References and Notes

1. S. P. Grossman, *Science* **132**, 301 (1960); A. E. Fisher and J. N. Coury, *ibid.* **138**, 691 (1962); J. W. Wagner and J. DeGroot, *Amer. J. Physiol.* **204**, 483 (1963).
2. R. Velluti and R. Hernández Peón, *Exp. Neurol.* **8**, 20 (1963); R. Hernández Peón, G. Chavez-Ibarra, P. J. Morgane, C. Timo-Iaria, *ibid.*, p. 93.
3. A. E. Fisher, *Sci. Amer.* **210**, 60 (1964).
4. ——— and J. N. Coury, in *Thirst: Proceedings of the First International Symposium on Thirst in the Regulation of Body Water*, M. J. Wayner, Ed. (Pergamon Press, New York, 1964), p. 515.
5. S. P. Grossman, *ibid.*, p. 487.

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19 August 1966

Reovirus-Specific Polyribosomes in Infected L-Cells

Abstract. *Polyribosomes were isolated from reovirus-infected L-cells. Virus-induced RNA isolated from these polyribosomes was single-stranded, heterogeneous in size, and capable of extensive hybridization with viral double-stranded RNA. Reovirus RNA, therefore, specifies in infected cells the formation of a single-stranded messenger RNA for virus-specific protein synthesis.*

Reovirus is unique among mammalian viruses and bacteriophages in that its genetic material is double-stranded RNA (*1*). Very few features about its replication have been established. Some 7 hours after infection of L-cells, a virus-induced synthesis of RNA commences, and this synthesis probably involves prior formation of at least two new proteins (*2*). Approximately 40 percent of the newly synthesized RNA is double-stranded and resembles the RNA of the virion in physical characteristics; the remaining RNA is single-stranded and heterogeneous in size, being distributed between 30*S* and 10*S* molecules in sucrose-gradient analyses (*3*). We now describe experiments showing that part of this single-stranded RNA is associated with polyribosomes in infected cells and is probably a messenger RNA (mRNA) involved in virus-directed protein synthesis.

Before infection, exponentially growing suspension cultures of L-cells were centrifuged; the cells were suspended to a concentration of 5×10^5 per milliliter in Eagle's minimal medium containing 2 percent bovine fetal serum. After the culture was incubated for 1 hour, actinomycin D (0.5 μg/ml) was added, and the culture was incubated 1 hour longer to ensure suppression of host RNA synthesis. A stock suspension of reovirus, type 3, was then added to give a multiplicity of infection of 5 to 10 plaque-forming units (PFU) per cell (*3*).

For isolation of polyribosomes from infected or uninfected cultures, approximately 10^8 cells were centrifuged, washed three times with ice-cold phosphate-buffered saline (*4*), and resuspended in 1.5 ml of extraction buffer containing 0.01*M* tris-HCl at *p*H 7.4, 0.1*M* NaCl, and 0.015*M* $MgCl_2$. All procedures were carried out at 4°C. After 15 minutes the detergent, BRIJ-58, was added to a final concentration of 0.5 percent. The cells were then ruptured in a tight-fitting Dounce homogenizer, and nuclei were removed by centrifugation at top speed in a clinical centrifuge. Sodium deoxycholate was added to the supernatant cytoplasmic fraction to a final concentration of 0.5 percent. The material was then layered on a 15- to 30-percent linear sucrose gradient prepared in the same buffer used for extraction, and centrifuged for 110 minutes at 24,000 rev/min and 4°C in a Spinco centrifuge. Under these conditions polyribosomes were present in fractions 5 to 22 (Fig. 1), while virions were sedimented to the bottom of the tube.

Functional polyribosomes in reovirus-infected cultures were demonstrated by treating the cultures with C^{14}-labeled algal-protein hydrolyzate for 5 minutes, at 10.5 hours after infection. The cytoplasmic fraction was then prepared and analyzed by sucrose-gradient sedimentation (Fig. 1).

Few polyribosomes were seen by optical-density measurements in uninfected cultures after treatment with actinomycin D, and there was only a small increase in the number after infection. Moreover, in the uninfected culture only a small amount of label was observed in the polyribosome region.

