Generation of a Translational Extract from Eukaryotic Cells that have Grown as Monolayers

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Summary

The present article relates to the rapid and efficient generation of an in vitro translational extract that is obtained from the cytoplasmic fraction of eukaryotic cells that have grown as monolayers. The procedure is totally devoid of the use of animals or animal fluids. The cytoplasmic extract that is obtained allows efficient protein synthesis of exogenously added mRNAs. The latter mRNAs can be purified from prokaryotic or eukaryotic cells, or can be transcribed in vitro by employing any convenient RNA polymerase (for example, the bacteriophage SP6, T3 or T7 RNA polymerase). The described cytoplasmic preparation appears to be applicable to a large number of different eukaryotic cell lines. The cytoplasmic extract can be prepared freshly on a daily basis, or can be frozen and subsequently thawed for further use, without loss of activity. The preparation of the translation extract does not require living animals.

Zusammenfassung: Herstellung eines Translations-Extraktes aus als Monolayer gewachsenen Eukaryonten-Zellen

Der vorliegende Artikel schildert die schnelle und effiziente Herstellung eines in vitro Translations-Extraktes aus der zytoplasmatischen Fraktion von als Monolayer gewachsenen Eukaryonten-Zellen. Das Verfahren benötigt weder Tiere noch tierische Prodikte. Das gewonnene Zytoplasmaextrakt erlaubt eine effiziente Proteinsynthese mit exogen zugegebenen mRNAs. Diese RNAs können aus prokarytotischen oder eukaryotischen Zellen gewonnen werden oder sie können in vitro unter Verwendung einer herkömmlichen RNA-Polymerase transkribiert werden (z.B. mit Bakteriophagen SP6, T3 oder T7 RNA-Polymerase). Die beschriebene Zytoplasma-Präparation kann offenbar bei einer großen Zahl verschiedener eukariotischer Zellinien verwendet werden. Der Zytoplasma-Extrakt kann täglich frisch präpariert, aber auch ohne Aktivitätsverlust gefroren und für den weiteren Gebrauch aufgetaut werden. Zur Präparation des Translations-Extraktes müssen keine Tiere verwendet oder getötet werden.

Keywords: translational extract, protein synthesis, eukaryotic cell lines, animal free

1 Introduction

Several cell-free protein-synthesizing systems have been used in recent years for the translation of prokaryotic as well as eukaryotic messenger ribonucleic acids (mRNAs). Of these, the rabbit reticulocyte lysate, the Krebs ascites fluid and the wheat germ extract have received the most attention. The rabbit reticulocyte lysate is obtained from the red blood cells of encaged, anemyzed living rabbits that have been previously treated with phenylhydrazine (Pelham and Jackson, 1976). Moreover, mice, as an other „living material", provide the so-called Krebs ascites fluid. This extract is prepared from laboratory induced tumors after sacrifice of the animals. These two procedures are unethical in the sense that the animal, rabbit or mouse, is solely considered a factory for the generation of experimental fluids. Finally, the use of the wheat germ extract does not successfully translate highly-complexed messenger RNAs: this translation system is unfortunately thus limited in it's applications. In addition to these widely used systems, extracts from a variety of eukaryotic cell types have been generated for the translation of mRNAs or in order to study aspects of the regulation of protein synthesis. Most of the latter systems are 10000-30000 g supernatants which have been preincubated or nuclease-treated (Pelham and Jackson, 1976) to eliminate endogenous mRNAs and then dialysed or treated with Sephadex™) G-25 to standardize ionic conditions. The cells from which the latter supernatants are prepared are usually either grown as large-scale suspension cultures (usually ranging between 1 to 5 liters) or as ascites tumours in mice. The latter yields larger amounts of material more easily but cannot be readily manipulated to examine translational responses to changes in cellular physiology.

To date, the generation of an efficient protein-synthesizing system obtained from eukaryotic cells that have grown as monolayers has been challenging. There is one report in the literature claiming evidence for the efficient translation of exogenously added mRNAs in such a system (Mohammadi *et al.* 1996). However, in the latter translational system derived from hepatoma cells, solely homologous liver specific mRNAs or the viral brome mozaic virus (BMV) RNA were translated; the latter BMV RNA contains a poorly structured 5'untranslated leader of ΔG of -13.1 (Pogue and Hall, 1992) which is efficiently translated *in vitro*. For this reason, it would desirable to develop an efficient translation system that has been generated from eukaryotic cells that have

grown as monolayers, and that is efficient in translating exogenously added mRNAs. To this end, a novel experimental procedure has been developed.

