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A Poly(A) Tail-Responsive In Vitro System for Cap- or IRES-Driven Translation From HeLa Cells

Christian Thoma, Antje Ostareck-Lederer, and Matthias W. Hentze

Summary

In cells, the poly(A) tail stimulates translation from messenger RNAs bearing a cap structure or viral IRES elements. This 3' end-mediated stimulation of translation is not reflected in commonly used commercial cell-free translation systems prepared from rabbit reticulocytes or wheat germ. We describe a simple procedure to generate poly(A) tail-responsive translation extracts from HeLa cells. We suggest that this procedure should be adaptable to many animal cell lines.

Key Words

Poly(A) tail; cap structure; internal ribosome entry site (IRES); translation; RNA stability.

1. Introduction

Translation initiation of cellular messenger RNAs (mRNAs) bearing a 5' ^{7}mGpppG cap structure is greatly stimulated by the 3' poly(A) tail ***(1–3)***. In eukaryotic cells, the cap structure and the poly(A) tail synergize to drive translation initiation ***(1***,***2***,***4)***. This synergism involves the cap-binding protein eIF4E, besides eIF4G a component of the eIF4F translation initiation complex, and the poly(A)-binding protein Pabp1/PABP. A simultaneous interaction of eIF4G with eIF4E and Pabp1/PABP was first described in yeast ***(5)*** and has subsequently been reported for plant and mammalian cells ***(6***,***7)***. As an alternative to cap-driven translation initiation, protein synthesis can also initiate in a 5' end-independent way, mediated by internal ribosome entry sequences (IRES; **refs. *8–12***). IRESes are commonly located within long 5'-untranslated regions and

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can occur in (picorna-) viral as well as cellular mRNAs. It has been reported that the poly(A) tail can also stimulate translation driven by viral IRESes ***(13–15)***, although the exact mechanism underlying this effect of the poly(A) tail is not yet fully understood.

In vitro translation systems have been used for decades to enable cell-free protein synthesis and to investigate the mechanisms of translation. Initial in vitro studies of translation were performed in extracts derived from rabbit reticulocytes and wheat germ ***(16,17)***. However, the contribution of the poly(A) tail to cap-dependent translation could not be recapitulated in these extracts. The first in vitro translation system that could reflect the synergism between the cap structure and the poly(A) tail was derived from *Saccharomyces cerevisiae* ***(18)***. To date, several in vitro translation systems that recapitulate this synergy have been reported, including extracts derived from *Drosophila melanogaster* embryos ***(19)***, HeLa cells ***(14)***, rabbit reticulocytes ***(13)***, and Krebs II ascites cells ***(15)***. In this chapter, we describe a cell-free translation system based on HeLa cell extracts, which is simple to prepare and which displays a strong poly(A) tail contribution to the cap-dependent and IRES-mediated initiation of translation ***(14)***.

2. Materials

2.1. HeLa Extracts

1. HeLa cells S3 (ATCC, Rockville, MD).
2. Joklik Media (Biochrom, Berlin, Germany).
3. Fetal bovine serum, penicillin/streptomycin, glutamine (Gibco, Karlsruhe, Germany).
4. Lysis buffer: 10 m*M* HEPES, pH 7.6 (Biomol, Hamburg, Germany); 10 m*M* potassium acetate (Merck, Darmstadt, Germany); 0.5 m*M* magnesium acetate (Merck, Darmstadt, Germany); 5 m*M* dithiothreitol (Biomol, Hamburg, Germany), two tablets Protease inhibitor (ethylenediamine tetraacetic acid free, Roche, Mannheim, Germany; *see* **Note 1**).
5. Phosphate-buffered saline: 140 m*M* NaCl; 2.7 m*M* KCL; 10 m*M* Na_2HPO_4; 1.8 m*M* KH_2PO_4, pH 7.3.
6. Dounce homogenizer (Landgraf, Langenhagen, Germany).

2.2. Transcription

1. ^{7}mGpppG (Kedar; Warsaw, Poland).
2. ApppG (NEB; Frankfurt, Germany).
3. RNAsin, 5X transcription buffer, T3/T7 RNA polymerase (Stratagene, La Jolla, CA).
4. Dithiothreitol (Biomol, Hamburg, Germany).
5. Cytidine triphosphate (CTP), adenosine triphosphate (ATP), uridine triphosphate (UTP), guanosine triphosphate (GTP), [^{32}P]UTP (PB10163, 400 Ci/mmol, 10 mCi/mL, Amersham, Freiburg, Germany).

