A STUDY OF THE COMPLEXING OF VIRAL RNA WITH CELL PROTEINS

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Data have been obtained regarding certain physical and chemical properties of chick fibroblast proteins which form in vitro complexes with the RNA of Venezuelan equine encephalitis virus. Soluble cell proteins which are not sedimented by centrifugation at 105,000 g were shown to form complexes with viral RNA. These proteins firmly adsorb on DEAE cellulose at pH 6.8 and are eluted only in 0.3 M NaCl. Sedimentation analysis revealed three classes of proteins forming complexes with RNA. It was determined that the process of complexing of viral RNA with cell proteins proceeds most effectively in solutions with low and medium ion strength (0.01 and 0.1 M NaCl). The fact that protein-RNA complexing is inhibited by high salt concentrations (0.5 and 1 M NaCl) suggests that electrostatic forces play a role. A relationship was found between the sedimentation rate of the resulting ribonucleoprotein complexes and the amount of protein complexing with RNA.

Two tables. Three illustrations. Eighteen bibliographic entries.

I has been shown that cytoplasmic extract of animal cells contains proteins

of high molecular weight which form *in vitro* complexes with various species of RNA and in particular with messenger RNA [3, 4, 6, 11, 12, 17]. The resulting ribonucleoprotein structures are similar in their physical and chemical properties to the informosomes isolated from animal cell homogenates [1]. The latter appear to play a substantial role in preserving and transmitting genetic information [7, 8, 14, 15] and may participate in the process of initiating translation [13, 16].

The phenomenon of the complexing of viral RNA with cell proteins is of special interest. Viral RNA belongs to the special group of template RNAs and differs from cellular RNA in a variety of biological, physical and chemical properties. We showed earlier that, when infectious RNA of Venezuelan equine encephalitis (VEE) virus is introduced into chick fibroblast homogenate, and also when the microsomal-mitochondrial fraction extracted from VEE-infected cells is incubated, hybrid ribonucleoprotein complexes consisting of viral RNA and cell proteins are formed [2-4]. The hybrid ribonucleoproteins (RNP) have an infectious property which is not neutralized by virus-specific serum. At the same time, the different techniques employed to identify the infectiousness of RNP complexes and of viral RNA permits these structures to be easily differentiated [17].

Little study has been devoted to the physical and chemical properties of cell proteins forming hybrid RNP complexes, or to the nature of their interaction with viral RNA. Some data on these questions are contained in the literature [9, 11] and are presented in the present study.

MATERIALS AND METHODS

Viruses and cells. All experiments were conducted with VEE virus (SPF strain) and chick embryo fibroblasts (CEF). Culture and purification methods have been described earlier [10, 18].

In order to obtain virus labelled with ^{3}H- and ^{14}C-uridine, the virus was cultured in a depleted culture medium (medium No. 199 diluted three times with Hanks' saline) containing radioactive precursors in a concentration of 5 μCi/ml.

RNA isolation. Cultured and purified virus was used to isolate viral RNA, and CEF cells to isolate ribosomal RNA. Extraction was carried out three times with phenol in the presence of 0,5% sodium dodecyl sulfate at 65° [5]. RNA was precipitated with ethanol, cooled in an acetone-dry ice mixture and stored at -20°. Before use RNA samples were reprecipitated with alcohol. The absorption spectrum of the extracted RNA was found to be SF-16.

Preparation of cell proteins. Trypsinized CEF cells were sedimented by centrifugation at 2,000 RPM for 15 minutes. The sediment was washed with a large volume of 0,1 M phosphate-sodium buffer (PB) (pH 7,2), and the cells were reprecipitated in the same manner. The precipitate was homogenized in 20-3- ml of 0,01 M PB at pH 7,2. The cell debris was centrifuged at 15,000 RPM for 20-30 minutes. The supernatant was centrifuged at 105,000 g for one hour in an angle rotor. The resulting supernatant (S-105 fraction) was drawn off by pipette from the bottom of the test tube to avoid admixture of the upper layer of lipids. The proteins thus obtained were stored at -20°. A portion of them

