COMPLETE TRANSLATION OF ENCEPHALOMYOCARDITIS VIRUS RNA AND FAITHFUL CLEAVAGE OF VIRUS-SPECIFIC PROTEINS IN A CELL-FREE SYSTEM FROM KREBS-2 CELLS

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1. Introduction

There are numerous reports describing translation of encephalomyocarditis (EMC) virus RNA in cell-free systems (c.f. [1–10]). The products formed in these systems, however, did not coincide, upon polyacrylamide gel electrophoresis, with 'mature' virus-specific polypeptides found in EMC virus-infected cells. The difference between the in vitro and in vivo products seemed to be primarily due to two factors, non-complete translation of the RNA template and inefficient cleavage of precursor polypeptides formed. Indeed, the major products of in vitro synthesis corresponded to high-molecular-weight polypeptides containing amino acid sequences of capsid proteins, which implied that only a 5′-terminal region of EMC virus RNA was predominantly, or exclusively, translated. The reasons for such an incomplete translation were thought to be either premature termination at certain regions of the template or template degradation, or both [5,6,10]. In systems where these problems were partially overcome, the resulting product did contain, as minor components, polypeptides corresponding to the non-capsid 3′-terminal portion of viral genome but only in a high-molecular-weight uncleaved form [11].

The present report describes conditions under which the entire genome of EMC virus appears to be translated and proper cleavage of polypeptide precursors takes place in vitro. The combination of these events results in formation of almost all 'mature' virus-specific polypeptides. Although the formation of these polypeptides can be observed in extracts from uninfected Krebs-2 cells, a cell-free system derived from EMC virus-infected cells yields a pattern of products which is more similar to that observed in vivo. Two main methodological changes were responsible for a drastic improvement of the efficiency of in vitro translation of EMC virus RNA: the use of a Ca^{2+}-dependent nuclease for elimination of endogenous templates [12] and the choice of appropriate ionic conditions. Some additional information on this system is in [13].

2. Materials and methods

2.1. *Isolation of template RNAs*

Purified [14] preparations of EMC virus were suspended in a solution containing 0.5% sodium dodecyl sulphate (SDS), 50 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, pH 8.0, and were treated with a chloroform/phenol/isoamyl alcohol mixture (50:50:1). Viral RNA was precipitated with ethanol. Isolation of total poly(A)-containing RNA from the cytoplasm of Krebs-2 cells is in [15].

2.2. *Cell-free systems*

Non-infected and EMC virus-infected (10 p.f.u./cell) Krebs-2 cells were suspended in Eagle's medium (2×10^7 cell/ml) and were incubated for 18 h at 4°C, then for 4 h at 37°C [16]. S_{30} extracts from these cells were prepared essentially as in [3]. Hypotonic solution (10 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris–HCl, pH 7.5) 1.5 vol., was added to the pelleted cells and, after 20 min, the swollen cells were dis-

rupted in a Dounce homogenizer, the concentration of salts was increased by adding 0.11 vol. solution containing 1 M KCl, 37.5 mM $MgCl_2$, 0.21 M Tris–HCl, pH 7.5, and the homogenate centrifuged at 30 000 × *g* for 10 min. Nuclease treatment of the supernatants was performed as recommended [12]. Micrococcal nuclease (Schwartz/Mann) and $CaCl_2$ were added to the S_{30} extract to final conc. 10 μg/ml and 0.75 mM, respectively. After incubation for 15 min at 20°C, Ca^{2+} was chelated by adding ethylene glycol-bis-(2-aminoethylether)-*N,N'*-tetracetate (EGTA) to a final concentration of 2 mM. The treated extract was again centrifuged at 30 000 × *g* for 10 min. The supernatant was supplemented with glycerol (to 10%) and was stored at −70°C. Before use, the extract was thawed and chromatographed through a column of Sephadex G-25 equilibrated with a solution containing 65 mM KCl, 3.3 mM $MgCl_2$, 0.1 mM EGTA, 30 mM Tris–HCl, pH 7.5. The resulting extract (40–60 A_{260} units/ml) was used for the translation of exogenous RNAs.

The translation samples (0.1 ml) contained 60 μl of S_{30} fraction and the following concentrations of other components: 4 mM $MgCl_2$; 30 mM Tris–HCl, pH 7.5; 1.5 mM ATP; 0.1 mM GTP; 0.6 mM CTP; 10 mM dipotassium creatine phosphate; 80 μg/ml creatine kinase; 19 unlabeled amino acids, 0.04 mM each; 0.008 mM [^{14}C]lysine (340 mCi/mmol, Amersham) as well as RNA and KCl as indicated in the figure legends. After an appropriate incubation (see figure legends), radioactivity in the acid-insoluble material was determined; if the samples were to be analysed by electrophoresis, the radioactivity was determined in 15 μl aliquots.

