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Cytoplasmic Fractions Associated with Semliki Forest Virus Ribonucleic Acid Replication

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When actinomycin D-treated chick fibroblasts were labeled with ^{3}H-uridine for varying periods during the log phase of Semliki Forest virus infection, radioactivity was found associated with different cytoplasmic fractions. After a 1-min period of labeling, it appeared in a large cytoplasmic structure which was seen in electron micrographs of infected cells. Sediments of sucrose density gradients of cytoplasmic extracts of these cells also contained these structures. Three forms of viral ribonucleic acid (RNA) were associated with this cytoplasmic structure: a ribonuclease-sensitive 42*S* form identical to the RNA of the mature virus, a ribonuclease-sensitive 26*S* form, and a ribonuclease-resistant 20*S* form. After a 5- to 10-min labeling period, radioactivity was associated with a ribonuclease-sensitive 65*S* cytoplasmic fraction which contained only the 26*S* RNA form. Finally, after a 1-hr labeling period, a 140*S* ribonuclease-resistant particle was the most prominent radioactive structure in the cytoplasm. This particle contained only 42*S* viral RNA. Negative-contrast electron micrographs of the 140*S* particle and the virion demonstrated structural differences between them. The base compositions of the 42*S* and 26*S* viral RNA forms were not significantly different. The base composition of the 20*S* form differed significantly from that of the other two viral RNA forms, but the values obtained for the mole fractions of the bases present in the 20*S* form differed, and depended on the period during the virus growth cycle in which ^{32}P was present. These results suggested that viral RNA originated in the large cytoplasmic body. The 20*S* RNA appeared to be a structure engaged in viral RNA replication and the 140*S* particle appeared to be a virus precursor.

Several forms of virus ribonucleic acid (RNA) other than that appearing in the virion have been discovered in cells during the process of replication of animal RNA viruses, but the relationship between these RNA forms and the production of mature virus is poorly understood (13). Even less is known about the relationship between these forms of viral RNA and the cell structures on which the replication processes take place.

Although most studies on the replication of animal RNA viruses have been carried out on picornaviruses, the process of replication of the group A arbovirus Semliki Forest virus (SFV) has recently been studied in some detail (26). At least three forms of viral RNA were present during replication of SFV in chick embryo fibroblasts (CEF): (i) a ribonuclease-sensitive 42*S* form identical to the RNA of the mature virus; (ii) a ribonuclease-sensitive 26*S* form; and (iii) a ribonuclease-resistant 20*S* form. Pulse-labeling studies showed that isotope was first associated with the 20*S* form, then with the 26*S* form, and, finally, with the 42*S* form (7). In studies with tritiated input viral RNA, radioactivity rapidly became associated with the 20*S* and 26*S* RNA forms (7).

In this study, the presence of viral RNA in three different cytoplasmic fractions was noted. New viral RNA was first associated with a rapidly sedimenting body which was seen in electron micrographs of sediments obtained by zone centrifugation of cytoplasmic extracts of virus-infected cells. Similar cytoplasmic particles were seen in electron micrographs of thin sections from infected cells but not from uninfected controls. This body was found to contain all three forms of viral RNA. With longer pulse periods, a 65*S* ribonuclease-sensitive fraction was labeled with isotope. This fraction contained only 26*S* RNA. With still longer pulses, a ribonuclease-resistant 140*S* particle became labeled. This particle contained only 42*S* RNA. Base ratio analysis of the three RNA forms showed that the 42*S* and 26*S* forms had a similar base composition. The 20*S*

form had a base composition which differed from that of the 42S and 26S forms.

MATERIALS AND METHODS

Cells, media, and virus. The method of preparation of CEF monolayers and the media employed have been described in detail (27). The preparation of high-titered pools of the Kumba strain of SFV in CEF has also been described (8). Virus purification was by Mecs' (16) modification of Cheng's method (3).

Virus growth. The method of infection of the CEF employed has been described in detail (7). Briefly, CEF were infected for 80 min at 37 C at a virus-cell ratio of 40:1. The cells were then incubated overnight at 4 C in medium containing 1 μg/ml of actinomycin D (ACM). When the plates were warmed to 37 C, virus replication began almost immediately; the log phase of virus growth occurred 3 to 5 hr after warming. Most studies were carried out beginning at 4 hr after warming to 37 C. In studies on base composition, cells were infected overnight at 4 C, and then warmed to 37 C. In this case, the log phase of virus growth was 4 to 6 hr after warming to 37 C.

Preparation of cytoplasmic particles. CEF monolayers were washed five times with 0.85% saline, removed from plastic plates by scraping into RSB (23) [0.01 M KCl, 1.5×10^{-3} M $MgSO_4$, 0.01 M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.4], and disrupted with three strokes of a Dounce homogenizer. Sufficient sucrose was added to adjust the concentration to 5%, and two additional strokes applied. More than 95% of the cells were broken by this procedure, but nuclei remained intact. The disrupted cells were sedimented ($800 \times g$) for 10 min at 3 C. Since this sediment contained less than one-fourth of the radioactivity of the cytoplasmic supernatant fluid, it was not further studied. The cytoplasmic supernatant fluid was then layered over a 15 to 30% sucrose gradient in RSB and was sedimented in a swinging bucket rotor as indicated under Results. Sedimentation values were estimated by the method of Martin and Ames (15), with the use of either chick ribosomes (74S) or ribosomal RNA (28S and 16S) as reference markers.

