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IDENTIFICATION OF VIRAL RIBONUCLEOPROTEINS IN THE CYTOPLASM OF MURINE CELLS CHRONICALLY INFECTED BY A RETROVIRUS

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SUMMARY

Ribonucleoproteins were isolated from the cytoplasm of Friend-Eveline cells which produce the Friend virus complex, after a short labelling with [^{3}H] uridine. These particles moved with a sedimentation coefficient of 53S in sucrose gradient and had a buoyant density of 1.46 g/cm^3 in CsCl gradient. Analysis of their RNA content showed that they possessed a 35S major species having the size of the viral genome subunit. Moreover, a positive hybridization was observed when RNA of the 53S particles was annealed with viral complementary DNA. No such particles were found in cultures of uninfected murine cells suggesting that 53S RNPs have a viral origin.

INTRODUCTION

The multiplication of retroviruses in mammalian cells involves the synthesis by reverse transcriptase of a DNA intermediate, the provirus, which gets integrated into the cellular genome. It is then transcribed into RNA molecules which have been characterized in the cytoplasm (1,2,3,4) : genome-sized 35S RNA, which has been demonstrated to code only for GAG and POL genes products, and 20S RNA which expresses the ENV gene information (5, 6,7,8). In infected cells, the coexistence of viral and cellular mRNAs on polysomes suggests that both behave similarly if not identically during the events involved in the protein synthesis. In eukaryotic cells, cytoplasmic mRNAs are associated with proteins under the form of ribonucleoprotein complexes. Some of them, called informosomes by A.S. Spirin (9, 10) exist under a free state, while others are associated to polyribosomes as messenger ribonucleoproteins (11,12). The function of informosomes and the nature of the relationship between the two types of RNPs are still a matter of controversy (13,14,15,16,17).

We were interested in the characterization of cytoplasmic ribonucleoproteins which have a viral specificity. In this work, when virus producing cells were incubated for a short time with [^{3}H]uridine, a free 53S RNP population could be isolated, which contained viral RNA species as judged by its size and its capacity to hybridize with [^{3}H]DNA complementary to the Friend virus genome.

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MATERIAL AND METHODS

Solution : Tris buffer : 10mM Tris-HCl, pH 7.4 ; 100mM NaCl ; 2mM Mg acetate ; 3 µg/ml Sodium Polyvinylsulfate (PVS).

Cells : culture and labelling: Murine Friend-Eveline cells were grown in suspension cultures and maintained at a 2-8.10^5 cells/ml concentration range (3,18). For labelling experiments, exponentially growing cells were concentrated five fold and incubated for 30 min. with 50 µCi/ml of [^{3}H] uridine (29 Ci/mmole, from the CEA, Saclay, France). Parallel experiments were performed using uninfected D55 murine cells.

Preparation of cytoplasmic extracts: At the end of incubation with [^{3}H] uridine, cells were disrupted in Tris buffer containing 1 % Nonidet P_{40} (3). The postmitochondrial supernatant was processed by two alternative methods. Procedure 1 : the postmitochondrial supernatant was spun at 50.000 rpm for 60 min., in order to pellet the polyribosomes (19). The postpolyribosomal supernatant was further centrifuged at 50.000 rpm for 5 hours and the postpolysomal pellet was resuspended in Tris buffer. Procedure 2 : the postmitochondrial supernatant was layered on a 75 % sucrose cushion in a SW50 rotor and run at 50.000 rpm for 5 hours. The upper part was discarded and the sucrose fraction was recovered and dialysed against Tris buffer.

Analysis of cytoplasmic particles: 0.6-1.0 $O.D_{260}$ unit of the different extracts prepared as reported above was centrifuged through a 10-40 % sucrose linear gradient in Tris-buffer for 16 hours at 22.000 rpm at 4°C in a Spinco SW27 rotor. [^{14}C]-labelled 40S and 60S ribosomal subunits were added as markers. Aliquots of the gradient fractions were precipitated with 10 % trichloracetic acid and filtered on nitrocellulose membranes which were dried and counted in a scintillation Tricarb spectrometer. Particles isolated in sucrose gradients were analyzed in CsCl equilibrium gradients according to Spirin (20).

