Volume 4 Number 10 October 1977 Nucleic Acids Research

Highly efficient translation of messenger RNA in cell-free extracts prepared from L-cells

Danial Skup and Stewart Millward

Department of Biochemistry, McGill University, Montreal, Quebec H3G 1Y6, Canada

Received 29 July 1977

ABSTRACT

Micrococcal nuclease was used to eliminate endogenous protein synthesis in extracts prepared from L cells. The nuclease can be inhibited subsequently with 2'-deoxythymidine-3', 5'-diphosphate. Nuclease-treated extracts primed with exogenous reovirus mRNA, synthesized full length polypeptides with linear kinetics for almost two hours leading to stimulation of the order of 10^4 times over endogenous background. On the average, between 40 and 50 molecules of polypeptide were synthesized per molecule of mRNA.

INTRODUCTION

In vitro translational systems derived from eukaryote cells have been reported from a number of cultured mammalian cells (1), reticulocytes (2), wheat germ (3), rye embryo (4) and many other sources. Whereas these systems are of use in assaying RNA molecules for messenger activity, they all present disadvantages when used to study the actual mechanisms of translation *in vitro*. This is due mainly to the fact that extracts prepared from mammalian cells all show high levels of endogenous protein synthesizing activity. Although preincubation of an extract lowers endogenous activity it also decreases the response to exogenous mRNAs to extremely low levels. The highest ratio of molecules of protein produced per mRNA in such systems is approximately 2 (5). An exception is the reticulocyte system where high activity can be obtained; however, the endogenous synthesis of globin remains high. Extracts derived from plants, while giving low levels of endogenous activity and high rates of stimulation, have definite built-in limitations for the study of translation in mammalian cells. The possibility of making extracts efficient in translating exogenously added mRNA *in vitro*, from a wide variety of cells would be invaluable in the study of specificity and control of translation (see 6 for review).

In the present paper we describe a method of preparing cell-free extracts from L-cells which translate reovirus and globin mRNAs with high efficiency. The method involves pre-treatment of the extracts with micro-

coccal nuclease (EC 3.1.4.7), a Ca^{+2} dependent enzyme (7) which inactivates endogenous mRNA. The nuclease is subsequently inhibited by the addition of 2'-deoxythymidine-3', 5'-diphosphate (pTp) (8). Extracts prepared in this way, (1) translate exogenous mRNAs with high efficiency and (2) are stimulated approximately 10^4 times, over endogenous activity.

Materials and Methods

Cells, Reovirus and Reovirus mRNA. Reovirus, type 3 (Dearing strain) was grown in mouse L-cells and purified as described by Smith *et al.*, (9). Reovirus mRNA was synthesized *in vitro* as described previously (10) except that radioactive precursors were not used. Following the transcriptase reaction, viral cores were pelleted, the supernatant made 1% with sodium dodecyl sulphate (SDS) and extracted 3 times with 90% redistilled phenol. The pooled aqueous phases were precipitated with ethanol and left at -20°C overnight. The resulting pellet of RNA and nucleotide triphosphates was redissolved and then precipitated twice from 2 M NaCl, for 24 hours each time. The RNA pellet resulting from the final NaCl precipitation was dissolved in water, made 0.3 M with potasium acetate (pH 5.5) and precipitated with ethanol. This process was repeated, and the pellet dried, dissolved in sterile double-distilled water and stored in aliquots at -90°C. Immediately prior to use aliquots of reovirus mRNA were thawed then heated for 2 min at 50°C to reduce aggregation.

Preparation of Globin mRNA from Rabbit Reticulocyte Lysates. Reticulocyte lysates were obtained commercially (Gibco, Grand Island, N.Y.). Globin mRNA was purified from polyribosomes dissolved in SDS buffer as described by Krystosek *et al.*, (11). Globin mRNA prepared in this way was highly active in protein synthesis in extracts from wheat germ (data not shown) and in the L-cell extracts described here.

Preparation of S-10 Extract from L-Cells. L-cells at a concentration of about 7×10^5 cells/ml were pelleted and washed twice with phosphate buffered saline (PBS). After the second wash the cells were resuspended in a small volume of PBS and pelleted again to determine the packed volume of cells. Packed cells were suspended in 2 volumes of buffer containing; 10 mM HEPES, pH 7.5, 10 mM KCL, 1.5 mM $Mg(OAc)_2$ 2 mM dithiothreitol (DTT) and allowed to swell on ice for 10 min. Cells were disrupted with 40-50 strokes in a tight fitting Dounce homogenizer. The homogenate was centrifuged for 10 min at 800 g, the pellet was discarded and the supernate centrifuged for 20 min at 10,000 g. The supernate (S-10 extract) was fast frozen in aliquots and stored at -90°C until required.

