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Semliki Forest Virus-Specific RNAs Synthesized In Vitro by Enzyme from Infected BHK Cells

MARCEL R. MICHEL AND PETER J. GOMATOS
Division of Virology, The Sloan-Kettering Institute, New York, New York 10021

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When Semliki Forest virus (SFV)-infected BHK cells were disrupted 4 h after infection, 75 to 90% of the total virus-specific RNA synthesizing enzyme was found in the large particle fraction, along with 75 to 90% of the in vivo-synthesized double-stranded RNAs. The RNA products of this enzyme-template complex in an in vitro system were double-stranded RNAs sedimenting predominantly at 18*S*, and single-stranded RNAs sedimenting at 42*S*, 26*S*, and 22*S*. The various virus-specific SFV RNAs synthesized in vitro were associated with different sized structures, and thus each was separable by differential centrifugation. Kinetic and pulse-chase experiments showed that the double-stranded RNAs were the precursors to the single-stranded RNAs. There were several double-stranded RNAs identified both in the in vitro product and also in extracts from infected cells. The major replicative form had a molecular weight of 4.4×10^6.

There are at least four different virus-specific RNAs found in group A arbovirus-infected cells: the predominant single-stranded RNAs sedimenting at about 42*S* and at 26*S*, a replicative form (RF) reported as sedimenting at about 20*S*, and a replicative intermediate (RI) sedimenting faster than 20 to 22*S* (4, 10, 18, 33-35, 37, 39). Three additional species of single-stranded RNAs have been described, two sedimenting at 38 and 33*S* (20), and a third sedimenting from 20 to 22*S* (18, 20).

An RNA-synthesizing activity has been detected in the cytoplasm of arbovirus-infected chicken embryo fibroblasts (14, 20, 24, 36) or baby hamster kidney cells (23, 29). It was found in the large particle fraction of the cytoplasm of infected cells after nuclei and cytoplasmic debris had been removed by low-speed centrifugation. This virus-specific enzyme supported the in vitro synthesis of only 20*S* double-stranded RNA (20, 23, 24). When a similar enzyme preparation was further purified through sucrose density gradients, it supported the synthesis of small amounts of virus-specific, single-stranded RNAs in addition to double-stranded RNAs, but the enzyme activity was low (36).

The present paper analyzes the RNA synthesizing capacity of two cellular fractions which together represent the total cell homogenate of Semliki Forest virus (SFV)-infected BHK-21 cells. It was the intent to characterize the SFV RNA synthesizing enzymes in these fractions, to determine the various species of RNA synthesized in vitro, and to establish any relationship which exists between the various species of SFV RNAs. This paper was presented in part at the Annual Meeting of the American Society for Microbiology, Philadelphia, Pa., 23-28 April 1972.

MATERIALS AND METHODS

Cell culture. BHK-21 cells, clone 13, a continuous cell line derived from baby hamster kidney cells were grown at 37 C in Eagle minimal essential medium (MEM) supplemented with 5% fetal bovine serum in cylindrical bottles on rollers (18).

Virus. Semliki Forest virus, prototype strain obtained from J. R. Henderson, Yale University School of Medicine, was cloned and used as stock virus after two passages. Growth of virus and the determination of its infectivity by plaque assay were as described before (18).

Chemicals and isotopes. Actinomycin D was purchased from Merck, Sharp, and Dohme (West Point, Pa.). Unlabeled ribonucleoside triphosphates were obtained from P-L Biochemicals, Inc. (Milwaukee, Wis.). GTP labeled with ^{32}P in the α position was obtained from International Chemical and Nuclear Corp. (City of Industry, Calif.) or from New England Nuclear Corp. (Boston, Mass.). Sodium dodecyl sulfate (Matheson, Coleman and Bell, East Rutherford, N.J.) was recrystallized according to Mandel (22). Sodium deoxycholate (DOC), enzyme grade, was obtained from the Mann Research Laboratories (New York, N.Y.); Tween 40 was from the Amend Drug Company (New York, N.Y.); BRIJ 58 was from Atlas Chemical Industries, Inc. (Wilmington, Del.); polyvinyl sulfate, potassium salt, was from General Biochemicals (Chagrin Falls, Ohio). *N,N,N',N'*-tetra-

methylethylenediamine (TEMED), acrylamide, and *N,N'*-methylene bisacrylamide were purchased from Eastman Organic Chemicals (Rochester, N.Y.). RNase, crystallized once, and DNase, electrophoretically purified, were purchased from Worthington Biochemicals (Freehold, N.J.). Pyruvate kinase and phosphoenolpyruvate, potassium salt, were obtained from the Boehringer Mannheim Corp. (New York, N.Y.)