were used for further fractionation in a column containing fibrous DEAE cellulose. Regeneration of the exchangeant was carried out by the usual method. Elution was performed by the method described in [11]. Twenty ml of the S-105 fraction (protein concentration = 27 mg/ml) was introduced into a column measuring 1.5 x 18 cm which had been previously neutralized with 0.02 M PB at pH 7.4. The excess of uncombined protein was eliminated by passing 5-6 volumes of 0.02 M PB at pH 6.8 through the column. (Testing for absorption was conducted at 260 and 280 nm.) The proteins were eluted with a stepwise NaCl salt gradient (0.3 and 1 M) prepared on the same buffer. The fractions were collected in 7 ml test tubes and absorption was determined at 260 and 280 nm. To obtain higher concentrations the proteins were salted out with 70% (of saturation) $(NH_4)_2SO_4$ at 4° for 24 hours and centrifuged at 5,000 RPM for ten minutes. The precipitate was suspended in 0.01 M PB at pH 7.2 and dialyzed for 48 hours against 1,000 volumes of PB in a magnetic mixer. The proteins thus obtained were stored at -20°.

Preparation of complexes of viral RNA with cell proteins. The S-105 fraction and proteins purified in the column were added to the RNA isolated from cultured and purified virus.

Complexing was carried out for 10 minutes at 0° in a 1 ml volume. The amount of RNA and proteins varied according to the purpose of the experiment.

Adsorption of ribonucleoprotein complexes on Millipore filters. In order to adsorb complexes of labelled viral RNA with cell proteins, HVFS membrane filters (Czechoslovak SSR) with pore size of 0.3-0.5 microns were used. The filters were first soaked in a solution of yeast RNA with a concentration

of 1 mg/ml in order to reduce the adsorption of free viral RNA [11]. The RNP complexes were precipitated in a membrane filter without adding trichloroacetic acid (TCA) and casein to the sample. The filters were washed with 10 ml of Tris buffer (0,1 M Tris, 0,01 M NaCl) or 0,01 M PB at pH 7,2. In these conditions the adsorption of free viral RNA on the filters did not exceed 5% of the quantity introduced. The filters were dried at room temperature, then placed in flasks containing toulene scintillator (PPO + POPOP) and the radioactivity of the samples was counted in a Packard-Tricarb liquid scintillator counter (USA).

Centrifugation of proteins in sucrose density gradient. The S-105 fraction and proteins purified in the column were layered over a 3-20% (by weight) linear sucrose gradient prepared in 0,1 M NaCl 0,01 M PB pH 7,2. Hemoglobin with a sedimentation constant of 4,5 S was used as a marker [6]. Centrifugation was carried out in a 3 X 32 ml bucket rotor of a Superspeed-50 ultracentrifuge (MSE, England) at 23,000 RPM for 16½ hours. The fractions were collected from the bottom of the test tube by extrusion using a system consisting of a compressor, an LKB collector, a recording Uvicord spectrophotometer (λ = 254 nm). Specimens were taken from each sample to measure absorption at 260, 280 and 410 nm (maximum absorption of hemoglobin) and to determine the complex-forming activity of the fractions. For this purpose all fractions of the sucrose gradient were dialyzed for 24 hours at 4° against 0,1 M NaCl 0,01 M PB at pH 7,2. Then 100-200 μg of labelled viral RNA (1,000-2,000 CPM) isolated from the culture virus were added to the samples, and the smaples were precipitated in membrane filters having a pore diameter of 0,2-0,5 microns, as described above. The filters were dried and the radioactivity in them was counted.

Centrifugation of viral RNA and ribonucleoprotein complexes in sucrose density gradient. The material was layered onto a 10-30% (by weight) sucrose gradient prepared on Tris buffer (0.01 M Tris, 0.1 M NaCl, 10^{-4} M EDTA). Centrifugation was carried out in a 3 x 23 ml bucket rotor of a Superspeed-50 ultracentrifuge (MSE, England) at 18,000 RPM for 13½ hours. The fractions were collected from the bottom of the test tube by the system described above.

