CHARACTERIZATION OF A PARTICULATE REPLICATIVE STRUCTURE IN SINDBIS VIRUS INFECTED CELLS

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1. Introduction

The investigation of the cytoplasmic viral cycle of Arboviruses is made easier because the synthesis of viral RNA is insensitive to actinomycin D. Thus cytoplasmic species of viral RNA and its replicative structures can be selectively labelled and characterized. Some replicative intermediates seem to be bound to membranes [1–3] but a considerable amount also appears in the ribosomal pellet after subcellular fractionation. Thus, the question has arisen as to the occurrence of a transcription–translation complex similar to the one postulated by Hotham-Iglewski [4] in the case of an RNA bacteriophage. Our present results suggest that the replicative intermediate may not be associated with ribosomes but co-sediments with ribosomes and polysomes by virtue of its density and particulate structure. Dissociation of polysomal structures in vivo in presence of aurintricarboxylic acid [5] allows the isolation of such 150 S nucleoprotein particles.

2. Materials and methods

Fibroblast cultures were set up from 11 day-old chick embryos, as described elsewhere [6]. In all experiments, 2 day-old primary cultures were infected with Sindbis virus in the presence of 1 μg/ml of actinomycin D. Treatment by aurintricarboxylic acid (ATA) and radioactive labelling of RNA were performed as indicated in legends. As a rule, a non-infected fibroblast culture, labelled with ^{32}P provided labelled ribosomes and ribosomal RNA marker.

2.1. *Subcellular fractionation*

Infected cells were scraped from the culture vessels maintained in the cold, washed with culture medium, and ground in a Teflon-glass Potter–Elvehjem homogenizer with the following buffered solution: 10 mM TEA, pH 7.2; 50 mM KCl; 5 mM Mg acetate; 1M sucrose. The homogenate was centrifuged at 20 000 *g* for 20 min; nuclei, cell debris and mitochondria were rejected. The supernatant (= S 20) was layered on a discontinous D_2O–sucrose gradient as described elsewhere [7], and centrifuged for 20 hr at 56 000 rev/min in a Spinco rotor 65 at 4°C. The polysome–ribosome pellet was then resuspended in the initial buffer solution.

2.2. *Analytic procedures*

The polysome–ribosome pellet was analyzed by conventional sucrose density gradient centrifugation. RNA was extracted by the phenol–SDS method described elsewhere [6]. Purified RNAs were analysed by sucrose-density gradient centrifugation. Proteins were analysed by SDS-poly-acrylamide gel electrophoresis by the method of Waehneldt [8] modified as to obtain a SDS concentration of 0.1%.

2.3. *Assay of viral RNA replicase activity*

Extracts from Sindbis-virus infected, non-labelled fibroblasts were fractionated by the D_2O–sucrose den-

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* Abbreviations used: SDS, sodium dodecyl sulphate.

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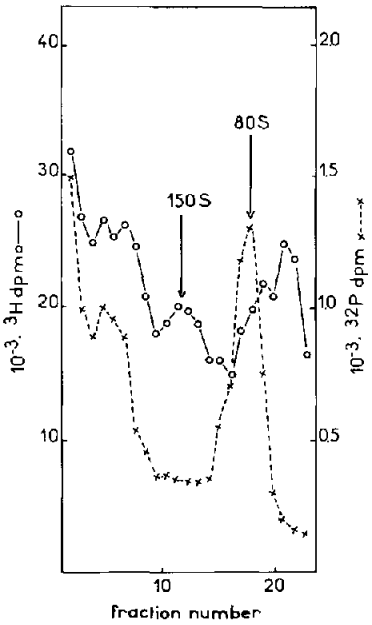

Fig. 1. Sucrose gradient centrifugation of the polysomal fraction. Linear density gradients with concentration between 10 and 30% are established and 0.1 to 0.2 ml of polysomal fraction was centrifuged at 4° C during 4 hr at 22 000 rev/min in a Spinco rotor SW25 (o——o) ^{3}H radioactivity from the viral ribonucleoproteins after 2 hr labelling (2 hr 30 min infection). (X——X) ^{32}P radioactivity from chicken marker ribosomes.