Preparation of cellular extracts. BHK-21 cells in the logarithmic stage of growth were infected at a multiplicity of infection of 40 to 100 with virus in MEM containing 0.2% bovine plasma albumin. Actinomycin D, 0.15 μg/ml, was present at all times after addition of virus to cells. The cells from 12 cylindrical bottles, each containing approximately 200 × 10^6 cells, were used as the source of enzyme. Four hours after infection, the growth medium was removed, the cells were washed with ice-cold PBS-PVS, phosphate-buffered saline (7) containing polyvinyl sulfate, 20 μg/ml, and subsequently with RSB-PM (0.01 M Tris, pH 7.4, 0.01 M KCl, 0.0015 M $MgCl_2$ containing 0.001 M 2-mercaptoethanol, and polyvinyl sulfate, 20 μg/ml). The cells were scraped into RSB-PM and disrupted with 25 strokes of a tight-fitting Dounce homogenizer. The homogenate was centrifuged for 10 min at 250 × *g*, separating it into two fractions: (i) supernatant no. 1; and (ii) a large particle fraction, P-250 × *g*, which sedimented to the pellet. Supernatant fluid no. 1 from the cell homogenate was 40 to 50 ml, and the large particle fraction was resuspended in 15 ml of RSB-PM.

Assay condition optimal for synthesis of single- and double-stranded viral RNAs. The standard reaction mixture for the enzyme assay contained the following in a volume of 2.4 ml: 1.6 ml of either supernatant fluid no. 1 containing 1.1 to 2.4 mg of protein per ml, or the large particle fraction containing 4.8 to 8.8 mg of protein per ml; 240 μmol of Tris hydrochloride, pH 8.1; 16 μmol of 2-mercaptoethanol; 19.2 μmol of magnesium chloride; 19.4 μmol of potassium phosphoenolpyruvate; 160 μg of pyruvate kinase; 24 μg of actinomycin D; 5 μmol of the three unlabeled ribonucleoside triphosphates, ATP, CTP, and UTP, and 2.5 μmol of GTP labeled with ^{32}P in the α position and used at a specific activity of 12,000 to 27,000 counts per min per nmol. The incubation was at 28 C. Actinomycin D was present in the assay mixture even though the amount and distribution of viral RNAs synthesized in its presence were identical to those found in its absence.

Extraction and analysis of virus-specific RNAs. Before centrifugation, all fluids were made 0.02 M in EDTA, 1.95% in sodium dodecyl sulfate, and 0.5% in BRIJ 58 (SDS-BRIJ-EDTA mixture). The treated samples were layered on linear 15 to 30% sucrose density gradients in TNE buffer (0.01 M Tris hydrochloride, pH 7.4, 0.1 M NaCl, and 0.001 M EDTA) and centrifuged in an SW27 rotor at 76,000 × *g* for 16 to 17 h at 15 C. Fractions, each containing 1 ml, were collected from below, and absorbancy was determined in a Zeiss PMQ II spectrophotometer. Two equal samples were taken. One sample was left untreated, whereas the other was treated with 3 μg of pancreatic RNase per ml in 1.0 × RSB and 1.7 × KKC (1 × KKC: 0.15 M potassium chloride, 0.015 M potassium citrate) and incubated for 30 min at 37 C. To the untreated and RNase-treated sample was added 50 μg of bovine plasma albumin as carrier and the samples were made 5% in trichloroacetic acid. The resulting acid-insoluble material was collected on membrane filters and the amount of radioactivity on the dried filters was determined in a TriCarb scintillation spectrophotometer as described before (18).

