Formation of Mengovirus-like Particles in Cell-Free Extracts from Virus-Infected Cells

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^{3}H-labeled particles with the density of intact mengovirus in CsCl were detected following the incubation of cell-free extracts from mengovirus infected cells with ^{3}H-UTP in a RNA polymerase reaction mixture. The ^{3}H-particles contained complete strands of ^{3}H-labeled 35 S mengovirus RNA. The viral-like particles were found in the region of a sucrose gradient (150-250 S) where viral-specific RNA polymerase activity is detected.

Viral-specific RNA polymerase activity from cells infected with foot-and-mouth disease virus (1-3), mengovirus (4,5), and poliovirus (6,7) is found in rapidly sedimenting structures, termed the replication complex (8). These structures sediment between 100-300 S following treatment with detergents, such as sodium deoxycholate, NP-40, and Triton X-100. Mengovirus structural polypeptides and polypeptide ϵ [the precursor to capsid polypeptides β and δ (9)] are associated with or cosediment with the detergent treated polymerase structures isolated by sucrose gradient centrifugation (10). Poliovirus structural polypeptides have also been detected in the poliovirus-specific polymerase complex (7). If virion polypeptides and their precursors are indeed associated with the RNA polymerase, then virion RNA synthesis and assembly may be closely linked. If this is the case, then it may be possible to form or complete virus particles *in vitro*. We provide evidence in this report that virus-like particles with the density of mengovirus and containing intact viral RNA are formed during *in vitro* viral RNA synthesis.

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RESULTS

The S-5 extract was incubated for 60 min at 37°C in the RNA polymerase mixture containing ^{3}H-UTP. The incubated extract was treated with detergent, and fractionated on a 10-50% linear sucrose gradient. The fractions were divided into two equal portions for CCl_3COOH precipitation and binding to nitrocellulose filters in TMN buffer (Fig. 1A). The results show two classes of CCl_3COOH-insol-