sity gradient procedure as described. Aliquots from the different fractions obtained were tested in an incubation mixture of the following composition: 60 mM triethanolamine-HCl (TEA) buffer, pH 8.2; 4 mM Mg acetate; 6 mM β-mercaptoethanol; ATP, UTP and CTP, 2 mM each; 10 mM phosphoenolpyruvate; pyruvate kinase 10 mg/ml; actinomycin D, 1 μg/ml; 0.15 ml of enzymatic fraction and [^{3}H] GTP 12.5 μCi for a final volume of 0.25 ml. The mixture was incubated 1 hr at 37°C and the reaction was stopped in ice. The whole mixture was pipetted on filter papers, thoroughly washed with 10% trichloroacetic acid (TCA), and the radioactivity measured in the scintillation spectrometer Intertechnique ABAC, SL40.

3. Results

The diagram of ribosomal ^{32}P-labelled particles from virus infected, actinomycin-treated cultures analysed by sucrose-density gradient centrifugation, shows a peak of 80 S monosomes, and a poly disperse polysomal region. The viral tritium-labelled material is polydisperse. The part of the material with sedimentation coefficients higher than 80 S, corresponding to the polysomal region, increase towards the bottom, but a distinct peak of 150 S can be observed (fig. 1).

After isopycnic sedimentation in CsCl, the viral polydispersed tritium-labelled material showed buoyant densities between 1.45 g/cm^{3} and 1.56 g/cm^{3}, with a peak at 1.53 g/cm^{3}. The ^{32}P-labelled ribosomes showed a density of 1.56 g/cm^{3} (fig. 2). The figure shows that only viral-RNA bearing structures of densities higher than 1.43 g/cm^{3} occur in the polysome-ribosome fraction; free informosomes (densities between 1.40 and 1.45 g/cm^{3}) are scarcely represented, and membranous structures are totally absent.

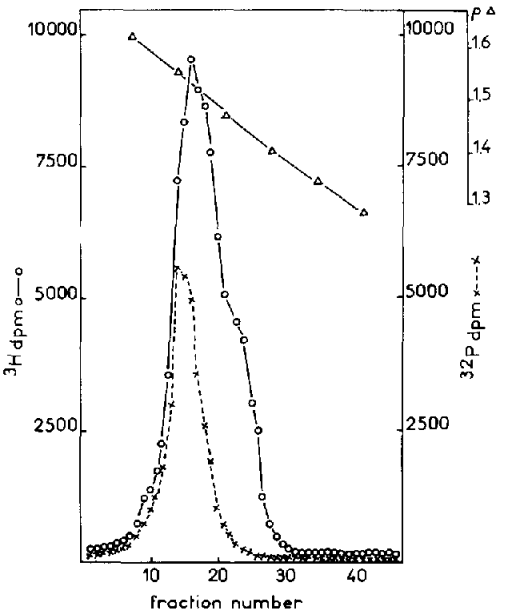

Fig. 2. Isopycnic sedimentation in CsCl–linear CsCl gradients were preformed with densities between 1.25 and 1.65 g/ml. The polysomal fraction is fixed with formaldehyde (3%) layered on top of the gradient in a volume of 0.2 – 0.4 ml and centrifuged during 14 hr at 10°C and at 45 000 rev/min in the Spinco rotor SW65. Two-drop fractions are recovered for radioactivity measurement. (o——o) ^{3}H radioactivity from the Actinomycin D-resistant viral ribonucleoproteins after 2 hr labelling (2 hr 30 infection). (X——X) ^{32}P chicken marker ribosomes.

