

Analysis of Arbovirus Ribonucleic Acid Forms by Polyacrylamide Gel Electrophoresis

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Viral ribonucleic acid (RNA) from Semliki Forest virus- and Sindbis virus-infected cells was analyzed by electrophoresis on polyacrylamide gels. In contrast to earlier results obtained by sucrose density gradient centrifugation, all of the known viral RNA forms (i.e., the 42S, 26S, replicative form, and replicative intermediate) were very clearly separated. The high resolution of the electrophoretic method permitted the identification of two new single-stranded RNA species. In addition, the replicative form was shown to be heterogeneous and to consist of at least two forms. The results suggested that the replicative forms occur in vivo although in relatively small amounts.

Recently several studies were published on the viral ribonucleic acid (RNA) forms produced during arbovirus infections (13, 25, 26). These studies employed sucrose density gradient analysis as their chief method for separating and quantitating the viral RNA forms present. The development of techniques for electrophoretic analysis of RNA on polyacrylamide gels has, however, introduced an extremely sensitive method for separating viral RNA forms (1, 14). It has been used successfully to analyze the products of Q β replicase (21), the multiple RNA forms present in myxo- and reoviruses (23, 24) and in poliovirus-infected HeLa cells (20). As pointed out previously, analysis by acrylamide gel electrophoresis should be especially useful in the case of the arboviruses because of the small differences in the sedimentation values of some of the RNA forms present in infected cells (5).

To date, three and possibly four forms of viral RNA have been identified in arbovirus-infected cells. These are: (i) a 42S single-stranded form, the only RNA form present in the virion; (ii) a 26S single-stranded RNA whose function is unknown; (iii) a polydisperse partly double-stranded replicative intermediate (RI) form which is thought to be the template on which new RNA is produced; and, possibly, (iv) a 20S replicative form which is analogous to the resistant core produced by ribonuclease treatment of the RI. The replicative form has not actually been shown to be present in infected cells and may be produced only by ribonuclease treatment of the RI (3, 13, 26, 27).

In the present study, viral RNA extracted from Semliki Forest virus (SFV)- and Sindbis virus-infected cells was analyzed on polyacrylamide gels. All of the previously characterized forms, including the replicative form, could be identified in the patterns obtained. In addition, at least two distinct replicative forms were found and two previously unknown minor species of single-stranded RNA were identified.

MATERIALS AND METHODS

Materials. Actinomycin D was a gift from Merck, Sharp, and Dohme Research Laboratories (Div. of Merck & Co., Inc., Rahway, N. J.). Adenosine-2,8-³H (7.0 Ci/mole) and uridine-5-³H (19.9 Ci/mole) were purchased from New England Nuclear Corp. (Boston, Mass.); ³²P-phosphoric acid (carrier free) was obtained from Tracerlab (Waltham, Mass.); and ³H-guanosine triphosphate (1.0 Ci/mole) was purchased from Schwarz BioResearch Inc. (Orangeburg, N. Y.). Adenosine triphosphate, uridine triphosphate, and cytidine triphosphate were purchased from General Biochemicals, Inc. (Chagrin Falls, Ohio). Pancreatic ribonuclease, Pronase, phosphoenolpyruvate, pyruvate kinase, and dithiothreitol were obtained from Calbiochem (Los Angeles, Calif.). *N,N,N',N'* tetramethylethylenediamine, acrylamide, and *N,N'*-methylene bisacrylamide were purchased from Eastman Organic Chemicals (Rochester, N. Y.). The acrylamide and bisacrylamide were recrystallized according to Loening (14). Agarose (Seakem) was obtained from Bausch and Lomb Inc. (Rochester, N. Y.). Ribonuclease-free sucrose was purchased from Mann Research Laboratories (Orangeburg, N. Y.). Triton X-100 was obtained from Packard Instrument Co., Inc. (Downers Grove, Ill.) and toluene-Liquifluor was obtained from New England Nuclear Corp.

Cells and virus pools. The procedures for prepara-

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tion of chick embryo fibroblasts and SFV virus pools and the media employed have been described (13). Identical procedures were used for preparation of Sindbis virus (Oie strain) pools and for growth of Sindbis virus in chick cells. MA-308 cells (human diploid fibroblasts), passage number 26, were purchased from Microbiological Associates (Bethesda, Md.).

Virus strain. The RLGI strain of Semliki Forest virus was obtained from J. Sonnabend (Dept. of Microbiology, The Mt. Sinai School of Medicine, New York, N. Y.). It had been plaque purified six times and was in its fourth passage in chick cells after plaque purification. The Oie strain of Sindbis virus was obtained from S. Baron (National Institute of Allergy and Infectious Diseases, National Institutes of Health).

Preparation of RNA. To prepare ^3H -labeled SFV RNA, monolayers of chick embryo fibroblasts were infected with virus at a multiplicity of 40:1 in the presence of $1\ \mu\text{g}$ of actinomycin D per ml. After 1 hr at 37 C, medium containing ^3H -adenosine and ^3H -uridine (each $50\ \mu\text{Ci/ml}$) was added, and the incubation continued for another 4 hr. When ^{32}P -labeled SFV RNA was prepared, cells were infected as usual, then washed with phosphate-free medium, and incubated overnight at 4 C with ^{32}P -phosphoric acid ($50\ \mu\text{Ci/ml}$) in medium otherwise phosphate-free. The cells were warmed to 37 C in the morning and incubated for 6 hr. After the appropriate incubation period, the infected cells were suspended in buffer containing 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.2), and 0.001 M ethylenediaminetetraacetate (EDTA). Viral RNA was extracted with 1% sodium dodecyl sulfate (SDS) and phenol as previously described (12), except that the extraction was performed at room temperature.

^{32}P -labeled cell RNA was prepared by overnight incubation of chick embryo fibroblasts at 37 C with ^{32}P -phosphoric acid (0.7 mCi) in medium otherwise phosphate-free. The cells were washed and RNA was extracted from the whole cell suspension with SDS and phenol at room temperature.