Some of the major advantages of such a procedure are the following:

- The preparation of the translation extract does not require living animals.
- The cells can be grown as monolayers by employing small volumes of cell culture medium before the generation of the translational extract.
- The cells can be: preincubated with hormones, toxins, ions, etc pretreated with chemical or other external stimuli before the generation of the translational extract.
- The extract can be prepared by employing inexpensive compounds that are available from commercial sources.
- The cytoplasmic extract that is generated translates endogenous mRNAs with very high efficiencies, as seen with the incorporation of [35S] methionine (or any other radiolabeled amino acid(s)) into newly synthesized polypeptides. Moreover, after hydrolysis of the endogenous mRNAs with micrococcal nuclease, an important synthetic activity on added exogenous mRNAs can be obtained with this *in vitro* system.
- The proteins synthesized *in vitro* in the cell-free extract that is not treated with micrococcal nuclease are an accurate reflection both qualitatively and quantitatively of the proteins synthesized by the whole cell prior to extract preparation.
- The extract can be frozen and subsequently thawed for further use, without loss of translational activity.

In summary, the procedure described below is rapid and efficient. Translation pro-

Example: Translation of HIV-1 TAR-containing mRNA

Background

Translation of mRNAs of human immunodeficiency virus type 1 (HIV-1) has been shown to be mediated by cis-acting sequences responsive to the gene product, the trans-acting responsive (TAR) region, which is located immediately adjacent to the site of transcription initiation (Rosen et al., 1985). The TAR sequence is therefore located at the 5' end of all viral mRNAs. It has been proposed that the TAR sequence and flanking 3' region played a role in the regulation of translation of HIV-1 mRNAs by inhibiting this translation (Parkin et al., 1988). However, in the latter study, the authors, by translating HIV-1 TAR-containing mRNAs in a rabbit reticulocyte lysate or in a cytoplasmic extract of eukaryotic HeLa cells that have grown in suspension cultures, or by microinjecting HIV-1 TAR-containing mRNAs in Xenopus oocytes, predict that the block to translation of viral mRNAs by their 5' untranslated region (UTR) must somehow be overcome to allow for efficient viral structural protein synthesis and viral replication during viral infection.

Moreover, it has been shown that the tat-responsive region RNA of HIV-1 can prevent activation of the double-stranded-RNA-activated protein kinase PKR (Gunnery et al., 1990). In this study, the authors concluded that the TAR RNA may serve to neutralize a cellular defense mechanism during HIV infection, namely the interferon response. It is known that HIV-1 replication is sensitive to interferon, and that in addition to immunological abnormalities, AIDS is accompanied by a suppression of the interferon-mediated defense system (Pomerantz et al. 1987). Thus, the inhibition of PKR activation by TAR RNA is to be seen as an aspect of disarming the host defenses during HIV infection.

Hypothesis

The results published thus far might not fully represent the fate of HIV TAR-containing mRNA in *in vitro* translation. HIV TAR-containing mRNAs might be indeed efficiently translated *in vitro*. Moreover, even though PKR is not activated in presence of the TAR RNA sequence, it might be possible to somehow inhibit the TAR-mediated translation *in vitro* in a PKR-independent fashion.

Material and Methods

A cytoplasmic extract from monkey Cos-1 cells has been obtained by following the procedure described above for generating a translational extract from cells that have grown as monolayers. The translational extract has been thawed and has not been treated with micrococcal nuclease.

Messenger RNA consisting of a molecule containing the HIV-1 TAR structure upstream of the sequence coding for the polypeptide chloramphenicol acetyl transferase (CAT) has been transcribed *in vitro* from linearized plasmid pSP64/TAR(+111)CAT by employing commercial (Pharmacia™) SP6 RNA polymerase (Dideoxy sequencing using a SP6 oligonucleotide confirmed that the plasmid employed was the correct one). Translation reactions in a final volume of 20µl were performed in absence or presence of 0.1µg of capped TAR(+111) CAT mRNA (ie: 5 µg per ml of translation reaction), and with [35S] methionine as a source of radiolabeled amino acid. Incubations were for 60 min at 30°C. 10 µl from each translation reaction were analyzed by SDS-PAGE followed by fluorography of the gel using En3Hance (Dupont™).

