Benchmarks

A technique to increase protein yield in a rabbit reticulocyte lysate translation system

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Rabbit reticulocyte lysate (RRL) is a mammalian cell-free system for protein production. However, one of the limitations of this system is its low protein yield. Inclusion of recombinant virus proteins and specific viral structures on target mRNA could enhance protein production in RRL. Here we show that simultaneous addition of influenza A virus NS1 protein and inclusion of the encephalomyocarditis virus (EMCV) internal ribosome entry site (IRES) in the target mRNA facilitate translation initiation and increase protein yield over 10-fold, improving the translation capacity of RRL.

Cell-free translation systems (CFTSs) are powerful tools in protein research. CFTSs were originally used for deciphering the genetic code (1). Recent advances in CFTSs have significantly broadened their application, specifically for large-scale protein production, overcoming many obstacles associated with protein production in cell culture. CFTSs allow the production of toxic proteins, proteins with low solubility (e.g., membrane proteins), incorporation of stable isotope-labeled or non-canonical amino acids, on-chip protein synthesis, and synthesis of pharmaceutical polypeptides. In addition, CFTSs allow studies of protein folding and post-translational events (2). Thus, the CFTS is an attractive tool for structural biology, functional genomics, drug screening, and vaccine production.

Rabbit reticulocyte lysate (RRL) is a mammalian CFTS with two key advantages: the lysate is easy to prepare and, importantly, it produces mammalian post-translational protein modifications. For these reasons, it is actively used in protein research. However, low protein yield is a considerable limitation of RRL (2). Possible ways to enhance protein production in this system include the addition of factors that up-regulate translation.

Viruses have developed multiple strategies to regulate the translation of the host cells using viral proteins and RNA elements. The addition of such virus factors could be used to enhance the translation capacity of RRL (3). For example, during influenza A virus (IAV) infection the viral NS1 protein targets multiple factors to prevent translational shut-off, which is mediated via eIF2α phosphorylation (4). Non-phosphorylated eIF2α modulates recruitment of the initiator tRNA^Met^ to the 40S ribosome subunit, which is a critical step in the translation initiation of most mRNAs, including IAV mRNAs (5). Thus, NS1 could be used in RRL to inhibit endogenous eIF2α phosphorylation and facilitate translation initiation. Hepatitis C (HCV) and encephalomyocarditis (EMCV) viruses use internal ribosome entry sites (IRESs) at the 5' ends of their mRNAs to harness ribosomes independently of other initiation factors, allowing preferential translation of viral mRNA (6). However, efficient translation initiation from HCV and EMCV IRESs is still dependent on non-phosphorylated eIF2α (7). In addition, translation is stimulated by the 3' untranslated regions (UTRs) of HCV or simian vacuolating virus 40 (SV40) mRNAs (8), which retain ribosome complexes during translation termination to facilitate subsequent translation re-initiation. Thus, engineered IRES and/or UTR structures on the target mRNAs and addition of influenza NS1 protein to the RRL could enhance protein production.

In the current study, we attempted to optimize the translation efficacy of an RRL system by supplementing it with viral enhancers of translation. We have shown previously that purified recombinant NS1 proteins from H5N1 and H5N2 (but not A/H1N1pdm09) influenza viruses enhance translation in RRL (9). The effect was dependent on H5N1 and H5N2 NS1 protein concentration, and the optimal concentration of NS1 was 4 µM. Importantly, NS1 mutants R38A and K41A (NS1R38A/K41A) from H5N1

Method summary:

In the present method, we enhanced the protein synthesis in rabbit reticulocyte lysate by adding purified recombinant NS1 protein of influenza A virus and target mRNA with the encephalomyocarditis virus internal ribosome entry site. This increased target protein production over 10-fold. This technique can be used for large-scale protein production in the rabbit reticulocyte lysate system and in other applications.
and H5N2 viruses, which lack RNA binding activity, were also active in the RRL system. These mutants have increased protein solubility and a longer shelf life. Therefore, we used H5N1 NS1<sup>RK/AA</sup> at 4 µM throughout the study.