Polyacrylamide gel electrophoresis of RNA. Preparation of 2.2% polyacrylamide, 0.5% agarose gels (0.6-cm internal diameter, 8 cm long) was carried out by combining 15 ml of melted 1% agarose (w/v) with 15 μl of *N,N,N',N'* tetramethylethylenediamine, and 15 ml of the following mixture: 0.72 ml of water; 5.28 ml of a stock solution containing 15% acrylamide (w/v) and 0.75% *N,N'*-methylene bisacrylamide (w/v); and 12 ml of gel buffer (0.04 M Tris, 0.06 M sodium acetate, 0.003 M EDTA, adjusted to pH 7.2 with glacial acetic acid). To prevent solidification of the agarose, the reagents were mixed at 45 C. After evacuation of air from the mixture, 0.3 ml of a fresh solution of 10% ammonium persulfate (w/v) was added and the mixture was rapidly transferred to glass tubes. Since solidification of the agarose occurs more rapidly than polymerization of the acrylamide, the gel forms with an uneven surface at both ends. Before use one of the agarose tips was sliced off with a scalpel. The gels were stored for 1 week (or even 2 weeks) in a tightly sealed glass vessel containing a wet piece of cotton.

The electrophoresis buffer (with 0.5% SDS) and the conditions for removal of excess catalyst have been described (1, 19). Prior to electrophoresis a piece of dialysis tubing containing the buffer was placed over the lower end of the glass tube so that the gel would not slip out (1). The sample (10 to 50 μl), containing a few dissolved crystals of sucrose, was applied to the gel and run at room temperature for 4 hr at a constant current of 6 ma per gel. After electrophoresis the gels were transferred to test tubes containing 5% trichloroacetic acid at 4 C and within 40 min the RNA had precipitated. The gels were washed twice with 5% trichloroacetic acid and were then sliced into 1.3-mm segments (7). Each slice was placed in a scintillation vial and incubated overnight at 55 C with 50 μl of 30% hydrogen peroxide. Ten milliliters of scintillation fluid (625 ml of toluene, 332 ml of Triton X-100, 40 ml of toluene-Liquifluor, and 100 ml of water) was added to each vial. The samples were counted in a liquid scintillation spectrometer (Packard Instrument Co., Inc.). Where necessary, the ^3H values were corrected for 5% crossover of ^{32}P into the ^3H channel.

All of the results shown are representative of several runs. In almost all cases identical results to those reported were obtained in experiments in which the isotope of the marker RNA (usually ^{32}P) and of the experimental RNA (usually ^3H) were reversed. Virtually all of the acid precipitable radioactivity applied to the gels was recovered.

RESULTS

Analysis of RNA forms present in infected cells.

Chick cells were treated with actinomycin D (1 $\mu\text{g/ml}$) and infected with SFV. After 1 hr, Eagle's medium containing ^3H -uridine and ^3H -adenosine was added. At 5 hr after infection, the RNA from these cells was extracted with SDS and phenol. The ^3H -labeled RNA was analyzed by polyacrylamide gel electrophoresis together with a marker of ^{32}P -labeled RNA from uninfected chick cells.

The resulting electropherogram is shown in Fig. 1A. Six peaks of radioactivity (I to VI) were repeatedly observed, in addition to some radioactivity which failed to enter the gel. The peaks of radioactivity seen in the RNA pattern of uninfected cells are the 45S, 28S, and 18S RNA forms. In actinomycin D-treated cells which were not infected with virus (*data not shown*), only the 45S ribosomal precursor RNA and the small molecular weight species of RNA present in region VI were observed as distinct peaks. In some experiments no peak VI was observed (Fig. 5) and we are uncertain as to its nature. When the RNA of SFV-infected cells was treated with ribonuclease before electrophoresis, two or possibly three slowly migrating peaks of radioactivity could be identified (Fig. 1B). This observation is discussed below in greater detail.

In order to identify the various peaks of radio-

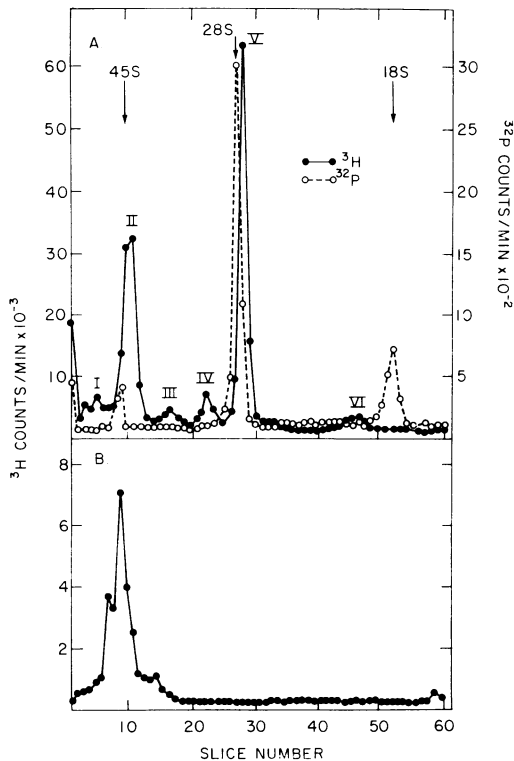


FIG. 1. Polyacrylamide gel electrophoresis of ^3H -labeled RNA extracted from Semliki Forest virus (SFV)-infected chick embryo fibroblast cells. ^3H -labeled SFV RNA and ^{32}P -labeled RNA from uninfected chick cells were prepared as described in Materials and Methods. Electrophoresis of a 25- μl sample containing 23 μl of ^3H -labeled SFV RNA and 2 μl of ^{32}P -labeled cell RNA (A) and a 25- μl sample of ^3H -labeled SFV RNA treated with ribonuclease (1 $\mu\text{g}/\text{ml}$) for 30 min at 37 C prior to electrophoresis (B) was performed in separate polyacrylamide gels as described in Materials and Methods. (●), ^3H ; (○), ^{32}P .

active RNA present in Fig. 1A, known SFV RNA markers were required. For this purpose it seemed appropriate to fractionate RNA from SFV-infected cells by sucrose density gradient centrifugation and to use the resulting fractions, already characterized by previous studies (3, 13, 25-27), as the source of SFV RNA species. Fig. 2A illustrates the profile obtained after sedimentation of a 50- μl sample of the ^3H -labeled RNA preparation used in Fig. 1A. As expected, two discrete peaks corresponding to the 42S and 26S RNA forms were found. However, there was also a small but significant intermediate peak at fraction 17 (see below). Treatment of another small sample of viral RNA with ribonuclease followed