RNA analysis: Appropriate fractions recovered from the sucrose gradient were pooled and adjusted to 1 % SDS at room temperature for 30 min. RNA was then precipitated by ethanol with 50 µg yeast RNA as a carrier and stored overnight at -20°C. It was analyzed by electrophoresis in 1.7 % polyacrylamide - 0.5 % agarose composite gels (21).

Hybridization assays of RNA from particles with viral complementary synthetic DNA: The conditions of viral cDNA synthesis and RNA isolation have been described elsewhere (3). Annealing reactions were performed in small vials containing 0.6M NaCl ; 0.02M Tris-HCl, pH 7.4 ; 1mM EDTA ; 0.1 % SDS and 12 µg/ml of denatured calf thymus DNA. 0.1 ml of mineral oil was added to prevent evaporation during incubation for 48 hours at 68°C. The hybrids were assayed for S_1 nuclease resistance (3).

RESULTS

I - Isolation of subribosomal particles in the cytoplasm of Friend-Eveline cells : To isolate viral RNA containing particles, Friend-Eveline cells were disrupted at a 2mM Mg^{++} concentration which prevents the dissociation of polysomes. Cells were labelled with [^{3}H] uridine for periods not exceeding 30 min., since newly synthesized ribosomal RNA had not yet entered the cytoplasm at this time. This point was verified by isolating cytoplasmic RNA from cells incubated for various periods of time. It was found that radioactivity occured in the 18S region of sucrose gradients after 35 to 40 min.

Friend-Eveline cells were labelled for 30 min., and a postmitochondrial supernatant was prepared. It was fractionated into polysomal pellet and postpolyribosomal supernatant (procedure 1 of Materials and Methods). Each fraction was then analyzed by velocity sedimentation in 10-40 % sucrose gradients. Figure 1 shows the presence of radioactivity sedimenting in a peak located between the 60S and 40S ribosomal markers. The apparent sedimentation coefficient value of this peak was estimated to be 53S. Other heterogeneous labelled material were visible in the lighter regions (10-30S) of the gradients.

Although the material of 53S peak was reproducibly found in the postpolyribosomal supernatant, its importance varied greatly from one experiment to another, suggesting that it could be lost by degradation or sedimented in the polysomal fraction. Indeed, 53S material co-sedimenting with undissociated polysomes was found in the polysomal pellet (result not shown). In order to get quantitative recoveries, another method was used in which polysomes were not separated from the subribosomal structures. The postmitochondrial supernatant was centrifuged on a 75 % sucrose cushion (procedure 2 in Materials and Methods). Then, the sucrose cushion content was analyzed in a 10-40 % sucrose gradient (Fig. 2). Again a peak of radioactivity was resolved in the 53S region of the gradient. Since a better recovery in the isolation of the 53S material was obtained using this second procedure, it was adopted for all the following experiments.

The above results were obtained starting from retrovirus-infected cells. The same experimental procedures were repeated using murine D55 cells which do not contain any viral RNA in their cytoplasm (Ravicovitch et al. - personal communication). Analysis of the postpolyribosomal supernatant obtained by the procedure 1 as well as that of the extract resulting from the procedure 2 did not show any radioactivity in the 53S region, but only material sedimenting in the 10-30S area.

II - Caracterization of the 53S particles.

a) Analysis of the RNA moiety by electrophoresis in polyacrylamide gels: The RNPs of the sucrose cushion were centrifuged in a 10-40 % sucrose gradient. Fractions containing 53S ribonucleoproteins were pooled and treated by SDS. The RNA was subjected to gel electrophoresis using [^{14}C]-labelled 28S and 18S rRNA as markers. The results are presented in Figure 3. A predominant peak moving at a slower rate than the 28S rRNA was consistantly observed. A more precise examination of its mobility showed that it migrated exactly at the position of the 35S RNA isolated by denaturation of 70S viral