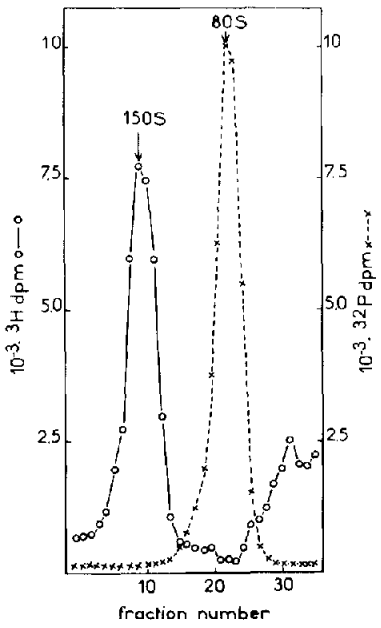

Fig. 3. Sucrose gradient centrifugation of the polysomal fraction. The cells have been treated by 100 μg/ml of ATA, 10 min after infection. Conditions identical to those of fig. 1. (○——○) ^{3}H radioactivity from viral ribonucleoproteins after 2 hr labelling (3 hr infection). (X——X) ^{32}P-radioactivity from chicken marker ribosomes.

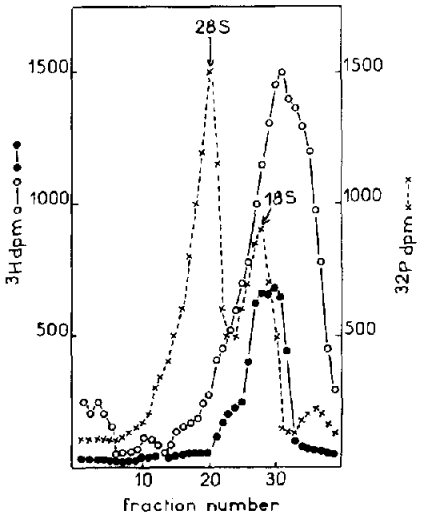

Fig. 4. Sucrose density gradient analysis of RNA from 150 S viral particles, isolated in ATA treated cultures. (○——○) ^{3}H radioactivity from the viral RNA; (●——●) ^{3}H radioactivity from the RNAase-resistant viral RNA core. (X——X) ^{32}P radioactivity from marker ribosomal RNA. RNAase resistance was assayed after sedimentation of the viral RNA and fractionation of the gradient. An aliquot of each fraction was adjusted at 0.1 M. Tris–HCl, pH 7.6, and incubated with 1 μg/ml pancreatic RNAase at 37°C during 1 hr. TCA-insoluble radioactivity remaining after RNAase treatment, was measured.

Following a treatment by ATA 10 min after infection, the polysomes disappeared entirely leaving a tall peak of viral RNA label in the position corresponding to 150 S. Viral and cellular polysomes were completely dissociated and ^{32}P radioactivity of the ribosomes was concentrated entirely in the 80 S peak (fig. 3).

The tritium-labelled 150 S peak which contains viral RNA was isolated from the gradient and the RNA extracted. Analysed by sucrose gradient sedimentation, the RNA was polydisperse with sedimentation coefficients mainly of 12 S to 26 S, and was partially RNAase resistant mainly in the 16–22 S region (fig. 4). 150 S particles were solubilized by SDS and the proteins electrophoresed on SDS-polyacrylamide gel. The densitogram seen in fig. 5 shows 2 bands: a major band of M.W. 40 000 and a minor band of M.W. 22 000. These two bands are very similar or identical to those of the two nucleocapsid proteins from the virion.

The polysome fraction contains about 6% of the

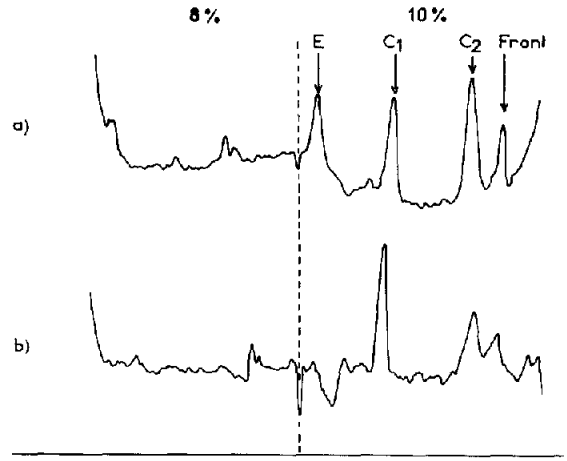

Fig. 5. SDS-polyacrylamide gel electrophoresis of viral proteins. Densitogram of the Coomassie blue stained gels. a) Proteins from the purified virion; b) proteins from the 150 S viral particles.

