REPLICATION OF SEMLIKI FOREST VIRUS: THREE FORMS OF VIRAL RNA PRODUCED DURING INFECTION

By Robert M. Friedman, Hilton B. Levy, and William B. Carter

NATIONAL CANCER INSTITUTE AND NATIONAL INSTITUTE OF ALLERGY AND INFECTIOUS DISEASES, NATIONAL INSTITUTES OF HEALTH, BETHESDA, MARYLAND

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The mechanism for the replication of animal RNA viruses has been the subject of intense investigation. With few exceptions (Gomatos and Tamm, 1963; Sonnabend *et al.*, 1964), however, these studies have been on picornaviruses, specifically poliovirus (Baltimore, Becker, and Darnell, 1964) or members of the encephalomyocarditis group (Montagnier and Saunders, 1963). This report will present studies on the replication pattern of Semliki Forest virus, a group A arbovirus. Semliki Forest virus has some properties advantageous to the study of animal virus replication, including a particle-to-infectious-virus ratio close to 1 (Cheng, 1961), wide host range, rapid growth to high titers in tissue culture, and little or no pathogenicity for man (Andrews, 1964). In this paper the forms of viral RNA produced in the cytoplasm of infected chick embryo fibroblasts are considered.

Materials and Methods.—Virus: The Kumba strain of Semliki Forest virus (SFV) employed in these studies was originally obtained from Dr. Alick Isaccs, Mill Hill, London. Pools of virus were prepared by infection of actinomycin D (ACM)-treated (0.5 μg/ml) chick embryo fibroblasts with low multiplicities of virus. Virus was grown in Eagle's medium, harvested after 14 hr by the methods of Taylor (1965), and assayed by a plaque titration method (Friedman, 1964).

Radioactive virus was grown in chick cells treated with ACM (0.5 μg/ml) in the presence of H^3-uridine and H^3-adenosine. Fluids were harvested after 18 hr and virus was purified by the method of Cheng (1961) with the addition of a final dialysis to remove small tritiated products. The specific activity of the final preparation was usually about 1 cpm/2000 infectious units. The RNA extracted from this virus preparation by the method of Cheng (1958) had a sedimentation value of 42*S* and was ribonuclease-sensitive. Sedimentation velocities of the tritiated virus performed on sucrose gradients indicated a peak of radioactivity at about 350*S* which corresponded to the peak of virus infectivity (cf. Cheng, 1961). Radioactivity and infectivity were also present in the pellet, possibly due to viral aggregation.

Cells: One-day-old primary chick embryo monolayers (CE) were prepared in 100-mm plastic Petri plates by previously described methods (Taylor, 1965); these cells were employed in all studies. When confluent, these monolayers contained about 2.5×10^7 cells/plate.

Pulse-labeling and viral RNA extraction: Pulse-labeling was carried out by adding to the cell cultures 1 ml of tissue culture fluid containing the isotope. After an appropriate pulse period, the monolayers were washed five times with cold phosphate buffered (0.01 *M*, pH = 7.2) 0.85% saline and once with potassium phosphate buffer (0.1 *M*, pH = 7.1). Cells were removed from plastic Petri dishes with a rubber stopper and suspended in 1 ml of potassium phosphate buffer.

The cells were disrupted with a Dounce homogenizer, and the homogenate was centrifuged at 600 *g* for 10 min. The remaining cytoplasmic fraction was extracted with 5% sodium dodecyl sulfate (SDS) (Sonnabend *et al.*, 1964).

Sucrose density gradients: These were prepared and analyzed by the method of Dalgarno and Martin (1965). Sedimentation values were estimated by the method of Martin and Ames (1961). Radioactive RNA, precipitated on Whatman no. 2 filter paper strips, was counted in a Packard Tri-Carb 3003 liquid scintillation spectrometer with a background activity of 4 cpm.

Infectious RNA assay: Infectious RNA was assayed on CE by the method of Richter and Wecker (1963). After incubation for 15 min in hypertonic buffer, Eagle's medium was added, and the monolayers were incubated for 24 hr. The fluids were then assayed for mature SFV by plaque titration.

