THE PHENOMENON OF HYBRID RIBONUCLEO-PROTEIN COMPLEXES ("PSEUDOVIRUSES") FORMATION

F. I. Ershov, et al

Army Medical Research Institute of Infectious Diseases
Frederick, Maryland

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F. I. Ershov, V. M. Zhdanov, L. V. Uryvaev, A. S. Agabalyan

Summary

During reproduction of some RNA viruses or after exposure of the infectious RNA isolated from the virions with various cytoplasmatic fractions of the animal cells the formation of hibrid RNP-complexes ("pseudoviruses") is recorded. These present characteristic biological and physico-chemical properties (infectivity, resistance to antiviral sera, partial sensitivity to the action of ribonuclease, sedimentation constants ranging between 80S and 160S, buoyant density in the Cesium gradient from 1.30 to 1.43 g/cm³).

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The Phenomenon of Hybrid Ribonucleoprotein Complex ("Pseudovirus") Formation

F.I. Ershov, V.M. Zhdanov, L.V. Uryvaev, A.S. Agabalyan - D.I. Ivanov Virological Institute, AMN USSR, Moscow

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We demonstrated earlier that virus-specific RNA and protein are synthesized in the preformed subcellular structural system isolated from infected cells (F.I. Ershov, et al, 1969; V.M. Zhdanov, et al, 1969, 1970). Within this very system reliable growth of infectious titers has been observed 90-99 times (F.I. Ershov, et al, 1970).

The group A - Venezuelan Equine Encephalomyelitis (VEE), causing severe illness in horses after infection by mosquitoes or through the air, provided the basic model for our experiments. Ribonucleic acid (RNA), enclosed within the inner protein and outer protein-lipid shell, formed its genetic material.

Materials and methods. The method described by us earlier was used: VEE virus and fibroblasts of chicken embryo (F.I. Ershov and V.M.Zhdanov, 1968).

The following procedure was used: cultivation, concentration, purification of the virus; separation of RNA; determination of its infective activity; cell fractionalization; sedimentation analysis; centrifugation in a cesium chloride (CsCl) gradient, etc. A detailed description of these methods appears in published works (F.I. Ershov, et al, 1968-1970; V.M. Zhdanov, et al, 1969, 1970; L.V. Uryvayev, et al).

Formation of two ribonucleoprotein types on preformed subcellular structures

It has been shown during incubation of so-called mitochondria-microsomal fraction (MF-15) isolated from cells infected with VEE virus, active synthesis of virus-specific material can be observed. Moreover, virtually all the RNA-polymerizing activity (Martin and Sonnabend; F.I. Ershov, et al, 1970) and replicating

complex (V.M. Zhdanov, et al, 1969) of this virus appear in this fraction. The final product formed during MF-15 fraction incubation in an optimal cultivative medium is infectious ribonucleoprotein (RNP), which proved heterogenic in terms of sedimentation and density. The basic tests followed the plan given below. Cells previously treated with actinomycin D were infected with a massive dose of VEE virus (50-100 BOE/ml) and incubated for 2½-3 hours. Then, the culture was rapidly cooled and washed with Hank's solution and the MF-15 fraction was isolated and incubated in No. 199 medium at 37°. At various intervals H^3-uridine (20 microcuries/ml) and C^{14}-amino acid (2 microcuries) were added. The product was investigated in a linear density gradient of saccharose or in an equilibrium density gradient of CsCl.