Table 1
Distribution of RNA-replicase activity in cytoplasmic subfractions

Cytoplasmic fraction	Infected cells cpm [^{3}H] inc. Exp. 1	Exp. 2	Non-infected cells cpm [^{3}H] inc.
A	2130	5770	130
B	9830	13 940	180
C	4340	4360	90
D	120	300	80
E	1220	1340	75

Six ml of $S_{2\,C}$ from Sindbis-virus infected or non-infected fibroblasts were layered over a discontinuous D_2O–sucrose density gradient formed by 3 ml of D_2O–sucrose solution of density 1.29 g/cm^3, and 3 ml of D_2O–sucrose of density 1.26 g/cm^3, and were centrifuged at 56 000 rpm for 20 hr in a Spinco rotor 65 at 4° C [7]. After centrifugation, the following layers were removed from top to bottom: A, 4 ml, soluble fraction; B, 3 ml, smooth microsomes; C, 3 ml, rough microsomes; D, 2 ml, free informosomes; E, pellet resuspended in 1 ml of buffer, polysomes, ribosomes and '150 S particles'. Three aliquots of 0.15 ml from each fraction were assayed for RNA replicase activity as described in methods. Table 1 reports the calculated total activity in each fraction expressed as cpm [^{3}H]GTP incorporated in TCA-insoluble material.

whole RNA-replication activity of the cytoplasmic extract. The bulk of the remaining activity is located in membrane fractions (table 1).

4. Discussion

A highly purified polysome–ribosome fraction, sedimented through a D_2O–sucrose layer of density 1.29 g/cm^3, has been isolated from Sindbis virus-infected fibroblasts. It contained viral particles with a sedimentation coefficient of 150 S. Treatment in vivo with the initiation-blocking agent aurintricarboxylic acid, promotes depolymerization of the whole polysome population and unmasks the 150 S viral particles, which can be isolated from the sucrose gradient in a highly pure form bearing exclusively viral RNA and viral capsid proteins. The viral RNA extracted from purified 150 S particles had heterogeneous sedimentation coefficients of 12 S to 26 S and bore a RNAase-resistant core in the 16 S to 22 S region. These are characteristics of arbovirus replicative intermediates [9,10].

The polysome–ribosome fraction also showed a distinct RNA-replicase activity. While the bulk of the RNA-replicase activity from total cytoplasm was shown by membrane fractions, about 6% of the total replicase activity from the cytoplasmic extract was contained in the polysomal fraction. This replicase activity may be associated with the 150 S particle and its replicative intermediate, although this fact could not yet be proved.

Particles of similar signification, containing the replicative intermediate and sedimenting at 130 S have been shown in picornavirus-infected cells. In this case, the particles appear early in the replication cycle and evolve toward 250 S particles, containing higher structured replicative intermediates [11,12].

Friedman et al. [1] and Grimley et al. [2,3] described 140 S particles, visible by electronmicroscopy and interpreted them as virion precursors particles. A replication complex of sedimentation coefficient 250 S, associated with membranes and bearing virus specific proteins has been described by Qureshi et al. [13] in the case of St. Louis encephalitis virus. These results can be combined and show that arbovirus-infected cells contain well-defined particles of homogenous size, which have a density similar to that of ribosomes. They contain RNA with replicative intermediate structure, and proteins from the viral capsid which perhaps associate with the nascent RNA chains.

This structure does not seem to be associated with ribosomes in a transcription–translation complex as it is the case for some bacteriophages. The 150 S particles pre-exist in a free state in the polysomal fraction and are unmasked by the aurintricarboxylic acid treatment.

Acknowledgements

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