Reagents: Actinomycin D was kindly supplied by Merck, Sharp and Dohme. Tritiated uridine 5-T (20.4 c/Mm) and uniformly labeled adenosine (1.8 c/Mm) were purchased from the Radiochemical Centre, Amersham, England.

Results.—Forms of viral RNA: CE were infected with 0.5 ml of the SFV pool at a virus-to-cell multiplicity of 20–40 to 1 for 80 min. Ten ml of cold tris buffered Gey's medium with 10% fetal calf serum and ACM (1 μg/ml) were added and the plates refrigerated for 14 hr at 4°C (Taylor, 1964). This medium was removed, 5 ml of Eagle's medium with 10% fetal calf serum with ACM (1 μg/ml) were added, and the plates replaced at 37°C. Under these conditions the log phase of viral growth occurred 3–5 hr after warming to 37°C.

Figure 1 illustrates the results of sucrose density gradient analysis of RNA obtained from cytoplasmic extracts of CE pulsed with 30 μc of tritiated uridine 4–5 hr after infection with SFV. Confirming previously reported observations (Sonnabend *et al.*, 1965), two peaks of radioactivity were observed, one at about 42*S*, the other at about 22*S*. The 42*S* peak corresponded to the single peak obtained from the RNA extracted from purified virus. In uninfected cells under these conditions the only RNA synthesized sedimented in the 4*S* region. Treatment of the cytoplasmic extracts with pancreatic ribonuclease (1 μg/ml, 0°, 10 min, in 0.05 *M* phosphate buffer) before sedimentation eliminated the 42*S* peak and a large part of the 22*S* peak, the remaining peak of radioactivity now appearing at about 20*S*.

The fractions containing the peaks of radioactivity (42*S* and 22*S*) were precipitated with added ribosomal RNA by 2 vol of alcohol and 0.1 *M* NaCl, and the precipitate was resuspended in hypertonic buffer (1 *M* NaCl, 0.1 *M* tris, pH 8.2). Both were infectious in a hypertonic assay for infectious RNA. Dilutions were made in hypertonic buffer; the 42*S* RNA had a titer of 10^5/0.2 ml, the 20*S* RNA, a titer of 10^4/0.2 ml.

The broadness of the 20*S* peak and its shift to a somewhat lower sedimentation value after ribonuclease treatment suggested that it might contain more than one component. Accordingly, the cytoplasmic extract was sedimented for a longer

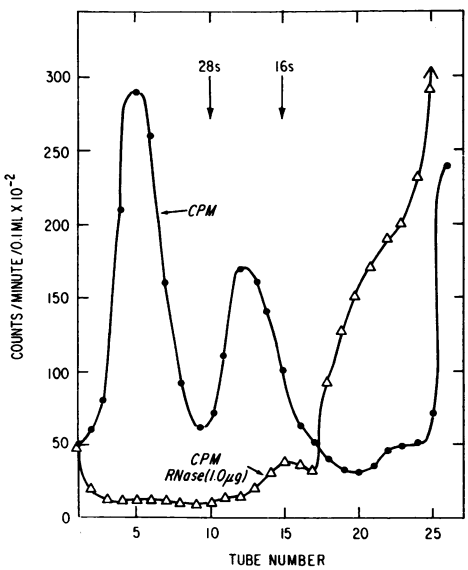

FIG. 1.—Actinomycin D-treated (1 μg/ml) CE (5×10^7 cells) were infected with SFV at a multiplicity of 40:1 (see *Methods*). During the log phase of virus growth, the cells were pulsed with 30 μc of H^3-uridine for 1 hr. Cytoplasmic RNA was extracted from cells. The extract was mixed with ribosomal RNA. A portion of the extract was treated with ribonuclease (1 μg/ml, 0°, 10 min), and extracts were layered over a 6–30% sucrose gradient prepared in 0.01 *M* Tris, 0.001 *M* EDTA, and 0.1 *M* KCl. The gradient was sedimented for 3 hr at 38,000 rpm in an SW39 rotor, and fractions were collected by puncturing the bottom of the tube. The optical density of fractions was measured—the designations 28*S* and 16*S* refer to the peaks of optical density; 0.1 ml of each fraction was dried on strips of Whatman no. 2 filter paper; the papers were washed with 0.25 *M* perchloric acid and counted in a Packard Tri-Carb liquid scintillation spectrometer.