In contrast, a significant amount of label was seen in the polyribosomes of infected cells, an indication that viral-directed protein synthesis was in-

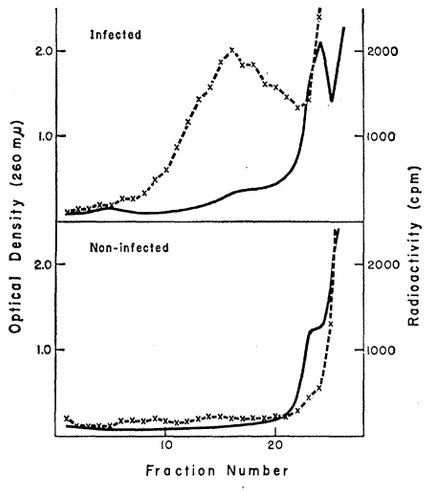

Fig. 1. C^{14}-Amino acid incorporation into polyribosomes. At 9.5 hours after infection, 200 ml of cell culture (5×10^5 cell/ml) were centrifuged, and the cells were resuspended in 50 ml of growth medium containing 1/40 of the normal amino acid concentration and 2 percent fetal calf serum. After incubation for 1 hour at 37°C, 50 μc of C^{14}-labeled algal-protein hydrolyzate was added. Uptake of label was stopped 5 minutes later by rapid chilling of the cells, and the cytoplasmic fraction was prepared and analyzed by sucrose-gradient sedimentation. Optical density in the gradient was monitored continuously with a Gilford recording spectrophotometer. Fractions collected from the gradient were precipitated with ice-cold trichloroacetic acid on glass-fiber filters, and the radioactivity of the filter was determined by liquid scintillation counting in a Tricarb spectrometer. Optical density (——); Radioactivity (x - x - x).

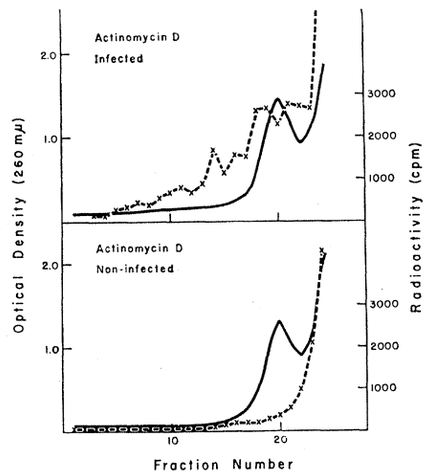

Fig. 2. H^3-Uridine incorporation into polyribosomes. Ten hours after infection, H^3-uridine (4770 μc/μmole, final concentration, 1 μc/ml) was added to 200 ml of cell culture. After 30 minutes, uptake of label was terminated by rapid chilling of the culture; the polyribosomes were then isolated. Optical density (——) and radioactivity (x - x - x) were determined as described in Fig. 1.

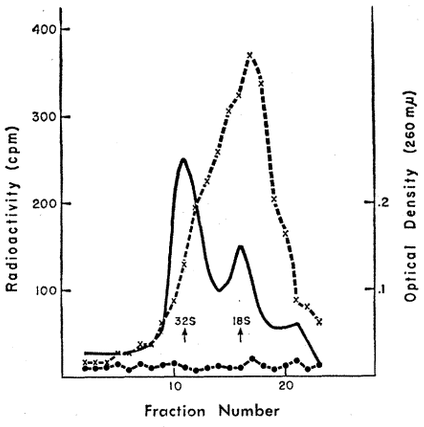

Fig. 3. RNA extracted from polyribosomes of infected cells. RNA was extracted from sedimented polyribosomes with either phenol–sodium dodecyl sulphate at 4°C or with sodium dodecyl sulphate alone. Identical results were obtained with both extraction procedures. The RNA in buffer was layered on a sucrose gradient (15 to 30 percent) containing 0.14*M* LiCl, 0.01*M* tris-HCl (*p*H 7.4), and 0.001*M* $MgCl_2$. After centrifugation for 15 hours at 24,000 rev/min and 4°C, the optical density along the gradient was determined and fractions were collected. Each fraction was divided into two parts; one portion was treated with 2 μg of ribonuclease per milliliter for 30 minutes at 37°C. The fractions were then precipitated with 10 percent trichloroacetic acid, and radioactivity was determined. Optical density (——), total radioactivity (x-x-x), radioactivity after treatment with ribonuclease (•-•-•).

deed occurring. Treatment of the cytoplasmic extract with 0.1 μg of ribonuclease per milliliter for 15 minutes at 0°C before sedimentation resulted in a complete loss of label from polyribosomes and transfer of the label to single ribosomes. These results are consistent with the idea that virus-induced polyribosomes are present in the infected cell culture.

It was next shown that virus-specified RNA was serving as messenger in these polyribosomes. For these experiments, the infected cell culture was labeled with H^3-uridine for 0.5 hour, beginning 10 hours after infection. The cytoplasmic fraction was then prepared and analyzed (Fig. 2). In uninfected cells all of the radioactivity precipitable by acid was at the top of the gradient. In infected cultures, however, the radioactivity was associated with the sedimenting small polyribosomes, as well as with the region of single ribosomes and smaller structures.