Nuclease Treatment of S-10 Extract. Micrococcal nuclease (PL Biochemicals Inc.) and $CaCl_2$ were added to 1 ml of S-10 extract to final concentrations of 75 U/ml and 1 mM respectively, and the mixture incubated at room temperature for the times indicated. At the end of the incubation, pTp (PL Biochemicals Inc.) was added to a final concentration of 1.2×10^{-4} M and the S-10 extract was stored in ice until required for *in vitro* protein synthesis.

In Vitro Protein Synthesis in S-10 Extracts from L-Cells. Reaction mixtures in a final volume of 50 µlitres contained the following components; 30 µlitres of nuclease treated, S-10 extract, 1 mM ATP (neutralized with KOH), 10 mM creatine phosphate, 1.2 mg/ml creatine phosphokinase (EC 2.7.3.2), 30 mM HEPES buffer, pH 7.5, 80 mM KCL, 4 mM $Mg(OAc)_2$, 1 mM DTT, 0.5 mM GTP, 5 µM of each amino acid in a mix minus methionine, 300 µCi/ml [^{35}S] methionine (New England Nuclear, spec. act. 380-460 Ci/mmole), 5-7 pmoles of reo-

virus or globin mRNA and water or other additions such as aurin tricarboxylic acid (ATA) to a final volume of 50 µlitres. Incubation was carried out at room temperature. At various times, aliquots of 5 µlitres each were removed and spotted on Whatman 3 MM filter paper discs and washed for 10 min in ice-cold 10% trichloroacetic acid (TCA) containing unlabeled methionine. The filters were transfered to hot (90°), 5% TCA for another 10 min then washed twice in ice-cold 5% TCA, once in ethanol, once in ethanol: ether (3:1) and once in ether alone. The filters were dried, placed in 5 ml of toluene-based scintillation fluid and the radioactivity determined in an Intertechnique SL30 liquid scintillation spectrometer.

The remaining portion of the incubation mixture was made 10% TCA, 3% Casamino acids and placed in ice for 10 min. The pellet, obtained by centrifugation, was suspended in 5% TCA, 3% Casamino acids, heated at 90° for 10 min, centrifuged again, washed three times with 5% TCA containing Casamino acids, once with acetone, dried and dissolved in sample buffer for analysis by polyacrylamide gel electrophoresis (PAGE).

Polyacrylamide Gel Electrophoretic Analysis of Polypeptides Synthesized *in Vitro*. Preparation of samples and gels and the procedure for PAGE was carried out as described by Laemmli (12). Autoradiography was done as described by Spandidos *et al.*, (13).

Results

The concentration of pTp required to inhibit micrococcal nuclease under the conditions described here was examined over the range 10^{-4} M to 6×10^{-8} M (data not shown). Although as little as 10^{-6} M pTp effectively inhibited the nuclease, increasing the concentration to 10^{-4} M increased the incorporation of [^{35}S] methionine into TCA precipitable material by about 15 percent.

Aliquots of S-10 extract were incubated with micrococcal nuclease for various times then 1.2×10^{-4} M pTp was added and the aliquots were assayed, (1) for residual endogenous incorporation of [^{35}S] methionine and (2) for their response to exogenous reovirus mRNA. As shown in Figure 1, the optimum time of exposure to the nuclease was 10 min. Shorter periods of incubation resulted in significant levels of endogenous activity while longer periods of incubation resulted in a significant decline in the ability of the S-10 extract to respond to exogenous mRNA. This could be due to destruction of tRNA or damage to ribosomes after prolonged nuclease treatment. Thus, treatment of the S-10 extract with micrococcal nuclease for 10 min at room temperature followed by the addition of pTp to 1.2×10^{-4} M are the standard conditions used for the rest of the work.