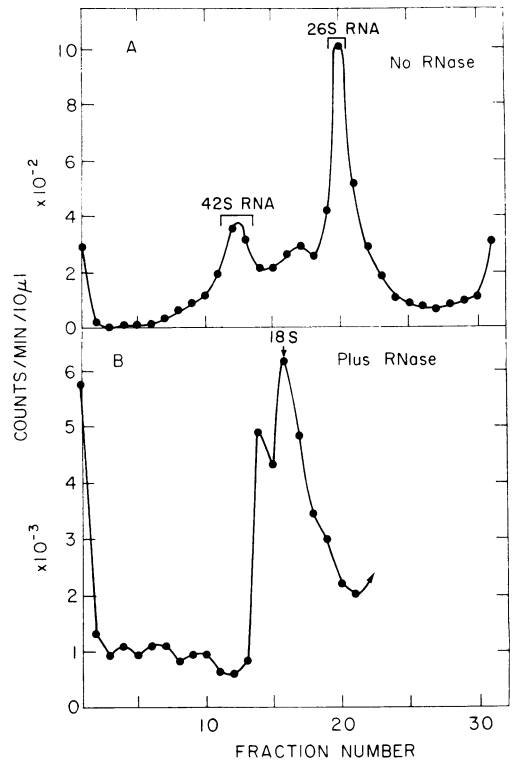


FIG. 2. Sucrose density gradient analysis of ^3H -labeled RNA from Semliki Forest virus (SFV)-infected chick embryo fibroblast cells. In (A), a 50- μl sample of ^3H -labeled SFV RNA was layered directly on a 4.4-ml, 6 to 30% linear sucrose gradient prepared in 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (pH 7.2), and 0.001 M ethylenediaminetetraacetic acid; in (B) another sample (50 μl) was treated with ribonuclease (0.1 $\mu\text{g}/\text{ml}$) for 30 min at 37 C and then applied to a separate gradient. Under these conditions viral RNA is hydrolyzed to the same extent with ribonuclease concentrations of 0.1 $\mu\text{g}/\text{ml}$ or 1 $\mu\text{g}/\text{ml}$ (reference 10; R. M. Friedman, unpublished observation). Centrifugation was for 1 hr (A) or 2 hr (B) at 300,000 $\times g$ in a Spinco SW 65 rotor. After collecting 0.15-ml fractions, 10- μl portions of each fraction were spotted on filter paper discs and precipitated with 1.7% perchloric acid by methods described previously (11). In (B) only fractions 1 to 21 were precipitated with acid since the fractions at the top of the gradient have large amounts of acid-soluble radioactivity which cannot be adequately removed by the washing procedures employed. Each filter paper disc was dried and counted in 10 ml of toluene-Liquifluor scintillation fluid in a Packard liquid scintillation spectrometer. In Fig. 2A 42S RNA and 26S RNA refer to the previously determined location of the major species of SFV RNA in a sucrose density gradient (13); in Fig. 2B 18S refers to the location of this species of ribosomal RNA in sucrose density gradients sedimented under the conditions of Fig. 2B but in the absence of ribonuclease.

by prolonged centrifugation in a sucrose gradient revealed two peaks of radioactivity (Fig. 2B). Analysis of small samples of ^{32}P -labeled RNA from SFV-infected cells gave essentially the same results as those shown in Fig. 2A and 2B, and in subsequent experiments the ^{32}P - and ^3H -labeled SFV RNA preparations are used interchangeably.

Single-stranded RNA forms. The patterns resulting from co-electrophoresis of labeled RNA from SFV-infected cells with selected sucrose gradient fractions are shown in Fig. 3. In Fig. 3A and B the gradient fractions were labeled with ^{32}P and the unfractionated RNA with ^3H ; in Fig. 3C and D the labels were reversed. The arrows indicate the species of RNA which is enriched in a particular gradient fraction, and the Roman numerals are given for comparison with the peaks of RNA seen in Fig. 1A.

Peak II was found to migrate with sucrose gradient fraction 11, the 42S RNA form (Fig. 3A) and peak V, with the major species present in fraction 20, the 26S viral RNA form (Fig. 3B). The minor peaks III and IV were enriched in fractions 15 (Fig. 3C) and 17 (Fig. 3D), respec-

tively. No distinct peak of radioactivity comparable to peak III has been seen in sucrose gradients; however, RNA found in this region of the gradient would have a sedimentation value of 38S (17). A minor peak corresponding to peak IV is sometimes seen on sucrose density gradient analysis of SFV-infected cell extracts if, as in Fig. 2A, small sample volumes are used and numerous fractions are collected. The sedimentation value of fraction 17 (Fig. 2A) can be calculated to be 33S.

Since peaks III and IV appeared to be ribonuclease-sensitive single-stranded RNA forms (cf. Fig. 1A and B) which had not previously been identified, it was important to demonstrate as well as possible that they are associated with the SFV infection itself and not due to an extraneous actinomycin D-resistant agent present in the chick cells or virus pool used for these experiments. As a first approach to this problem, a pool of ^3H -labeled SFV virus was partially purified by high speed sedimentation; viral RNA was extracted and then analyzed on polyacrylamide gels. As shown in Fig. 4, only one homogeneous peak, corresponding to the 42S RNA of