Fractions were collected by puncturing the bottom of the centrifuge tube and collecting the effluent. The RNA of the fractions was estimated by measuring optical density (260 mμ) either by pumping the effluent through a Gilford recording spectrophotometer or by readings on individual samples. Radioactivity of samples was measured by drying 0.1- or 0.2-ml samples on filter paper strips and washing with perchloric acid and then with alcohol by the method of Dalgarno et al. (5). Counting was performed in a Packard Tri Carb scintillation spectrometer model 3003.

RNA extraction. RNA was extracted by adding sodium dodecyl sulfate (SDS) to a concentration of 0.5% to cytoplasmic fractions (22). The RNA thus extracted was alcohol-precipitated with added unlabeled ribosomal RNA carrier. After 16 hr at −20 C, the precipitate was dissolved in Tris buffer (0.1 M KCl, 0.01 M Tris, 0.001 M ethylenediaminetetraacetate, *p*H 7.1), layered on a 6 to 30% sucrose gradient, and sedimented as indicated below.

Base ratio analysis. Actinomycin D-treated CEF monolayers, containing 5×10^8 cells, were exposed to 7 mc of ^{32}P in otherwise phosphate-free medium for at least 4 hr during SFV infection. For base ratio analysis, RNA was extracted from cytoplasm or from purified SFV with 3% SDS at 37 C, and then with 2 volumes of water-saturated phenol at 60 C. The extraction was carried out in 0.05 M potassium phosphate buffer (*p*H 7.1). The extracted RNA was then alcohol-precipitated twice, dissolved in 1 ml of 0.01 M sodium acetate (*p*H 5.1), and passed through a Sephadex G-25 column. The fractions containing RNA were alcohol-precipitated, dissolved in Tris buffer, and fractionated in a 6 to 30% sucrose density gradient. Fractions containing the form of viral RNA under study were alcohol-precipitated and again sedimented in a sucrose gradient; two or three fractions containing the RNA species under analysis were pooled, acid-precipitated, hydrolyzed in 0.3 N KOH, and again acidified with perchloric acid. After the second sedimentation in sucrose, each of the RNA forms under study was present as a single, sharp peak of radioactivity. Base ratios were determined by the method of Sebring and Salzman (25). Specimens analyzed for 20S RNA were treated with 2 μg/ml of ribonuclease (0.05 M KCl, 37 C, 10 min, *p*H 7) after SDS, but before phenol extraction. Under these conditions, the 42S and 26S viral RNA were hydrolyzed to acid-soluble or small acid-precipitable fractions.

Electron microscope preparations. Cells were concentrated by centrifugation, and the resulting pellet was fixed for 45 min with 3% gluteraldehyde in 0.1 M sodium phosphate buffer (*p*H 7.3). The pellets were washed with buffer containing 0.3 M sucrose, postfixed in 1% buffered osmium tetroxide for 1 hr, rapidly dehydrated in graded alcohol solutions, and embedded in Epon (14). Sections were cut on an LKB microtome with glass knives, mounted on uncoated grids, stained in uranyl acetate, and examined in an RCA EMU-3F microscope.

For negative staining studies, a drop of the preparation was placed on Formvar-coated grids for 1 to 2 min. Excess fluid was blotted, and a drop of 1% phosphotungstic acid (*p*H 5.3) containing 0.4% sucrose was added (10). This was again blotted, and the preparations were immediately examined. The electron micrographs were taken at an instrument magnification of 13,000 to 26,000.

Reagents. Uridine-5-^3H (21.0 c/mmole was purchased from Schwarz Bio Research, Inc., Orangeburg, N.Y. Actinomycin D was donated by Merck, Sharp, and Dohme, Rahway, N.J. Pancreatic ribonuclease was purchased from Calbiochem, Los Angeles, Calif.

RESULTS

Three cytoplasmic fractions associated with SFV replication. When SFV-infected CEF were pulse-labeled with ^3H-uridine for varying periods during the log phase of virus growth, studies of cell extracts showed association of tritium with different cytoplasmic fractions in the different periods studied (Fig. 1). With very short pulse-

labeling periods, all of the radioactivity was associated with sediment (Fig. 1A). In other experiments (Fig. 2), these counts were found to sediment under approximately the same conditions (3×10^5 g-min) as the virus-synthesizing body of poliovirus (22). With longer labeling periods (5 to 10 min), radioactivity in the cytoplasm was also associated with a fraction which sedimented with 74*S* chick ribosomes (Fig. 1B). Finally, in cultures incubated with ^{3}H-uridine for 60 min, radioactivity in the cytoplasmic extracts was predominantly associated with a particle which sedimented at about 140*S* (Fig. 1C). Cytoplasmic extracts from ACM-treated, but uninfected, CEF which had been incubated for 1 hr with ^{3}H-uridine failed to show significant radioactivity in any fraction (Fig. 1D).

Sedimented radioactivity. Cells were infected with SFV and were then incubated with ^{3}H-uridine for varying periods. Cytoplasmic extracts were prepared and sedimented through sucrose (25,000 rev/min, 1 hr, SW-39 rotor); the material sedimenting to the bottom of the tube was recovered. RNA was extracted from the labeled sediments with 0.5% SDS, and the distribution of radioactivity was analyzed by zone centrifugation.

