

Translational extracts active biologically in vitro obtained from eukaryotic monolayer cells: a versatile method for viral RNA studies

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Abstract

Preservation of enzymatic activities in biological samples, especially after freeze/thawing, is a crucial requirement in virological research. Theoretically, this preservation can be achieved with the presence of cryopreservative agents. In contrast to tedious methods, it was found that this might be readily achieved by using well-defined conditions, including sucrose in the samples. Hence, the generation of a translational extract obtained from eukaryotic cells that have grown as monolayers is described below. This versatile method could be used advantageously for the in vitro translation of messenger RNAs, added exogenously, including viral mRNAs. The translational extract can be prepared freshly on a daily basis, or more conveniently it can be frozen and thawed subsequently for further use, without loss of activity. It can replace the Krebs ascites fluid and the commercial rabbit reticulocyte lysate. The procedure employed for the preservation of the biological activity of the translational extract can be extended to various other biological samples. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Several protein-synthesizing systems have been used in recent years for the translation of viral, prokaryotic, as well as eukaryotic messenger ribonucleic acids (mRNAs) (Ochoa and de Haro,

1979). Of these, the rabbit reticulocyte lysate, the Krebs ascites fluid, and the wheat germ extract have received the most attention and are widely used, especially the former (Pelham and Jackson, 1976; Clemens, 1979). However, these systems are not representative of eukaryotic cells in the way they regulate translation. They cannot or do not respond to various physiological (e.g. hormones, toxins, ions), chemical, and other external (heat shock, magnetic fields) stimuli which are important regulators of cellular functions in nucleated

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cells, and they cannot be used for studies on viral infection in the context of relevant cellular interactions. In addition to these systems used widely, cell-free extracts originating from a variety of eukaryotic cell types have been generated for the translation of mRNAs or to study regulation aspects of protein synthesis. Unfortunately, most of the latter systems involve tedious processes including preparations of 10 000–30 000 g supernatants which have been preincubated or micrococcal nuclease-treated (Pelham and Jackson, 1976) to eliminate endogenous mRNAs and then dialysed or treated with Sephadex™ G-25 to standardize ionic conditions. Other translational extracts were prepared from large-scale suspension cultures (usually ranging between 1 and 5 l) (Skup and Millward, 1977), or as ascites tumors in mice. The latter yields larger amounts of material more easily, but cannot be readily manipulated to examine relevant translational responses to changes in cell metabolism.

The generation of an efficient protein-synthesizing system obtained from eukaryotic cells that have grown as monolayers has been hampered by the lack of translational ability of exogenously added mRNAs, and by the loss of biological activities after freeze/thawing. Here, it is shown that biologically active creatine kinase does play a crucial role in allowing translation of exogenous mRNAs in the translational extract. Using well-defined conditions this activity can be kept in extracts that can be frozen and thawed subsequently due to the presence of sucrose as a cryopreservative.

2. Materials and methods

Eukaryotic cells were grown as monolayers in Petri dishes in their respective optimal cell culture medium. For example, baby hamster kidney (BHK) cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with antibiotics and 10% heat-inactivated fetal calf serum (FCS). In vitro transcription on linearized plasmids was carried out essentially as described (Svitkin et al., 1994). Total cellular RNA can be prepared as described elsewhere (Chirgwin et al.,

1979). Translational extract was prepared essentially as described previously (Brown et al., 1983). Briefly, the cytoplasmic membranes of the cells were lysed by lysolecithin. Following the complete removal of lysolecithin from the Petri dish, the cells were scraped into extraction buffer containing 100 mM Hepes-KOH (pH 7.4), 120 mM potassium acetate (pH 7.4), 2.5 mM magnesium acetate, 1 mM dithiothreitol, 2.5 mM ATP, 1 mM GTP, 100 μ M S-adenosyl-methionine, 1 mM spermidine, 20 mM creatine phosphate, 40 U of creatine phosphokinase (Sigma) per ml, 40 μ M of each essential amino acid except methionine, or except methionine plus cysteine (Promega), and, importantly, additional 100 mM sucrose. The cells were then passed ten times through a 25-gauge needle, and the lysate was centrifuged at 4°C and 100 g for 2 min. The supernatant was collected to prepare the mRNA-dependent lysate; for this, the endogenous mRNAs were hydrolyzed by incubating the lysate at 20°C for 7–10 min in the presence of 10 U of micrococcal nuclease (Pelham and Jackson, 1976) (P-L Biotechnology) per ml and 1 mM CaCl₂. The enzyme was inhibited by adding 2.5 mM ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (pH 7) (EGTA). The volume of micrococcal nuclease plus CaCl₂ plus EGTA that was added represented 4% of the final volume of the translational extract. The presence of 120 mM potassium acetate in the extraction buffer was preferred to 90 mM KCl as described originally, because Cl⁻ might inhibit the initiation of translation in certain circumstances (Weber et al., 1975). A Petri dish of 10 cm-in-diameter provides 0.2 ml of translational extract obtained from about 10⁷ cells. The latter extract could be used immediately for translation of exogenously added mRNAs, or it could be frozen at -20 or even -70°C until further use. Additional 100 mM sucrose was present in the extraction buffer in order to preserve the biological activity of the creatine kinase and to allow the translation of the mRNAs added exogenously after freeze/thawing of the extract. In vitro translation in 20 μ l was carried out by mixing 15 μ l of the micrococcal nuclease-treated extract in which (³⁵S) methionine or (³⁵S) methionine/cysteine (translation grade; > 1000 Ci/mmol) and exogenous mRNA (final