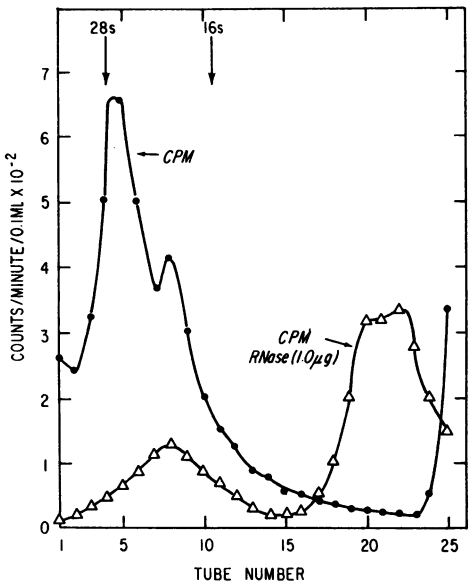

FIG. 2.—A cytoplasmic extract prepared by the same methods as that shown in Fig. 1 was centrifuged for 6 hr at 39,000 rpm in an SW39 rotor. Fractions were collected and analyzed as those shown in Fig. 1.

period of time (6 hr). The 22*S* material was resolved into two components, one sedimenting in the 26*S* region, the other in the 20*S* region (Fig. 2). The single peak of the ribonuclease-treated cytoplasmic extract corresponded exactly to the 20*S* peak.

Sequence of development of viral RNA forms: Additional information could be obtained by varying the duration of the pulse with radioactive precursor. The short time intervals of labeling required the use of 100 μc of uridine or adenosine. In Figure 3, the results of 10-, 5-, 3-, and 1-min pulses are shown. In the 10-min pulse a pattern similar to that of the 1-hr pulse (Fig. 1) was seen, except that the 22*S* peak was more prominent with the shorter labeling time. The 42*S* peak progressively disappeared in extracts of cells labeled with shorter pulses, only the 22*S* peak being prominent.

When a 10-min pulse-labeled extract was sedimented for 6 hr (Fig. 4), separation of the 22*S* peak into two components, 26*S* and 20*S*, was noted. Only the former (not shown) was ribonuclease-sensitive. In contrast, a fairly homogeneous 20*S* peak was noted in the 1-min pulse-labeled extract. This material was resistant to ribonuclease (Fig. 4).

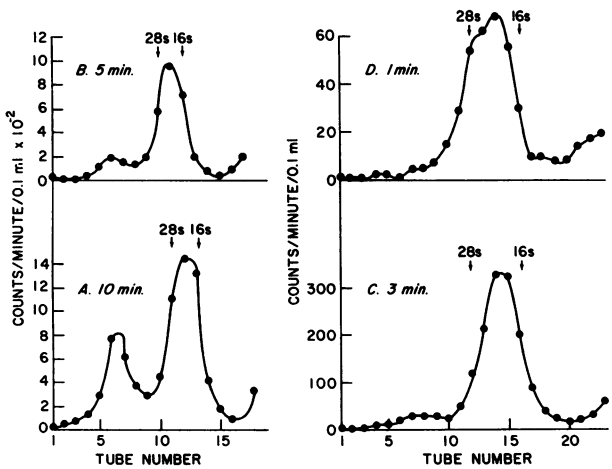

FIG. 3.—Actinomycin D-treated CE were infected with SFV at a multiplicity of 40:1. During the log phase of virus growth, cells were pulsed for (*A*) 10 min, (*B*) 5 min, (*C*) 3 min, and (*D*) 1 min with 100 μc of H^3-uridine. Cytoplasmic RNA was extracted and assayed as described in the legend to Fig. 1. The designations 28*S* and 16*S* refer to the peaks of optical density of an added preparation of ribosomal RNA.