A 1-min pulse labeled only the slower sedimenting viral RNA forms (Fig. 2A); however, more than 70% of radioactivity was resistant to 1 μg/ml of ribonuclease (37C, 10 min). With longer labeling periods, at least 40% of the slower sedimenting RNA was ribonuclease-resistant, and, in addition, some 42*S* viral RNA was present (Fig. 2B-D). As in previous studies (7), longer sedimentation (39,000 rev/min, 6 hr, SW-39 rotor) clearly separated the 16 to 28*S* material into a ribonuclease-sensitive 26*S* form and a ribonuclease-resistant 20*S* form.

Treatment of a 1-min pulse-labeled extract with 1% sodium deoxycholate (DOC) before sedimentation altered the distribution of radioactivity obtained upon subsequent zonal centrifugation analysis. The radioactivity after DOC was no longer found in the sediment (Fig. 2A), but was near the top of the gradient.

Electron micrographs of infected cells taken during the log phase of virus growth (Fig. 3A) showed a large cytoplasmic structure found only in infected CEF. Similar cytoplasmic structures have been reported in several arbovirus infections (18). Examination of the sediments from the

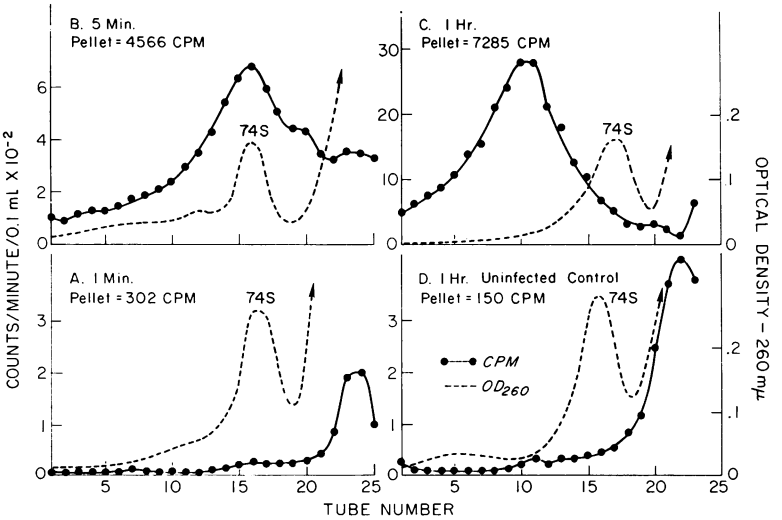

FIG. 1. *Sucrose density gradient analysis of cytoplasmic extracts from SFV-infected cells incubated with ^{3}H-uridine for varying periods. After 4 hr of SFV infection, 5 × 10^7 (B and C) or 7.5 × 10^7 (A and D) actinomycin D-treated (1 μg/ml) chick cells were incubated with 100 μc (A, B, and D) or 30 μc (C) of ^{3}H-uridine for the indicated periods. The monolayers were washed, scraped into RSB buffer (0.01 M KCl, 1.5 × 10^{-3} M $MgSO_2$, 0.01 M Tris, pH 7.4), and disrupted with a Dounce homogenizer. After sedimentation at 800 × g for 10 min, the cytoplasmic extract was layered over a 15 to 30% sucrose gradient prepared in RSB. The extracts were then sedimented at 38,000 rev/min for 1 hr in an SW-39 rotor. Fractions were collected by puncturing the bottom of the tube, and the collected fractions were analyzed for optical density at 260 mμ or for acid-precipitable radioactivity (see Materials and Methods). Monolayers were incubated with ^{3}H-uridine for (A) 1 min, (B) 5 min, or (C) 1 hr; (D) uninfected control treated with actinomycin D for 4 hr before incubation with ^{3}H-uridine for 1 hr. The bottom of the gradients is to the left. The optical density peak for the chick ribosomes has been assigned a value of 74S in this and succeeding figures.*