Casein and TCA were added to the fractions to a concentration of 5%. The samples were precipitated in membrane filters, washed with 5% TCA and 96% ethanol, dried, and the radioactivity in them was counted.

Determination of infectiousness of ribonucleoprotein complexes. The infectiousness of the RNP complexes was determined by titration using the plaque method. Dilutions of the material were prepared in 0.1 M PB at pH 7.2 and 0.2 ml of the dilution was placed on a CEF monolayer. After 40 minutes of adsorption at 20° the infected cells were covered with a layer of agar medium prepared with neutral red (1:10,000) in accordance with Melnik's formula and incubated in a thermostat at 37°. Results of the titration were assayed after 48 hours.

Reagents. ^{3}H- and ^{14}C-uridine (specific activity = 0.3 Ci/mmol) were obtained at the Radioisotope Center (Leningrad). Membrane filters with a pore diameter of 0.3-0.5 μ were obtained from the Synpor firm (Prague). Hemoglobin (from horse blood) was obtained from the Reanal firm (Hungary). DEAE cellulose in the form of cotton wool was produced by the Donetsk Chemical Reagent Factory.

RESULTS

Purification of cell proteins which form complexes with viral RNA on DEAE cellulose. It has been shown that cell proteins forming complexes with viral RNA are not precipitated by centrifugation at 105,000 g [17]. These data were confirmed in the present work. With a view to further purifying the S-105 fraction, chromatography of the proteins in a column containing

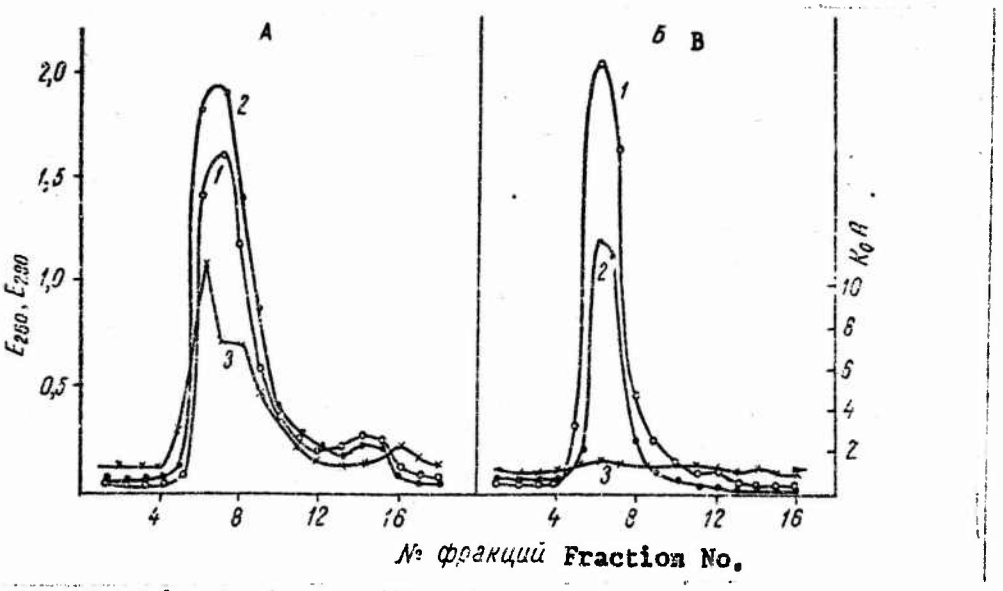

Fig. 1. Protein elution profiles in a column containing DEAE cellulose. Elution was carried out with a stepwise salt gradient: A -- 0.3 M NaCl, B -- 1 M NaCl. The elution profiles were determined by photospectrometry. 1 -- absorption at 260 nm; 2 -- absorption at 280 nm; 3 -- CFA of fractions. CFA was computed as the ratio of adsorption of RNP complexes on membrane filters (in CPM) to the adsorption of free viral RNA (in CPM).

