Structure and Infectivity of Picornaviral RNA Encapsidated by Cowpea Chlorotic Mottle Virus Protein

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Poliovirus and Mengo virus RNA were shown to associate efficiently with cowpea chlorotic mottle virus protein to form pseudovirions. The sedimentation coefficient for the pseudovirions was similar to that of poliovirus, and electron microscope observations showed the Mengo pseudovirions to be similar in size to Mengo virus. Such pseudovirions were infectious and were more resistant to ribonuclease than viral RNA; however, under our assay conditions, their infectivity was about equal to that of viral RNA.

Detailed studies by Bancroft have shown that cowpea chlorotic mottle virus (CCMV) protein will encapsidate most single-stranded polyribonucleic acids with the formation of virus-like nucleoprotein particles (1, 2, 12). Our investigation shows that poliovirus and Mengo virus RNA can also be encapsidated by CCMV protein. This system was studied to see whether such protein encapsidation can increase the nuclease resistance and the uptake by mammalian cells of single-stranded polyribonucleotides. The infectivity of these pseudovirions was compared with that of their RNA constituents to measure any increase in cell penetration or resistance to breakdown. The structure of these pseudovirions was also compared with that of poliovirus and Mengo virus.

MATERIALS AND METHODS

Cell and virus sources. Cell and virus stocks were kindly provided as follows: HeLa S₃ cells by B. Woodson, Department of Microbiology, U.C. Medical School, San Francisco. HeLa cells and poliovirus-type II stocks by C. E. Schwerdt, Department of Medical Microbiology, Stanford University Medical School. CCMV and bushy stunt virus (BSV) purified by differential centrifugation by C. A. Knight, Department of Molecular Biology, U.C. Berkeley. Additional CCMV preparations by C. W. Kuhn, Department of Plant Pathology, University of Georgia, Athens, Ga., and by J. H. Tremaine, Canadian Department of Agriculture, Vancouver, British Columbia.

HeLa cell monolayers were grown in an atmosphere of 5% CO₂ in minimum Eagle medium (MEM) containing 10% fetal calf serum. L-cell monolayers were grown in MEM containing 10% fetal calf serum

and 20 mM N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid (HEPES) buffer. HeLa S₃ cells were grown at a density of 2×10^5 to 6×10^5 /ml in Eagle spinner medium containing 7% fetal calf serum. Poliovirus stocks of 10^7 to 5×10^7 PFU/ml were prepared by infecting HeLa cell monolayers in 32-oz bottles (about 960 ml) and harvesting medium after cell lysis. High-titer poliovirus stocks (5×10^8 to 10×10^8 PFU/ml) were prepared in HeLa S₃ cells, as described by Levintow and Darnell (14). Mengo virus stocks were prepared by infecting L-cell monolayers in 32-oz bottles and harvesting after cell lysis.

Purification of viruses. HeLa S₃ cells were concentrated to 10^7 /ml, infected at 10 PFU/cell for 45 min and resuspended at 10^6 /ml in Eagle spinner medium containing 5 μ Ci of ³H-uridine per ml. Eight hours postinfection, cells were collected by centrifugation; resuspended in 0.01 M NaCl, 0.01 M Tris (pH 7.8), and 0.002 M CaCl₂; and frozen at 15 C. Thawed cells were then vortexed with 0.5 volume of Genesolv-D (Allied Chemical, Morristown, N.J.), and virus from the supernatant fluid was pelleted by centrifugation at $100,000 \times g$ for 2 h. Pelleted material was resuspended in 0.1 M Tris, pH 7.8, homogenized with 0.5 volume of Genesolv-D, and the supernatant fluid was centrifuged at $100,000 \times g$ for 15 h through a linear 1.1 to 1.4 g of cesium chloride density gradient per cm³ buffered with 0.1 M Tris, pH 7.8. Gradient fractions were collected, and the radioactivity of samples was determined; poliovirus was shown to be cleanly separated from cellular tritium-labeled components. Virus fractions were further purified with a polyacrylamide P-60 column equilibrated with 0.1 M Tris, pH 7.8, and polio RNA was isolated by phenol extraction at 0 to 4 C in the presence of Bentonite, followed by ether extraction and ethanol precipitation. The RNA precipitate was dissolved in 0.01 M Tris, pH 7.8, and centrifuged at $80,000 \times g$ for 30 min to remove residual Bentonite.