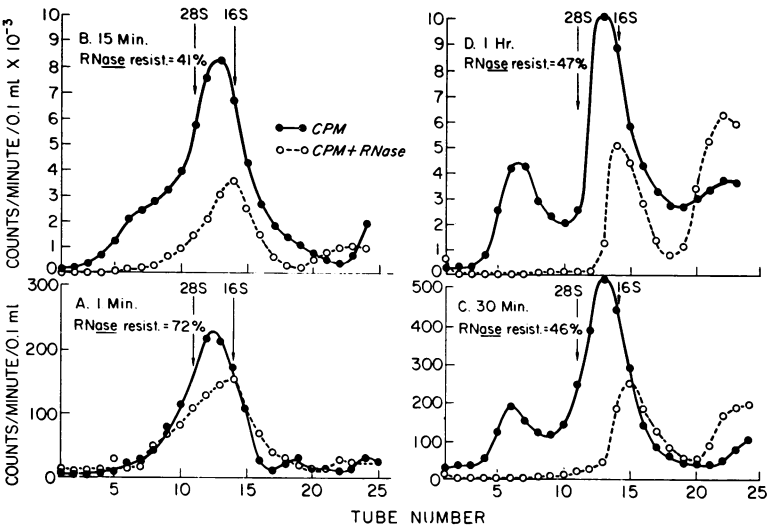

FIG. 2. *Sucrose density gradient analysis of RNA extracted from a large cytoplasmic structure. After 4 hr of SFV infection, 5 × 10⁷ actinomycin D-treated cells were incubated with 100 μc (A and B) or 30 μc (D and C) of ³H-uridine for the indicated periods. The cells were suspended in RSB, disrupted with a Dounce homogenizer, and sedimented at 800 × g for 10 min. The cytoplasmic extracts thus obtained were layered over a 15 to 30% sucrose gradient prepared in RSB and sedimented at 25,000 rev/min for 1 hr in an SW-39 rotor. The fluid was removed from the centrifuge tubes, and the sediment was extracted at 37 C with 0.5% sodium docecyl sulfate in 0.5 M potassium phosphate buffer (pH 7.1). The RNA was precipitated at −20 C in alcohol with added ribosomal RNA, and after 16 hr the precipitate was dissolved in Tris buffer (0.1 M KCl, 0.01 M Tris, 0.001 M ethylenediaminetetraacetate, pH 7.1), layered on a 6 to 30% sucrose gradient prepared in Tris buffer, and sedimented in an SW-39 rotor for 3 hr at 38,000 rev/min. Ribonuclease (RNase, 1 μg/ml) treatment was for 10 min at 37 C just before sedimentation. Fractions were collected and analyzed as described in Materials and Methods. Ribonuclease resistance was measured as a percentage of total 16 to 28S radioactive RNA. Monolayers were pulsed for (A) 1 min, (B) 15 min, (C) 30 min, (D) 1 hr. The designations 28S and 16S show the optical density (260 mμ) peaks of added ribosomal RNA. The bottom of the gradients is to the left.*

cytoplasm of SFV-infected cells showed a similar virus-specific structure (Fig. 3B).

Cytoplasmic fraction sedimenting at 65S. The radioactivity of a cytoplasmic fraction sedimenting at 65*S* was prominent in sucrose gradients made from cells which had been exposed to 100 μc of ³H-uridine for 5 to 10 min (Fig. 4). This fraction was ribonuclease-sensitive; after exposure to ribonuclease, the resultant gradients showed a polydisperse radioactive distribution (40*S* to 65*S*) instead of a homogeneous peak at 65*S* (Fig. 5).

When RNA was extracted from the 65*S* fraction, only a single species of viral RNA was present, the 26*S* form (Fig. 6A). Prolonged sedimentation failed to fractionate the peak (Fig. 6B). The 26*S* RNA was ribonuclease-sensitive; however, unlike the 42*S* RNA (Fig. 8), the 26*S* material was not completely broken down to acid-soluble fractions. A polydisperse material (8*S* to 16*S*) remained after ribonuclease treatment (Fig. 6B). The RNA extracted from ribonuclease-treated 65*S* RNA (fractions labeled "a" in Fig. 5) closely resembled this 8*S* to 16*S* polydisperse RNA when sedimented in sucrose gradients.

The 140S particle. Incubation of ACM-treated, infected CEF for 1 hr with 30 μc of ³H-uridine revealed a single prominent radioactive peak of 140*S* on sucrose density gradient analysis of the cytoplasmic extract (Fig. 7). This fraction was not sensitive to ribonuclease under the conditions employed, but became partially sensitive after treatment with 1% DOC. The only radioactive RNA extracted from the 140*S* particle proved to be the 42*S* ribonuclease-sensitive form (Fig. 8).

Negative-contrast electron microscope studies of the 140*S* fraction (Fig. 9A) revealed that it contained a particle 34 to 40 mμ in diameter. Similar studies on mature SFV gave results like those previously reported (3); the virion was found to measure 50 to 53 mμ in diameter (Fig. 9B). The 140*S* particle appeared to lack the outer envelope with projections which was present in mature SFV.

Other differences between the 140*S* particle and the virion were noted. The 140*S* particle was noninfectious in the routinely employed plaque