DEAE cellulose was carried out. Figure 1 shows the elution profiles of the S-105 fraction. As may be seen, the material eluted from the column by 0.3 M NaCl (Fig. 1A) contains basically proteins ($E_{280}/E_{260} \sim 1.3$), while that eluted by 1 M NaCl (Fig. 1B) contains basically RNA ($E_{260}/E_{280} \sim 2$).

The complex-forming activity (CFA) of the fractions was determined by their capacity to retain labelled viral RNA in the membrane filters. For this purpose labelled viral RNA was added to the fractions from the column and the samples were precipitated in the filters under the conditions in which RNP complexes were adsorbed on them (see Materials and Methods). Figure 1 shows that the proteins eluted from the column with 0.3 M NaCl formed complexes with viral RNA, while the material eluted with 1 M NaCl was only slightly active in this regard.

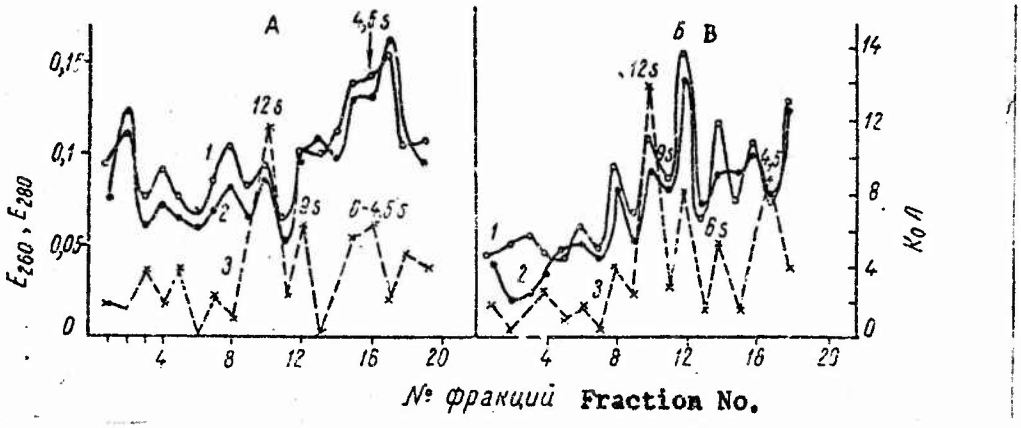

Fig. 2. Distribution of protein in a 3-20% sucrose density gradient. Centrifugation was carried out in a 3 x 23 ml bucket rotor of a Superspeed-50 ultracentrifuge (MSE, England) for 16 hours at 23,000 RPM. Distribution curves were determined at 260 nm (1) and 280 nm (2). Complex-forming activity of the fractions (3) was computed as described in Fig. 1.

Fractionation of cell proteins forming complexes with viral RNA in a sucrose gradient. Data found in the literature [11] give reason to suppose that a heterogeneous class of soluble cell protein forms complexes with viral RNA. It is therefore of interest to study the distribution of proteins in a sucrose density gradient and to determine their capacity to form complexes with viral RNA. Figure 2 shows the sedimentation distribution of the proteins

of the S-105 fraction and of the protein purified in the column. The complexes with viral RNA are seen to form three basic classes of proteins: 12 S, 9 S and 6-4.5 S, the complex-forming activity of each class being different. Similar classes were not found when the material eluted from the column by 1 M NaCl was studied in the sucrose density gradient.

Table 1

The effect of NaCl concentration on the formation of RNP complexes

Material	RNA retention on filters with NaCl concentrations of: 0.01 M		0.1 M		0.5 M		1.0 M	
	CPM	*	CPM	*	CPM	*	CPM	*
RNA	93	1	98	1	103	1	83	1
RNA + fraction	2367	26	1773	18	235	2	265	3
RNA + column-purified protein	1167	13	1380	14	367	4	317	4

Note. To 1 mg of proteins were added 100 μg (2839 CPM) of ^{3}H-Uridine-marked viral RNA isolated from culture virus. The adsorption of free viral RNA did not exceed 3.5%.