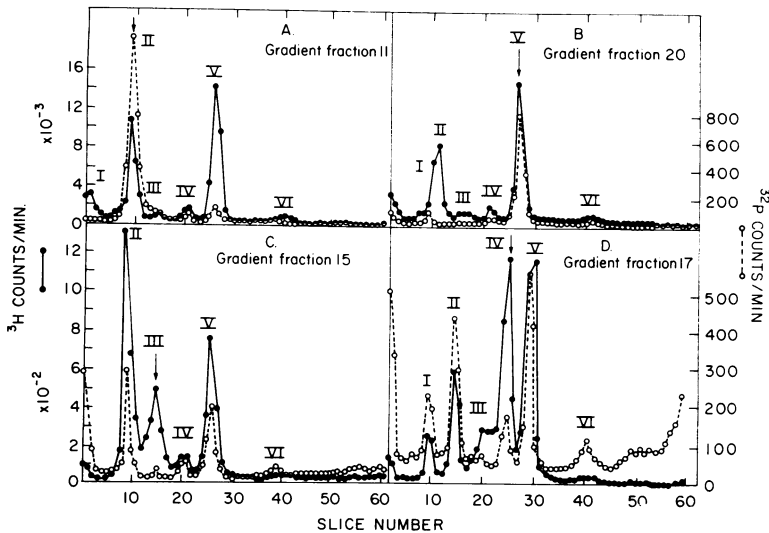


FIG. 3. Polyacrylamide gel electrophoresis of sucrose gradient fractions. Fraction 11 (A) and fraction 20 (B) were obtained by centrifuging a 0.1-ml sample of ^{32}P -labeled RNA from Semliki Forest virus (SFV)-infected chick embryo fibroblast cells in a 6 to 30% sucrose gradient by the procedures detailed in the legend to Fig. 2. The sedimentation profile was similar to that shown in Fig. 2A. Fractions 15 (C) and 17 (D) were from the sucrose gradient illustrated in Fig. 2A. Electrophoresis was performed on 50- μl samples containing 40 μl of the specified ^{32}P -labeled fraction and 10 μl of marker ^3H -labeled SFV RNA from chick cells (A) and (B) or 48 μl of the specified ^3H -labeled fraction and 2 μl of marker ^{32}P -labeled SFV RNA from chick cells (C) and (D). Procedures used for polyacrylamide gel electrophoresis are given in Materials and Methods. Arrows indicate the species of RNA which are enriched in a particular gradient fraction, and the Roman numerals indicate the peaks of RNA previously shown in Fig. 1A. Samples in (C) and (D) were run at different times and thus the ^{32}P -labeled marker had less radioactivity in (C) than in (D). Since peak II of (C) was found before gel slice number 10, the absence of peak I in (C) indicates that the RNA of peak I either did not fully enter the gel or was not resolved from that of peak II. (●), ^3H ; (○), ^{32}P .

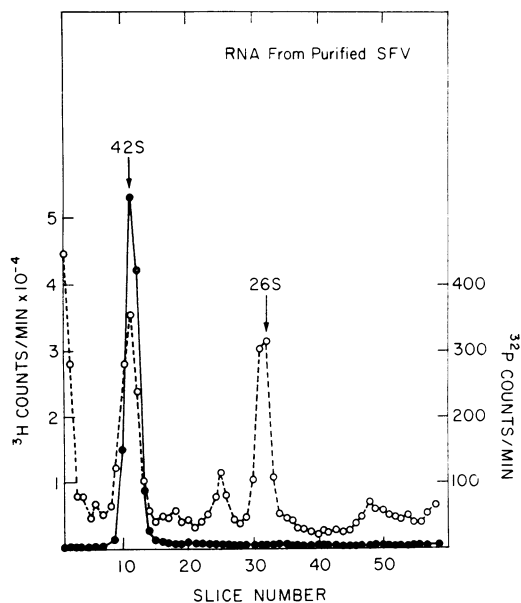


FIG. 4. Polyacrylamide gel electrophoresis of viral RNA from partially purified ^3H -labeled Semliki Forest virus (SFV). Chick embryo fibroblast cells were infected with SFV as described in Materials and Methods. After 1.5 hr at 37 C, medium containing ^3H -adenosine and ^3H -uridine (each 25 $\mu\text{Ci/ml}$) was added and the incubation continued for another 7 hr. Medium was poured off from the plates and then centrifuged at 1,000 rev/min to remove cells. Extracellular fluid (30 ml) was added to a centrifuge tube containing 8 ml of 10% sucrose dissolved in 0.1 M NaCl, 0.01 M tris(hydroxymethyl)aminomethane (Tris) (pH 7.8), 0.001 M ethylenediaminetetraacetate (EDTA), Tween 80 (2 $\mu\text{g/ml}$), and 0.1% bovine serum albumin and was sedimented for 2 hr at 254,000 \times g in a Spinco 60 Ti fixed angle rotor. The pellet was resuspended in 4 ml of the above buffer and was sedimented on a 1-ml cushion of 10% sucrose in a Spinco SW 65 rotor for 2 hr at 300,000 \times g. The resulting clear pellet was resuspended in buffer containing 0.1 M NaCl, 0.01 M Tris (pH 7.2), and 0.001 M EDTA and was extracted with sodium dodecyl sulfate and phenol as described in Materials and Methods. A 25- μl iter sample containing 23 μl iters of ^3H -labeled SFV RNA from partially purified virions and 2 μl iters of marker ^{32}P -labeled RNA from SFV-infected chick embryo fibroblast cells was subjected to analysis by polyacrylamide gel electrophoresis. (●), ^3H ; (○), ^{32}P .

the SFV virion, was present. This suggested that SFV was the only virus in the SFV pool.

To rule out the possibility of contamination of the SFV pool by another virus, pools of SFV were prepared under conditions which would tend to enrich the titer of contaminating agents present. In one experiment (*data not shown*) the virus was treated with a rabbit anti-SFV anti-

serum (prepared 4 yr earlier against the Kumba strain of SFV) and the neutralized SFV pool was passaged in the presence of the antiserum. This procedure was repeated four times. In another experiment the virus pool was treated sequentially with ether (to which arboviruses are sensitive) and with antiserum and the pool was then grown in the presence of the antiserum. This too was repeated four times. The residual virus was, in each case, then grown up to a pool in a single passage in chick cells. Actinomycin D-treated chick cells were infected with each pool, respectively, and ^3H -labeled RNA was prepared. In both experiments analysis of the RNA on acrylamide gels showed essentially the same pattern as was seen in Fig. 1A. These results again suggested that the virus pool used contained only SFV.

To determine whether peaks III and IV (Fig. 1A) could be detected in cells infected with another arbovirus, ^3H -labeled Sindbis RNA was prepared in the same manner as ^3H -labeled SFV RNA (Fig. 1). As shown in Fig. 5A when ^3H -labeled RNA from Sindbis-infected chick cells was subjected to polyacrylamide gel electrophoresis, all of the six RNA peaks previously observed in SFV-infected cells (Fig. 1A) could be identified. Comparison with the marker ^{32}P -labeled SFV RNA indicated coincidence of all peaks with the exception of peak IV, which consistently migrated slightly more slowly than the corresponding peak of SFV RNA.