L-cell monolayers grown in roller bottles were infected with Mengo virus stocks at pH 8.0, and 3 h later 2 μ Ci of 3 H-uridine per ml was added. After 48 h at 34 C, cells and medium were frozen and thawed, and the cell debris was removed by centrifugation. The cell pellet was sonically treated and vortexed with Genesolv-D and then centrifuged at $10,000 \times g$ for 10 min. The supernatant fluids were combined and made 10% (wt/vol) with polyethylene glycol 6000 and 0.5 M with NaCl. After 16 h at 0 C, the precipitate was collected by centrifugation at $5,000 \times g$ and dissolved in 0.15 M NaCl and 0.05 M Tris buffer, pH 7.8. Mengo virus was then further purified by differential centrifugation and two cesium chloride density gradient equilibrium centrifugations.

CCMV was purified by density gradient centrifugation (10 to 40% sucrose containing 0.1 M sodium acetate, pH 5.0). Virus zones were removed and centrifuged for 4 h at $100,000 \times g$ on a linear 1.1 to 1.4 g of CaCl gradient per cm^3 containing 0.1 M sodium acetate, pH 5.0. Virus zones were then dialyzed twice against 2 liters of 0.1 M sodium acetate, pH 5.0, and stored at 4 C. CCMV protein was prepared by dialyzing virus against 1 M NaCl, 0.02 M Tris, pH 7.8, and 10^{-3} M dithiothreitol (disassembly buffer), followed by centrifugation to pellet RNA (12). A_{280}/A_{260} ratios for CCMV protein preparations were about 1.7 ($<0.25\%$ RNA).

Assembly of polio pseudovirions. Polio RNA and CCMV proteins were mixed together at a 1:12 weight ratio, respectively, in disassembly buffer (0.2 to 0.6 ml) and then dialyzed for 2 h at 0 C against 0.01 M Tris, pH 7.8, 0.01 M KCl, 5×10^{-3} M MgCl_2 , and 10^{-3} M dithiothreitol (TKM buffer; reference 12).

Density gradient sedimentation analysis. Polio RNA or pseudovirions were sedimented on linear 4.5 ml 10 to 40% sucrose gradients containing TKM buffer at 4 C in a Spinco SW 50.1 rotor. Gradients were then fractionated by collecting drops through the bottom of the tubes, and samples of these fractions were analyzed for radioactivity, absorbancy, or infectivity.

Infectivity analysis. Polio virus, RNA, or pseudovirions in 0.5-ml samples were assayed on confluent HeLa monolayers in 60-mm Falcon plates. Polio RNA or pseudovirions were diluted in MEM containing 100 μ g of DEAE-dextran per ml and then assayed on HeLa monolayers washed once with PBS (8). After adsorption at 37 C for 1 h, plates were overlaid with MEM containing 0.95% agar and further incubated at 37 C in an atmosphere of 5% CO_2 . After 2 to 3 days, plates were stained with neutral red, and plaques were counted.

Radioactivity analysis. Samples of 3 H-uridine labeled viruses or RNA were solubilized with 1 ml of Nuclear Chicago Solubilizer (diluted 1:3 with scintillation solution), followed by 5 ml of scintillation solution (42 ml of Liquifluor per liter toluene; New England Nuclear Corp., Boston, Mass.). Density gradient fractions or samples thereof were added to Triton-X scintillation solution (42 ml of Liquifluor, 500 ml of Triton-X, and 458 ml of toluene). Radioactivity was then determined by liquid scintillation spectrometry.

Electron microscopy. Virus samples were pre-

pared for observation by negative staining of unfixed suspensions. Copper grids (500 mesh) were covered with a support film of 2% parlodian in amylacetate. The film was stabilized by coating the grids with a layer of carbon in an Elion DV-502 evaporator. Mengo pseudovirion suspensions purified by sucrose density gradient centrifugation were placed undiluted on grids for 1 min, rapidly washed twice with 0.1 M sodium acetate, and then stained twice for 1 min with 5% uranyl acetate (17). Mengo virus suspensions in 0.1 M Tris buffer, pH 7.8, were placed on grids for 1 min and then stained twice for 1 min with uranyl acetate. Grids were examined in a Philips 200 electron microscope at 80 kV.