* Here and in Table 2: the ratio of the adsorption of RNP complexes on Millipore filters (in CPM) to the adsorption of free viral RNA (in CPM).

The influence of ion strength on the complexing of viral RNA with cell protein. The nature of RNA's interaction with protein is unclear, but electrostatic forces may be assumed to play a role. It was of interest to study the effect of ion strength on the formation of RNA complexes. For this purpose labelled viral RNA in solutions with various concentrations of NaCl (0.01, 0.1, 0.5 and 1 M) was added to the S-105 fraction and protein purified in the column.

Four experiments were performed and yielded similar data. Table 1 shows the results of one of these experiments. As is seen, complexing of viral RNA with cell proteins is observed in solutions with low and medium ion strength. Increasing the salt concentration to 0,5 and 1 M significantly lowers the adsorption of labelled viral RNA on Millipore filters.

The effect of protein concentrations on the formation of RNP complexes. The next series of experiments studied the effect of the concentration of proteins forming complexes with viral RNA on the amount of RNA adsorption on membrane filters. For this purpose dilutions of protein purified in the column were used. A constant amount of labelled viral RNA was added to the proteins and the samples were sedimented in Millipore filters under the conditions in which RNP complexes were adsorbed. The results are shown in Table 2. As the concentration of proteins added to viral RNA is decreased, the adsorption of RNA on Millipore filters may be seen to increase. A definite relationship may be considered to exist between the amount of RNA adsorbed on the filter and the amount of protein which has complexed with it.

In studying the effect of protein concentration on the sedimentation rate of viral RNA in a sucrose density gradient it was possible to show a relationship between RNA weight and the amount of protein complexing with RNA. For this purpose various concentrations of protein were added to marked viral RNA and, after complexing took place under optimal conditions, centrifugation in a sucrose gradient was carried out. The results are shown in Fig. 3. In the first case the RNA:protein weight ratio was 1:6 and in the second it was 1:1.5. Distribution of viral RNA in the gradient served as control (Fig. 3B). As is seen, the radioactivity peak of viral RNA was displaced in these cases toward

the bottom of the gradient and sedimented in positions 85 S and 57 S respectively; i.e. the sedimentation rate of the RNA after complexing grew by 2,1 and 1,4 times. The position of the radioactivity peak of the heavy viral RNA corresponded to maximum infectivity, which was shown by titrating the material by the method used to titrate virus and constituted 4,0-4,2 PFP/ml in both cases.

Table 2

Adsorption of viral RNA on Millipore filters as a function of the amount of protein complexing with the RNA

Protein dilution	Experiment No. 1		2		3	
	CPM	*	CPM	*	CPM	*
Undiluted	399	13	265	8	252	8
1:2	383	12	271	8	210	7
1:4	339	11	243	7	149	5
1:8	139	5	104	3	54	2
Control RNA	30	1	30	1	30	1

Note. Fifty μg of ^{3}H-uridine-labelled viral RNA isolated from culture virus were added to the protein dilutions. Protein concentrations in the experiments equalled 0,23, 0,25 and 0,2 μg/l respectively.

DISCUSSION

In the present work the formation of complexes of viral RNA with cell proteins was examined [1] in terms of their capacity to be precipitated on membrane filters with a pore diameter of 0,4 μ [2], their sedimentation constant [3], and the infectivity of the complexes on tissue culture, determined