Sedimentation coefficients were estimated by the method of Martin and Ames (25). For single-stranded RNAs, BHK-21 ribosomal RNAs, 29*S* and 19*S*, were used as reference. For double-stranded RNAs, reovirus double-stranded RNAs were used as reference. The sedimentation coefficients of the three size classes of reovirus RNAs were kindly determined in the analytical ultracentrifuge by M. J. Hamilton. The average values, $s^0_{20,w}$, at infinite dilution in TNE or PBS obtained in three different experimental runs were 15, 13, and 11.4*S*.

Isolation of RF and RI of SFV. Fractions from sucrose gradients containing the virus-specific RNAs were pooled as described in the text and were exposed to 2 M lithium chloride (LiCl) at 0 C for 16 h. RNA insoluble in 2 M LiCl was collected as a pellet by centrifugation in an SW27 rotor at 4 C for 1 h at 76,000 × *g* and suspended in 1/10 × SSC (1 × SSC: 0.15 M sodium chloride, 0.015 M sodium citrate). RNA remaining in the supernatant fluid, and thus soluble in 2 M LiCl, was collected after precipitation with two volumes of ethanol and resuspended in 1 × TNE.

To have the RNAs in the same ionic environment, both RNAs, soluble and insoluble in 2 M LiCl, were chromatographed through Sephadex G-50 in 1/10 × SSC. The fractions containing the RNAs were pooled, lyophilized, and suspended in the electrophoresis buffer described below or in 1 × SSC.

Polyacrylamide gel electrophoresis. Polymerization of gels (0.4 by 14 cm) in buffer A (0.14 M Tris hydrochloride, pH 7.8, 0.02 M sodium acetate, 0.002 M EDTA) containing 3% acrylamide, 0.15% bisacrylamide, and 0.4% agarose was catalyzed with 0.1% ammonium persulfate in the presence of 0.1% TEMED. Before application of sample, gels were prerun for 1 h at 2 mA per gel. The RNA sample in buffer A, 100 μliters, was made 10% in sucrose and 0.15% in SDS and layered onto the gels. Electrophoresis was at 53 V and 2 mA per gel in the presence of buffer A containing 0.2% SDS. After electrophoresis, the gels were cut into slices approximately 1-mm thick which were placed 2 each into vials and dissolved at 70 C in 0.5 ml of 30% hydrogen peroxide. Fifteen milliliters of the following mixture was then added: Omnifluor, 16.8 g; toluene, 2 liters; anhydrous Triton X-100, 1 liter; water, 250 ml. Radioactivity was then determined in a Packard TriCarb scintillation spectrometer.

Electron microscopy. Material in the large particle fraction or supernatant fluid no. 1 which sedimented to the pellet in 2 h at 76,000 × *g* was fixed for 30 to 60 min at 0 C with 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, and postfixed with 1% osmium tetroxide for 60 min. After dehydration in graded alcohols, the pellets were embedded in Epon 812. Thin sections, cut on an LKB Ultrotome III, were

stained with uranyl acetate and lead citrate. All specimens were examined in a Philips 300 electron microscope.

RESULTS

Analysis of viral RNAs synthesized in vitro. The cell homogenate of SFV-infected BHK cells was separated by centrifugation at 250 × *g* into two fractions, both of which were used as enzyme sources: the large particle fraction which was pelleted and the supernatant fluid no. 1. Electron microscopic analysis revealed that the pelleted fraction contained rough and smooth endoplasmic reticulum, vesicles of various sizes with rough or smooth membranes, nuclei, and formed organelles of the cytoplasm. The supernatant fluid no. 1 contained all of the above components except for nuclei and mitochondria. In addition, supernatant fluid no. 1 contained the cytopathic vacuoles, CPV-1, described by Grimley et al. (16). The pelleted material at 250 × *g* did not contain the cytopathic vacuoles, neither CPV-1 nor CPV-2. We do not know whether this resulted from disruption of these vacuoles during preparation for electron microscopy or because BHK cells were harvested for analysis at 4 h after infection, a time early in exponential growth of virus.