Experiments employing tritiated Semliki Forest virus (H^3-SFV): When ACM-treated (0.5 μg/ml, 2 hr) CE were infected with H^3-SFV, sedimentation patterns revealed increasing amounts of new forms of radioactive RNA with increasing time intervals after infection. At 30 and 60 min after infection (Fig. 5), a single peak at 42*S* was seen, and in addition some radioactivity was present in the 28*S* to 16*S* region of the gradient. By 2 hr after infection (Fig. 6), distinct peaks were present at the 16–28*S* region in addition to a 42*S* peak. Ribonuclease treatment of the 2-hr extract before sedimentation yielded a single 20*S* peak.

Figure 7 illustrates the results of sucrose gradients performed on the cytoplasm of cells extracted 4 hr after H^3-SFV infection, early in the log phase of virus growth. A sedimentation pattern was noted similar to that seen when infected cells were pulse-labeled for 1 hr (Fig. 1).

Experiments employing tritiated viral RNA: These results suggested that a progression occurred in SFV-infected cells in which the parental viral RNA passed from a form which sedimented at 42*S* (corresponding to the RNA of the purified virus) to forms which sedimented at 20*S* and 26*S*. It was of interest to determine how efficiently this process was carried out, once the viral RNA was uncoated. Since the SDS extraction procedure employed does extract viral RNA in low yields from whole virus (Friedman, unpublished observation), the 42*S* peaks noted in

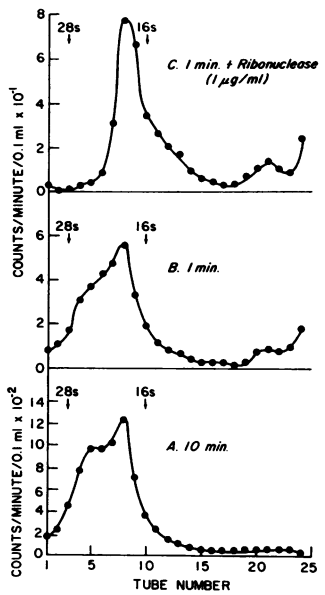

FIG. 4.—CE were infected, pulsed, labeled, and extracted as in Figs. 1 and 2. The extracts were layered over a 6–30% sucrose gradient and sedimented for 6 hr at 38,000 rpm in an SW39 rotor. The fractions were collected and analyzed; the peaks of optical density of added ribosomal RNA are indicated by the designations 28*S* and 16*S*.

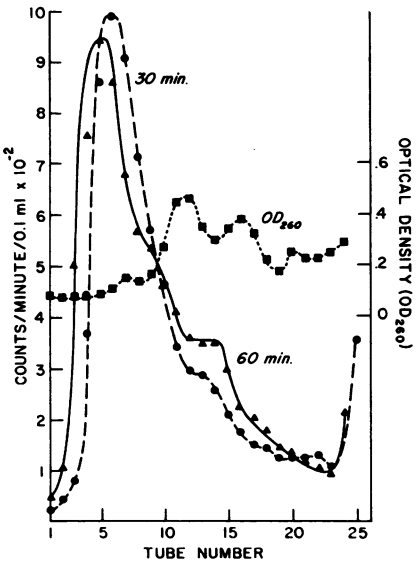

FIG. 5.—Actinomycin D-treated (0.5 μg/ml) CE were infected with tritiated SFV at a multiplicity of 40:1. After 30 or 60 min, cytoplasmic RNA was extracted from the cells and analyzed in 6–30% sucrose gradients as previously described (38,000 rpm for 3 hr). The optical density pattern of ribosomal RNA added to the 30-min sample is shown. The 60-min sample's carrier RNA sedimentation pattern was almost identical.

