Stability and Function of Mengovirus RNA in Cell-Free Protein Synthesis*

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Summary:

Cell-free protein synthesis utilizing mengovirus RNA as message occurs on large (200S) polyribosomes similar in size to polyribosomes formed by viral message in intact Ehrlich ascites tumor (EAT) cells. However, most viral RNA in the cell-free system is not associated with rapidly sedimenting structures, but rather sediments in a 40S peak.

As measured by function, viral RNA is remarkably stable to incubation in the cell lysate. Message function is preserved for over 30 min under conditions where synthesis is reversibly blocked by lack of ATP and GTP. However, 40% of added radio actively-labeled viral RNA is rapidly adsorbed onto the incubation vessel. These results indicate that viral RNA as isolated may seem homogenous by sedimentation, yet be functionally heterogenous, as measured by stability and by involvement in protein synthesis.

Introduction:

Cell-free protein-synthesizing systems from cultured animal cells have been developed recently which utilize messenger RNA isolated from cardiovirus virions (1,3,5). Addition of purified viral RNA to suitably pre-incubated cell lysates has been shown to cause as much as a 50-fold stimulation of aminoacid incorporation into virus-specific polypeptides (1,3). In the course of studies involving mengovirus RNA in a system derived from EAT cells we became interested in the fate of the purified viral RNA added to the cell lysate. In this communication we provide evidence that the viral RNA which serves as template for protein synthesis in this cell-free system is incorporated into polyribosomes of predominantly 200S sedimentation coefficient (about 8-10 ribosomes). However, only a small proportion of

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viral RNA is utilized in protein synthesis. Much of the added RNA becomes rapidly adsorbed to the tube walls, and most of the remainder sediments at about 40S and is not incorporated into polyribosomes.

Materials and Methods:

The growth of EAT cells, L cells and mengovirus has been described (2). To purify mengovirus, L cells that were infected with 20 PFU/cell in monolayer were harvested 11-12 hr post-infection and a lysate prepared by freeze-thawing 3X. After clarification by centrifugation at 12000xg for 30 min, deoxyribonuclease and ribonuclease were added to 30 ug/ml and 10 ug/ml respectively and incubation carried out for 30 min at 37°. Trypsin was added to 100 ug/ml and incubation continued for 30 additional min. Aliquots of 7.5 ml of the enzyme-treated solution were layered over 5ml of CsCl solution of ρ=1.33 g/cm^3 and centrifuged at 40,000 rpm for 20-40 hr in the Spinco SW41 rotor. The virus banding at ρ=1.33 was pooled, diluted 1:1 with CsCl solution and rebanded in the SW50.1 rotor at 40,000 rpm for 24-40 hr. After desalting on Sephadex G-25 the virus had a 260nm/280nm absorbance of 1.58-1.66.

For viral RNA extraction, sodium dodecylsulfate was added to 5mg/ml and potassium ethylenediaminetetraacetate (EDTA) to 25 mM. The suspension was heated to 45° for 3 min, cooled, and extracted 3X with 2 vol of phenol saturated with buffer (Tris, 1mM, pH 7.3). The RNA-containing aqueous phase was extracted 2X with ether, followed by treatment with dry N_2. After cooling to 4°, NaCl was added to 0.5 M followed by 2 vol of cold ethanol. The solution was kept at 4° overnight, then centrifuged at 10,000xg for 30 min and the pellet dissolved in TE