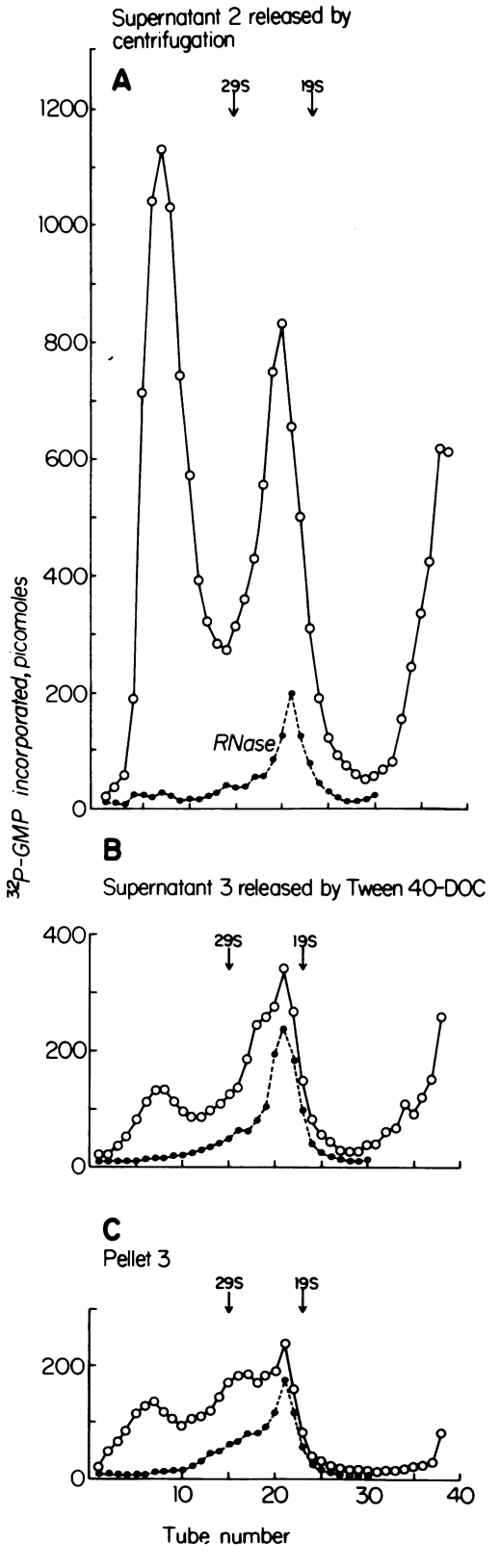
When supernatant fluid no. 1 was used as enzyme source, both single- and double-stranded newly synthesized RNAs could be directly extracted intact from the assay mixture by treatment with the SDS-BRIJ-EDTA detergent mixture. Both virus-specific single- and double-stranded RNAs were also synthesized in vitro by the large particle fraction, but they were hydrolyzed or fragmented during extraction by nucleases present in the large particle fraction which were released by the detergent treatment. The RNAs were recovered intact if they were sequentially removed from the large particle fraction in three steps: (i) after incubation, large structures were removed by centrifugation at 250 × *g* to yield supernatant fluid no. 2; (ii) the pelleted material was resuspended in 0.8% Tween 40 and 0.4% DOC in RSB-PM, and recentrifuged to yield supernatant fluid no. 3; and (iii) the resulting pelleted material was resuspended in high salt buffer containing 20 μg of polyvinyl sulfate per ml and 100 μg of DNase per ml, and incubated at room temperature for 5 min to yield the suspension of pellet no. 3. The RNA in supernatant fluids no. 2 and no. 3 and the suspension of pellet no. 3 could then be extracted with the SDS-BRIJ-EDTA detergent mixture.

Figure 1 shows the distribution in the three subfractions of the RNAs newly synthesized by the large particle fraction. More than 70% of the single-stranded RNAs synthesized by the large particle fraction and identified by their RNase sensitivity was found in supernatant fluid no. 2 obtained by low-speed centrifugation (Fig. 1A). The newly synthesized double-stranded RNAs were distributed about equally between the three subfractions. In all subsequent experiments, for clarity in presentation, the newly synthesized RNAs present in the large particle fraction are presented as the composite of all three portions of the large particle fraction.