6. Linearized plasmid.
7. RQ DNAse (Promega, Mannheim, Germany).
8. Phenol, phenol:chloroform:isoamyl alcohol (25:24:1) (Amresco, Solon, OH).
9. Chromaspin 100 columns (Clontech, Heidelberg, Germany).
10. Sodium acetate, ethanol (Merck, Darmstadt, Germany).
11. Tris-borate/ethylenediamine tetraacetic acid electrophoresis buffer.
12. Agarose.

2.3. Translation

1. HeLa extracts.
2. 2 m*M* Amino acids (Sigma, Taufkirchen, Germany).
3. 5 m*M* Spermidine (Sigma, Taufkirchen, Germany).
4. 100 m*M* solution ATP, 100 m*M* solution GTP (Amersham, Freiburg, Germany).
5. HEPES (Biomol, Hamburg, Germany).
6. RNAse Inhibitor (Eppendorf, Hamburg, Germany).
7. Creatine phosphate, creatine kinase, micrococcal nuclease (Roche, Mannheim, Germany).
8. Magnesium acetate, potassium acetate (Merck, Darmstadt, Germany).
9. H_2O.
10. Luciferase Assay System (Promega, Mannheim, Germany).

3. Methods

The methods described below outline the extract preparation ***(14)***, the in vitro transcription ***(20)***, and the in vitro translation (***14***; Thoma, C., Bergamini, G., Galy, B., Hundsdörfer, P., and Hentze, M. W., manuscript in preparation) procedures.

3.1. Preparation of HeLa Cell Extract

For large-scale production of translation extracts, HeLa cells (S3) growing in suspension are used. The total procedure requires approx 6 d. The starting culture is transferred into complete Joklik's medium and grown in suspension at 37°C on a magnetic stirrer. The culture is expanded to maintain a cell density between 2.5 and 5×10^5 cells/mL. Eight liters of suspension culture are collected by centrifugation at 700*g* for 15 min at 4°C and washed three times with ice-cold phosphate-buffered saline before disruption. The cells are disrupted in a hypotonic HEPES-based lysis buffer. The cell pellet is resuspended in an equal volume of ice cold lysis buffer. After 5-min incubation on ice, the lysate is transferred into a Dounce homogenizer (working capacity: 15 mL) and homogenized by applying 18 strokes. The lysed extracts are centrifuged at 13,000*g* for 5 min at 4°C. The supernatants are pooled, snap frozen in liquid nitrogen and stored at –80°C. These extracts usually have a protein concentra-

tion of 15–20 mg/mL. In our experience, frozen extracts remain active for several months, and can be freeze-thawed up to two times without profound loss of activity.

3.2. In Vitro Transcription Protocol

^{7}mGpppG-capped mRNAs for in vitro translation are transcribed from *BamH*I-linearized LUC template plasmids. A-capped mRNAs are transcribed from Not-1 linearized-EMCV-LUC or -Ecl136II-linearized BiP-LUC templates in 25-µL reactions (*see* **Note 2**). The RNA is purified by phenol and chloroform:isoamylalcohol (24:1) extractions after DNAse treatment with 1 U/µg plasmid DNA and RQ DNAse for 15 min at 37°C. To remove the nonincorporated ^{7}mGpppG or ApppG, respectively, the transcripts are centrifuged twice through two Chromaspin-100 columns (Clontech), followed by ethanol precipitation. The pellet is washed in 70% ethanol and resuspended in water. mRNA concentration and integrity are assessed by trace labeling and agarose gel electrophoresis.

3.3. In Vitro Translation Protocols

3.3.1. Cap-Dependent Translation

The poly(A) tail contribution to cap-dependent translation is an important feature of translation in vivo and can be recapitulated in the HeLa cell-derived translation system. **Figure 1** illustrates the known interaction between the poly (A) tail and the cap structure.