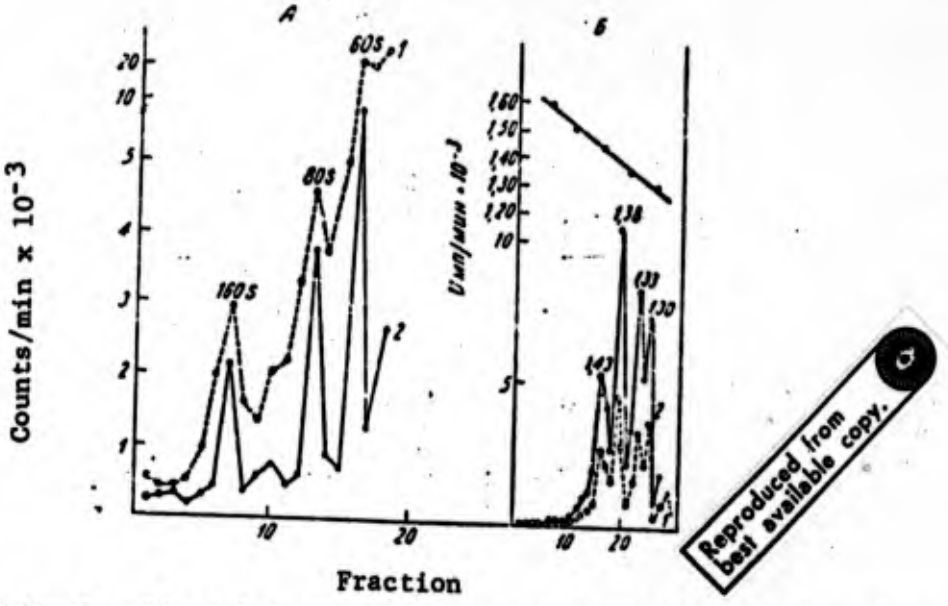

Fig. 1. Distribution of freshly synthesized RNA (1) and protein (2)

A-in fractions of a density gradient of 15-60% saccharose after centrifugation at 25,000 rpm for 2 hrs of MF-15 fraction obtained 2½ hrs after infection of the cells and incubated again for 3 hrs in the presence of H^3-uridine and C^{14}-amino acid; B - after centrifugation of the same material in a CsCl equilibrium gradient.

It was noticed during these investigations that along with RNP, which has sedimentation (160 S) and density (ρ=1.43 g/cm^3) characteristics which precisely correlate with RNP parameters of VEE virions, in subcellular structures the same synthesis and formation of RNP occurs, having other sedimentation (80 S and lower) and density (ρ=1.38, 1.33 and 1.30 g/cm^3) characteristics (Fig. 1). The result serves as the basis for assuming that along with virus-specific RNP, during incubation of MF-15 fraction as a result of complexing of viral RNA with cellular protein hybrid RNP is formed. Experiments to determine the infective ability of the RNP obtained and tests of its sersitivity to the neutralizing effect of antiviral serum were necessary to back up this hypothesis. The results of these tests are summarized in Table 1.

It follows from the data in Table 1 that all types of RNP synthesized

in the MF-15 fraction possess infective ability. Moreover, for all practical purposes virus-specific antiserum neutralizes only RNP with a buoyant density of 1.43 g/cm^3, e.g. RNP the parameters of which correlate with those of the virion. The composition of the remaining types of RNP (1.30, 1.33, and 1.38 g/cm^3) apparently include cellular protein on which antiserum has no effect.

In situ formation of "pseudovirus"

To the extent that formation of RNP consisting of viral RNA and cell protein occurs in MF-15 fraction separated from infected cells together with virus-specific RNP, interest has been expressed in explaining if similar formation of hybrid RNP-complexes occurs in situ. Infectious RNA isolated from VEE virus was, with this in mind, added to various fractions obtained from homogenate of uninfected cells, and after contact at 0° for 30 minutes the infective activity of the product was determined. It must be stressed that, as we noted earlier, the method for explaining infective activity permits differentiation of infective RNA from mature virus (V.M. Zhdanov, et al, 1969). During titration of RNA preliminary treatment of cells with hypertonic solution (method No. 1) is necessary while the usual agar coating (method No. 2) is used during VEE virus titration.

Table 1

Infective activity and sensitivity to the neutralizing effect of antiserum of various types of RNP formed during incubation of MF-15 fraction

Density (g/cm^3)[1]	Infectivity titer (in BOE/ml)		lg neutralization
	original	after serum treatment	
1.30	5.6	5.3	0.3
1.33	6.3	6.1	0.2
1.38	6.1	5.7	0.4
1.43	7.1	2.3	4.8

1 In the experiment CsCl gradient of the fraction was taken which correlated with the densities obtained after centrifugation at 35,000 rpm for 16 hrs.

Table 2

Infectivity of the product formed in fractions of cell homogenate after application of infectious RNA from VEE virus

No	Fraction	BOE in 1 ml		in 3 tests
1	800g (10 min)	2.4	3.4	2.9
2	15,000g (20 min) (MF-15)	2.6	3.7	2.8
3	Supernatant after No. 2	2.8	4.2	3.3
4	Cellular fluid[1]	0.1	0.2	0.0
	Original RNA (control)[2]	2.0	3.1	2.5

1 Obtained after centrifugation No. 3 at 150,000 g for 3 hrs.

2 For titration of RNA method No. 1 was used with preliminary treatment of cells with 1M NaCl

Sensitivity of VEE virus, hybrid RNP, VEE-RNA to ribonuclease

Material	Fraction No[1]	Infectivity: Before treatment[2]	Infectivity: After treatment	Infectivity: After inactivation
		BOE/ml		
VEE virus	--	8.9	8.8	--
Hybrid RNP	1	3.6	2.7	0.9
" "	2	3.8	2.1	0.7
" "	3	3.5	2.8	0.7
RNA of VEE virus	--	3.1	0	3.1

1 Specifications of the fraction the same as in Table 2.
2 Treatment consisted of 20 microns of enzyme in 1 ml for 30 min. at room temperature.