Results

Interestingly, the capped TAR(+111)CAT RNA is very efficiently translated in this system (Fig.: eukaryotic cell extract, lane 2, „CAT"), when compared to the control reaction that was performed without exogenously added RNA (lane 1). This result taken into consideration, it has been determined, whether the HIV-1 TAR-mediated translation might be somehow inhibited. One interesting and putative inhibitor might be „compound X" (Daniel Favre: personal hypothesis). „Compound X" is a heat-stable molecule that is neither a peptide nor a nucleic acid.

When „compound X" is added to the reaction, the translation of the capped TAR(+111)CAT RNA is drastically inhibited (lane 3: 10000 arbitrary units of

ducts can be readily analyzed within two hours considering the following steps:

- the preincubation of the cells, 20 min,
- the preparation of the translation extract, in less than 10 min,
- the mixing of the various components to be translated, about 5 min,
- the translation reaction itself, 60 min.

2 Materials and Methods

Eukaryotic cells are grown as monolayers in Petri™ dishes in their respective optimal cell culture medium. For example, baby hamster kidney (BHK) cells or monkey Cos-1 cells are grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat-inactivated (30 min, 56°C) fetal calf serum. Prior to preparation of the translational extract, cell monolayers are incubated in deprived DMEM for about 20 min. All subsequent steps are performed at 4°C. The cells are permeabilized by using a convenient detergent and then directly resuspended in reaction mix©. The cells are then disrupted within 10 sec. by using mechanical means. Following disruption, the nuclei are removed by pelleting them for 30 sec. at 100 g in an Eppendorf™ benchtop centrifuge. The cell-free supernatant fluid that is obtained represents the translational extract. A petri dish of 10 cm-in-diameter provides 0.2 ml of translational extract obtained from about 10^7 cells. The latter extract can be held on ice until used, or directly frozen at -70°C until further use. The cell-free extract can be rendered mRNA-dependent by treatment with micrococcal nuclease by following the method described elsewhere (Pelham and Jackson, 1976).

„compound X" per µl of translation reaction; lane 4: 1000 arbitrary units per µl; lane 5: 100 arbitrary units per µl; lane 6: 10 arbitrary units per µl; in the latter case, translation of capped TAR(+111) CAT RNA is inhibited by more than 90% when compared to the translation performed in absence of „compound X", as shown in lane 2). Translation of capped TAR(+111)CAT RNA recovers when „compound X" is present at 1 arbitrary unit per µl (lane 7). Interestingly, „compound X" at high concentrations might also have an effect on cap-dependent translation, as seen with the inhibition of translation of the endogenous mRNAs.

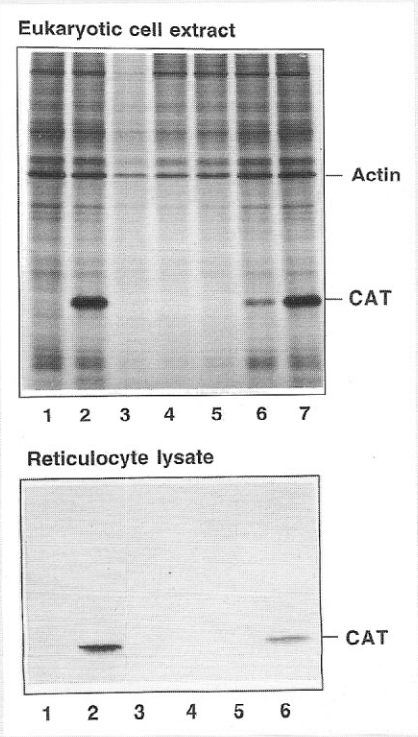

Figure
Translation of HIV-1 TAR-containing mRNA *in vitro*. Translation in eukaryotic cell extract (upper panel) and in rabbit reticulocyte lysate (lower panel). Polypeptides were labeled with [35S] methionine for 1 h and analysed by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); the gel was then treated for fluorography using EN3Hance (Dupont™). CAT: chloramphenicol acetyl transferase. Lanes 1: without exogenous mRNA; lanes 2 to 7: in presence of exogenous HIV-1 TAR-containing mRNA coding for the CAT polypeptide, and with decreasing concentrations of „compound X".