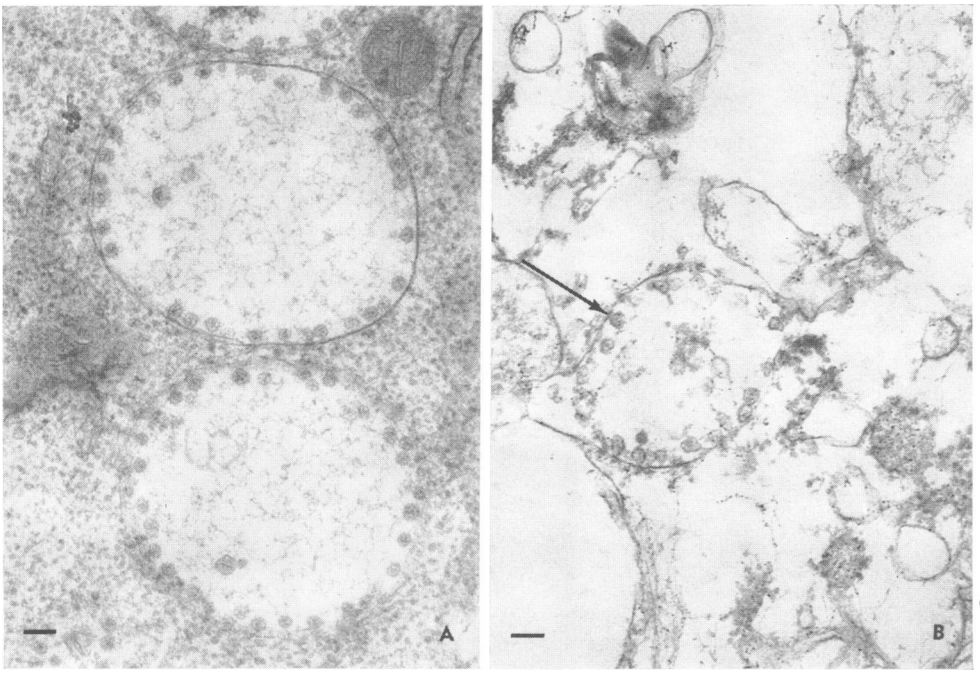

FIG. 3. *Electron micrographs of a large cytoplasmic structure in Semliki Forest virus (SFV) infection. Scale marker is 0.1 μ. (A) Cytoplasm of chick fibroblasts 4 hr after SFV infection. A large vacuolar structure is present. (B) Structure present in thin section of sucrose density gradient sediment.*

assay used for SFV. Finally, the virion sedimented in a 300 to 350*S* fraction under conditions identical to those under which the 140*S* value had been determined for the cytoplasmic particle.

Base ratio analysis of viral RNA forms. Purified samples of 42*S* viral RNA from either 140*S* cytoplasmic particles or from purified SFV, or of 26*S* RNA from cytoplasm, or of ribonuclease-resistant 20*S* viral RNA from the large cytoplasmic particle were employed to determine the mole fractions of the various bases present (Table 1). The base composition of 42*S* viral RNA extracted from CEF incubated with ^{32}P throughout the virus growth cycle was not significantly different from that of purified SFV and was similar to that determined for the RNA of a related arbovirus, Sindbis (24). The base compositions of the 42*S* form and the 26*S* form also did not differ significantly. The 20*S* ribonuclease-resistant viral form, extracted from CEF labeled from 0 to 4 hr after infection (20*S* RNA "early") had a base composition similar to that calculated for a triplex containing two strands of 42*S* RNA and one strand complementary to 42*S* RNA. The base composition of the "early" 20*S* viral RNA was significantly different from that of the 42*S* and 26*S* RNA, or that calculated for a duplex base paired structure containing one 42*S* RNA strand together with one complementary strand.

The base composition of the 20*S* RNA extracted from CEF incubated with ^{32}P from 0 to 4 hr after infection also differed from that of 20*S* ribonuclease-resistant RNA extracted from cells incubated with ^{32}P from 2 to 6 hr after infection (20*S* RNA "late"). The mole fractions of the bases of the "late" 20*S* viral RNA resembled those of the 42*S* and 26*S* viral RNA more than did those of "early" 20*S* RNA.

DISCUSSION

When SFV-infected CEF were pulse-labeled with ^{3}H uridine for varying periods, radioactivity was found to be associated with different cytoplasmic particles. Short pulses labeled only a deoxycholate-sensitive rapidly sedimenting structure probably equivalent to the particle seen in electron micrographs of the cytoplasm of arbovirus-infected cells or in the sediment of sucrose density gradient analyses of these cells. Previous studies employing Western equine encephalitis virus had suggested that virus was formed in structures or membranes of a post-mitochondrial fraction (28). The first viral RNA form to be

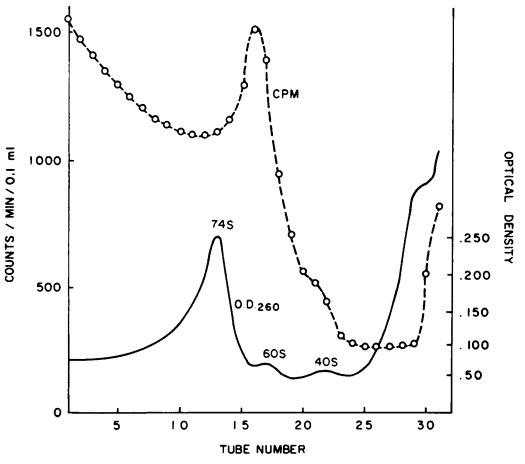

FIG. 4. *Sucrose density gradient analysis of cytoplasm extracted from SFV-infected, actinomycin D-treated cells incubated with ^{3}H-uridine for 10 min after 4 hr of SFV infection; 10^8 chick cells were incubated with 100 μc of ^{3}H-uridine. The cytoplasm was extracted as previously described (Materials and Methods) and sedimented through a 15 to 30% sucrose gradient for 20 hr at 18,000 rev/min in an SW-25 rotor. Optical density at 260 mμ was estimated by pumping the effluent through a Gilford recording spectrophotometer. Acid-precipitable radioactivity of fractions was measured in a liquid scintillation counter (see Materials and Methods). The bottom of the gradient is to the left. The optical density peaks at 74S, 60S, and 40S are those, respectively, of chick ribosomes and of two ribosomal subunits.*