We first tested whether NS1<sup>RK/AA</sup> could enhance the synthesis of viral and/or cellular proteins in RRL. For this, we programmed the RRL (Promega, Fitchburg, WI) with 0.2 µg/µl RNA extracted from influenza A/WSN/33 virus-infected Madin-Darby canine kidney (MDCK) and adenocarcinomic human alveolar basal epithelial A549 cells at different times post infection using an RNeasy kit (Qiagen, Hilden, Germany) and supplied the reactions with NS1<sup>RK/AA</sup> and [35S]-methionine. Control reactions used buffer lacking NS1<sup>RK/AA</sup>. After 2 h of incubation at 30°C, the proteins were resolved in a 4%–20% gradient SDS-PAGE, and incorporation of [35S]-methionine into newly-synthesized proteins was analyzed by autoradiography and quantified by densitometry analysis using ImageJ software. Figure 1A shows that NS1<sup>RK/AA</sup> enhanced production of both cellular and viral proteins in RRL by 1.3–2 fold, indicating that the effect of NS1<sup>RK/AA</sup> on translation is not specific to viral or cellular transcripts.

To further determine the effect of NS1<sup>RK/AA</sup> on translation in RRL, we produced reporter mRNA by transcribing luciferase control DNA (Promega) in the presence of [32P]-ATP (Perkin Elmer, Waltham, MA) using an mMessage mMachine kit (Ambion, Life Technologies, Carlsbad, CA). This template mRNA allowed us to monitor the fate of the mRNA by autoradiography and reporter protein synthesis by luminescence. The RRL reactions were programmed with the reporter mRNA and supplemented with NS1<sup>RK/AA</sup> or TN buffer (Tris-HCl pH 7.5, 50 mM KCl). Importantly, the RRL could be diluted up to 2.5 times without loss of translation capacity (data not shown). After a 2 h incubation at 30°C, 400 µL aliquots of the reaction mixtures were loaded on top of 10%–30% sucrose gradients containing 20 mM Tris-HCl pH 7.5, 50 mM KCl, 5 mM MgCl<sub>2</sub>, and sedimented for 3 h at 131000×g at 4°C. Gradient fractions were analyzed by agarose gel electrophoresis followed by autoradiography. We observed that, in the presence of NS1<sup>RK/AA</sup>, reporter mRNA and rRNA were more pronounced in the polysomal fractions than in the control reactions. Accordingly, in the NS1<sup>RK/AA</sup>-supplemented reaction, we observed an increase in luciferase activity at the top of the gradient (Figure 1B). These results suggest that the fraction of ribosomes associated with mRNA is greater in RRL reactions containing NS1<sup>RK/AA</sup>, most probably indicating that NS1<sup>RK/AA</sup> secures translation initiation (4,9).

We next studied the effect of adding viral RNA regulatory elements to the 5' and 3' ends of the target mRNA on NS1-mediated translation in RRL. For this, we evaluated the translation of
luciferase-encoding mRNAs with a 5′-7-methylguanosine (m7G) cap or IRES and 3′-poly(A) or a viral UTR (Figure 2A). Expression constructs were cloned in a pGL3 vector as described previously (6,10,11). The corresponding mRNAs were translated in RRL with or without NS1RK/AA, and luminescence was used as readout. We observed that NS1RK/AA enhanced translation of all studied mRNAs by at least 4-fold (Figure 2B). In particular, translation of mRNAs containing HCV IRES and 3′-poly(A) or HCV 3′ UTR and mRNAs containing a 5′-m7G cap and 3′-poly(A) or a SV40 UTR was enhanced 4.3–4.8 fold in the presence of NS1RK/AA. Translational mRNA containing EMCV IRES and poly(A) appeared to be most robustly enhanced by NS1RK/AA (11.2-fold increase). The later also resulted in the highest luminescence level compared to other mRNA templates. These data suggest that supplementing RRL with NS1RK/AA in combination with the mRNA of interest containing a 5′-EMCV IRES and 3′-poly(A) can represent a beneficial strategy to improve the protein yield of the RRL translation system. Thus, we have developed an easy and efficient technique to enhance protein synthesis and obtain significantly higher yields of the protein of interest in RRL by supplementing the translation system with viral factors. In particular, we used purified IAV NS1 protein and incorporated an EMCV IRES structure in the target mRNA. Adding both factors significantly enhanced protein synthesis in RRL in comparison with a single enhancer (9,12). Our technique is cost effective, as NS1 can be efficiently produced and easily purified from E. coli and retains its activity for months. mRNA containing a 5′ IRES can be readily synthesized using T7 in vitro transcription systems. It should be noted that the multifunctional IAV NS1 protein and the EMCV IRES could be involved in a number of interactions that might potentially interfere with the synthesis of a protein of interest in RRL (12,13). This method can be improved in the future by screening for even more efficient combinations of translation enhancers, such as small molecules, other viral factors, or modified versions of published enhancers (9,14).

**Author contributions**
MA, IT, and DK planned and performed experiments. MA, SB, and DK wrote the manuscript.

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Competing interests
The authors declare no competing interests.

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