IN VITRO SYNTHESIS OF STRUCTURAL PROTEINS OF SEMLIKI FOREST VIRUS DIRECTED BY ISOLATED 26 S RNA FROM INFECTED CELLS

J. C. S. CLEGG and S. I. T. KENNEDY
Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, England

Received 11 March 1974

1. Introduction

Cells infected with Semliki Forest virus (SFV), an alphavirus, contain four species of single-stranded RNA [1]. These are the 42 S RNA found in the virus particle, a 26 S RNA, and much smaller quantities of 38 S and 33 S RNAs. There is indirect evidence that certain of these molecules act as messenger RNAs during the course of virus replication. Since 42 S RNA extracted from the virus is infectious, it is presumed to code for at least a part of the virus RNA polymerase found in the cytoplasm of infected cells. We have recently shown that all four single-stranded RNA species contain poly A sequences [2], a characteristic of most of the types of mRNA functioning in eukaryotic cells examined so far. Better evidence of messenger function is the demonstration that 26 S, 33 S, and possibly 42 S RNA species are found associated with the polysomes of infected cells [3,4]. However, there has been, to date, no direct demonstration of the messenger RNA activity of these RNAs. Here we show that 26 S RNA, isolated from infected cells by zonal centrifugation and chromatography on oligo(dT)-cellulose, stimulates the synthesis of material with immunological and electrophoretic properties of SFV structural proteins.

2. Methods

2.1. *Isolation of 26 S RNA*

Ten roller bottles of BHK cells infected with SFV, labelled with [^{3}H]uridine (2 bottles, 50 μCi ml^{-1}) between 5$\frac{1}{2}$ and 7 hr post-infection were washed and harvested as described [2]. The cells were lysed by gentle homogenisation in 50 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM EDTA buffer containing 1% naphthalene-1,5-disulphonate. The nuclei were removed by centrifugation (800 *g*, 10 min), washed in the same buffer, and the combined supernatants made 1.5% in SDS, and extracted at room temperature twice with buffer-saturated phenol and once with chloroform–octanol (24:1 v/v). After washing with ether, RNA was precipitated at 4°C by addition of 2.5 vol of ethanol, and dissolved in 50 mM Tris–HCl (pH 7.5), 100 mM LiCl, 1 mM EDTA, 0.1% SDS (TLES). Up to 25 mg RNA were fractionated on a 5–20% sucrose gradient in TLES centrifuged for 16 hr at 25 000 rpm in a B XIV zonal rotor. The fractions containing 26 S RNA were pooled and passed over a 6 × 90 mm column of oligo(dT)-cellulose in four separate applications. The column was washed and eluted as described [2] and the pooled poly A-containing material precipitated at −20°C by addition of NaCl to 0.4 M and 3 vol of ethanol. The precipitate was washed in 70% ethanol containing 50 mM NaCl, dissolved in water at a concentration of 1 mg ml^{-1} and stored frozen in small aliquots at −70°C.

2.2. *Cell-free protein synthesis*

A pre-incubated S-10 extract was prepared from LS cells following the methods described [5–7] except that Tris–HCl was replaced by Hepes potassium salt at the same concentration and pH. A standard 50 μl reaction mixture for amino acid incorporation contained 30 mM Hepes buffer (pH 7.4), 5 μCi [^{35}S]methionine (100–150 Ci mmol^{-1}), 50 μM each of the other 19 amino acids, 10 mM potassium phosphoenolpyruvate, 1 mM ATP, 0.1 mM GTP,

0.6 mM CTP, 50 μg ml^{-1} pyruvate kinase and 0.3 A_{260} units of S-10 extract, supplemented where indicated by 1–2 μg mRNA. The final concentrations of K^+ and Mg^{2+} were brought to 60 mM and 5 mM respectively by addition of concentrated solutions of KCl and $Mg(CH_3COO)_2$. Incubations were for 100 min at 30°C. Hot TCA-precipitable radioactivity was determined by addition of 1 ml 5% TCA containing 0.5% lactalbumin hydrolysate and heating at 90°C for 5 min. The precipitate was collected on Whatman GF/C filters, washed with 5% TCA, then ethanol–ether (1:3 v/v), and dried and counted by scintillation in toluene containing 0.4% PPO. For immunoprecipitations, 5 μl samples were diluted with 800 μl phosphate-buffered saline (PBS) containing 1 mM methionine and 2% Triton N-101 and mixed with 2 μl rabbit SFV-specific antiserum [8] or pre-immune serum. After 30 min at 30°C, 40 μl sheep anti-rabbit globulin was added, and the incubation continued for a further 30 min at 30°C, and then at 4°C overnight. The precipitate was gently washed in the PBS mixture, transferred to GF/C filters, dried and counted as for the hot TCA precipitates.