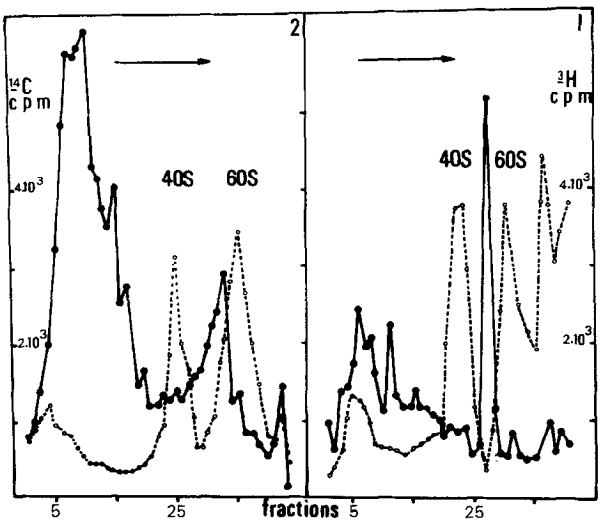

Fig. 1 : Velocity sucrose gradient of postpolysomal supernatant of Friend-Eveline cytoplasmic extract. A postpolysomal extract was isolated from cells labelled for 30 min. with [^{3}H] uridine and analyzed in a 10-40 % sucrose gradient (Beckman SW27 rotor-22 000 rpm - 16 hours at 4°C). Aliquots of each sample were assayed for acid-insoluble radioactivity (●——●). The [^{14}C] labelled markers were centrifuged in the same tube (o- - -o).

Fig. 2 : Velocity sucrose gradient of the sucrose fraction (procedure 2) from Friend-Eveline cells. A subribosomal particles extract was prepared according to the procedure 2 and analyzed in the same conditions as in Fig. 1. ^{3}H-profile of RNPs (●——●). [^{14}C]-labelled markers run in the same tube (o- - -o).

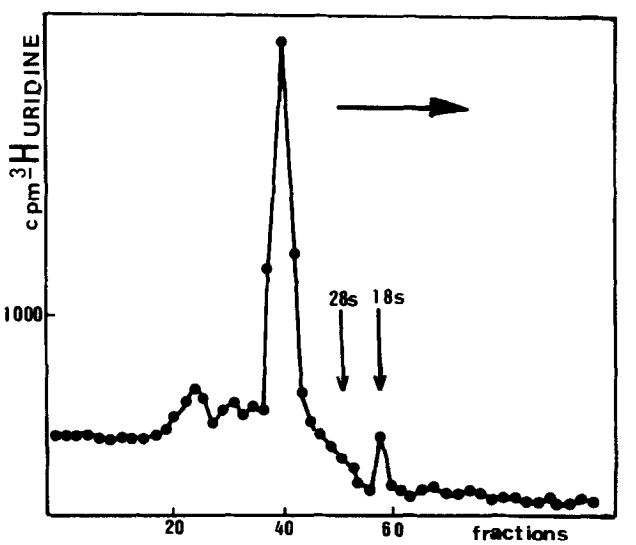

Fig. 3 : Gel electrophoresis of RNA extracted from 53 RNPs. RNA was extracted from 53S material of the sucrose cushion and subjected to electrophoresis on a 1.7 % polyacrylamide-0.5 % agarose gel. 1.5mm slices were incubated with water for 16 hours at 37°C and the radioactivity of the eluate determined as described (21).

RNA. Hence, it can be pointed out that this rapidly labelled major RNA species contained in the 53S RNPs has the size of the viral 35S mRNA described by others (1,4). No such 35S RNA was found in the sucrose cushion of D55 uninfected cells (not shown). Electrophoretic patterns indicated the presence of 5-18S RNA species suggesting that RNPs labelled in D55 cells contain the same RNA species reported by others for mammalian cells (19).

b) Hybridization of total RNA of 53S RNP with viral cDNA: A cushion extract was prepared starting from unlabelled cells and fractionated in a sucrose gradient. Fractions were pooled along the gradient and their RNA was purified (see Materials and Methods) and hybridized with equal quantities of Friend virus cDNA. The cDNA hybridized with RNA located in three regions of the sucrose gradient (Figure 4). A peak was evidently present in the 40-60S region with its maximum centered around 53S. The hybridization in the heaviest part of the gradient most likely corresponded to viral RNA bound to polysomes, while the lighter part could represent degraded viral RNA or other lighter mRNAs.

c) Centrifugation in CsCl density gradients: The 53S particles recovered from a sucrose cushion as shown in figure 2 were pooled, fixed, and banded in CsCl density gradient as described in Materials and Methods. Major part of the radioactivity was concentrated in a single peak at a 1.46 g/cm^3 density (not shown). Existence of free ribonucleoproteins banding in the 1.39-1.47 g/cm^3 range has been reported in previous works (19,22,23,24).

