the human cells-derived *in vitro*-protein expression systems are unique with fascinating values than compared with other cell-free systems. However, the extract-dependent system contains unknown substances, and proteases in the extract are particularly problematic, since synthesized proteins would be degraded. In this regard, the PURE system (27), which comprises purified translation factors and ribosomes from *E. coli* is an ideal system, and a humanized PURE system should be developed as soon as possible.

Author details

Kodai Machida
Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, Himeji, Japan
Molecular Nanotechnology Research Center, Graduate School of Engineering, University of Hyogo, Himeji, Japan

Mamiko Masutan
Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, Himeji, Japan

Hiroaki Imataka*
Department of Materials Science and Chemistry, Graduate School of Engineering, University of Hyogo, Himeji, Japan
Molecular Nanotechnology Research Center, Graduate School of Engineering, University of Hyogo, Himeji, Japan
RIKEN Systems and Structural Biology Center, Tsurumi-ku, Yokohama, Japan

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* Corresponding Author

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