In the in vitro translation assay, the mRNA is added to the HeLa cell extract mix and incubated for 30 min at 37°C. It is optional to remove endogenous mRNAs by previous treatment of the extracts with micrococcal-nuclease

(Thoma, C., Bergamini, G., Galy, B., Hundsdörfer, P., and Hentze, M. W., manuscript in preparation). If desired, a micrococcal–nuclease treatment of the extracts prior to the translation reaction is performed by incubation of 4 µL of HeLa extracts with 0.04 U of micrococcal-nuclease and 1 m*M* calcium acetate per reaction for 6 min at 26°C. The reaction is stopped by adding ethylenebis(oxyethylenenitrilo) tetraacetic acid (EGTA) to a final concentration of 2 m*M*. **Table 1** shows the detailed protocol of the in vitro translation assay of cap-dependent mRNAs. These values are optimal for a 10-µL translation reaction. The reaction mix is incubated at 37°C for 30 min and stopped by snap-freezing in liquid nitrogen. In **Fig. 2**, the contribution of the poly(A) tail to cap-driven translation is shown. Cap-driven translation is stimulated approximately fivefold by the poly(A) tail in micrococcal-nuclease treated extracts. We commonly observe some batch to batch variation ranging from a four- to sixfold effect of the poly(A) tail. Luciferase expression was determined using the luciferase assay system (Promega).

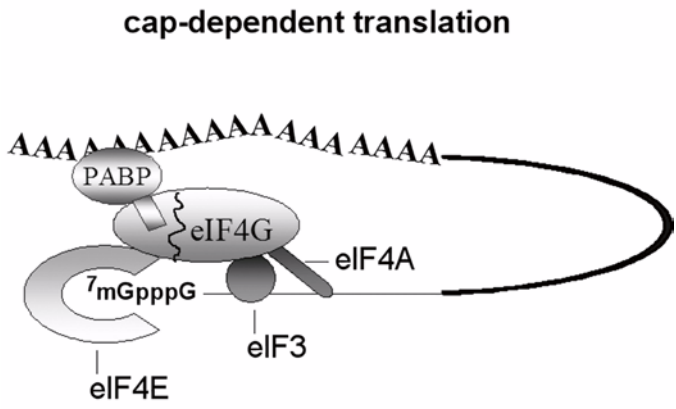

Fig. 1. Scheme representing the poly(A) tail contribution to cap-dependent initiation of translation.

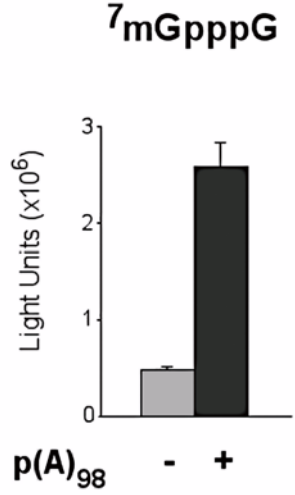

Fig. 2. Stimulative effect of the poly(A) tail on cap-driven translation in micrococcal nuclease-treated HeLa cell extracts. Translation assay of Luc mRNAs modified with either a ^{7}mGpppG cap alone or a ^{7}mGpppG cap and a 98-residue poly(A) tail. The amount of luciferase expression was determined using the luciferase assay system (Promega). For additional information, *see also* **ref. *14*** and Thoma, C., Bergamini, G., Galy, B., Hundsdörfer, P., and Hentze, M. W., manuscript in preparation.

Table 1
Components of Translation Reactions

Reagent	Volume (μL per reaction)
Cell extract	4
2 m*M* Amino acids	0.3
100 m*M* ATP	0.08
10 m*M* GTP	0.1
1 *M* HEPES, pH 7.6	0.16
Prime RNAse inhibitor	0.2
1 *M* Creatine phosphate	0.2
10 mg/mL Creatine phosphokinase	0.04
5 m*M* Spermidine	0.1
125 m*M* Magnesium acetate	0.2
2 *M* Potassium acetate	0.2
10 ng mRNA template	1.0
H_20 added to 10 μL	

3.3.2. Viral IRES-Mediated Translation

IRES-driven initiation of translation is best understood for the EMCV-IRES (**Fig. 3**). The HeLa cell-derived in vitro system is a useful tool to investigate the effect of the poly(A) tail on IRES-mediated translation. Using the HeLa cell system, a stimulatory effect of the poly(A) tail on the EMCV IRES-mediated translation can be observed (**Fig. 4**; ***14***). The poly(A) tail contribution enhances translation approx threefold in untreated extracts. For the EMCV-IRES, the optimal concentration of magnesium and potassium is slightly different from cap-dependent translation: 4 m*M* magnesium acetate and 80 m*M* potassium acetate (final concentration). EMCV-IRES (25 ng) bearing mRNA is used. For the other components, *see* **Table 1**.