2.3. *Electrophoresis*

Aliquots of translation samples were supplemented with 2 vol. solution containing 3% SDS, 6 M urea (deionized), 7.5% 2-mercaptoethanol, 15% glycerol, 0.005% bromophenol blue, and 75 mM Tris–HCl, pH 6.8. Before electrophoresis the samples were heated at 100°C for 3 min and were applied onto gradient (8–20%) polyacrylamide gel slabs. Electrophoresis was performed in the SDS-containing buffer system [17] for about 18 h. The gels were treated with dimethylsulfoxide and PPO fluor as in [18]. The dried gels were exposed to RM-1 X-ray films (Svema, USSR) at −70°C.

3. Results

3.1. *General characteristics of the system*

Cell-free extracts prepared by the method described, displayed a fairly efficient translation of exogenous RNA templates. Optimal ionic concentrations determined [13] at 37°C, 105 mM KCl and 4 mM $MgCl_2$ for the translation of EMC virus RNA and up to 75 mM KCl and 3.3 mM $MgCl_2$ for the translation of poly(A)-containing RNA from the cytoplasm of uninfected Krebs cells, the optimal concentration of KCl being dependent in the latter case on the RNA concentration used. The dependence of polypeptide synthesis on exogenous mRNAs is illustrated in fig.1. The rate and maximal values of

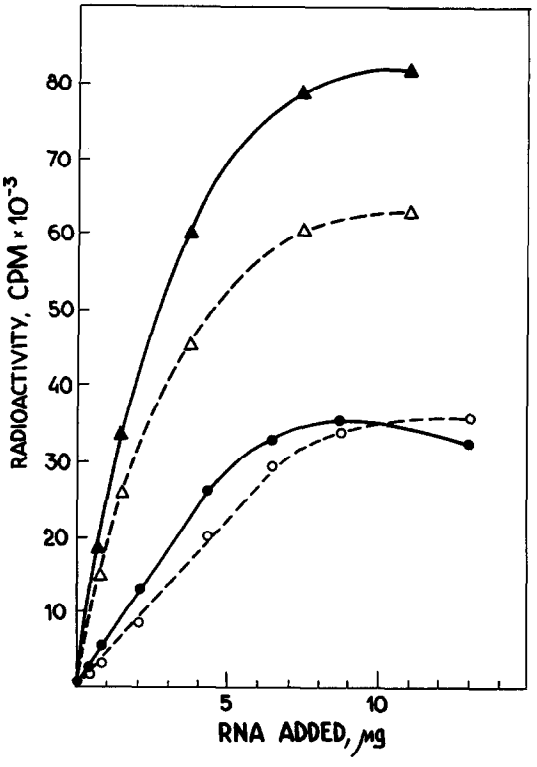

Fig.1. Effect of mRNAs concentration on the incorporation of [^{14}C]lysine in extracts from uninfected (solid lines) and EMC virus-infected (broken lines) cells. Translation of viral RNA (triangles) was carried out at 105 mM KCl and 4.0 mM $MgCl_2$; translation of cellular mRNA (circles) was carried out at 65 mM KCl and 3.3 mM $MgCl_2$. All samples were incubated at 37°C for 90 min. Endogenous incorporation without added mRNA in any sample did not exceed 700 cpm.

amino acid incorporation were greater with EMC virus RNA compared to those with cellular mRNA. The extracts from uninfected and infected cells did not differ significantly from each other with respect to the translational capacity, although the translation of EMC virus RNA in the latter appeared to be somewhat less efficient than in the former. There was no evidence for selective inhibition of template activity of cellular mRNA in the extract from infected cells. All these features are similar to those found with other cell-free systems [7,16].

A remarkable and distinct property of the present system, however, was very low background incorporation which amounted to as little as less than 1% of the incorporation in the presence of saturating concentrations of exogenous mRNA. Moreover, this background incorporation displayed a minor, if any, dependence on either ionic conditions or incubation time (data not shown).

The lowering of the incubation temperature to 30°C resulted in some increase of optimal KCl concentration which, in the case of EMC virus RNA, was found to be 145 mM. Under these conditions, the extent of amino acid incorporation increased approximately by 25% compared to the optimal incorporation at 37°C and the rate of incorporation was essentially constant for at least 2.5 h.