A wide variety of cell types is sensitive to infection with Sindbis virus, so that it was possible to examine the RNA produced by infection of a different cell line. Accordingly, ^3H -labeled RNA was extracted from Sindbis-infected MA-308 cells (human diploid fibroblasts), and then co-electrophoresed with ^{32}P -labeled SFV RNA (Fig. 5B). The electrophoretic pattern of the Sindbis RNA was qualitatively similar to that of the SFV RNA and also corresponded to the Sindbis RNA peaks seen in Fig. 5A.

It is apparent from Fig. 5A, however, that in contrast to SFV-infected cells, Sindbis virus-infected cells contain relatively large amounts of 26S RNA (peak V) and rather small amounts of the RNA present in peaks I, III, and IV. The altered distribution of Sindbis virus RNA forms is even more striking in Fig. 5B. These results indicate that the relative amounts of arbovirus RNA forms can vary with the virus as well with the cell type.

The data discussed so far show that of the six peaks of radioactivity present in SFV- or Sindbis virus-infected cells, peak II corresponds to the 42S RNA of the virion; peak V to the 26S RNA previously identified in arbovirus-infected cells; and peaks III and IV to 38S and 33S RNA,

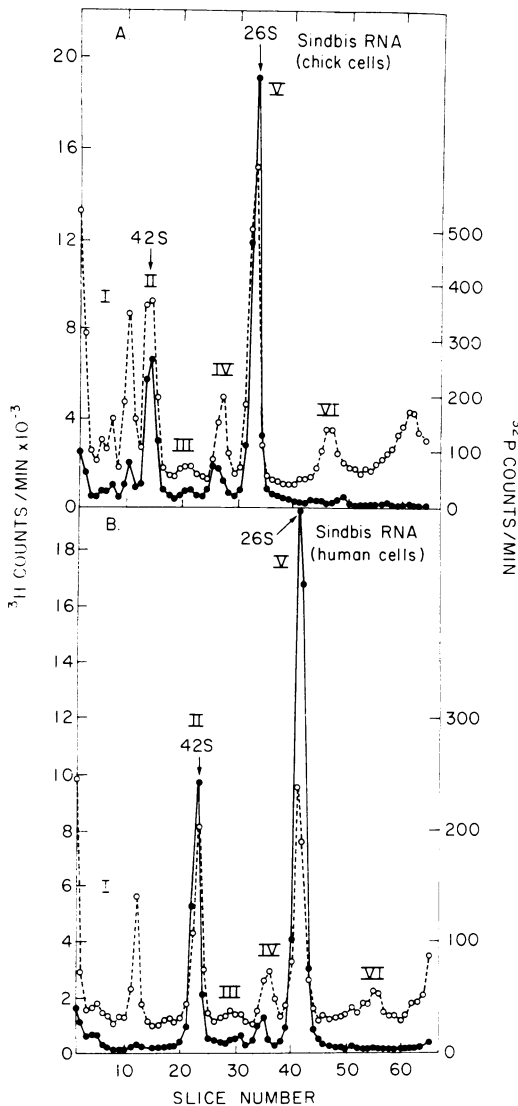


FIG. 5. Polyacrylamide gel electrophoresis of ^3H -labeled RNA from Sindbis virus-infected cells. Sindbis virus RNA was prepared from chick embryo fibroblast cells (A) or MA-308 cells, human diploid fibroblast cells (B) using the procedures given for extraction of RNA from Semliki Forest virus (SFV)-infected cells except that in (A) the medium contained $5 \mu\text{Ci}$ each of ^3H -adenosine and ^3H -uridine per ml and in (B) the medium contained $25 \mu\text{Ci}$ of ^3H -uridine per ml. The sample (25 μliters) analyzed in (A) contained 21 μliters of ^3H -labeled Sindbis RNA and 4 μliters of marker ^{32}P -labeled SFV RNA from chick embryo fibroblast cells; in (B) the sample (50 μliters) contained 40 μliters of ^3H -labeled Sindbis RNA and 10 μliters of the same marker ^{32}P -labeled SFV RNA. Roman numerals indicate the peaks of RNA previously shown in Fig. 1A. (●), ^3H ; (○), ^{32}P .

respectively. The production of these latter RNA forms also appears to be directed by the arbovirus genome.

Complex RNA forms. The mechanism of replication of arboviruses is not yet fully understood. Although it appears to resemble in some respects the mechanism described for the RNA bacteriophages (2), the existing knowledge of arbovirus RNA synthesis is not as great as the use of the terms RI and replicative form might imply. The adoption of this usage here is not meant to define a particular mechanism for the replication process, but simply to utilize terms generally accepted to describe the structures involved in viral RNA synthesis.

As mentioned earlier, the RI is presently considered to be a partly double-stranded, polydisperse RNA form. In contrast, the replicative form of arboviruses has until now been assumed to be a homogeneous RNA species since only one peak of ribonuclease-resistant RNA can be demonstrated in sucrose gradients under the usual sedimentation conditions (3, 13, 26, 27). However, as illustrated in Fig. 2B, when small volumes of ribonuclease-treated RNA were subjected to prolonged sedimentation at high speeds in sucrose density gradients, two peaks of radioactivity were seen. Moreover, when RNA from virus-infected cells was treated with ribonuclease before electrophoresis, the resulting electropherogram clearly showed two and possibly three peaks (Fig. 1B). These observations taken together strongly suggested that the ribonuclease-resistant RNA of arboviruses is not homogeneous.

In order to characterize further the ribonuclease-resistant SFV RNA, the two peaks of radioactivity from a sucrose gradient similar to the one shown in Fig. 2B were pooled, treated with Pronase, and co-electrophoresed with ^3H -labeled RNA from SFV-infected cells. Two peaks of radioactivity were observed (Fig. 6A) corresponding to the polydisperse peak I of the marker RNA. More prolonged electrophoresis (6 hr) led to even greater separation of the two peaks of radioactivity, although no additional peaks were observed. A similar result was obtained when unfractionated ^{32}P -labeled SFV RNA was treated with ribonuclease and then subjected to electrophoresis for 8 hr (Fig. 9). These results supported the conclusion that the replicative form of arboviruses is heterogeneous, consisting of at least two forms. Other data suggest that as many as four replicative forms may be produced (see below).