RESULTS

Formation and structure of polio and Mengo virus pseudovirions. Efficient production of pseudovirions occurs when CCMV protein is mixed at a weight ratio of 3:1 with single-stranded RNA of 10^6 molecular weight or less and dialyzed against low-ionic strength TKM buffer (1, 12). Studies with larger RNA such as TMV RNA (2.0×10^6 molecular weight) showed that CCMV protein-to-RNA ratios of 10:1 are required to produce highly infectious pseudovirions (18). Since the molecular weight of the poliovirus or Mengo virus genome is probably somewhat greater than that of TMV (5), the protein-to-RNA weight ratio was increased to 12:1. As reconstitution of the poliovirus or Mengo virus genome into pseudovirions was efficient under these conditions, no further experiments were conducted to determine the optimal ratio of picornaviral RNA to CCMV protein for pseudovirion formation.

Polio RNA (75 μ g/ml) and CCMV protein (890 μ g/ml) in a volume of 0.6 ml were dialyzed against 100 ml of TKM buffer for 2 h at 0 C. The reconstitution product and undialyzed polio RNA were then sedimented through paired sucrose density gradients, and the gradient fractions assayed for radioactivity and infectivity. The results (Fig. 1) show that the reconstitution of the polio genome within a CCMV protein shell is a highly efficient process, as might be expected from Bancroft's studies on plant virus reconstitution. The zone of radioactivity (polio RNA) is shifted from the top of the sucrose gradient to near the bottom after the assembly process. Also, the reconstitution product of polio RNA and CCMV protein appears to be fairly homogeneous. In a similar experiment, a cushion of dense cesium chloride was placed at the bottom of the sucrose gradient; after fractionation, no radioactivity was found at the sucrose-cesium chloride interface, indicating an absence of rapidly sedimenting aggregated or precipitated protein-RNA complexes. Little

FIG. 1. Sedimentation and infectivity of polio pseudovirions and RNA. Polio pseudovirions (○) and polio RNA (●) were prepared as described in the Materials and Methods section and placed on separate 10 to 40% sucrose gradients. Centrifugation was for 2 h at $130,000 \times g$ and 4 C. Fractions were collected by puncturing the bottom of the centrifuge tube and 100- μ liter samples were assayed for radioactivity or infectivity.

overt nucleolytic degradation of polio RNA occurs during reconstitution, as shown by the small amount of low molecular weight radioactivity remaining at the top of the gradient. The pseudovirion fractions were infectious only when assayed in the presence of DEAE-dextran; however, as shown in Fig. 1, the specific infectivity of the RNA or pseudovirion gradient fractions was about the same. (The infectivity observed in the pseudovirion gradient is not due to residual undegraded polio virus since, when fraction 3 was assayed on HeLa monolayers without DEAE-dextran, no infectivity could be detected). It is still possible that if RNA were reisolated from gradient-purified pseudovirions, its specific infectivity would be lower than that of RNA before reconstitution, implying that CCMV protein encapsidation can increase the infectivity of polio RNA. The small amount of

material available in these experiments, however, did not allow for this control.

The size and structure of the pseudovirions were investigated next. Cosedimentation of polio pseudovirions with bushy stunt virus (BSV) indicated (Fig. 2) that the particles clearly had a sedimentation coefficient in excess of 132S (that of BSV)—probably in the area of 150 to 160S, as estimated by Verduin and Bancroft for their pseudovirions formed from TMV RNA and CCMV protein (18). Although the sedimentation coefficient of polio pseudovirions is similar to that of poliovirus (160S), this agreement may be fortuitous.

In another experiment, polio pseudovirions were prepared as described previously and purified by sucrose density gradient centrifugation. The peak fractions representing the bulk of radioactivity and having a sedimentation coefficient of about 150S were examined spectrophotometrically. The results in Fig. 3, comparison of the ultraviolet spectra of poliovirus and polio pseudovirions, show a greater degree of

FIG. 2. Cosedimentation of polio pseudovirions and bushy stunt virus. Conditions were the same as in Fig. 1 except BSV was added to the pseudovirions before centrifugation. Fractions were assayed for absorbance (○) and then radioactivity (●).

light scattering and a lower $A_{260/280}$ ratio for the pseudovirions, which is consistent with a larger particle containing a lower percentage of RNA or with the presence of light-scattering protein aggregates.