The RNA products synthesized in vitro by both the pelleted fraction and supernatant fluid no. 1 contained all the virus-specific RNAs found in infected BHK cells (18). The predominant single-stranded RNAs were those which sedimented at 42, 26, and 22*S*. In only one in vitro experiment shown later (Fig. 9) was there any indication that the 38 and 33*S* RNAs may be discrete viral single-stranded RNAs. The 42*S* RNA is the viral RNA and is easily identified by its sedimentation value and its RNase sensitivity. The 26 and 22*S* single-stranded RNAs can be separated by more prolonged centrifugation than shown in Fig. 1A. The 26*S* single-stranded RNAs and the RI's sedimenting at about 26*S* are identified by their mobilities on polyacrylamide gel electrophoresis (PAGE). During PAGE, the RI's do not enter the gels under conditions where the 26*S* single-stranded RNA does (20). The 22*S* single-stranded RNA is LiCl-insoluble and RNase-sensitive, whereas the RF is LiCl-soluble and RNase-insensitive.

In TNE buffer, the SFV RF sedimented faster than the 19*S* ribosomal RNA and was coincident in sedimentation rate with the 22*S* single-stranded RNA. In the same buffer, the SFV RF sedimented at 18*S* relative to reovirus double-stranded RNAs as sedimentation markers. Since the sedimentation coefficient of double-stranded RNA is unaffected by salt concentration whereas that of single-stranded RNA is (6, 38), the SFV RF has a sedimentation value of 18*S*, even though relative to the ribosomal RNAs it seemed to sediment at 22*S*.

Distribution of viral RNA polymerase in the pelleted fraction and supernatant fluid no. 1. To determine the relative amounts of virus-specific RNA-synthesizing enzyme present in the cell homogenate of SFV-infected BHK cells, samples of the pelleted fraction and supernatant no. 1 were incubated at 28 C for 30 min in the standard assay. The upper panel of Fig. 2 shows the sedimentation profile of the viral RNAs synthesized in vitro by the pelleted fraction, and the lower panel shows those synthesized in vitro by sypernatant fluid no. 1. Both the pelleted fraction and supernatant fluid no. 1 synthesized in vitro all species of virus-specific



RNAs and in approximately the same proportion to each other. Of the total virus-specific RNA-synthesizing enzyme present in the cell homogenate, 75 to 90% was found in the pelleted fraction. Also, in the pelleted fraction was 75 to 90% of the total double-stranded RNAs synthesized within the cell during infection. Thus, in any study of synthesis in vitro of SFV RNAs by fractions from BHK cells, investigation of the pelleted fraction is mandatory.

Association with formed structures of viral RNAs synthesized in vitro by the pelleted fraction. As shown in Fig. 1, more than 70% of the single-stranded RNAs and about one-third of the double-stranded RNAs newly synthesized in vitro by the pelleted fraction were separated from the enzyme-template complex by centrifugation for 10 min at 250 × *g* and did not sediment with the enzyme-template complex to the pellet. To determine if any of the RNAs newly synthesized in vitro were free, or if all were associated with formed structures, the material in the pelleted fraction after synthesis was centrifuged at 76,000 × *g* for 5.25 h through a linear sucrose density gradient formed on a cushion of 45% sucrose. In the experiment shown in the upper panel of Fig. 3, 60% of the RNAs newly synthesized in vitro by the large particle fraction was associated with formed structures which sedimented in the ultracentrifuge to the pellet. Most of the remaining RNA products were also associated with formed

FIG. 1. *Analysis by sucrose density gradient centrifugation of the SFV RNAs synthesized in vitro by the large particle fraction. BHK cells, harvested 4 h after infection, were disrupted by Dounce homogenization and centrifuged for 10 min at 250 × g to yield a pelleted, large particle fraction. The large particle fraction was resuspended in RSB-PM and incubated at 28 C for 30 min in the standard assay mixture containing* ^{32}*P-GTP. After incubation, the large particle fraction was centrifuged at 250 × g for 10 min to yield supernatant fluid no. 2. The pelleted material was treated with 0.8% Tween 40 and 0.4% DOC in RSB-PM and recentrifuged at 250 × g for 10 min to yield supernatant fluid no. 3. The resulting pellet, pellet no. 3, was resuspended in a high salt buffer (0.28 M NaCl, 0.028 M* $MgCl_2$*, 0.01 M Tris hydrochloride, pH 7.4) containing 20 μg of PVS per ml and 100 μg of DNase per ml and incubated at room temperature for 5 min. All three samples, supernatant fluids no. 2 and no. 3 and pellet no. 3, were treated with the SDS-BRIJ-EDTA detergent mixture and were layered over separate 15 to 30% sucrose gradients in TNE. Centrifugation was at 15 C for 16 h in an SW27 rotor at 6,000 × g. Fractions were collected from below, and each fraction was analyzed for absorbancy at 260 nm, total RNA (solid line), and RNase-resistant RNA (broken line). RNase treatment was at 3 μg/ml for 30 min at 37 C in 1.7 × KKC and 1 × RSB.*