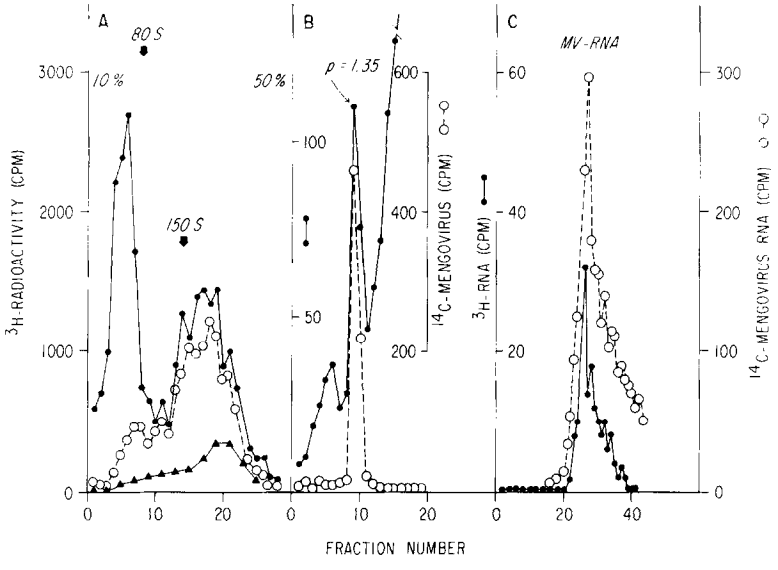

Fig. 1 Analysis of the S-5 extract following incubation in a cell-free RNA polymerase assay mixture containing ^{3}H-UTP. Baby hamster kidney (BHK) cells (1×10^{9} cells) were infected in roller bottle cultures with ten plaque forming units (p.f.u.) per cell of mengovirus and the cells harvested 5 hrs after infection as described (2). The cells from two confluent cultures were homogenized in 3 mls of 0.01 M Tris, 0.001 M MgCl (TM) and then an equal volume of a solution containing 0.2 M KCl and 0.50 M sucrose was added. After removal of the nuclei and cell-debris (5,000 x g for 10 min), a 0.7 ml aliquot (2.1 mg of protein) of the supernatant (S-5 extract) was incubated for 60 min at 37°C in 2.1 ml of RNA polymerase reaction mixture (4) containing 50 μCi of ^{3}H-UTP at a specific activity of 5,000 μCi/μmole. The mixture was chilled, diluted to 3.0 ml, and adjusted to 0.5% Triton X-100 (Beckman Instruments, Palo Alto, California). Panel A. Sucrose gradient centrifugation. The solid line, closed circles represents CCl_3COOH-insoluble ^{3}H-radioactivity; dashed line, open circles is nitrocellulose filter-bound ^{3}H-radioactivity; solid line with filled triangles is nitrocellulose filter bound RNA polymerase activity. The reaction mixture was applied to a 34 ml 10-50% linear sucrose gradient buffered in TM. The sample was centrifuged at 13,000 r.p.m. in a SW-27 rotor for 16 hrs at 4°C. Fractions were processed by either a CCl_3COOH precipitation procedure (2) or by binding to nitrocellulose filters. The binding to nitrocellulose filters (Type B-6, Schleicher and Shuell, Keene, N. Y.) was accomplished by dilution of the gradient fractions (2-fold) in 0.01 M Tris, pH 7.5, 0.05 M

uble products. The faster sedimenting component has a sedimentation rate of about 250 S. The slower sedimenting acid-insoluble material sediments at less than 80 S. The sucrose gradient fractions 14-22 (Fig. 1A) contain ^{3}H-labeled replicative intermediate (RI) RNA and a heterogeneous fraction of single stranded viral RNA ranging from 35 S to low molecular weight RNA as determined by sodium dodecyl sulfate (SDS)-agarose gel electrophoresis (11).

Analysis of the fractions by binding to nitrocellulose filters in TMN buffer showed that the 250 S component almost quantitatively binds to nitrocellulose filters whereas the slowly sedimenting structure had little tendency to bind to nitrocellulose filters (Fig. 1A). The inability of the CCl_3COOH-insoluble material from the slowly sedimenting peak to bind to the nitrocellulose filters may be explained by the fact that free viral RNA is not bound to the filters under these conditions but ribonucleoprotein (RNP) particles are. For example, ^{14}C-uridine labeled mengovirus (4) (10-20 μg) is quantitatively retained on the filter in TMN binding buffer whereas the viral ^{14}C-RNA is not. Of 2,400 cpm of CCl_3COOH-insoluble ^{14}C-mengoviral RNA applied to the filter, only 50 cpm is retained on the filter. Thus, the rapidly sedimenting structure contains ^{3}H-

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NaCl, and 0.001 M $MgCl_2$ (TMN), passing the solution through the filters, and washing 5 times with TMN buffer. The material bound to the nitrocellulose filters in TMN buffer was examined for viral RNA polymerase activity as follows. The S-5 extract was incubated 30 min at 37°C in the polymerase mixture in the presence of only unlabeled nucleoside triphosphates. The mixture was detergent treated and fractionated on a sucrose gradient as described above. After passing the fractions through the nitrocellulose filters, the filters were incubated in 0.7 ml of the polymerase mixture containing ^{3}H-UTP. The filters were rinsed three times in 0.1 M sodium pyrophosphate containing 0.1 mM UTP and washed on a filter grid five times with cold 5% CCl_3COOH and counted as usual (13). Panel B. Cesium chloride gradient centrifugation. Sucrose gradient fractions 19-21 from Panel A (volume 5.5 ml) were mixed with purified ^{14}C-uridine labeled mengovirus (4) and applied to 25 to 53% (w/w) linear 12 ml CsCl gradient buffered in TM and centrifuged at 23,000 r.p.m. for 22 hrs in a SW-27 rotor at 4°C. The fractions (0.9 ml) were processed through a CCl_3COOH wash procedure as in Panel A. The direction of sedimentation is from left to right. Panel C. SDS-agarose gel electrophoresis. Two parallel gradients as in Panel B were processed and the regions co-banding with the ^{14}C-mengovirus were pooled, diluted ten-fold with 0.01 M Tris, pH 7.5, 0.1 M NaCl, 0.001 M EDTA (TNE) buffer, and centrifuged at 100,000 x g for 60 min. The pellet was rinsed with TNE buffer, and extracted with 1% SDS-phenol-chloroform as described by Perry et al. (12). The RNA sample was fractionated on a 10 ml SDS-agarose gel (0.75% w/v) as described by Arlinghaus et al. (11). Ribosomal RNA (28 S and 18 S) migrated to gel slices 30-32 and 36-40, respectively.