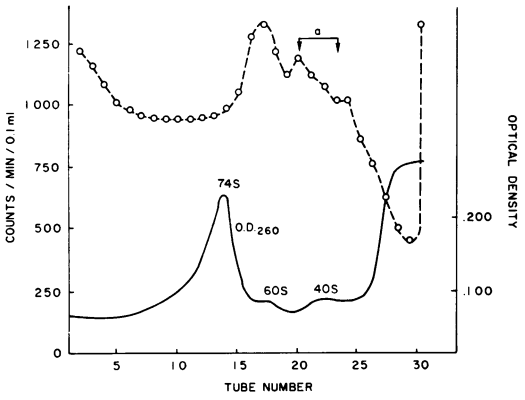

FIG. 5. *Effect of ribonuclease treatment on 65S radioactive fraction in the cytoplasm of SFV-infected and actinomycin D-treated cells. The 65S fraction was extracted as in Fig. 4 but was treated with ribonuclease (1 μg/ml, 37 C, 10 min) just before sedimentation. Sedimentation and analysis of RNA (260 mμ) and radioactivity were performed as in Fig. 4; "a" designates fractions extracted for RNA (see text). The bottom of the gradient is to the left.*

labeled in this heavy particle (indeed, the first viral form to be associated with radioactivity) was a ribonuclease-resistant 20S form. At all times studied, the large particle contained at least 40% of this RNA. The 20S RNA has been shown to undergo a thermal denaturation with a T_m of 97 C (0.02 M KCl) in our laboratory and of 103 C (0.15 M NaCl) by E. M. Martin and J. A. Sonnabend (*personal communication*).

The base composition analysis of the "early" 20S RNA extracted by the method employed is consistent with its being a triplex containing two

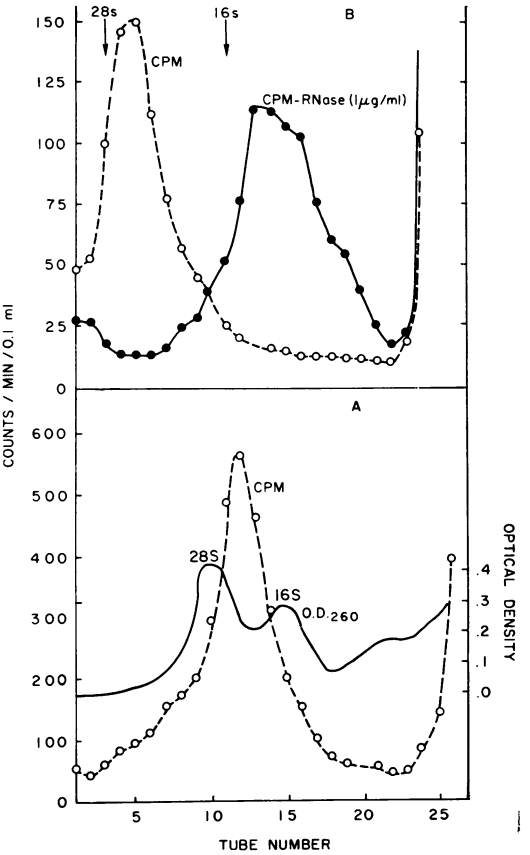

FIG. 6. *Sucrose density gradient analysis of RNA extracted from 65S radioactive fraction in the cytoplasm of SFV-infected, actinomycin D-treated cells. RNA was extracted with 0.5% SDS and precipitated for 16 hr at −20 C with alcohol and carrier ribosomal RNA. The precipitates were resuspended in Tris buffer and sedimented in a 6 to 30% sucrose density gradient in an SW-39 rotor at 39,000 rev/min for (A) 3 hr and (B) 6 hr. Ribonuclease (1 μg/ml) treatment (in B) was at 37 C for 10 min just before sedimentation. Optical density was measured at 260 mμ. The bottom of the gradient is to the left. The values 28S and 16S designate the optical-density peaks of the added ribosomal RNA.*

strands of viral RNA with one strand of RNA complementary to the viral RNA, a structure similar to the replicative intermediate proposed for some other RNA viruses (1, 20). Viral RNA might be synthesized on such a structure. It is of interest that a viral RNA polymerase which synthesized only ribonuclease-resistant RNA was also associated with a large, easily sedimented particle from the cytoplasm of SFV infected CEF (E. M. Martin and J. A. Sonnabend, *personal communication*). The addition of ^{32}P later in the course of infection with SFV yielded a "late" 20*S* ribonuclease-resistant viral RNA which had a base composition closer to that of 42*S* viral RNA than did the above-described, possibly triplex, RNA. This result suggested that asymmetrical labeling had taken place; possibly, late in the course of infection viral RNA was synthesized in a triplex structure, that is, on a stable duplex template.

A radioactive cytoplasmic fraction sedimenting at 65*S* was prominent in cells labeled with a 5- to 10-min pulse. This fraction contained only 26*S* viral RNA. The 26*S* viral RNA form had several properties characteristic of interjacent RNA (E. M. Martin, *personal communication*), proposed intermediary forms of RNA sedimenting between the mature virus and the replicative intermediary. Like previously reported interjacent RNA forms (2, 4, 6, 12, 26), SFV 26*S* RNA was ribonuclease-sensitive and sedimented in sucrose gradients between the replicative intermediary and the RNA of the mature virus. The 26*S* form was