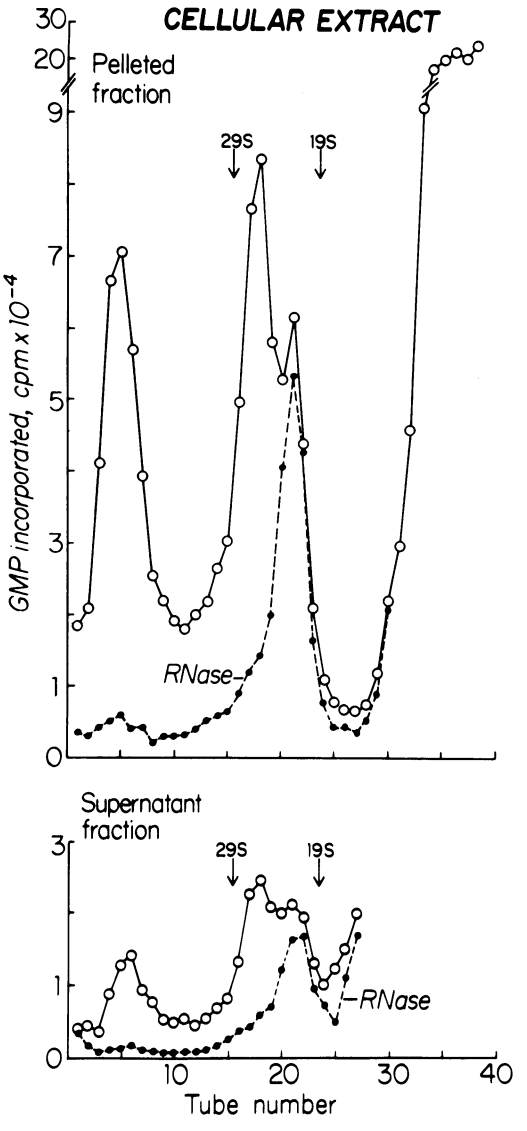


FIG. 2. *Distribution of SFV RNA polymerase in the large particle fraction and supernatant fluid no. 1. Sucrose density gradient analysis of total RNA products synthesized in vitro by the pelleted, large particle fraction, upper panel, and by the supernatant fluid no. 1, lower panel. The pelleted, large particle fraction and supernatant fluid no. 1 were obtained from BHK cells harvested at 4 h after infection and contained, respectively, a total of 87 and 105 mg of protein as determined by the method described by Lowry et al. (21). After incubating a sample of each in the standard assay mixture containing ^{32}P-GTP for 30 min at 28 C, the supernatant fluid no. 1 was directly treated with the SDS-BRIJ-EDTA detergent mixture. The large particle fraction was separated into the three subfractions described in Fig. 1 and then treated with the SDS-BRIJ-EDTA detergent mixture. All samples were layered over separate 15 to 30% sucrose gradients in TNE. Centrifugation and analysis were as described in Materials and Methods. The results of the RNAs synthesized by the large particle fraction are the composite of its three subfractions.*