3.2. Products of translation of EMC virus RNA

It was found, in preliminary experiments, that the set of polypeptides formed in response to the addition of EMC virus RNA to the extracts depended on the ionic conditions of incubation. Figure 2 presents the results of an electrophoretic analysis of the products formed in extracts from uninfected cells at different concentrations of KCl (lanes 1–7). The following main conclusions can be made. At low (65 mM) concentrations of KCl (lane 2), the major band corresponded to a polypeptide with est. mol. wt 112 000. Two less prominent bands were identical, with respect to electrophoretic mobility, to polypeptides A and B found in EMC virus-infected cells (lane 8). Some additional polypeptides, especially in the high-molecular-weight region of the slab, were present in trace amounts. The increase in KCl concentration to 85 mM resulted in appearance of additional clearly visible bands having electrophoretic mobilities of virus-specific polypeptides C, D, E, F and I or H

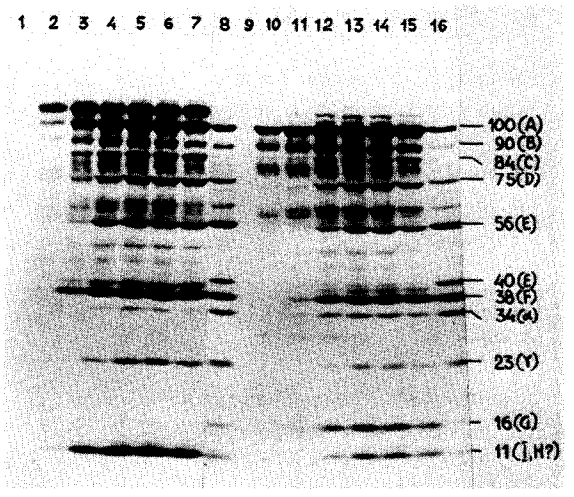

Fig.2. Products of translation of EMC virus RNA in extracts from uninfected (lanes 1–7) or virus-infected (lanes 9–15) cells. The extracts were incubated at 30°C for 150 min either in the absence of exogenous RNA (lanes 1, 9) or in the presence of 6.5 μg EMC virus RNA (lanes 2–7, 10–15) at 4.0 mM $MgCl_2$ and the following concentrations of KCl: 1–65 mM (71 cpm); 2–65 mM (3897 cpm); 3–85 mM (8454 cpm); 4–105 mM (13 951 cpm); 5–125 mM (18 167 cpm); 6–145 mM (19 141 cpm); 7–165 mM (14 454 cpm); 9–65 mM (105 cpm); 10–65 mM (2992 cpm); 11–85 mM (5479 cpm); 12–105 mM (10 061 cpm); 13–125 mM (13 331 cpm); 14–145 mM (13 293 cpm); 15–165 mM (10 622 cpm); in parentheses, the radioactivities of acid-insoluble material in 15 μl aliquots corresponding sample are given. EMC virus-specific polypeptides found in the infected cells at 4 h infection are shown in lanes 8, 16. On the ordinate mol. wts × 10^{-3} and viral protein designations are indicated.

(lane 3); trace amounts of polypeptide G could also be seen. Further increase in KCl concentration (lanes 4–7) led to a markedly greater accumulation of the label in these bands and to appearance of new bands apparently comigrating with EMC virus-specific polypeptides α and γ as well as a number of minor bands between the polypeptides D and F. The concentration of KCl at which all these bands were most distinct was in the region of 125–145 mM.

Results obtained with extracts from virus-infected cells (lanes 9–15) were, in general, similar to those just described, but several points of difference could be noted.

(1) There was no preferential accumulation of 112 000 dalton polypeptide at any concentration

of KCl tested, the major product being polypeptide A.

(2) Several polypeptides, namely B, G and several minor ones, seemed to be present in relatively higher amounts.

(3) A band with an electrophoretic mobility close to, or identical with, that of EMC virus protein ϵ was evident.

4. Discussion

Conditions are reported here under which an almost complete set of EMC virus-specific polypeptides known from in vivo experiments, can be formed in vitro. The notable exceptions are capsid polypeptides β and δ, but their formation by cleavage of precursor polypeptide ϵ is a late event in the infectious cycle related to the maturation of virions [19].

According to current concepts [19], there is only one translation initiation point on the EMC virus RNA template, and 'polyprotein' synthesized in vivo is cleaved, while still being attached to the ribosomes, into three 'primary' products, A, F, and C, which are then cleaved into 'mature' polypeptides. The appearance in vitro of all three 'primary' products found in vivo, strongly suggests that translation of the entire EMC virus genome takes place in our system. Translation of the 3′ end-adjacent region of viral RNA may be explained by either overcoming weak termination signals supposedly present in this RNA [20,21] or initiation at weak internal initiation sites (cf. [22]). Whatever mechanism is responsible for the complete translation, it is evidently highly dependent on salt concentrations. In fact, at low KCl neither polypeptide C (encoded in the 3′-terminal region of the genome) nor polypeptide F (central part of the genome) could be detected. With a related picornavirus, poliovirus, it was also shown that the increase in KCl concentration resulted in a more complete translation of viral RNA in vitro as judged by the formation of an uncleaved polypeptide of more than 200 000 daltons [23].

