A COMPARATIVE STUDY ON TRANSLATION OF FLAVIVIRUS AND PICORNAVIRUS RNAs IN VITRO

Apparently different modes of protein synthesis

Yu. V. SVITKIN, V. N. LYAPUSTIN, V. A. LASHKEVICH and V. I. AGOL
Institute of Poliomyelitis and Viral Encephalitides, USSR Academy of Medical Sciences, Moscow Region, 142782 and *Moscow State University, Moscow 117234, USSR*

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1. Introduction

In eukaryotic cells, mRNAs are believed to be, at least predominantly, monocistronic and thus to possess a single initiation site of translation [1,2]. The mRNA species of certain RNA-containing viruses that replicate in these cells, may each code for several different proteins, but in relatively well studied cases, e.g., picornaviruses, alphaviruses, retroviruses and some others, each molecule of viral mRNA appears again to possess only one functional initiation site, the ultimate protein products being formed by proteolytic cleavages of high molecular weight polypeptide precursors. In this respect, the mode of translation of flavivirus genome, a single-stranded RNA molecule of about 4×10^6 mol. wt [3], seems to be of profound interest since some indirect evidence obtained in vivo suggests that this RNA may be translated from multiple internal initiation sites. In fact, different flavivirus proteins appeared to be initiated simultaneously after relieving the block of initiation of translation in the virus-infected cells and no indications for the formation of the major flavivirus proteins by the cleavage of high molecular weight precursors was obtained [4–7]. The possibility that different virus-specific polypeptides are encoded in different subgenomic monocistronic mRNA species seems unlikely because no viral mRNA other than 42–45 S virion RNA can be detected in the infected cells [8,9].

The existence of multiple initiation sites in the flavivirus RNA can be verified using cell-free protein synthesizing systems. We are aware of no previous reports on in vitro translation of the flavivirus genome. We wish to describe here:

(i) The kinetics of appearance of different proteins in a cell-free system from Krebs-II cells [10] programmed by virion RNAs isolated from either tick-borne encephalitis (TBE) virus, a flavivirus, or encephalomyocarditis (EMC) virus, a picornavirus.

(ii) The differential effects of ionic conditions on the synthesis of flavivirus proteins in vitro.

The results obtained support the hypothesis of the existence of multiple initiation sites of translation on flavivirus RNA.

2. Materials and methods

TBE virus, strain Sophin, was grown, concentrated and partially purified as in [11]. Viral material from the peak fractions of sucrose gradients was pelleted by centrifugation and suspended in a solution containing 0.12 M NaCl, 0.01 M Tris–HCl (pH 7.3), 0.001 M EDTA and 0.5% sodium dodecyl sulfate. The viral RNA was isolated from this material by two extractions with a phenol–chloroform mixture (1:1). After precipitation with ethanol, it was purified by sedimentation in a 5–20% sucrose concentration gradient. The material from the 35–45 S region of the gradient was precipitated with ethanol and used for the experiments.

As a source of labeled structural proteins of TBE

virus, the interphase obtained during the phenol–chloroform extraction of the purified virus grown in the presence of ^{14}C-labeled *Chlorella* hydrolysate was used. For labeling virus-specific proteins in vivo, the SPEV cells infected with TBE virus at a multiplicity of about 1 p.f.u./cell were incubated in medium 199 for 44 h, then actinomycin D (5 μg/ml) was added and after 3 h the concentration of NaCl was increased (190 mM excess); the latter procedure was known to inhibit preferentially the synthesis of cellular rather than viral proteins [5,12]. After 20 min of incubation in this medium, ^{14}C-labeled *Chlorella* hydrolysate was added and the cells were lysed in a sample buffer for electrophoresis [10] 3 h later.

Methods for the isolation of EMC virus RNA, the preparation of the cell-free system, and the electrophoresis in polyacrylamide gels were as in [10] except for two modifications:

(i) A mixture of ^{14}C-labeled lysine, leucine and valine was used.