DISCUSSION

In the present work, using pulse-labelling conditions with a RNA precursor, we found that the cytoplasm of chronically infected cells contains a population of particles moving at 53S in a velocity sucrose gradient. The 1.46 g/cm^3 density value observed in CsCl gradient approximately corresponds to a relative content of 75 % proteins and 25 % RNA (25).

Gel electrophoresis of the rapidly labelled RNA from the 53S particles revealed that most of the high molecular weight radioactivity was located in a very homogenous peak comigrating with the 35S RNA of the subunit present in the extracellular virions (in the form of a 70S complex). Moreover, hybridization tests using viral complementary DNA identified viral RNA sequences. These results suggest that 53S particles contain viral genome-sized RNA or the viral genome itself. It should be noticed that our hybridization protocol could detect other RNA than the 35S species vizualized by pulse labelling, since we used the total RNA extracted from the 53S particles.

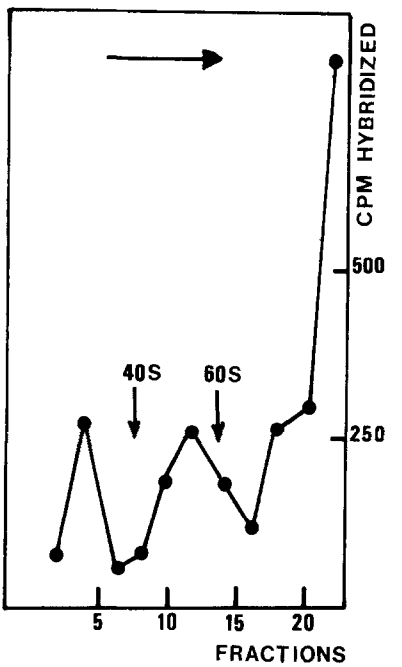

Fig. 4 : Hybridization of the Friend virus cDNA with the RNA contained in the cytoplasmic RNPs of Friend-Eveline cells. An extract (procedure 2) was obtained from unlabelled cells and fractionated in a sucrose gradient as in Fig. 2. ^{14}C - labelled 40S and 60S ribosomal subunits were sedimented in a parallel tube. Adjacent fractions were pooled two by two along the gradient. Each combined sample was deproteinized by a SDS-phenol procedure (3) and precipitated by 2 volumes ethanol in the presence of 20 µg of yeast RNA. RNA was dissolved in pure water and annealed with approximatively 800 cpm cDNA (see materials and methods). At the end of the incubation at 68°C, each sample was analyzed by S_1 nuclease (3).

Cytoplasmic extracts of non infected cells prepared under the same experimental conditions did not contain a well resolved peak of 53S particles. RNA analysis of this region of the gradient showed the only presence of molecular species not exceeding the 18-20S size range. The absence of 35S RNA confirms the viral nature of the peak found in Friend-Eveline cells.

The function of the 53S RNPs is presently unknown. It is tempting to consider that these particles represent a population of RNPs which are involved in the synthesis of the viral proteins. Their density is compatible with such a function, since the existence of RNPs with densities in the 1.39-1.47 g/cm^3 range has been reported. All the experiments presented here were performed in conditions where polysomal RNPs are not released. The 53S

RNPs recovered in the postpolyribosomal supernatant could be considered as viral informosomes. However, the release of some polysome-associated RNPs during the course of their preparation cannot be excluded. In that case, the 53S RNPs would represent a mixture of polysomal mRNPs and informosomes.

Alternatively, 53S RNPs could be a precursor of the viral core at a very early step of its synthesis. Presence of 35S RNA would be compatible with this hypothesis. In this case, polypeptides belonging to the core should be already associated with viral RNA in 53S particles. Further analysis of their protein content should distinguish between these possibilities.

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