The second feature of our system which merits attention, is an adequate processing of polypeptide precursors in the sense that almost all 'mature' virus-specific proteins are accumulated. The faithful cleavage of the precursors was demonstrated in extracts from uninfected cells. This fact indicates that the cleavage is carried out, at least partially, by pre-existing cellular proteases or it is accomplished by a kind of autocatalytic proteolysis (cf. [8,23,24]). It is not clear from the present experiments whether the increased accumulation of cleaved products at higher KCl concentrations was due solely to the enhanced synthesis of the corresponding precursors or to stimulating effect of the salt on the cleavage itself.

Reasons for certain differences in sets of polypeptides formed in extracts from uninfected and virus-infected cells programmed by the same template RNA, are as yet unknown. As a likely explanation, a more efficient cleavage of precursors in the latter may be suggested. This can account for the almost complete disappearance of polypeptide of 112 000 daltons (which is known to be a precursor of polypeptide A and consequently of all capsid proteins [8,9]) as well as for the increased accumulation of some polypeptides of a lower molecular weight. Alternatively, the use of different initiation or termination sites on viral RNA in extracts from uninfected and infected cells cannot be rigorously excluded.

References

[1] Mathews, M. B. and Korner, A. (1970) Eur. J. Biochem. 17, 328–338.
[2] Dobos, P., Kerr, I. M. and Martin, E. M. (1971) J. Virol. 8, 491–499.
[3] Aviv, H., Boime, I. and Leder, P. (1971) Proc. Natl. Acad. Sci. USA 68, 2303–2307.
[4] Boime, I., Aviv, H. and Leder, P. (1971) Biochem. Biophys. Res. Commun. 45, 788–795.
[5] Boime, I. and Leder, P. (1972) Arch. Biochem. Biophys. 153, 706–713.
[6] Kerr, I. M., Brown, R. E. and Tovell, D. R. (1972) J. Virol. 10, 73–81.
[7] Lawrence, C. and Thach, R. E. (1974) J. Virol. 14, 598–610.
[8] Lawrence, C. and Thach, R. E. (1975) J. Virol. 15, 918–928.
[9] Hunt, L. A. (1976) Virology 70, 484–492.
[10] Hunt, L. A. (1976) J. Virol. 18, 788–792.
[11] Esteban, M. and Kerr, I. M. (1974) Eur. J. Biochem. 45, 567–576.
[12] Pelham, H. R. B. and Jackson, R. J. (1976) Eur. J. Biochem. 67, 247–256.

[13] Svitkin, Yu. V. and Agol, V. I. (1978) Dokl. Akad. Nauk SSSR in press.
[14] Kerr, I. M. and Martin, E. M. (1972) J. Virol. 9, 559–561.
[15] Ugarova, T. Yu., Svitkin, Yu. V., Ginevskaya, V. A. and Agol, V. I. (1973) Dokl. Akad. Nauk SSSR 211, 996–999.
[16] Svitkin, Yu. V., Ugarova, T. Yu., Ginevskaya, V. A., Kalinina, N. O., Scarlat, I. V. and Agol, V. I. (1974) Intervirology 4, 214–220.
[17] Laemmli, U. K. (1970) Nature 227, 680–685.
[18] Bonner, W. M. and Laskey, R. A. (1974) Eur. J. Biochem. 46, 83–88.
[19] Rueckert, R. R. (1976) in: Comprehensive Virology (Fraenkel-Conrat, H. and Wagner, R. eds) vol. 6, pp. 131–213, Plenum Press, New York.
[20] Lucas-Lenard, J. (1974) J. Virol. 14, 261–269.
[21] Paucha, E., Seehafer, J. and Colter, J. S. (1974) Virology 61, 315–326.
[22] Celma, M. L. and Ehrenfeld, E. (1975) J. Mol. Biol. 98, 761–780.
[23] Villa-Komaroff, L., Guttman, N., Baltimore, D. and Lodish, H. F. (1975) Proc. Natl. Acad. Sci. USA 72, 4157–4161.
[24] Chatterjee, N. K., Polatnick, J. and Bachrach, H. L. (1976) J. Gen. Virol. 32, 383–394.