labeled RNP structures whereas the slowly sedimenting structure may be free ^{3}H-RNA.

The rapidly sedimenting structure bound to nitrocellulose filters was shown to contain RNA polymerase activity (Fig. 1A). The maximum polymerase activity cosedimented with the rapidly sedimenting peak of nitrocellulose-bound RNP structures labeled in vitro. This nitrocellulose filter-bound polymerase activity has the same sedimentation characteristics as the 250 S viral RNA polymerase (2-4). This enzyme activity was not found in the uninfected cell, and peak activity appeared after infection at a point in time coinciding with the peak of mengovirus-specific RNA synthesis in whole cells. Polymerase activity is completely resistant to Actinomycin D (5 μg/ml) added during in vitro incubation. These data indicate that the nitrocellulose filter-bound polymerase activity is mengovirus-specific.

To determine if the ^{3}H-RNA associated with the rapidly sedimenting RNP structures is viral-specific, ^{3}H-RNA containing 90% RI RNA was examined by competition hybridization experiments with 35 S mengovirus RNA. This ^{3}H-RNA preparation is about 65% resistant to pancreatic RNase and Ti-RNase (Table 1). After denaturation, the ^{3}H-RNA is 8% resistant to these RNases. Upon reannealing, 51% remains resistant to RNase. Addition of 100 μg of BHK cell ribosomal RNA had no effect on the reassociation of the ^{3}H-labeled RNA. Whereas 100 μg of 35 S mengovirus RNA reduced the RNase resistance to 19%. These results provide evidence that ^{3}H-RNA synthesized in the S-5 extract and associated with rapidly sedimenting RNP structures contains sequences similar to 35 S mengovirus RNA.

Since the rapidly sedimenting structures contain virus-specific RNP particles, it was of interest to examine these RNP particles by banding in isopycnic CsCl gradients. Three regions of the sucrose gradient (Fig. 1A) were examined on preformed linear CsCl gradients (25-53% w/w) buffered in TM (Fig. 1B). The bulk of the ^{3}H-labeled RNA-containing structures from sucrose gradient fractions 5-6, 13-15 and 19-21 were found in the pellet of the CsCl gradient (80%-

TABLE I

Reannealing of ^{3}H-RNA associated with the 250 S RNP particles in presence and absence of 35 S mengovirus RNA

Conditions	Resistance to RNase
	(%)
^{3}H-RNA	65
Denatured ^{3}H-RNA	8
Reannealed ^{3}H-RNA	51
^{3}H-RNA reannealed with ribosomal RNA	52
^{3}H-RNA reannealed with 35 S viral RNA	19