Longer periods of labeling, up to 2 hours, did not alter the pattern. Similar results were obtained whether the extraction procedure was carried out in buffer alone, buffer plus BRIJ, or buffer plus BRIJ followed by deoxycholate. Thus, unlike the situation with poliovirus polyribosomes in HeLa cells (*5*), reovirus polyribosomes do not appear to be associated with a lipid-containing particulate.

RNA was isolated from virus-induced polyribosomes by pooling the region of the sucrose gradient between single ribosomes and the bottom of the tube. The polyribosomes were then centrifuged for 8 hours at 24,000 rev/min and 4°C. RNA, extracted from the sedimented polyribosomes with either phenol–sodium dodecyl sulphate at 4°C, or simply by resuspending it in a buffer containing 0.5 percent sodium dodecyl sulphate in 0.01*M* tris-HCl (*p*H 7.4) and 0.1*M* NaCl (*6*), gave results on sucrose-gradient analysis (Fig. 3). The labeled RNA from polyribosomes of infected cells had sedimentation coefficients ranging from 30*S* to 10*S*; all of this RNA was sensitive to ribonuclease and was therefore single-stranded.

As a further test that the polyribosomal RNA was virus-specified, hybridization experiments between it and double-stranded viral RNA were carried out. Figure 4 shows a reannealing curve for denatured double-stranded viral RNA at 60°C under different salt concentrations. In 0.3*M* NaCl 90 percent of the RNA became annealed in 3.5 hours.

When H^3-labeled polyribosomal RNA from infected cells was incubated with unlabeled, denatured, double-stranded viral RNA in 0.3*M* NaCl for 4 hours at 60°C, 41 percent of the label became ribonuclease-resistant. This figure represents a lower limit on the amount of single-stranded RNA which will hybridize due to the rapid, competitive reannealing of double-stranded RNA (Fig. 4).

Polyribosomal RNA from infected cells incubated under the same conditions without double-stranded RNA remained totally ribonuclease-sensitive. This result might suggest that no complementary RNA strands are present in the single-stranded polyribosomal RNA and therefore that only one strand of the viral RNA is used as template for RNA synthesis. This conclusion is a tentative one, however. The concentration of virus-specific RNA in the polyribosomal extracts could not be determined owing to the presence of cellular ribosomal RNA, and may have been too low in the self-annealing tests to reveal some complementary RNA.

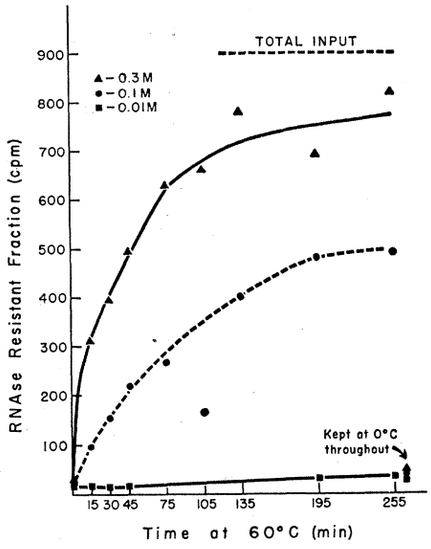

Fig. 4. Reannealing of melted viral RNA. Double-stranded H^3-labeled RNA extracted from infected cells purified on a methylated-albumin–keiselguhr column (*3*) was dissolved to a concentration of 10 μg/ml in buffer containing 0.01*M* NaCl, 0.01*M* tris-HCl (*p*H 7.4), and 0.01*M* EDTA. After heating at 100°C for 10 minutes to denature the RNA and quick chilling on ice, fractions were removed and brought to the indicated NaCl concentration. These fractions were then placed at 60°C, and 0.1 ml samples were removed at the times indicated. The samples were chilled to 0°C and treated for 30 minutes at 0°C with ribonuclease (5 μg/ml) dissolved in the appropriate salt. A drop of 0.5 percent human serum albumin was added, the sample was precipitated with trichloroacetic acid, and the precipitate was collected on Millipore filters for determination of radioactivity.

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References and Notes

1. P. J. Gomatos and I. Tamm, *Proc. Nat. Acad. Sci. U.S.* **49**, 707 (1963).
2. H. Kudo and A. F. Graham, *Biochem. Biophys. Res. Commun.* **24**, 150 (1966).
3. ——, *J. Bacteriol.* **90**, 936 (1965).
4. R. Dulbecco and M. Vogt, *J. Exp. Med.* **99**, 167 (1954).
5. S. Penman, Y. Becker, J. E. Darnell, *J. Mol. Biol.* **8**, 541 (1964).
6. W. Gilbert, *ibid.* **6**, 389 (1963).
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