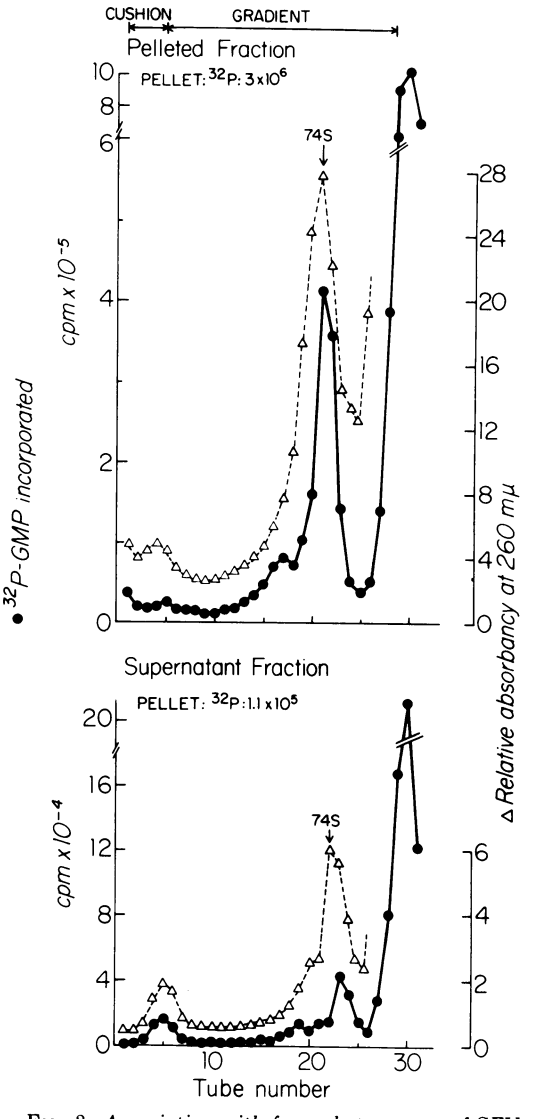


FIG. 3. *Association with formed structures of SFV RNAs synthesized in vitro by the large particle fraction or supernatant fluid no. 1 from infected BHK cells. The pelleted, large particle fraction and supernatant fluid no. 1 were obtained from BHK cells harvested 4 h after infection. A sample of each was incubated at 28 C for 30 min in the standard assay mixture containing ^{32}P-GTP. After incubation, the assay mixture containing supernatant fluid no. 1 was layered over a 15 to 30% sucrose density gradient in TNE, formed on a cushion containing 45% sucrose. Centrifugation was at 4 C for 5.25 h in an SW 27 rotor at 76,000 × g. Before centrifugation, the large particle fraction was separated into its three subfractions as described in Fig. 1, and each was layered over separate 15 to 30% sucrose density gradients in TNE. After centrifugation, fractions were collected from below, and each fraction was analyzed for absorbancy at 260 nm (broken line) and total RNA (solid line). The results for the large particle fraction (upper panel) are the composite of its three subfractions determined separately. The 74S BHK ribosomes served as sedimentation markers.*