Therefore, in all the tests described below for differentiating experimentally obtained hybrid RNP from the original RNA, the RNP was titrated the same as for mature virus, i.e. method No. 2.

The results of three experiments in which viral RNA was added to whole homogenate after preliminary removal of nuclei, debris, and undestroyed cells, and to mitochondrial fraction (MF-15), and supernatant after removal of this fraction and cell fluid are given in Table 2.

As is apparent from these data, after application of infective RNA to the indicated fractions, formation of hybrid RNP ("pseudovirus"), which is titrated like mature virus, is observed. After contact with cell fluid, pseudovirus formation is not observed, since cellular fluid apparently contains no proteins complexing with viral RNA.

The sensitivity of hybrid RNP to ribonuclease was determined in the following tests. The original VEE virus RNA and whole virus were used as controls.

As follows from Table 3, nature virions are not sensitive to enzymes, while the infective activity of RNA, conversely, completely ceases after treatment with ribonuclease. Hybrid RNP is only partially sensitive to enzyme digestion.

After treatment of hybrid RNP with specific anti-serum, neutralization of its infective activity was barely noticed.

During comparison in a saccharose gradient of the coefficients of sedimentation of the original viral RNA and hybrid RNP formed after contact of viral RNA with cytoplasmic extract, a reliable increase in the sedimentation constant by 1.5-2 times was observed. When the virion RNA of VEE had a sedimentation constant of 40 S, the hybrid RNA generally peaked in the 65-80 S region(Fig. 2).

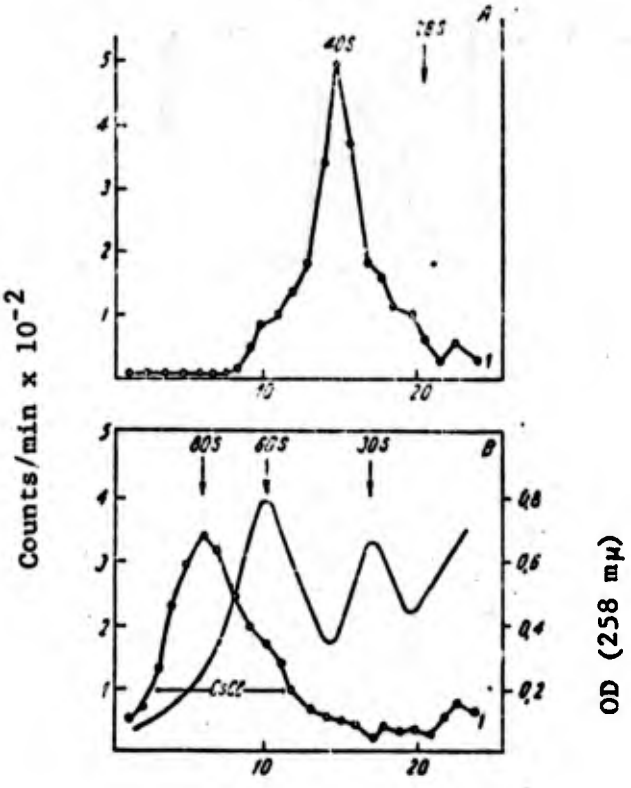

Fig. 2. Distribution of optical density of marker and H^3-radioactivity of tagged RNA of VEE virus (1) during centrifugation in a linear density gradient of saccharose of 5-30% at 37,000 rpm for 2½ hours. A - tagged (by H^3-uridine) RNA of VEE virus before putting in gradient diluted in buffer; arrows indicate the position of the marker (28 S) - cellular RNA; B - "loading" phenomenon - mixing of peak RNA of VEE virus after its contact with homogenate from uninfected cells.

The probability density function of hybrid RNP in a CsCl gradient was analyzed in special experiments. Fractions correlating to peaks which formed as a result of viral RNA interaction with soluble protein of cytoplasmic extract were selected from a saccharose gradient for this purpose. As is apparent from Fig. 3, hybrid RNP peaks 4 times, the buoyant densities consisting of 1.30, 1.33, 1.38, and 1.42 g/cm^3. Each type of RNP shown here is infectious. Thus, hybrid RNP, formed in situ by putting infectious RNA in homogenate of uninfected cells corresponds to various types of RNP, formed during incubation of MF-15 fraction isolated from infected cells.