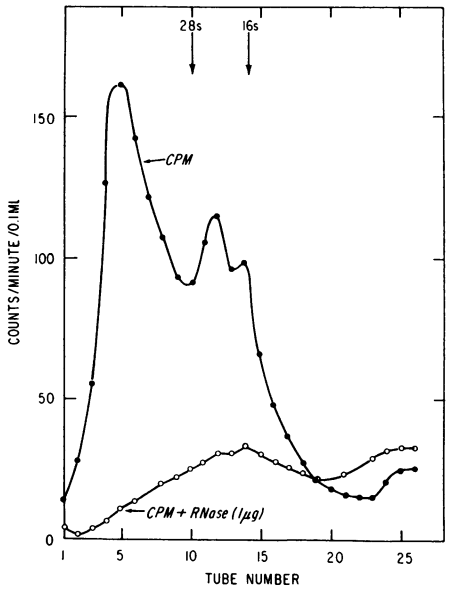

FIG. 6.—CE were treated with actinomycin D (0.5 μg/ml) for 2 hr and infected with tritiated SFV. After 2 hr incubation, cytoplasmic RNA was extracted and ribonuclease was added to an aliquot of the extract. The extracts were layered over a 6–30% sucrose gradient, sedimented for 3.5 hr at 38,000 rpm, and the collected fractions analyzed for optical density and counts.

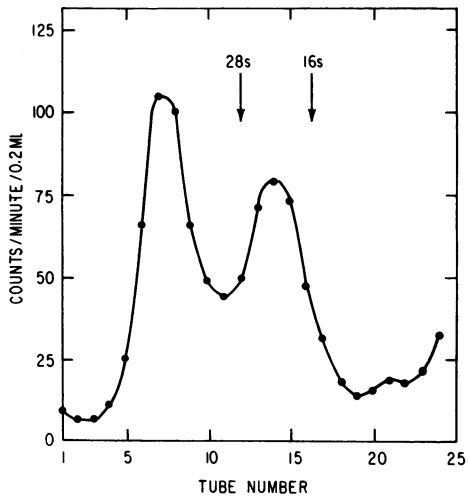

FIG. 7.—CE (5×10^7) were treated with actinomycin D (0.5 μg/ml) and infected with tritiated SFV. After 4 hr cytoplasmic RNA was extracted, layered over a 6–30% sucrose density gradient, sedimented for 3 hr at 38,000 rpm, and the collected fractions were analyzed for optical density and radioactivity.

Figures 5–7 could represent either RNA of eclipsed virus or uneclipsed virus RNA which had been extracted by SDS.

In order to distinguish between these alternatives, the fractions containing the 42*S* RNA peak obtained from a sucrose gradient of the cytoplasmic extract of SFV-infected CE were alcohol-precipitated and resuspended in hypertonic buffer. CE (5×10^7 cells) were infected with this tritiated infectious viral RNA containing about 8000 cpm. The cytoplasm of these cells was extracted 90 min after removal of infectious H^3-RNA and sedimented in a sucrose gradient. Prolonged counting was necessary to obtain significant numbers of counts, but the results (Fig. 8) indicated that the uncoated virus had entered very efficiently into the slower-sedimenting forms and therefore that the 42*S* peaks seen in Figures 5–7 probably represented in great part viral RNA extracted by SDS from uneclipsed SFV.

Discussion.—The results obtained with 1-hr pulse-labeling experiments agree with previously reported findings of Sonnabend *et al.* (1965). Two peaks of radioactivity were present, one of which was partially resistant to ribonuclease treatment. On prolonged sedimentation the slower-sedimenting RNA region contained two peaks, one at 26*S*, the other at 20*S*. The latter was the ribonuclease-resistant material. Sonnabend, Martin, and Mecs (personal communication) have also noted the resolution of the 22*S* peak into two RNA forms. In addition, three forms of viral RNA have been found during infection with foot-and-mouth disease virus