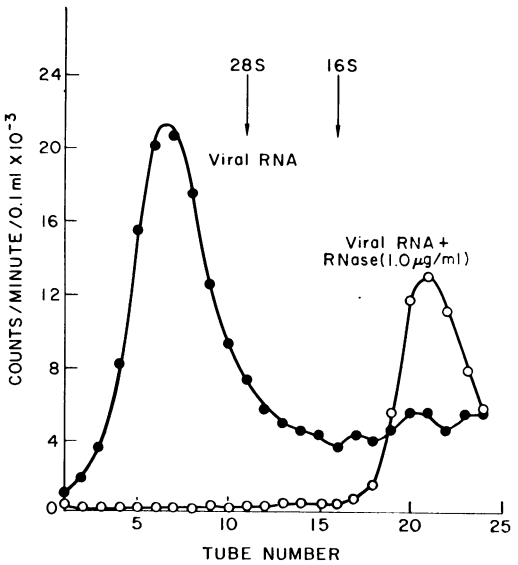

FIG. 8. *Sucrose density gradient analysis of RNA extracted from 140S radioactive fraction in the cytoplasm of SFV-infected and actinomycin D-treated cells. RNA was extracted with 0.5% SDS and alcohol-precipitated with carrier ribosomal RNA. The precipitates were resuspended, half of the volume was treated with 1 μg/ml of ribonuclease at 37 C for 10 min, and the untreated and ribonuclease-treated samples were sedimented in a 6 to 30% sucrose gradient at 38,000 rev/min for 3 hr; 28S and 16S designate the optical density peaks (260 mμ) of the marker ribosomal RNA. The bottom of the gradient is to the left.*

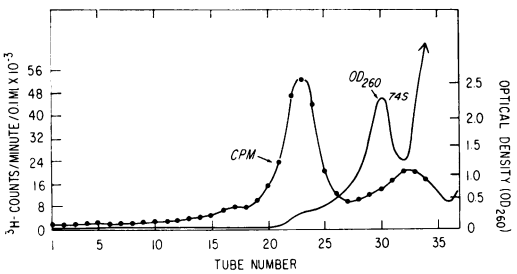

FIG. 7. *Sucrose density gradient analysis of ribonuclease-treated cytoplasmic extracts from SFV-infected and actinomycin D-treated cells incubated with ^{3}H-uridine for 1 hr. After 4 hr of SFV infection, 10^{8} chick cells were incubated with 30 μc of ^{3}H-uridine. The cytoplasm was extracted as described (Materials and Methods) and treated with 1 μg/ml of ribonuclease for 10 min at 37 C. The extracts were then sedimented through a 15 to 30% sucrose gradient for 100 min at 25,000 rev/min in an SW-25 rotor. Optical density (260 mμ) and radioactivity were estimated as previously described. The bottom of the gradient is to the left. The optical density peak of the chick ribosomes is designated as 74S.*

hydrolyzed by ribonuclease to acid-precipitable RNA sedimenting at 8 to 16*S* rather than being broken down to small acid-precipitable (<5*S*) or acid-soluble forms, as was the case with 42*S* RNA. This suggested that the 26*S* form might have ribonuclease-resistant regions, possibly due to some secondary structure. The base composition of the 26*S* RNA was not significantly different from that of 42*S* RNA, indicating that it was closely related to this form despite the marked differences noted in the sedimentation of these RNA forms in sucrose. As might be expected, however, studies have indicated that the 42*S* and 26*S* RNA have similar behavior in cesium sulfate equilibrium gradients (E. M. Martin and J. A. Sonnabend, *personal communication*).

It has been suggested that interjacent forms may in some instances be viral RNA with a messenger function (12). Recent reports have indicated that messenger RNA may be associated with a 40*S* ribosomal subunit before being associated with polysome structures (11). The 65*S* cytoplasmic fraction could be a structure contain-