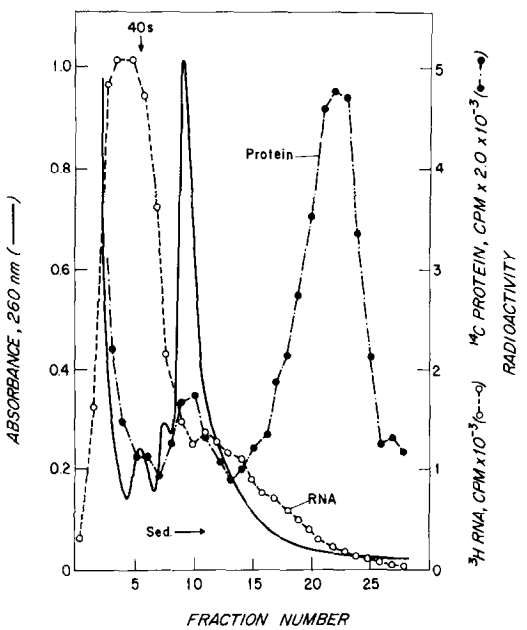

Figure 1: Incubations were carried out as described in the text. Aliquots (0.5 ml) of the incubation mixtures were layered on 20 to 50% sucrose gradients and run at 41,000 rpm for 2.5 hr in the Spinco SW41 rotor and fractions collected and processed for radioactivity. See references 2 and 4 for details.

buffer (1mM Tris, pH 7.3; 0.1 mM EDTA). Viral RNA purified in this manner sedimented as a single peak at about 38S in sucrose gradients. For the preparation of 3H-viral RNA, cells were infected and virus growth carried out in medium containing 3H-uridine.

The preparation of the pre-incubated 10,000xg supernatant (S-10) from EAT cells by hypotonic Dounce homogenization followed the procedure of Eggen and Shatkin (3).

The complete incubation mixtures contained: 1 mM Tris-HCl, pH 7.4; 100 mM KCl; 60 mM NH_4 acetate; 1.2 mM ATP; 0.3 mM GTP; 5 mM phosphoenol pyruvate; 50 ug/ml of pyruvate kinase; 6 mM 2-mercaptoethanol; 4 mM Mg acetate; 0.05 mM each of the 20 unlabeled amino acids, or for labeling, 0.05 mM of the 16 unlabeled amino acids plus 15 uci/ml of (14C) L-valine (s.a., 250 mci/mmole), 20 uci/ml of (14C) L-proline (s.a., 213 mci/mmole), 30 uci/ml of (14C) L-leucine (s.a., 300 mci/mmole) and 20 uci/ml of (14C) L-aspartic acid (s.a., 167 mci/mmole). S-10 was at 5 mg/ml and composed half the final mixture volume. Incubations were at 37°C.

Results:

Figure 1 shows the distribution on a sucrose gradient of acid-precipitable radioactivity produced by the incorporation of 14C-aminoacids during the first 15 minutes of incubation of lysate with viral RNA message. At least 55% of newly synthesized labeled polypeptides sediment in the polyribosome region with maximum radioactivity at about 200S. When incubation is carried out for 30 min, the 200S peak remains*. However, it now contains only 30% of the acid-precipitable label, the remainder being found toward the top of the gradient, presumably due to release from the polyribosomes.

When 3H-labeled viral RNA is incubated for 15 min in the system it is found to sediment mainly in a broad peak around 40S in size (Fig. 1, dashed line). Only some 15% sediments in the 200S region where active protein synthesis occurs. Other experiments have shown that the proportion of radioactive viral RNA present in the 200S region is similar at 15,30, and 60 min of incubation. Thus only a small proportion of added viral RNA is ever actually serving as template in the system.

In the course of incubating radioactive viral RNA for gradient analysis, it became evident that viral RNA was being lost from the incubation solution. To examine this further, 3H-labeled viral RNA was added to the complete system and after various periods aliquots were removed and assayed for total and acid-precipitable radioactivity (Table 1). It can be seen from measurements of total radioactivity (column 1) that about 40% of viral RNA is lost from the incubation

*Protein synthesis is linear for 90 minutes in this system.

Table 1

Adsorption and Degradation of Viral RNA

Length of Incubation (min)	Total (cpm)	Acid Precipitable (cpm)
0	13,100	12,000
15	8,200	7,500
30	7,800	6,000
60	7,500	5,800

Lysate S-10 and the incubation mixture with unlabeled aminoacids were incubated at 37°C with 3H-viral RNA and aliquots processed for total and acid precipitable (5% trichloracetic acid) counts at times indicated.

solution by 15 min and that further incubation does not appreciably increase this loss. The loss of total radioactivity from the solution implies adsorption of viral RNA to the tube walls, and this was confirmed by elution from the tubes with hot acid. Similar findings were obtained when incubations were conducted in glass tubes, silicone-treated glass tubes, polypropylene tubes, and polyallomer tubes. As the non-acid-precipitable radioactivity in the solution increases only slowly, the labeled viral RNA is apparently not being extensively hydrolyzed by nucleases. Varying the total concentration of viral RNA added from 0.1 to 10 times that normally used in routine incubations did not alter the proportion of labeled RNA adsorbed to the walls.