The structure of similar pseudovirions was examined further in electron micrographs. CCMV protein (1,070 $\mu\text{g/ml}$) and Mengo virus RNA (90 $\mu\text{g/ml}$) were dialyzed against TKM buffer for 2 h followed by dialysis against 0.1 M sodium acetate (NaAc), pH 5.7, for 2 h. This solution was then layered on a sucrose density gradient (10 to 40% wt/vol in 0.1 M NaAc, pH 5.7), and the pseudovirion peak was collected. A parallel gradient with a BSV marker showed

FIG. 3. UV spectra of polio pseudovirions and poliovirus. Poliovirus (●) and polio pseudovirions (○) were prepared as described in the Materials and Methods section. Pseudovirions were purified by sucrose density gradient centrifugation and spectrophotometric measurements made without prior dialysis.

that the Mengo pseudovirions sedimented at a rate similar to that of polio pseudovirions. Electron micrographs were prepared as described in the Materials and Methods section, and the results are shown in Fig. 4A. For comparison, electron micrographs of purified Mengo virus are also shown (Fig. 4B).

The diameter of the mengo pseudovirions, as shown in Fig. 4A, was slightly smaller (24 nm) than that of Mengo virus (25 nm) (Fig. 4B). Our value for the size of Mengo virus is in good agreement with an accurate value for particle diameter of 26.7 nm (16). The greater penetration of stain within the pseudovirions suggests that these particles may be more porous than Mengo virus. Our results argue against the possibility that the pseudovirions are larger in size than poliovirus or Mengo virus. The higher degree of light scattering observed for the polio pseudovirions relative to poliovirus (Fig. 3) is therefore most likely due to cosedimentating CCMV-protein aggregates.

The ribonuclease sensitivity of Mengo virus, Mengo pseudovirions, and Mengo virus RNA was next compared. Mengo pseudovirions were prepared as described previously but were not further purified by sucrose density gradient centrifugation. They were diluted in MEM containing 20 mM HEPES, pH 7.2, and 4 μg of RNase per ml or TKM buffer containing 4 μg of RNase per ml. Mengo virus and Mengo virus RNA were diluted in MEM containing 20 mM HEPES, pH 7.2, and 4 μg of RNase per ml and samples were incubated at 37 C for various lengths of time. The trichloroacetic acid-precipitable radioactivity was then determined; the results are shown in Fig. 5. The Mengo pseudovirions were degraded fairly rapidly compared with Mengo virus. The rate of degradation, however, was significantly less than that of Mengo RNA alone. In addition, the pseudovirions were somewhat less resistant to degradation in MEM than in TKM buffer, most likely because the higher ionic strength of the MEM caused the pseudovirion particles to swell. These results indicate that the Mengo or polio pseudovirions are similar in size to their original picornaviruses but are perhaps more porous and, therefore, subject to greater nucleolytic degradation.

Comparison of the infectivities of polio pseudovirions and polio RNA. Polio pseudovirions and RNA were diluted in MEM containing 100 μg of DEAE-dextran per ml, and samples were assayed on unwashed HeLa monolayers. The results are shown in Table 1A. The infectivity of polio RNA was extremely low relative to that of poliovirus, partly because the

FIG. 4. Electron micrographs of (A) *Mengo* pseudovirions and (B) *Mengo* virus. Suspensions of gradient-purified particles were placed on grids and stained with 5% uranyl acetate. Total magnification in both cases was $\times 190,000$.

HeLa monolayers were not prewashed to remove serum and cellular nucleases. It is of interest, however, that the infectivity of the polio pseudovirions assayed with DEAE-dextran was about 50-fold greater than that of polio RNA. Results similar to those in Table 1A were obtained when this experiment was repeated. Without DEAE-dextran, the polio pseudovirions were not infectious. These results suggested that the polio genome was protected from cellular and serum nucleases after encapsidation by CCMV protein. To increase the sensitivity of the infectivity assay, the previous experiment was repeated with prewashed HeLa monolayers with components made from new preparations of polio RNA and CCMV protein. As shown in Table 1B, the relative infectivity of polio RNA and pseudovirions was increased on prewashed monolayers, but the specific infectivity of pseudovirions was about half that of RNA. Such results would not be surprising if pseudovirions were more resistant to large amounts of cell and serum nucleases, whereas with low levels of nucleases the inhibitory action of DEAE-dextran would be sufficient.

It must also be noted that in our study the infectivity of the polio RNA relative to poliovirus was quite low compared with the best results reported by others (4, 15). This difference may be due to the relatively poor sensitiv-

ity of the HeLa monolayer assay compared with HeLa suspension cell assays.