The ^{3}H-RNA was prepared by incubation of the S-5 extract (0.7 ml or 2.2 mg of protein) for 10 min in the RNA polymerase incubation mixture. The reaction mixture was fractionated as in Fig. 1A. The ^{3}H-RNA (85%) was eluted from the nitrocellulose filters with 1% SDS buffered in TNE. The ^{3}H-RNA was extracted as described (12). The ^{3}H-RNA was analyzed by SDS-agarose gel electrophoresis (11) and found to be 90% RI RNA and 10% low molecular weight single-stranded RNA. The ^{3}H-RNA was heated to 100°C in H_2O for 2 min, quick-cooled in ice. Parallel samples were reannealed (14) in 0.05 ml of 2X SSC (SSC:150 mM NaCl, 15 mM sodium citrate, pH 7.4) at 69°C for 24 hrs in sealed ampules. BHK ribosomal RNA (100 μg) and 100 μg of 35 S mengovirus RNA (4) were added where indicated. To determine the precent of RNase resistance, one-half of the sample was precipitated with 5% CCl_3COOH. The other half was incubated with 10 μg/ml of pancreatic RNase and 50 units/ml of T_1 RNase for 30 min at 22°C in 0.3 M NaCl, 0.01 M Tris, pH 7.5, CCl_3COOH precipitated, and counted (13).

90%). However, fractions 13-15 (~150 S) and 19-21 (~250 S) contained a peak of ^{3}H-labeled material which co-banded with intact mengovirus particles (Fig. 1B). No such peak was found in the sucrose gradient fractions 5-6. These labeled particles represented about 10% of the ^{3}H-labeled material applied.

The material which co-banded with intact mengovirus (Fig. 1B) was pooled, diluted with 0.01 M Tris, pH 7.5, 0.1 M NaCl, 0.001 M EDTA (TNE) and isolated by centrifugation. The pellet was extracted with SDS-chloroform-phenol (12) and the RNA was analyzed by electrophoresis on 0.75% (w/v) agarose gels (11) (Fig. 1C). The results show that the material co-banding with mengovirus in CsCl gradient (Fig. 1B) contains RNA which migrates as intact viral RNA.

DISCUSSION

The results presented in this paper provide evidence that particles containing 35 S mengoviral ^{3}H-RNA and having the density of intact mengovirus are formed during in vitro synthesis of viral RNA in the S-5 extract. These labeled particles were found in 150-250 S region of the gradient, which also contains the mengovirus-specific RNA polymerase. The rapidly sedimenting structures also contain RI RNA, 35 S viral RNA, and the lower molecular weight single stranded RNA as determined by SDS-agarose gel electrophoresis.

The rapidly sedimenting structures almost quantitatively bind to nitrocellulose filters in TMN buffer. Under these conditions free viral RNA does not bind while mengovirus (20 μg) is quantitatively bound. Thus, the ^{3}H-labeled RNA components in the rapidly sedimenting structures have the properties of RNP particles. Competition hybridization experiments with RNase resistant ^{3}H-RNA isolated from the RNP particles indicate that sequences similar to 35 S mengovirus RNA are present in the rapidly sedimenting RNP particles. These RNP particles also contain mengovirus-specific RNA polymerase activity (Fig. 1A) as measured by its resistance Actinomycin D, its time of appearance in the infected cell, and its sedimentation rate in sucrose gradients.

Our previous results have shown that mengovirus (150 S), formed in the infected cell and present in Triton X-100 treated cytoplasmic extracts, sediments in a heterogeneous fashion in the 150-300 S region of a sucrose gradient buffered in TM (Loesch, Ph.D. Dissertation, 1973). Furthermore, the addition of the S-5 extract to purified ^{3}H-mengovirus causes an increase in the virus sedimentation rate from 150 S to 200-300 S. Thus, the finding of ^{3}H-RNA labeled mengovirus-like particles associated with or co-sedimenting with the 150-300 S polymerase fractions is not sufficient evidence to prove that these particles were formed in the rapidly sedimenting polymerase structure. However, this finding considered in light of the observed presence of virus precursor polypeptide ε and structural polypeptides α and γ in the rapidly sedi-

menting RNA polymerase complex (10), suggests that virion RNA synthesis and virus assembly may be closely linked.

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