3.3.3. Cellular IRES-Mediated Translation

The HeLa cell-derived translation system also recapitulates the function of the IRES of the BiP mRNA. **Figure 5** demonstrates the stimulatory effect of the poly(A) tail on BiP-IRES mediated translation. Translation is approx sixfold stimulated by the poly(A) tail for the BiP IRES in micrococcal-nuclease treated extracts. Magnesium and potassium acetate are used as described for the EMCV-IRES. The optimal amount of BiP mRNA is 50 ng in a 10-μL translation reaction. For the other components of the translation mix, *see* **Table 1**.

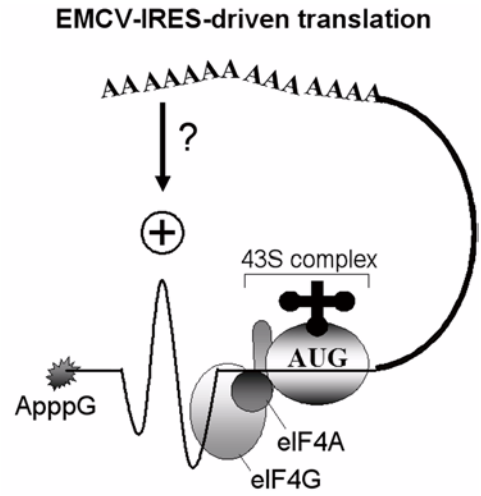

Fig. 3. Schematic representation of the poly(A) tail contribution to EMCV IRES-mediated initiation of translation.

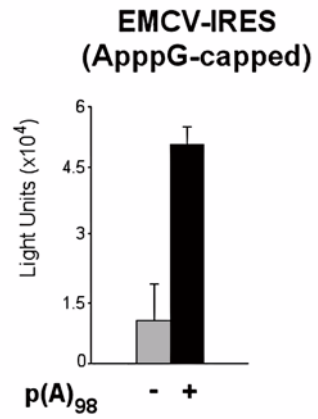

Fig. 4. Translation driven by the EMCV IRES is stimulated by the poly(A) tail in HeLa cell extracts. Translation assay of ApppG-capped, monocistronic EMCV IRES containing Luc mRNAs either with or without 98-residue poly(A) tail. The amount of luciferase expression was determined using the luciferase assay system (Promega). *See also* **ref. *14***.

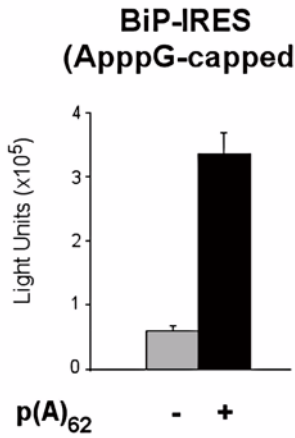

Fig. 5. Stimulative effect of the poly(A) tail on cellular IRES-driven translation in HeLa cell extracts. Quantitative analysis of luciferase expression from an ApppG-capped, monocistronic BiP IRES containing Luc mRNA either with or without a 62-residue poly(A) tail. The amount of luciferase expression was determined using the luciferase assay system (Promega). For additional information, *see also* Thoma, C., Bergamini, G., Galy, B., Hundsdörfer, P., and Hentze, M. W., manuscript in preparation.

The cell-free translation system described here appears to reflect important features of mRNA translation in vivo. In particular, it displays stimulation of cap-and IRES-driven translation by the poly(A) tail. We suggest that the procedure described here can be adapted to other cell lines growing in suspension, and modified to allow the preparation of extracts from cells that grow adhesively.

4. Notes

1. Filter and store on ice.
2. The transcription reactions contain 1 m*M* ATP, CTP, UTP; 10 m*M* DTT; 1 U Inhibit Ace (5Prime-3Prime, PA); 7 m*M* ^{7}mGpppG (Kedar, Warsaw, Poland) or 7 m*M* ApppG (NEB), 1 µL 1:50 α-[^{32}P]UTP (Amersham, PB10163, 10 mCi/mL) for trace-labeling, 1X transcription buffer with 60 U of T3 RNA-Polymerase (Stratagene, La Jolla, CA). After 5 min preincubation at 37°C, GTP is added to a final concentration of 1 m*M* for additional 60 min. For the transcription of "uncapped" mRNAs (e.g., for IRES-driven translation), we strongly recommend to replace the ^{7}mGpppG analog by ApppG, rather than simply omitting ^{7}mGpppG. "A-capped" transcripts are stable but cannot bind eIF4E.

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