Furthermore, it has been determined, that „compound X" could act on inhibiting HIV TAR-mediated translation even when the activation of PKR was inhibited. To test this hypothesis, a reaction has been performed in presence of the HIV TAR-containing mRNA and in presence of high concentrations of double-stranded RNA (poly(rI)-poly(rC); 20 µg per ml). The result showed that the HIV TAR-mediated translation was also totally abolished (not shown). Thus, the inhibition of HIV TAR-mediated translation by „compound X" does not require the activation of PKR. A similar translation reaction was performed by employing the commercial rabbit reticulocyte lysate (Promega Biotech™) that has been previously treated with micrococcal nuclease (Fig.1: reticulocyte lysate; bottom panel). Conditions for translation and analysis of the translated products were the same as for translations employing the eukaryotic cell extract. The result shows that translation on HIV-TAR-containing mRNA is less efficient in rabbit reticulocyte lysate than in eukaryotic cell extract. Lane numbers refer to the same numbers shown in the Figure, upper panel. HIV-1 TAR-containing mRNAs are poorly translated in this system. However, „compound X" is also active in inhibiting HIV-1 TAR-containing mRNA translation.

Conclusion

The HIV TAR structure on mRNAs is not inhibiting the translation of a reporter gene in the above described translational extract that is obtained from cells that have grown as monolayers.

It is not possible to obtain the above presented results by employing the rabbit reticulocyte lysate or any other *in vitro* translation system yet employed.

In vitro translation are carried out by addition of 15µl of the extract to tubes in which 35S] methionine (translation grade; >1200 Ci/mmol) and exogenous mRNA have been added. The final volume of the translation reaction is 20µl. Translation reaction is carried out at 30 °C for various times (usually 60 min). The reaction is stopped through the addition of 2X sodium dodecylsulfate (SDS)-sample buffer followed by boiling for 3 min; analysis of the polypeptides by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) is performed as described elsewhere (Laemmli, 1970).

3 Results

It has been determined that the extract could translate exogenous mRNA in presence or in absence of endogenous mRNA. Translation reactions were programmed with various exogenously added mRNAs such as:

- capped, polycistronic CAT (chloramphenicol acetyl transferase)-EMC (encephalo-myocarditis)-LUC (luciferase) mRNA, which is containing both a 5' cap (m7GpppG) and an internal ribosomal entry site (Pause et al. 1994)
- non-capped LUC mRNA from commercial source (Promega™)
- encephalomyocarditis (EMC) viral mRNA
- poliovirus mRNA
- human immunodeficiency virus (HIV) trans-acting responsive (TAR)-containing RNA,
- with or without pretreatment of the extract with micrococcal nuclease. The results have shown that all of these exogenously added mRNAs are efficiently translated. We will particularly focus our attention on the following example:

4 Discussion and perspectives

The article at hand, dealing with the generation of a cytoplasmic extract from eukaryotic cells that is allowing efficient *in vitro* protein synthesis, has an important potential for the following reasons:

- It can be employed in place of the commercial present available rabbit reticulocyte or wheat germ systems.
- It is ethical, since it does not require living animals for its generation. Various cell lines growing as monolayers can be employed.
- It is very efficient in allowing protein synthesis and it generates reproducible results.
- It can be prepared without following tedious and material-consuming protocols.
- It is easy to employ.
- It allows freeze/thawing without loss of activity.
- It is new in the sense that no other similar procedure is yet available for efficient translation of exogenous mRNAs obtained from various sources.
- It can be scaled up to prepare a commercial translation kit,
- it does not require the use of viral expression systems, such the vaccinia virus vector

Finally, several important improvements could further increase the potential of the method presented, namely:

- The generation of a one-tube, coupled transcription/translation system. This could be achieved by enabling the synthesis of proteins directly from protein-encoding deoxyribonucleic acid (DNA) sequences downstream of convenient RNA polymerase promoters.
- The low-to-middle scale production of valuable, biologically active polypeptides. This could be achieved by allowing prolonged incubation times of the translation reaction, followed by convenient purification procedures of the desired translated polypeptide.

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Dr. Daniel Favre is presently seeking a research position in either industry or at university

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