(ii) At the end of in vitro incubation, the extracts were treated with pancreatic RNase (50 μg/ml, 30°C, 10 min) in the presence of 10 mM EDTA, a procedure which was introduced largely for safety purposes but which, in addition, permitted the use of TBE RNA slightly labeled with ^{3}H as a template.

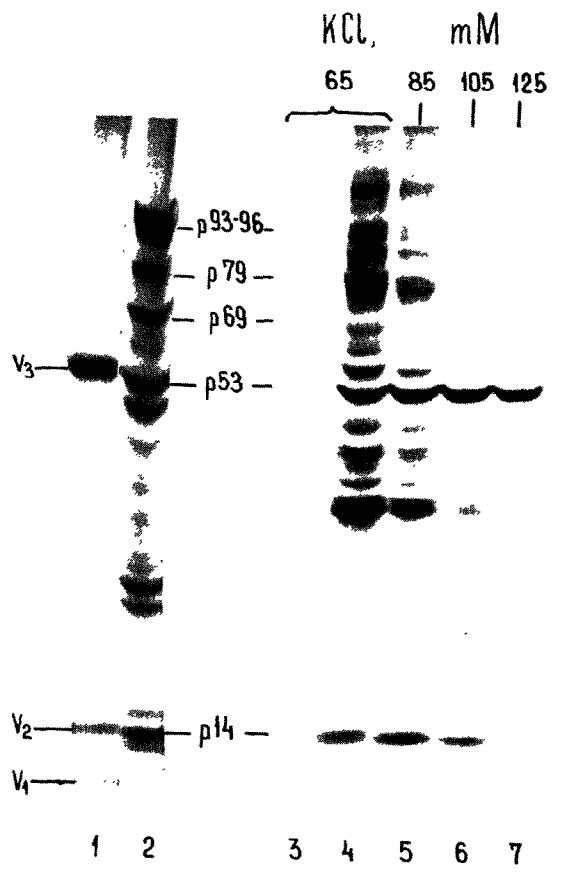

Fig.1. Translation of TBE virus RNA in vitro at different KCl concentrations. (1) Labeled proteins from the purified TBE virus; (2) labeled proteins synthesized in the TBE virus-infected cells; (3–7) products formed in the cell-free system: (3) a sample without exogenous RNA; (4–7) samples containing TBE virus RNA at 75 μg/ml. Concentrations of KCl (mM) in the incubation mixture are indicated over the slots; concentration of $MgCl_2$ in all samples was 3.3 mM. Molecular weights were calculated using EMC virus-specific polypeptides, run in the same slab, as markers (p*N* designates a polypeptide of an $N \times 10^3$ mol. wt).

3. Results

The addition of TBE virus RNA to micrococcal nuclease-treated extracts of Krebs-II cells results in a significant incorporation of labeled amino acids into the acid-insoluble material. The extent of incorporation depends on the quantity of RNA added. At 30°C, the optimal concentrations of $MgCl_2$ and KCl correspond approximately to 3 mM and 65 mM, respectively. Under these conditions, a number of discrete polypeptide chains are accumulated, as revealed by electrophoresis in polyacrylamide gels (fig.1, slot 4). Most of them correspond in electrophoretic mobility to labeled polypeptides synthesized in TBE virus-infected cells under hypertonic conditions (fig.1). It should be noted, however, that the relative ratios of different polypeptides synthesized in in vitro and in vivo systems differ markedly.

An increase in KCl concentration results in a dramatic inhibition of the accumulation of all virus-specific polypeptides except p53; this polypeptide is the only major product formed at 125 mM KCl, although trace amounts of other polypeptides, in particular p14, can be detected (fig.1, slots 4–7). Further increase in KCl concentration is accompanied by a progressive decrease of protein synthesis so that at 155 mM KCl the incorporation of amino acids is