(Brown and Cartwright, 1964) and WEE virus (Sreevalsan and Lockart, 1966). Very short pulse-labeling experiments indicated that the earliest form of the viral RNA to appear was the ribonuclease-insensitive (20*S*) form. Later there appeared a ribonuclease-sensitive form which sedimented at 26*S*. Last to appear was a 42*S* ribonuclease-sensitive form which corresponded to the RNA extracted with purified SFV. It is of interest that the ribonuclease-resistant 20*S* RNA was the only form of the viral nucleic acid synthesized in an RNA polymerase system from SFV-infected CE (Sonnabend, personal communication). At present, a most likely interpretation of these findings is that nascent RNA appears as a ribonuclease-insensitive, possibly double-stranded form. The viral RNA would then enter a configuration which sediments at 26*S* and finally appear in the 42*S* form, that of the RNA of infectious SFV. The 26*S* form of viral RNA appears to be an intermediate between single- and double-stranded RNA.

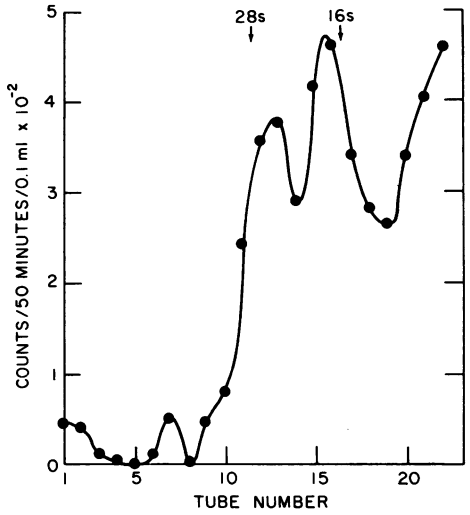

FIG. 8.—CE (5×10^7) were treated with actinomycin D and then infected for 20 min with tritiated SFV-RNA in 1 *M* NaCl, 0.1 *M* Tris, pH = 8.2. The input RNA contained about 8,000 cpm. The CE were then washed and growth medium was added for 90 min. Cytoplasmic RNA was extracted, layered over a 6-30% sucrose density gradient, sedimented for 3 hr at 38,000 rpm, and the collected fractions analyzed for optical density of added ribosomal RNA and for radioactivity.

Experiments performed with H^3-SFV and H^3-SFV RNA supported these kinetics in the development of viral RNA. The 42*S* parental RNA quickly and efficiently entered into the 26*S* and 20*S* forms which appeared to be associated with viral RNA replication. Preservation of some parental RNA in the early log phase of virus growth was indicated by the presence of label in the replicative RNA forms 4 hr after infection with H^3-SFV.

The presence of ACM in cell cultures eliminated the possibility that the 28*S* to 16*S* RNA forms found after infection with H^3-SFV represented reincorporation of viral breakdown products into cellular materials. That the appearance of 28*S* to 16*S* RNA forms was an event in the virus growth cycle was shown in previously reported experiments with interferon. With concentrations of interferon which blocked virus growth, the conversion of the 42*S* RNA of infecting virus into the 28*S* to 16*S* forms was markedly inhibited (Levy *et al.*, 1966).

Summary.—During the course of infection of chick cells with Semliki Forest virus, three forms of viral RNA were present. One form sedimented at 42*S* and was ribonuclease-sensitive, properties identical to those of RNA extracted from purified virus. Another form sedimented at 26*S* and was also ribonuclease-sensitive. The third sedimented at 20*S* and was ribonuclease-resistant. Short radioactive precursor pulse studies indicated that radioactivity was first associated with the 20*S*, form, then with the 26*S* form, and finally with the 42*S* form. Studies with

tritiated virus and viral RNA indicated that the parental 42*S* RNA passed rapidly and efficiently into the 20*S* and 26*S* forms.

It is suggested that during replication of viral RNA, nascent RNA first appears in the 20*S* possibly double-stranded form; then, in a 26*S* form intermediary between the single- and double-stranded forms; and finally, in the 42*S* form of the mature virus.

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