It is of interest to note that some RNA corresponding to peak I (Fig. 1A) is always present in analyses of viral RNA from whole cells (Fig. 1, 3-5). This suggests that some replicative form

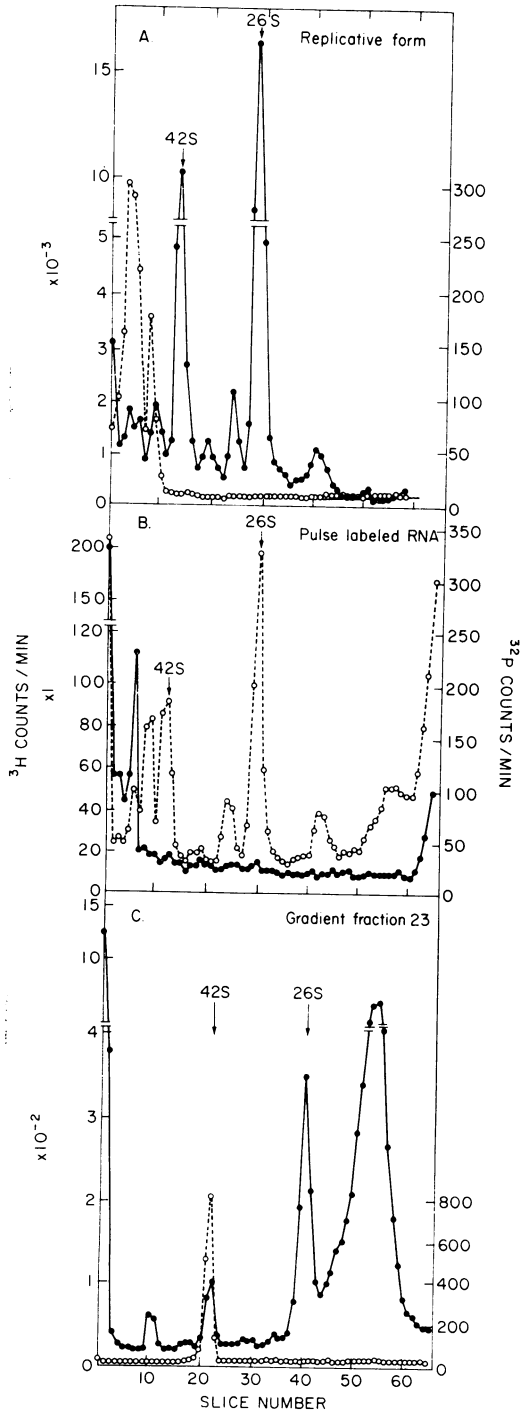


FIG. 6. Polyacrylamide gel electrophoresis of complex RNA species found in Semliki Forest virus (SFV)-infected chick embryo fibroblast cells. (A) Replicative form. ^{32}P -labeled SFV RNA was prepared from chick

is produced during the course of infection, although it is also possible that some or all of the replicative form seen is due to breakdown of the actual replicating structure during the extraction procedure.

Earlier studies have shown that when arbovirus-infected cells are given short pulses of radioactive RNA precursors, the RI is the major radioactive species (10, 13). In order to confirm this observation and to investigate further the question of the relative amounts of RI and replicative forms produced during infection, pulse-labeled ^3H -SFV RNA was analyzed on polyacrylamide gels (Fig. 6B). Although some replicative forms were present, the predominant species was a form which did not enter the gel. Thus, in analogy to the RI formed during poliovirus infection (20), the RI of arboviruses seems to be a very large molecule which does not enter the gel under the conditions employed. It should be noted, however, that the radioactivity at the top of the gel is greatly reduced when pulse-labeled viral RNA is treated with ribonuclease prior to electrophoresis (also cf. Fig. 1A and 1B).

The relative concentrations of RI and replicative forms were also examined in infected cells labeled for several hours. As already seen in Fig. 1 and 3 to 6, the replicative forms appeared to be a relatively minor species. In addition, frac-

embryo fibroblast cells as described in Materials and Methods. A 0.4-ml sample of this RNA was treated with ribonuclease (0.1 $\mu\text{g}/\text{ml}$) for 30 min at 37 C and then sedimented in a 6 to 30% sucrose density gradient by the procedures given in the legend to Fig. 2. Two peak fractions were pooled and treated with Pronase (100 $\mu\text{g}/\text{ml}$) for 1 hr at 37 C in order to remove residual ribonuclease. This preparation is referred to as ^{32}P -labeled replicative form. The sample (45 μl) used for electrophoretic analysis contained 40 μl of ^{32}P -labeled replicative form and 5 μl of marker ^3H -labeled RNA from SFV-infected chick embryo fibroblast cells. (B) Pulse-labeled RNA. Chick embryo fibroblast cells were infected with SFV as described in Materials and Methods. At 4.5 hr postinfection, the cells were incubated for 1 min with fresh medium containing ^3H -adenosine and ^3H -uridine (each 200 $\mu\text{Ci}/\text{ml}$). ^3H -labeled RNA was extracted with sodium dodecyl sulfate and phenol as described in Materials and Methods. Electrophoresis was performed on a 50- μl sample containing 45 μl of ^3H -labeled SFV pulse RNA and 5 μl of marker ^{32}P -labeled SFV RNA from SFV-infected chick embryo fibroblast cells. (C) Gradient fraction 23. Electrophoresis was performed on a 50- μl sample containing 48 μl of ^3H -labeled RNA (fraction 23 of the gradient shown in Fig. 2A) and 2 μl of marker ^{32}P -labeled SFV RNA extracted from virus particles purified by the Cheng procedure (6). (●), ^3H ; (○), ^{32}P .