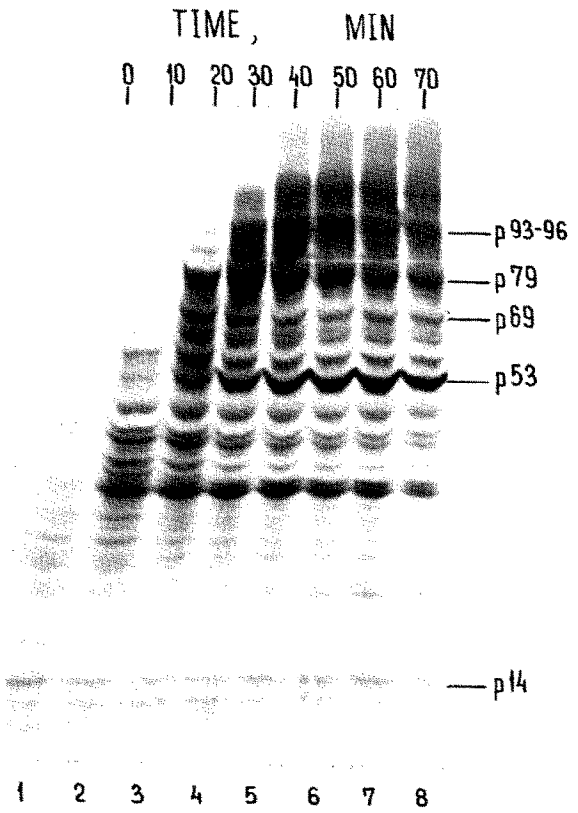

Fig.2. Kinetics of accumulation of polypeptides in the cell-free system programmed by TBE virus RNA. A complete incubation mixture, 0.25 ml, containing 12.5 μg TBE virus RNA was incubated at 30°C for 15 min at 65 mM KCl and 3.3 mM $MgCl_2$. Then the concentration of KCl was increased to 155 mM and at the time intervals indicated over the slots, 20 μl aliquots were withdrawn for the analysis (0 min corresponds to the time when concentration of KCl was increased)

nearly stopped. Changes in $MgCl_2$ concentration from 2–5 mM result in qualitatively similar alterations in the spectrum of polypeptides accumulated (data not shown). These effects of salt concentrations appear to be primarily due to variations in the efficiency of translation initiation, since the elongation step is known to be stimulated by the increase in KCl concentrations in the range studied ([13]; see also below).

For studying the kinetics of accumulation of TBE virus-specific polypeptides in vitro, we used an approach described in [14]; the synthesis was initiated at an optimal KCl concentration and then the salt concentration was increased. This procedure had a double purpose, to stop reinitiation and to enhance elongation. The results of such an experiment are presented in fig.2. It is seen that most, if not all, polypeptides with mol. wt $< 35 \times 10^3$ are synthesized during a 15 min incubation at 65 mM KCl, traces of larger polypeptides also being visible. Further incubation for 10–30 min leads to a consecutive accumulation of polypeptides with increasing molecular weights; no significant changes can be observed after a 30 min incubation at 155 mM KCl.