For SDS-acrylamide gel electrophoresis of cell-free products, samples of incubated cell-free systems were treated with 50 μg ml^{-1} pancreatic ribonuclease and 20 mM EDTA for 5 min at 30°C, and the proteins reduced and alkylated as described [8]. Material immunoprecipitated as above, was dissolved in 50 mM sodium phosphate (pH 7.2), 2% SDS and similarly reduced and alkylated. The material was analysed on 10% acrylamide gels [8] containing 0.05% SDS after mixing with dansylated SFV structural proteins. The positions of the radioactive bands were determined by autoradiography after drying down longitudinal gel slices [9].

3. Results

RNA was isolated from the cytoplasm of BHK cells infected with SFV 7 hr previously by a method (see legend to fig. 1) which avoided lysis of nuclei and extraction of DNA; the low viscosity of the resulting extract made handling the necessarily large volumes very much easier. It has also been found that the yield of virus-specific double-stranded RNA, which sediments close to the 26 S RNA in sucrose gradients, is very much reduced (J. Samuels and G. J. Atkins, unpublished). After centrifugation the region of the zonal gradient containing 26 S RNA (which migrates slower than 28 S rRNA in acrylamide gels [1]) was essentially free of 42 S, 38 S and double-stranded

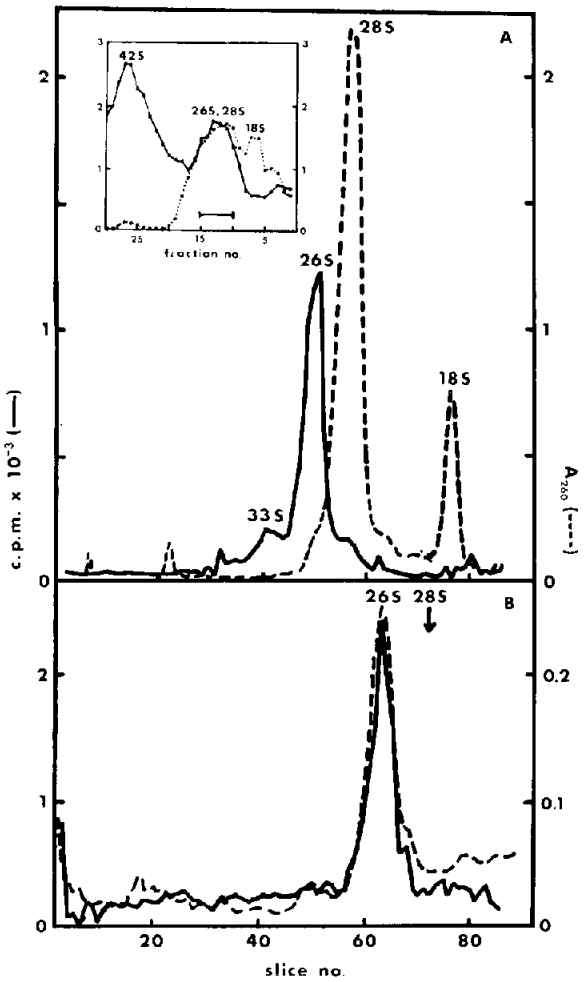

Fig. 1. Purification of 26 S RNA from infected cells. Up to 25 mg RNA were fractionated by zonal centrifugation in a B X IV rotor. The 22 ml fractions from the gradient were analysed for radioactivity and absorbance at 260 nm (insert). The fractions indicated by the bar were pooled and the poly A-containing material isolated by chromatography on oligo(dT)-cellulose. Samples of the RNA before (A) and after (B) the chromatography step were analysed on 2% acrylamide gels [11] which were scanned at 260 nm in a Gilford spectrophotometer and sliced to determine the distribution of radioactivity.

Table 1
Immunoprecipitation of the products of cell-free protein synthesis

Messenger	Cpm per 5 μl sample precipitated by: A Hot TCA	B pre-immune serum	C SFV-specific antiserum	D* Specific pptn. (%)
None	1996	302	380	4
EMC virus RNA (1 μg)	8300	574	792	4
SFV 26 S RNA (2 μg)	38 213	2215	19 551	45

* Specific precipitation = 100 × (C-B)/A.

viral RNAs, but detectable levels of 33 S RNA were found (fig. 1). The bulk of the RNA in the fraction, the cellular 28 S and 18 S ribosomal RNAs, was almost completely removed by oligo(dT)-cellulose chromatography. The material binding to the column consisted of a single major electrophoretic peak of coincident radioactivity and absorbancy, corresponding to 26 S RNA, together with very much smaller quantities of 33 S viral and 28 S ribosomal RNA. The 26 S RNA represented 1–2% of the total RNA extracted from the cytoplasm, and was used as messenger RNA in experiments described here.

Addition of this RNA to a pre-incubated cell-free protein synthesizing system from L cells [5–7] resulted in a 10- to 20-fold stimulation of the incorporation of labelled methionine. On a weight basis this stimulation is at least as efficient as that given by RNA from encephalomyocarditis (EMC) virus (a gift of Dr. I. Kerr) when it is assayed under the optimal ionic conditions [5,7] in this system. The stimulation was completely blocked in the presence of 5×10^{-5} M aurin tricarboxylic acid, an inhibitor of initiation of protein synthesis. The highest levels of activity were seen at potassium and magnesium concentrations of 60 mM and 5 mM, respectively. By contrast, addition of SFV 42 S RNA, extracted either from purified virus or from infected cells, to the cell-free system resulted in little stimulation of amino acid incorporation under a variety of conditions. This relative inefficiency in messenger function is presumably due to the secondary structure of the RNA, since the sequence of the 26 S RNA is included in that of the 42 S.