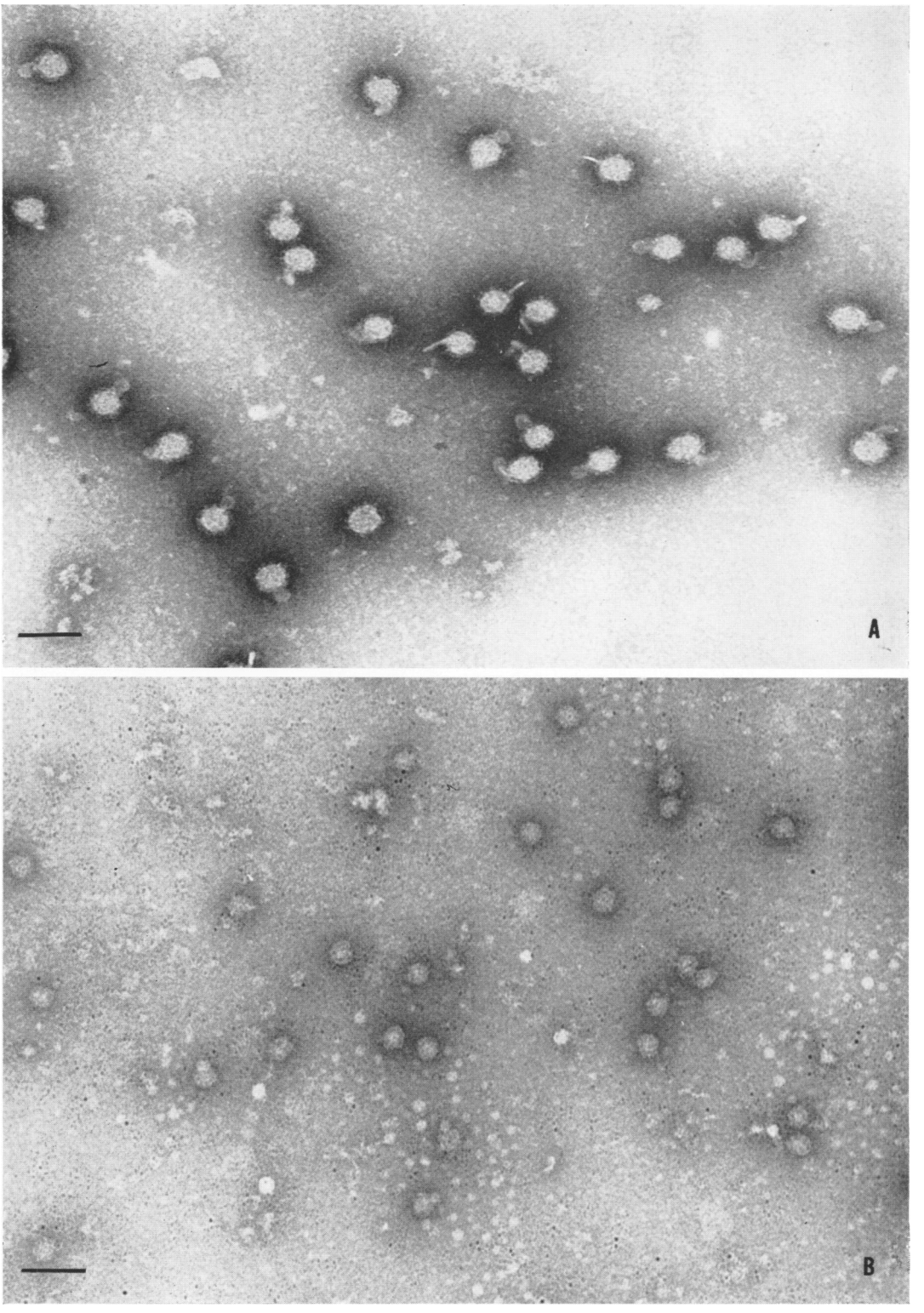

FIG. 9. *Negative-contrast electron micrographs of (A) Semliki Forest virus (SFV), and (B) 140S cytoplasmic particle from SFV infected chick cells 4 hr after infection. Scale marker is 0.1 μ.*

TABLE 1. *Base ratios of Semliki Forest virus RNA forms*

RNA form[a]	Source	Uracil	Guanine	Adenine	Cytosine
42S	Purified virus and cytoplasm	20.0 ± 0.2	27.2 ± 0.2	27.0 ± 0.2	25.9 ± 0.2
(Predicted complement)		(27.0)	(25.9)	(20.0)	(27.2)
26S	Cytoplasm	19.8 ± 0.1	27.0 ± 0.2	27.0 ± 0.3	26.2 ± 0.3
20S "late"	Cytoplasm	20.8 ± 0.3	27.6 ± 0.2	25.6 ± 0.2	25.9 ± 0.3
20S "early"	Cytoplasm	22.0 ± 0.2	27.0 ± 0.2	24.8 ± 0.1	26.2 ± 0.2
Calculated[b]:					
Triplex		22.3	26.8	24.7	26.3
Duplex		23.5	26.6	23.5	26.6

[a] All values given are the mean and standard deviation of the mean calculated from 8 to 16 separate determinations on two to four different preparations of the various RNA species. The 42S RNA was extracted from the cytoplasm of infected cells or from purified virus (no significant difference was observed between the 42S RNA samples from these sources); 26S RNA, from cytoplasm; and 20S RNA, from ribonuclease-treated (2 μg/ml, 37 C, 10 min) cytoplasm. All forms except the 20S "late" were produced in cells incubated with ^{32}P in otherwise phosphate-free medium from virus infection at 4 C to 4 hr (for 20S "early") or 6 hr (42S and 26S) after warming to 37 C. The 20S "late" RNA was produced in cells incubated with ^{32}P from 2 to 6 hr after warming to 37 C.

[b] The value for the duplex was calculated for a base-paired double strand containing one 42S strand and one strand of RNA complementary to 42S RNA. The value for the triplex was the duplex plus an additional strand of 42S RNA.

ing viral RNA and a 40S ribosomal subunit. Ribonuclease treatment of the 65S fractions gave rise to polydisperse material ranging from 65S to never less than 40S. A recent report, however, has raised some doubt about sedimentation values assigned to RNA molecules in the presence of cytoplasmic extracts, and so the nature of the 65S fractions is open to question (9). In preliminary experiments, we have been unable to locate any cytoplasmic particles in electron micrographs of the 65S fraction or to find an increase over control values in 16S ribosomal RNA in these fractions.

The most prominent radioactive fraction in the cytoplasm of cells pulsed for 1 hr was a 140S ribonuclease-resistant structure containing only 42S viral RNA. The 140S particle differed from mature virus in several respects: the 140S particle lacked the outer envelope with projections present in mature SFV; SFV sedimented with a 300 to 350S fraction under conditions similar to those in which the 140S sedimentation value had been obtained; and, finally, the 140S particle was not infectious under the usual assay conditions employed for SFV. In several respects, the 140S particle was similar to a 39-mμ structure obtained from SFV by treatment with caseinase C in phosphate buffer (21). This latter structure was also noninfectious and lacked the envelope structure of mature virus.

The 140S structure was probably identical to the intracellular immature arbovirus particles described by several workers (17, 19). We noted particles similar to the 140S particle in the cytoplasm and budding from the cell surface. As the mechanism of virus release appeared to be one of budding from the cell surface, it is likely that SFV acquired its outer envelope during this process.

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