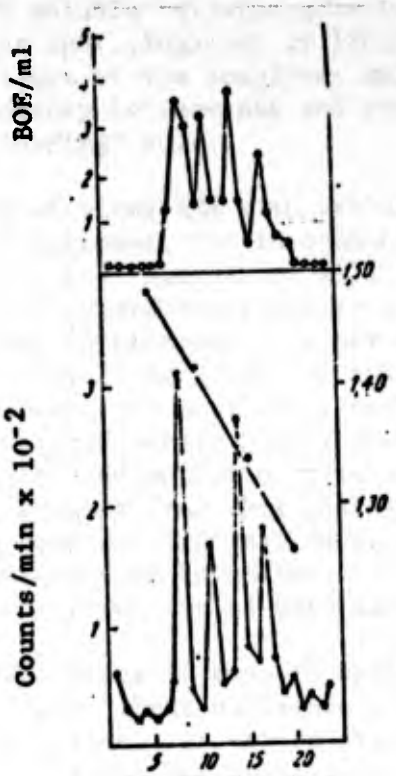

Fig. 3. Distribution of H^3 radioactivity (lower curve) and infectivity (upper curve) during centrifugation of the fraction from a saccharose gradient (selected fractions from third to 11th shown in Fig. 2, B by arrows) in equilibrium CsCl gradient. During titration of infectivity method No. 2. was used.

Structures formed in both cases, which we call hybrid RNP-complexes, possess infective properties, are insensitive to the neutralizing effect of antiserum, and are to a significant degree resistant to ribonuclease.

The above-described formation of particles resembling viruses was also observed during the use of other viral models (Semliki free virus, mouse encephalomyocarditus virus).

Investigation of the sedimentation properties of various hybrid RNP's showed that the complexing of viral RNA with components of cellular extracts leads to an approximate doubling of the rate of sedimentation. A similar RNA "loading" phenomenon was described earlier by Girard and Baltimore in relation to various types of cellular and viral RNA.

The density of hybrid RNP (1.3-1.4 g/cm^3) was always lower than the density of ribosome particles (1.5-1.6 g/cm^3). It may be hypothesized from this that ribosome particles do not enter into the composition of these virus-like particles. Evidently, in systems which have been studied, "loading" depends on the formation of complexes of viral RNA with soluble cellular proteins. The latter, as we showed, are removed from extracts after centrifugation at 150,000 G for 3-4 hours. Various probability density functions of the complexes may be indicated either by the variability in the relationship between RNA and protein or by the fact that various proteins can play a "loading" role.

Evidently, the complexing of viral RNA with cellular protein has the character of a general principle. Moreover, the informosome-type structure may be formed (L.P. Ovchirnikov, et al, A.S. Spirin, et al). The general "loading" phenomenon and concurring density characteristics in a CsCl gradient attest to the similarity of hybrid RNP and informosome. In our experiments the buoyant density of one of the stable peaks (1.42 g/cm^3) of hybrid RNP agrees with the density described for informosomes. It must be stressed that the buoyant density value for tested viral RNA is significantly higher (1.66 g/cm^3). Thus, after contact with cytoplasmic extract the sedimentation rate increased, but the buoyant density decreased. It may be assumed from this that hybrid RNP can be considered a specific stoichiometric RNA complex with less dense components of cell homogenate, which also plays a part in informosome formation. In other words, common mechanisms may lie at the basis of the formation of both hybrid RNP and informosomes.

The formation of hybrid RNP makes it easy to explain the reason for resistance of genetic material of the viruses, their nucleonic acid, to injurious actions of the cells and, above all, to nuclease. It may be assumed as well that formation of hybrid RNP lies at the basis of the mechanism for the development of chronic viral infection in an organism immune to viral protein. Pseudoviral particles, insensitive to antibodies, thus escape the effects of immunity.

A cell-free system, with which the results presented above were obtained, can be a convenient model for the study of chemotherapy of viral infections, since in the absence of cells in a developed system of preformed subcellular structures, the action of chemotherapy can be studied not only by reconstruction of whole viruses, but also in separate stages of their reproduction (transcription and translation of the viral templates), which permits testing of various materials in order to obtain information on their points of application.

Finally, the studied appearance represents an exceptionally important illustration of the mechanism governing transfer and preservation of viral genetic information in the cell.

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