DISCUSSION

Our results indicate that the poliovirus genome can be efficiently encapsidated with CCMV protein to form a nearly homogeneous population of pseudovirions. However, using this technique to facilitate greatly the uptake and expression of biologically active nucleic acids does not appear very promising. Recent evidence indicates that one of the poliovirus structural proteins (VP-4) increases the sensitivity of HeLa cells for infection by polio RNA (4). Moreover, inactivation of VP-4 on the polio virion by heat or diethylpyrocarbonate produces particles incapable of being adsorbed by HeLa cells, although they still contain RNA as infectious as that isolated from native poliovirus (4). Thus, it is not surprising that the polio pseudovirions examined in our investigation were not infectious in the absence of DEAE-dextran. It would be interesting to examine the infectivity of polio pseudovirions reconstituted in the presence of VP-4. As plant pseudovirions containing mixed coat proteins can be reconstituted from the capsomeres of CCMV and related plant viruses (19), it may be possible to form infectious polio pseudovirions where VP-4 protein

FIG. 5. Enzymatic hydrolysis of ^3H -uridine-labeled Mengo virus, Mengo pseudovirions, and Mengo virus RNA. All incubations were at 37 C with pancreatic ribonuclease (4 $\mu\text{g}/\text{ml}$). Symbols: ◆, Mengo virus; ▲, Mengo pseudovirions; ●, Mengo virus RNA in MEM with 20 mM HEPES buffer, pH 7.2; ■, Mengo pseudovirions in TKM buffer. Radioactivity precipitable by trichloroacetic acid was assayed by using standard techniques.

would be reconstituted with CCMV protein as part of the pseudoviral shell.

Although there have been recent studies on disassembly and reconstitution of poliovirus (6, 7) and polyoma (11), polynucleotides and proteins were not purified free from each other before reconstitution, as has been done for plant (1, 9, 10) and bacterial (13) viruses. Also, the efficiency of such animal virus reconstitutions was much lower than that observed for plant viruses. The study of Drzeniek and Bilello (6) showed that when poliovirus was dissociated with urea and then reassociated by stepwise dilution in PBS, about 50% of the infectivity of the RNA could be converted to viral infectivity. However, this reassociated poliovirus could only be detected by infectivity measurements in a sucrose density gradient, even though the RNA was labeled with ^3H -uridine to a very high specific activity. These observations may indi-

TABLE 1. Infectivities of polio RNA, pseudovirions, and virus^a

Expt.	Polio component	DEAE-dex-tran	Spec. infectivity (PFU per counts per min)	Relative infectivity
A	Virus	—	2.8×10^5	1.0
	RNA	+	3.7×10^{-4}	1.3×10^{-9}
	Pseudovirions	+	1.7×10^{-2}	6.0×10^{-8}
	Pseudovirions	—	0	0
B	Virus	—	2.5×10^6	1.0
	RNA	+	2.5	1.0×10^{-6}
	Pseudovirions	+	1.2	4.8×10^{-7}
	Pseudovirions	—	0	0

^a Poliovirus, RNA, or pseudovirions were assayed on confluent monolayers of HeLa cells with or without DEAE-dextran (100 $\mu\text{g}/\text{ml}$). Polio pseudovirions were prepared by dialyzing polio RNA and CCMV protein (1:12 wt/wt) TKM buffer. The same preparation of ^3H -uridine-labeled virus was used in each experiment for preparation and assay of the various components. The specific activity of the ^3H polio RNA was 2.3×10^6 counts per min per μg . In experiment B, the HeLa monolayers were prewashed with PBS before assay of infectivity whereas in experiment A they were not.

cate that a high level of specificity exists for the in vitro reassociation of poliovirus proteins with poliovirus RNA. In contrast, CCMV protein efficiently forms virus-like structures with most single-stranded polyribonucleotides, regardless of size or sequence.

Whether CCMV-polio pseudovirions are taken up intact by HeLa cells is also unclear. Considering the isoelectric point of CCMV (3), it would be expected that the pseudovirions would be negatively charged in MEM at pH 7.5 and, consequently, repelled by the HeLa cell membrane; addition of DEAE-dextran would help counteract this charge repulsion and therefore aid in penetration. Alternatively, the pseudovirions might be disrupted by the DEAE-dextran with the released infectious RNA entering the cell minus the CCMV capsomeres. Polio pseudovirions in which the CCMV protein is also radioisotopically labeled, and the use of a new, improved method for assaying the infectivity of polio RNA would help to resolve this question (15).

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