An antiserum to formaldehyde-fixed SFV, specific for the envelope proteins of the virus [8] was used to examine the relationship of the products of the cell-free system to authentic SFV protein. The immune complex was precipitated using an equivalent amount of anti-rabbit globulin raised in sheep (a gift of Dr. D. C. Kelly). Controls were run using pre-immune serum, with the products of cell-free systems containing no added messenger, and using EMC virus RNA as messenger. From the results presented in table 1 it is clear that only the products of synthesis directed by SFV 26 S RNA are specifically precipitated by the antiserum against SFV and, therefore, that at least a part of this RNA is being translated into virus-specific material.

The products of SFV 26 S RNA-directed protein synthesis, and the material precipitated in the presence of SFV-specific antiserum were compared on SDS-acrylamide gels with SFV structural proteins dansylated to make them fluoresce in UV light [8] (fig. 2). The proteins of the virus particle are the core (C) and the two components of the envelope (E1 and E2). It is apparent that the major product of the cell-free system has a mobility identical with that of the core protein. The relationship between the material migrating faster than the core protein and the virus structural proteins is unknown. Most of the material precipitated by the specific antiserum migrates slightly faster than the envelope proteins. This is consistent with the behaviour of authentic envelope proteins from which the sugar residues have been removed [8], and suggests that the glycosylation process is not operating effectively in the cell-free system. Gels of immune precipitates formed using pre-immune serum contain no detectable radioactivity.

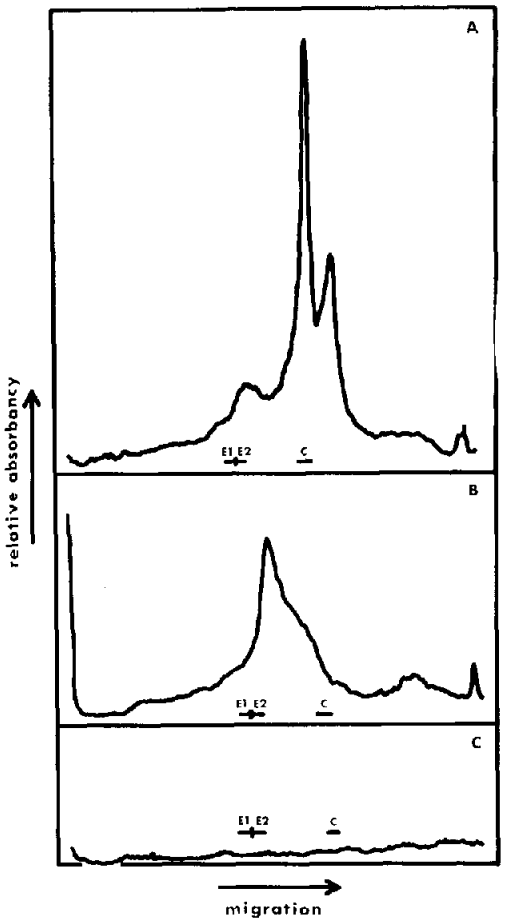

Fig. 2. SDS-acrylamide gel electrophoresis of cell-free products. Samples of incubated cell-free systems were treated and analysed as described in the text. Autoradiographs were scanned at 500 nm in the Gilford spectrophotometer. The positions of authentic SFV structural proteins indicated were those of dansylated proteins co-electrophoresed in the same gel. (A) SFV 26 S RNA-directed total incorporation products; (B) SFV 26 S-directed incorporation products precipitated by antiserum to SFV; (C) endogenous incorporation products.

We conclude that SFV 26 S RNA contains the structural information for the core protein and for at least one of the envelope proteins of the virus particle.

4. Conclusions

The abundance of 26 S RNA in infected cells relative to the other non-virion RNAs [1], its association with polysomes [3,4] and its behaviour in the cell-free protein synthesizing system described here all indicate that the functional role of this RNA species is as a messenger for the structural proteins of the virus. There is evidence that in intact cells these proteins are synthesized via high molecular weight precursors which are proteolytically cleaved to give the final products [10]. If this pathway is followed in the in vitro system the cleavage mechanism must be unusually effective since only the final products can be identified. By contrast, in a cell-free extract directed by EMC or Mengo virus RNA, most of the products are of much higher molecular weight than the structural proteins of the virion ([5,7] and our unpublished results). Finally, the fact that translation of SFV 26 S RNA occurs in a system derived from uninfected cells shows that no factors other than those required for host-controlled protein synthesis are necessary for the activity of this viral messenger.

Acknowledgements

We thank Miss C. J. Bruton for her excellent technical assistance and the Cancer Research Campaign for support for J.C.S.C.

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