A totally different pattern is observed when the

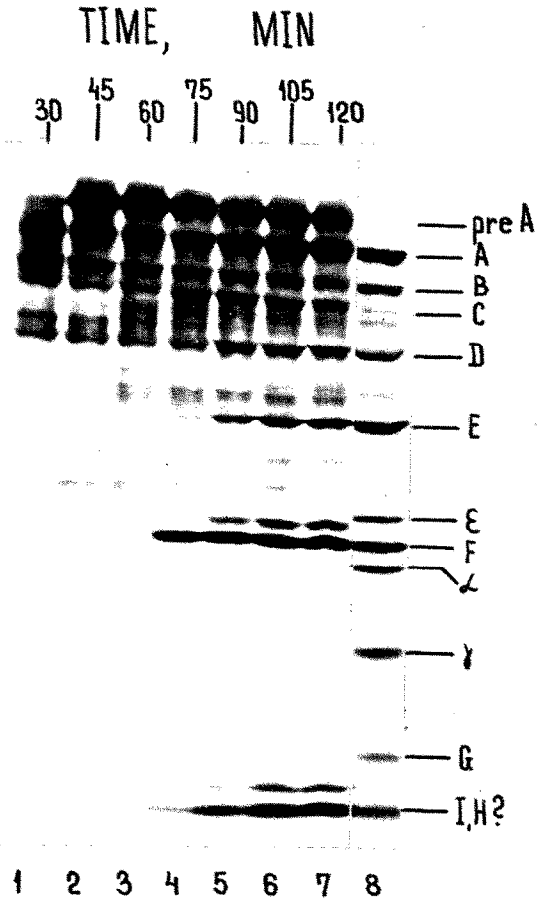

Fig.3. Kinetics of accumulation of polypeptides in the cell-free system programmed by EMC virus RNA. A complete incubation mixture, 0.25 ml, containing 20 μg EMC virus RNA was incubated at 30°C at 145 mM KCl and 4 mM $MgCl_2$. At the time intervals indicated over the slots, 20 μl aliquots were withdrawn for the analysis. Slot 8, labeled proteins synthesized in the EMC virus-infected cells.

kinetics of synthesis of EMC virus proteins in the same system is studied (fig.3). Here, the incubation was carried out at 145 mM KCl and 4 mM $MgCl_2$, the concentrations optimal for overall protein synthesis. It is seen that high molecular weight polypeptide precursors are synthesized first and only then the 'mature' polypeptides of lower molecular weights are sequentially accumulated.

4. Discussion

The profound difference in the kinetics of accumulation of TBE and EMC virus-specific polypeptides is a most interesting result of the present study. The sequential appearance of the 'primary' translation products preA(A), F and C followed by stepwise cleavages of preA(A) and C is a characteristic feature of the translation of the EMC virus genome. This pattern and similar results [15] are in accord with the genetic map of picornaviruses [16] as well as with the assumed single initiation site of translation on their RNA [1,17,18]. On the other hand, no evidence for the formation of TBE virus-specific polypeptides by cleavage of high molecular weight precursors is obtained. Furthermore, the time of synthesis of different polypeptides in this case appears to be roughly proportional to their molecular weight. It should also be noted that the time required for the formation of a complete set of TBE virus-specific polypeptides is significantly shorter (<45 min) than that needed for the translation of the entire EMC virus genome (>60 min) although the flavivirus genome is much larger than the picornavirus genome. At least two interpretations of such a pattern are possible:

(i) TBE virus RNA possesses multiple sites of translation initiation. The validity of this interpretation depends heavily on the assumption that different polypeptides formed in vitro are products of different TBE virus genes. Consistent with this assumption is the coincidence of electrophoretic mobilities of a number of polypeptides synthesized in vitro and in vivo. This seem to be true of the non-structural virus-specific proteins, e.g. NV5 (mol. wt 93–96 $\times 10^3$) and NV4 (69×10^3) as well as for the virion proteins V2 (14×10^3) and p53 which is likely to represent a non-glycosylated form of the envelope glycoprotein V3. It is clear, however, that a definite identification of the proteins requires more direct evidence. The multiple initiation sites hypothesis receives additional support from the fact that an increase in KCl concentration selectively inhibits the synthesis of some, but not all, TBE virus-specific polypeptides.

(ii) The TBE virus RNA possesses a single initiation site which is used for the synthesis of all polypeptides detected in our cell-free system. According to this interpretation, the formation in vitro of different polypeptides is a result of the functioning of multiple weak termination signals. It should be admitted that incomplete termination on some sites of TBE virus RNA cannot be excluded, since the total molecular weight of all polypeptides synthesized in vitro (including minor ones) exceeds the coding capacity of the flavivirus genome. However, an increase in KCl concentration (which should stimulate elongation [13] and help to overcome weak termination signals [10]) results in the inhibition, rather than stimulation, of the synthesis of high molecular weight TBE virus-specific polypeptides. This observation strongly contradicts the single initiation site hypothesis.

Thus the first alternative, i.e., the multiple initiation sites hypothesis, seems to be in better accord with the experimental findings. To be proven this hypothesis requires, however, more direct evidence.

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