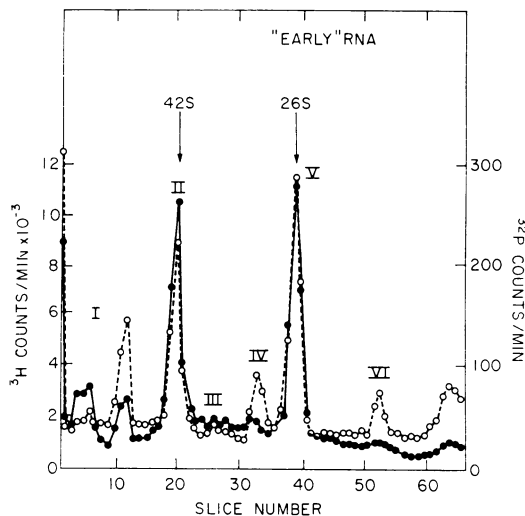


FIG. 7. Polyacrylamide gel electrophoresis of ^3H -labeled Semliki Forest virus (SFV) RNA formed early during infection of chick embryo fibroblast cells. Chick cells were treated with actinomycin D ($2 \mu\text{g}/\text{ml}$) for 1 hr at 37°C and then infected with SFV at a multiplicity of 80:1. After 30 min at 37°C Eagle's medium containing ^3H -adenosine and ^3H -uridine (each $50 \mu\text{Ci}/\text{ml}$) was added and the incubation continued for another hour. Viral RNA was extracted as described in Materials and Methods. A 50- μl iter sample containing 40 μl iters of ^3H -labeled RNA and 10 μl iters of marker ^{32}P -labeled SFV RNA extracted from chick cells 7 hr after infection was used for electrophoretic analysis. Roman numerals indicate the peaks of RNA previously shown in Fig. 1A. (●), ^3H ; (○), ^{32}P .

tion 23 from the sucrose density gradient shown in Fig. 2A was analyzed by gel electrophoresis since this fraction represents the region of the gradient in which double-stranded RNA forms are characteristically found when the sample is treated with ribonuclease before sedimentation (13). Fig. 6C shows that fraction 23 contains mostly RI and relatively little of the replicative forms. A large amount of 26S RNA and low molecular weight RNA (peak VI) were also observed.

Early RNA. In general the data presented thus far were obtained by analysis of RNA prepared from cells labeled for several hours after infection. However, the observation that the distribution of arbovirus RNA forms is not fixed but in fact varies with cell type or with the particular virus being studied (Fig. 5) prompted an investigation of the distribution of RNA forms in the same cell type at an earlier point in the replicative cycle. Thus, chick cells were infected with SFV and incubated with ^3H -labeled RNA precursors from 30 to 90 min after infection. The

RNA was extracted from these cells and then co-electrophoresed with SFV RNA prepared from chick cells labeled with ^{32}P -phosphoric acid between 1 and 7 hr after infection.

As shown in Fig. 7, such analysis revealed several differences between the early RNA and RNA produced later in infection. The early RNA consistently appeared to have a higher proportion of the slower migrating replicative forms and less of the more rapidly migrating replicative form. In addition, the minor species of RNA (peaks III and IV) were hardly in evidence in the early RNA. This was also a consistent observation and suggested that these peaks were probably not due to breakdown or association of the 42S or 26S RNA forms.

Analysis of RNA produced in vitro. Extracts of arbovirus-infected cells have been shown to incorporate nucleoside triphosphates into RNA species similar to viral RNA forms. In the case of Sindbis virus, reactions carried out with a partially purified mitochondrial fraction were reported to yield both 42S and 26S single-stranded RNA as well as RNA resistant to ribonuclease (29). However, with a crude mitochondrial fraction from SFV-infected cells only ribonuclease-resistant RNA appeared to be synthesized (16).

In the present study a partially purified membranous fraction of SFV-infected chick cells was used as the source of viral RNA polymerase (4), and the products of the enzymic reaction were analyzed on polyacrylamide gels. As may be seen in Fig. 8A, a heterogeneous mixture was found. The peaks of radioactivity corresponded to the replicative forms of the ^{32}P -labeled marker SFV RNA; very little RI was formed. Treatment of the reaction with ribonuclease prior to electrophoresis (Fig. 8B) yielded four distinct peaks of radioactivity. Similarly, electrophoresis of a ribonuclease-treated reaction for 8 hr (rather than the usual 4 hr) resulted in the appearance of three to five peaks of radioactivity (Fig. 9). The major peak (gel slice 11) coincided with one of the peaks of ribonuclease-treated ^{32}P -labeled SFV RNA from infected cells.

No peaks corresponding to the single-stranded SFV RNA forms were observed (Fig. 8A). This result may be due to the presence of ribonuclease in the enzyme preparation. Thus, when ^3H -labeled 42S SFV RNA was incubated with enzyme under the conditions of this experiment and the product analyzed on a polyacrylamide gel, very little of the added radioactivity could be detected in the usual position (peak II) for 42S RNA (J. G. Levin, unpublished observation). Alternatively, it is also possible that the in vitro reaction measures only the completion of RNA chains initiated during the in vivo infection.