An important indication of whether or not residual nuclease activity is still present in the extract following inhibition with pTp would be the duration of linear incorporation of labeled amino acid into hot TCA precipitable material. As shown in Figure 2, the response to exogenous reovirus

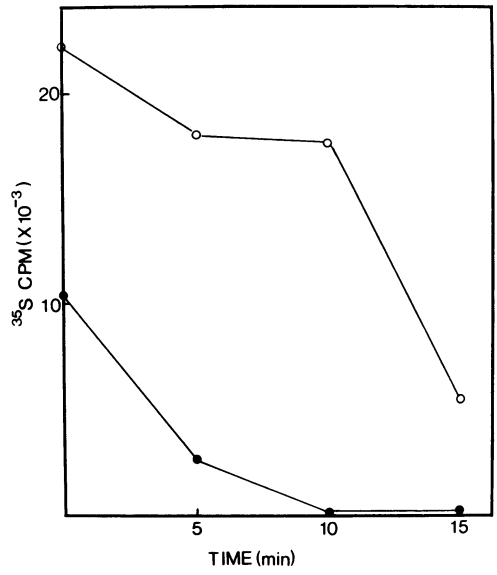

Figure 1. Influence of time of incubation with micrococcal nuclease on incorporation by S-10 extracts. S-10 extracts were prepared as described in Materials and Methods then incubated with 75 U/ml micrococcal nuclease in the presence of 1 mM $CaCl_2$. At the times indicated, pTp was added to duplicate samples of the extract to a concentration of 1.2 x 10^{-4} M. One sample received 7 pmoles of reovirus mRNA and the other sample received no mRNA and each was assayed for *in vitro* protein synthesis as described in Materials and Methods. Reaction mixtures were incubated for 30 min at 30° then aliquots of 5 µliters each were taken to determine the incorporation of [^{35}S] methionine into hot TCA precipitable material. Closed circles represent endogenous protein synthesis. Open circles synthesis with addition of 7 pmoles of reovirus mRNA.

mRNA is linear for almost 2 hr. This corresponds to a stimulation over endogenous of about 10^4 fold (Table 1). The response to exogenous rabbit globin mRNA during the same period of incubation was about 4 x 10^2 fold over endogenous. In both cases addition of ATA to 0.1 mM priot to addition of exogenous mRNA reduced incorporation to background levels (Table 1) showing that the response of the S-10 extract to exogenous mRNA was due to *de novo* initiation. Because of the low endogenous background in the present work, the expression 'fold stimulation' loses significance as a measure of efficiency of translation. A better measure of translational efficiency is the number of molecules of polypeptide synthesized per molecule of mRNA. In the case of reovirus where there are 10 different mRNA species this corresponds to an average of 40 to 50 molecules of viral polypeptide per molecule of mRNA.

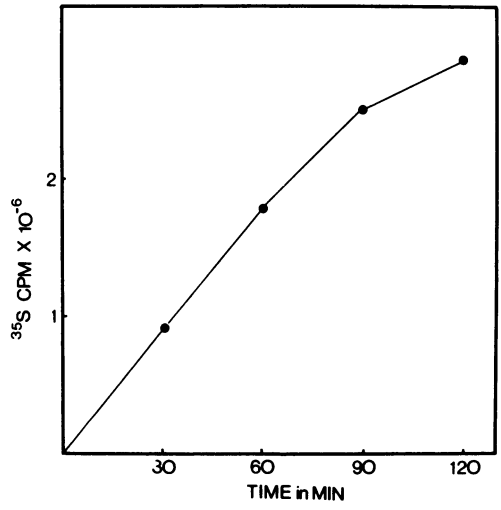

Figure 2. Kinetics of incorporation of [^{35}S] methionine in response to added reovirus mRNA. S-10 extracts were prepared, treated with micrococcal nuclease and pTp and added to a reaction mixture primed with 7 pmoles reovirus mRNA for *in vitro* protein synthesis, as described in Materials and Methods. At the times indicated, aliquots of 5 µliters each were taken to determine the incorporation of [^{35}S] methionine into hot, TCA precipitable material.

Table 1: Response of S-10 Extracts to Exogenous mRNAs.

mRNA Added	Additions	cpm
None	---	142
reovirus (7 pmol)	---	2.9 x 10^6
reovirus (7 pmol)	0.1 mM ATA*	109
rabbit globin$^+$ (5 pmol)	---	4 x 10^4
rabbit globin (5 pmol)	0.1 mM ATA*	110

Data taken from Figure 1 after 120 min of incubation.
\+ Incorporation during 120 min of incubation.
* Aurintricarboxylic acid at this concentration, specifically inhibits initiation of translation (14).