concentration, 5–10 µg per ml) have been added. Translation reactions were carried out at 30°C for 60 min. The reactions were stopped with the addition of 2X sodium dodecyl sulfate (SDS)-sample buffer followed by boiling for 3 min; the labeled polypeptides were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) followed by fluorography (En₃Hance, Dupont).

3. Results

Initial experiments were carried out by following an established protocol employed for *in vitro* protein synthesis (Brown et al., 1983). When sucrose was omitted during the generation of the translational extract, the efficient *de novo* translation of exogenous mRNAs could be achieved solely by adding biologically active creatine kinase, obtained from a commercially available glycerol stock, to the thawed translational extract. Moreover, translation of the exogenously added mRNAs in the micrococcal nuclease-treated extracts was preserved after freeze/thawing of the translational extracts in which sucrose was present during the preparation (Table 1).

Translation reactions were thus carried out with frozen and subsequently thawed extracts which have been generated in the presence of additional

sucrose, employed as a cryopreservative, during the preparation of the translational extract. The translation reactions were carried out with micrococcal nuclease-treated extracts which were programmed with various exogenously added mRNAs, such as, capped, polycistronic chloramphenicol acetyl transferase-(CAT), encephalomyocarditis-(EMC), luciferase-(LUC), mRNA (Pause et al., 1994), and capped, polycistronic CAT-[E1-ΔE2] hepatitis C mRNA (Kamoshita et al., 1997). This revealed that both cap-dependent (as shown by the presence of the CAT protein) and cap-independent *de novo* translation could be achieved on these RNAs (Fig. 1 lanes 2 and 3). Internal initiation of translation occurred efficiently on the encephalomyocarditis virus internal ribosome entry site (IRES) (lane 2). Internal initiation of translation was also successfully performed on the HCV IRES, and the resulting viral polyprotein was processed efficiently, even though no exogenous microsomal membranes have been added in the translational reaction (lane 3). Furthermore, poliovirus genomic mRNA, brome mosaic virus mRNA, and RNA from commercial source such as uncapped luciferase mRNA (Promega), for example, were also successfully translated (not shown). Similar results were obtained when human HeLa, HepG2, mouse L, monkey Cos and Vero cells were used for extract preparation (not shown).

Table 1

Translational ability of exogenously added mRNAs in nuclease-treated cytoplasmic extracts obtained from eukaryotic cells that have grown as monolayers^a

	Freshly prepared extract	Frozen and subsequently thawed extract
Without creatine kinase	– (+)	–
With creatine kinase without sucrose	+++	–
With creatine kinase with sucrose	+++	++/+++
Extract supplemented with creatine kinase, frozen without sucrose, thawed, and directly assayed	N.D.	–
As above, but with supplemented creatine kinase after thawing	N.D.	++/+++

^a Translational extracts were directly employed for translation after their preparation, or were frozen and subsequently thawed for further use. Various conditions for extract preparation and translation were assayed, as indicated. –, no translation on exogenous mRNAs; (+), labeled polypeptides barely detectable; ++, +++, efficient translation on exogenous mRNAs; N.D., not determined