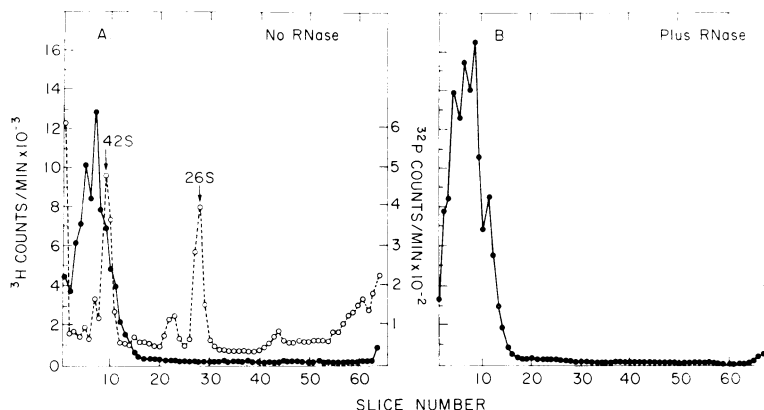


FIG. 8. Polyacrylamide gel electrophoresis of ^3H -labeled Semliki Forest virus (SFV) RNA formed *in vitro*. Duplicate reactions contained in a final volume of 0.1 ml: 0.05 M tris(hydroxymethyl) aminomethane (pH 8.0); 0.01 M MgCl_2 ; 0.001 M dithiothreitol; 0.025 μg of actinomycin D; 0.01 M phosphoenolpyruvate; 4 μg of pyruvate kinase; 10 nmoles each of adenosine triphosphate, uridine triphosphate, cytidine triphosphate, and ^3H -guanosine triphosphate (2.7×10^6 counts per min); and 118 μg protein of a partially purified SFV RNA polymerase. The enzyme was prepared from a membranous fraction of SFV-infected chick embryo fibroblast cells according to a modification of the fractionation procedure published by Caligiuri and Tamm (4); protein was determined by the method of Lowry *et al.* (15). Reactions were incubated for 30 min at 37 C. To one reaction (A), NaCl and sodium dodecyl sulfate (SDS) were added to final concentrations of 0.1 M and 0.5%, respectively. To the other reaction (B), NaCl and ribonuclease were added to final concentrations of 0.1 M and 1 $\mu\text{g}/\text{ml}$, respectively; the incubation was continued for another 30 min at 37 C, and SDS was then added to give a final concentration of 0.5%. In (A) the sample (50 μl iters) used for electrophoresis contained 45 μl iters of the deproteinized reaction mixture and 5 μl iters of marker ^{32}P -labeled SFV RNA from chick embryo fibroblast cells; in (B) a 50- μl iter portion of the deproteinized ribonuclease-treated reaction mixture was employed. (●), ^3H ; (○), ^{32}P .

DISCUSSION

Analysis of RNA forms by acrylamide gel electrophoresis gives far better resolution of a mixture of species than do sucrose density gradients. As suggested previously, this is especially useful in studies on the viral RNA forms of arboviruses since the polydisperse RI, the replicative form, and the 26S RNA are impossible to separate in sucrose gradients (5).

In addition to achieving the separation of these RNA forms, the analysis on acrylamide gels has revealed the probable presence of previously unrecognized arbovirus RNA species. The minor 38S and 33S species would be extremely difficult to resolve by sucrose density gradient analysis. Since the migration of RNA species on acrylamide gels is thought to be largely determined by their molecular weight (22), tentative values for the single-stranded SFV RNA forms can be given, based on the known molecular weights of 45S precursor ribosomal RNA and 28S and 18S ribosomal RNA (8). Thus, the tentative molecular weight for 42S RNA is 4×10^6 ; for 38S RNA, 3.1×10^6 ; for 33S RNA, 2.4×10^6 ; and for 26S RNA, 1.8×10^6 . The values for 42S and 26S RNA are in substantial agreement with previously

reported results based on gel electrophoresis (5, 9).

If the migration of RNA forms in polyacrylamide gels is indeed determined by molecular weight, then the various species identified in arbovirus-infected cells would be distinct RNA forms rather than configurational variants of one form. Since the base ratios of the 42S and the 26S RNA forms have been reported to be similar (11, 26, 28), it was suggested that the 42S and 26S RNA forms might differ only in their conformation (28). However, the 26S RNA employed in all of these studies was purified on sucrose gradients, and it is possible that the base ratio determined for the 26S RNA was really a composite of that of the 26S and 33S RNA forms. The sum of the tentative molecular weights of these RNA forms is close to that of the 42S RNA, suggesting that the 26S and 33S forms may be pieces of the whole genome.

Only the 42S viral RNA appears to be infectious (18). Several possible functions for the 38S, 33S, and 26S RNA forms can be considered, including among others: (i) RNA forms complementary in base composition to sections of the genome ("negative strands"); (ii) messenger RNA

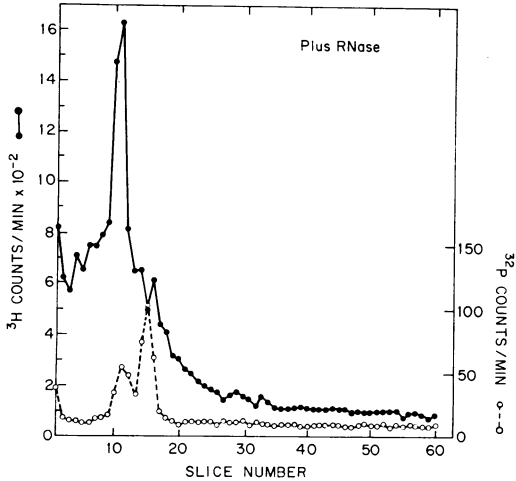


FIG. 9. Eight hour polyacrylamide gel electrophoresis of ribonuclease-treated Semliki Forest virus (SFV) RNA formed *in vitro* and *in vivo*. The *in vitro* reaction (0.1 ml final volume) contained the components given in the legend to Fig. 8 except that actinomycin D was 0.1 μ g, and 1.75 μ moles of β -mercaptoethanol were added instead of dithiothreitol. After incubation for 30 min at 28 C, the following additions were made: NaCl and ribonuclease to final concentrations of 0.1 M and 1 μ g/ml, respectively; and 20 μ liters of 32 P-labeled SFV RNA from infected chick cells. Incubation was continued for another 30 min at 37 C, and sodium dodecyl sulfate was then added to give a final concentration of 0.5%. A 50- μ liter portion of the final mixture was subjected to polyacrylamide gel electrophoresis for 8 hr. (●), 3 H (in *in vitro* SFV RNA); (○), 32 P (SFV RNA from infected cells).

species; or (iii) forms involved directly or indirectly in the regulation of virus protein synthesis. It is possible that some species have different functions than others. Preliminary studies have failed to show hybridization between the 42S viral genome and the other species. Since the messenger RNA of arboviruses has not yet been identified, it is difficult to rule out the possibility that some or all of these forms may be viral messenger RNA. The fact that the 38S and 33S forms are not prominent early in infection, however, may indicate their involvement in a late regulatory function.

The analysis by acrylamide gels also indicated that the replicative form is probably a naturally occurring RNA species. Somewhat to our surprise, the replicative form appeared to be heterologous, so that it is possible to speak of RFs. At least two replicative forms occur *in vivo*, whereas in the *in vitro* reaction, four forms could be identified after ribonuclease treatment. These results suggest that each single-stranded RNA

form may be produced on a specific template. It is interesting to speculate on the possible significance of the observations that four replicative forms and four single-stranded species may have been identified. The molecular weights of the respective replicative forms (calculated on the basis of their migration in the gels) are close to twice that of the respective single-stranded RNA species, if it is assumed that the largest replicative form represents part of the template on which the 42S RNA is formed and that the size of the template decreases in proportion to the size of the RNA it is producing.

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