Since viral RNA was being adsorbed from the incubation, it was important to determine whether adsorption was playing a role in the decline of initiation which occurs after 15-20 min of incubation. Table 2 shows the results when viral RNA stability in the system is measured functionally. Without the energy provided by the addition of ATP and GTP to the system, no incorporation occurs. Hence it is possible to add viral RNA to the system and incubate it for varying times in the absence of synthesis. Completion of the system by the addition of ATP and GTP allows protein synthesis to commence. The results in Table 2 indicate that,

Table 2

Stability of Viral RNA Function at 37°

Length of Pre-incubation (min)	Pre-incubated with ATP & GTP	Pre-incubated with viral RNA	Incorporation in 60 min after system completed (cpm)
0			16,000
15	-	-	15,000
15	-	+	14,600
30	-	-	14,000
30	-	+	13,700

The complete system utilized S-10 lysate and the aminoacid incorporating mixture described in the text with viral RNA at 10 ug/ml. As indicated, ATP-GTP and/or viral RNA was left out for a 30 min pre-incubation at 37°C. Subsequently ATP-GTP and/or viral RNA was added and the completed systems incubated for 60 min at 37°C.

despite the fact that 40% of the viral RNA is adsorbed on the walls in the first 15 min., the viral RNA which is serving as template is functionally stable for at least 30 min as regards its subsequent ability to initiate and continue protein synthesis.

Discussion:

In as much as over 95% of the amino acid incorporation in this preincubated system is virus-stimulated (ref. 3, and unpublished observation), the peak of labeled protein at about 200s must be due to ribosomes associated with viral RNA, corresponding to polyribosomes containing 8-10 ribosomes. We have found that virus-directed protein synthesis in the intact EAT cell involves polyribosomes of similar size, and others have reported picornavirus RNA-directed polyribosomes of 250-350s in Hela cells (6). However, our results indicate that less than 10% of added viral RNA (15% of the 60% not adsorbed to the walls) is in polyribosomes in the cell-free system. In addition, while the RNA which serves as template for protein synthesis is stable for at least 30 minutes (Table 2), a large fraction of the remaining RNA is subject to rapid adsorption to the walls of the tube (Table 1). Thus the viral RNA is functionally heterogenous. It is not clear whether this

functional heterogeneity is due to the partition of identical viral RNA molecules among several pathways, or is due to a microheterogeneity in the purified viral RNA, for instance the loss of a few critical nucleotides from the ribosome binding site of a majority of the viral RNA molecules. The possibility of microheterogeneity is suggested by the fact that we have found up to three-fold differences, on a per ug RNA basis, in the ability of different, but similarly produced, viral RNA preparations to stimulate protein synthesis (unpublished observation). Microheterogeneity would also explain the wide range over which the proportionality between active and inactive RNA is maintained. Thus, incorporation is proportional to input viral RNA up to 2 ug per 50 ul of lysate, implying that the same proportion of added RNA is utilized at each level, despite the fact that even at 2 ug the majority of ribosomes are not incorporated into functioning polyribosomes, and the system is therefore far from saturated with respect to mRNA over this range. (Attempts to saturate the system with mRNA are frustrated by the inhibitory effect of large amounts of viral RNA (ref 3,5,and unpublished observation)). However, a definitive demonstration of heterogeneity in the purified RNA requires a method for isolating active and inactive fractions, and this has not been possible to date.

In the system used, the initiation step in protein synthesis only continues for the first 15-20 min of incubation. However, synthesis by elongation occurs at a fairly constant rate from the 15th min through the 90th min of incubation. The evidence that a constant amount of viral RNA remains in the polyribosome region from the 15th to the 60th minutes is consistent with the observed kinetics of aminoacid incorporation.

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