The possibility that the S-10 extract was making less than unit length polypeptides due to cleavage of the mRNA was tested by PAGE of extracts primed with reovirus mRNA. Reovirus marker polypeptides were resolved as the usual three size classes called λ, µ and σ (9). Analysis of the S-10 extract primed with reovirus mRNA showed that virtually all the radioactivity coincided with the λ, µ and σ marker polypeptides and no fragmented (less

than unit length) polypeptides were observed. Furthermore no distinct regions of radioactivity could be discerned in unprimed S-10 extracts.

Discussion

We have demonstrated the usefulness of micrococcal nuclease for preparing extracts from L-cells which show very efficient mRNA-dependent incorporation of [^{35}S] methionine into full length polypeptides. While this work was in progress Pelham and Jackson (15) reported the use of micrococcal nuclease followed by inhibition of the nuclease with ethyleneglycol-bis-(β-aminoethyl ether) N N'-tetraacetic acid (EGTA) to reduce endogenous synthesis of globin in reticulocyte lysates. In our hands the use of EGTA to inhibit the nuclease in S-10 extracts from L-cells proved to be erratic and less efficient than when pTp was used. The effect of Ca^{2+} ions was also quite different in two systems: whereas in reticulocyte extracts exogenously added Ca^{2+} inhibited protein synthesis (15), this was not found to be the case in extracts derived from L-cells. Treating extracts with a mixture of EGTA and pTp showed no advantage over treatment with pTp alone (results not shown). Possible reasons for these differences could be different endogenous levels of Ca^{+2} ions or different sensitivity of the systems to these ions.

The procedure described here overcomes many of the difficulties involved in preparing efficient systems for *in vitro* protein synthesis from cultured cells. This should allow detailed study of many aspects of the regulation of protein synthesis in mammalian systems. For example, some authors have reported the existence of message-specific factors in different cell-types (6). One of the major objections to these results is that the work was done in extracts with very low efficiency of translation so explanations other than that of message specificity could be found (6). This work can now be confirmed using highly efficient translational systems from the cells in question. Comparative translation of the same mRNAs by different systems can also be undertaken, as can the study of translation in cells infected by virus. In reference to the latter, we have prepared an *in vitro* protein synthesizing system from L-cells infected by reovirus and found striking differences in the specificity of translation in this system as opposed to that from uninfected cells (manuscript in preparation).

Acknowledgement

This work was supported by a grant from the Medical Research Council (Ottawa). D.S. holds a MRC Studentship.

REFERENCES

1. Villa-Komaroff, L., McDowell, M., Baltimore, D., and Lodish, H.F. (1974) in "Methods in Enzymology". Eds: Moldave and Grossman 30, pp. 709-723. Academic Press, New York.
2. Adamson, S.P., Howard, G.A., Herbert, E. (1969) Cold Spring Harbour Symp. Quant. Biol. 34, 547-554.
3. Roberts, B.E., and Paterson, B.M. (1973) Proc. Natl. Acad. Sci. USA 70, 2330-2334.
4. Cartier, A.R., and Peumans, W.J. (1976) Biochim. Biophys. Acta 447, 436-444.
5. Metafora, S., Teruda, M., Dow, L.W., Marks, P., and Bank, A. (1972) Proc. Natl. Acad. Sci. USA 69, 1299-1304.
6. Lodish, H.F. (1976) Ann. Rev. Biochem. 45, 39-72.
7. Roberts, W.K., Dekker, C.A., Rushizky, G.W., and Knight, C.A. (1962) Biochem. Biophys. Acta 55, 664-673.
8. Cuatrecasas, P., Fuchs, S., and Anfinsen, F.B. (1967) J. Biol. Chem. 242, 1541-1559.
9. Smith, R.E., Zweerink, H.J., and Joklik, W.K. (1969) Virology 9, 791-810.
10. Faust, M., and Millward, S. (1974) Nucleic Acid Res. 2, 1329-1343.
11. Krystosek, A., Cawthon, M.L., and Kabat, D. (1975) J. Biol. Chem. 250, 6077-6084.
12. Laemmli, U.K. (1970) Nature 227, 680-685.
13. Spandidos, D.A., Krystal, G., and Graham, A.F. (1976) J. Virol. 18, 7-19.
14. Weber, L.A., Feman, B.R., and Baglioni, C. (1975) Biochemistry 14, 5315-5321.
15. Pelham, M.R.B., and Jackson, R.J. (1976) Eur. J. Biochem. 67, 247-256.