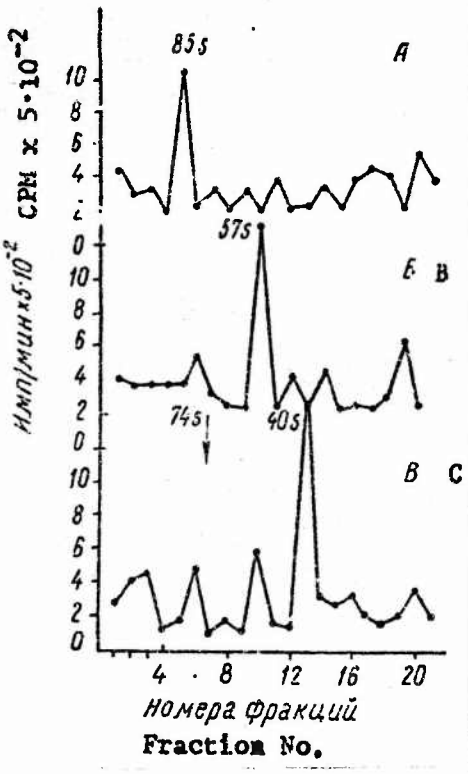

Fig. 3. Sedimentation rates of viral RNA in 10-30% (w/w) sucrose density gradient as a function of the amount of protein complexing with RNA. A -- RNA:protein ratio = = 1:6; B -- 1:1.5; C -- viral RNA. Centrifugation was carried out in a 3 x 23 ml bucket rotor of a Superspeed-50 ultracentrifuge for 13 hours at 18,000 RPM.

by the method used to titrate virus. Some preference was given to the first test, which is the simplest to perform and assures a good quantitative verification of complex formation. Study of the complex-forming activity of cell proteins showed that soluble CEF proteins of an apparently non-basic character, which are not sedimented by centrifugation at 105,000 g and adsorb solidly on DEAE cellulose at pH 6.8, form complexes with the RNA of VEE virus. Other authors [11] also obtained these data in studying the interaction of poliomyelitis virus with HeLa cell proteins. Sedimentation analysis revealed three classes of proteins -- 12, 9 and 6-4.5 S -- forming complexes with viral RNA. According to the literature [6, 9] rat liver proteins with a sedimentation constant of 9 S and sometimes 6-7 S form complexes with ribosomal RNA. This appears to reflect structural and functional differences between viral and ribosomal RNA. The conformation of RNA in solution is known to be determined to a considerable extent by ion strength. Our research indicates that the complexing of viral RNA with cell proteins occurs most effectively in solutions with low and medium ion strength and is inhibited in solutions with high ion strength. This may be explained by changes in the conformation of viral RNA. When the ion strength is decreased, the forces of electrostatic

repulsion between phosphate groups of like charge in the RNA molecule increase, which leads to unfolding of the chain. When the ion strength is increased, the charges are screened and the macromolecule folds up into a compact coil, inhibiting RNA-protein interaction.

The biological significance of the phenomenon of complexing of viral RNA with cell proteins is not ultimately clear. The possibility, demonstrated in the present work, that hybrid RNP complexes may be formed in isotonic solutions implies the existence of such structures in the infected cell. Study of the relationship between the weight of the viral RNA in the sucrose density gradient and the amount of protein complexing with the RNA permits the conclusion that when there is insufficient protein it is distributed evenly among all RNA molecules and an appreciable increase in the sedimentation rate of RNA does not occur.

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INVESTIGATION OF THE PHENOMENON OF COMPLEXING OF VIRAL RNA WITH CELL PROTEINS

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Information of some physico-chemical properties of chick fibroblast proteins forming in vitro complexes with Venezuelan equine encephalomyelitis virus RNA has been obtained. Soluble cell proteins which are not sedimented by centrifugation at 105,000 g have been shown to form complexes with viral RNA. These proteins adsorb solidly on DEAE-cellulose at pH 6.8 and are eluted from it only with 0.3 M NaCl. The sedimentation analysis revealed three classes of proteins according to the sedimentation constant which form complexes with RNA. It has been demonstrated that the process of complexing of viral RNA with cell proteins occurs most effectively in solutions of low and moderate ion strength (0.01 and 0.1 M NaCl). The fact that RNA complexing with protein is inhibited by high concentrations of salt (0.5 and 1 M NaCl) suggests participation in it of electrostatic forces. The relationship between the sedimentation rate of the resulting ribonucleoprotein complexes and the amount of protein complexing with RNA has been established.