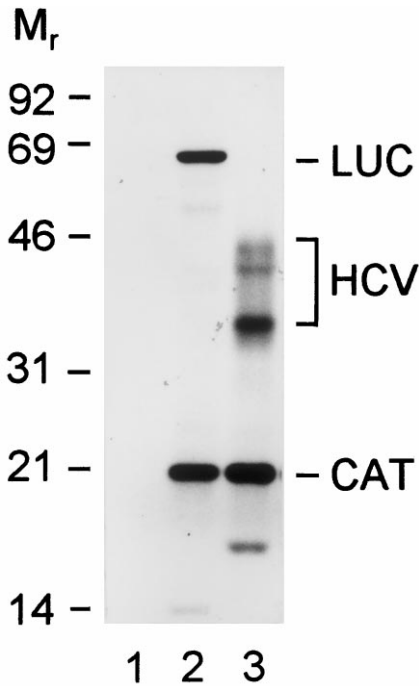


Fig. 1. In vitro translation in a micrococcal nuclease-treated cytoplasmic extract obtained from BHK cells that have been grown as monolayers. Translation of exogenous CAT-EMC-LUC (lane 2), CAT-HCV [E1- Δ E2] (lane 3) capped, polycistronic mRNAs. Translation without exogenous mRNA (lane 1). Chloramphenicol-acetyl-transferase (CAT), luciferase (LUC), HCV structural (HCV) polypeptides. No exogenous microsomal membranes were added in the translation reactions. M_r , relative molecular mass.

4. Discussion

Initial trials to obtain the in vitro translation of exogenous mRNAs in micrococcal nuclease-treated extracts, when the latter have been frozen and subsequently thawed, were always unsuccessful. To circumvent the lack of translational ability of these exogenous mRNAs, several well-established procedures were followed initially. For example, the addition of hemine (20–40 μ M) (Ochoa and de Haro, 1979), the use of other concentrations of GTP (0.05–2 mM) (Weber et al., 1975; Bader and Sarre, 1986) or creatine phosphate (up to 40 mM) (Morley and Jackson, 1985), the presence of additional tRNA (200 μ g/ml) (Pelham and Jackson, 1976), or higher concentrations of polyamines such as spermidine (10 mM)

(Atkins et al., 1975), the pretreatment of the cells in hypertonic buffer (200 mM KCl for 10 min at 37°C) (Yates and Nuss, 1982; Bader and Sarre, 1986) and the inhibition of micrococcal nuclease with 2'-deoxythymidine, 3'-5'-diphosphate (pTp) (Skup and Millward, 1977) instead of EGTA, were employed before or during the generation of the translational extract. However, all these mentioned conventional modifications were not successful and readily failed to allow the generation of a translational extract that was efficient for the initiation of protein synthesis on exogenously added mRNAs.

It finally appeared that the stumbling block in the translation of the exogenously added mRNAs was due to the loss of activity of the creatine kinase after freeze/thawing of the extract. To circumvent this loss of activity, the presence of a cryopreservative, such as sucrose, thus appeared to be highly beneficial. The preservation of the activity of the creatine kinase after freeze/thawing, with the addition of 10% glycerol (Molla et al., 1991), or 10% dimethylsulfoxide in the extraction buffer, is currently under investigation. The resulting frozen and thawed translational extract did allow both the cap-dependent and the cap-independent initiation of translation, as demonstrated for the first time by the translation of dicistronic mRNAs in such a translation system. Moreover, the ability of the translational extract to process viral polypeptides was successfully obtained without the addition of exogenous microsomal membranes, a feature that has not yet been obtained by employing other translational extracts resulting from HeLa cells that have been grown as large-scale suspension cultures (Kamoshita et al., 1997).

The measurement of the relative rates of translation of exogenous mRNAs, in terms of either amino acids per minute per active ribosome, or picomole of protein product synthesized per unit time per microliter of extract, and the comparisons with the activities of the rabbit reticulocyte lysate and the wheat germ extract, are currently in progress.

The generation of a translational extract obtained from eukaryotic cells that have been grown as monolayers has an important potential which

warrants further validation (i) it can be employed in place of the mostly used commercially available rabbit reticulocyte lysate; (ii) it is a priori preferable, since no living animals are required for its generation through experimental chronic anemia; (iii) the cells grown as monolayers in small volumes of cell culture medium, and not as large scale suspension cultures, can be preincubated with hormones, toxins, ions, thus allowing modulation of biological processes prior to extract preparation; (iv) the cells can be infected with viruses prior to extract preparation; (v) the translation extract allows both cap-dependent and cap-independent translation; (vi) in the presence of sucrose, it allows freeze/thawing without loss of activity, i.e. the translational ability of mRNAs added exogenously, a feature that can be extended to various biological samples for the preservation of their respective enzymatic activities. The relevance of such a method for viral translation studies as documented here on various mRNAs deserve special emphasis.

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