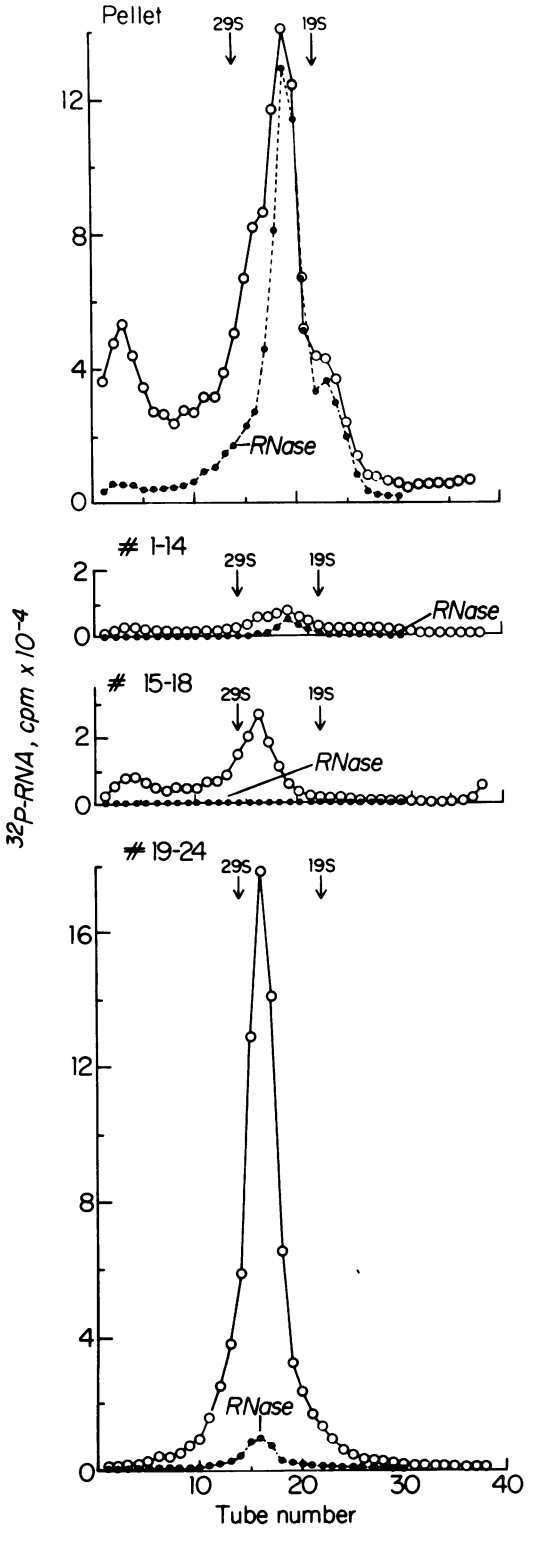


FIG. 4. *Sucrose density gradient analysis of the SFV ^{32}P-RNAs synthesized in vitro by the pelleted, large particle fraction and associated with the different sized structures shown in the upper panel of Fig. 3. The material in tubes 1 to 14, 15 to 18, and 19 to 24 of the upper panel of Fig. 3 were separately pooled, extracted with the SDS-BRIJ-EDTA detergent mixture, precipitated with two volumes of ethanol, and resuspended in TNE before centrifugation. The RNAs in the pellet of Fig. 3 were directly extracted with the SDS-BRIJ-EDTA detergent mixture. All samples were layered over separate 15 to 30% sucrose density gradients in TNE. Centrifugation and analysis were as described in Material and Methods.*

structures which sedimented in three distinct bands: (i) at the interface between the gradient and the cushion; (ii) slightly faster than the 74*S* monoribosomes; and (iii) essentially coincident in sedimentation rate with the 74*S* monoribosomes.

Figure 4 shows the various RNA species obtained by extraction of the pooled fractions of the upper panel of Fig. 3 with the SDS-BRIJ-EDTA detergent mixture. The results show that the various virus-specific RNAs were associated with structures differing in *S* values, and thus they were separable by centrifugation. Associated with large structures and sedimenting to the pellet and the interface between the gradient and 45% sucrose cushion were essentially all of the in vitro-synthesized double-stranded RNAs, along with 75% of the 42*S* single-stranded RNA, and 90% of the 22*S* single-stranded RNA. Little 26*S* single-stranded RNA was found in the pellet. Eighty percent of the total 26*S* single-stranded RNA newly synthesized in vitro sedimented with structures from 60 to 90*S* (tubes 19 to 24). A small amount of 42 and 26*S* single-stranded RNAs sedimented in structures from 95 to 120*S* (tubes 15 to 18). When the labeled material sedimenting at less than 60*S* in the experiment shown in the upper panel of Fig. 3 was analyzed, only RNAs sedimenting at 4*S* or less were present. Thus, the newly synthesized SFV RNAs were all associated with formed structures and none was found free.

Preliminary experiments show that some viral proteins newly synthesized during infection also were in structures which sedimented from 60 to 90*S* and from 95 to 120*S*. Whether the in vitro-synthesized 26*S* RNA was associated with the in vivo-synthesized protein in a structure which sedimented from 60 to 90*S* is not yet known. If the 42*S* RNA was associated with an in vivo-synthesized viral protein(s) in the structure sedimenting from 95 to 120*S*, it was not in an intact nucleocapsid, as nucleocapsids sediment at about 140*S* (9, 39). The lower panel of Fig. 3 shows the sedimentation characteristics of the structures to which the RNA newly

synthesized in vitro with supernatant fluid no. 1 as enzyme were associated. The profile is similar to that in the upper panel of Fig. 3 and the distribution of the various RNA species sedimenting at the different *S* values was identical to that in Fig. 4.

Kinetics of synthesis in vitro of SFV RNAs. To determine the sequence of synthesis of the various SFV RNA species, the pelleted fraction or supernatant fluid no. 1 was incubated at 28 C in the standard assay. Figure 5 shows the results obtained with supernatant fluid no. 1 as enzyme. At the times indicated, the in vitro synthesis of viral RNAs was terminated by the addition of the SDS-BRIJ-EDTA detergent mixture, the sample was analyzed by sucrose density gradient centrifugation, and the amount of each species was determined as described before.

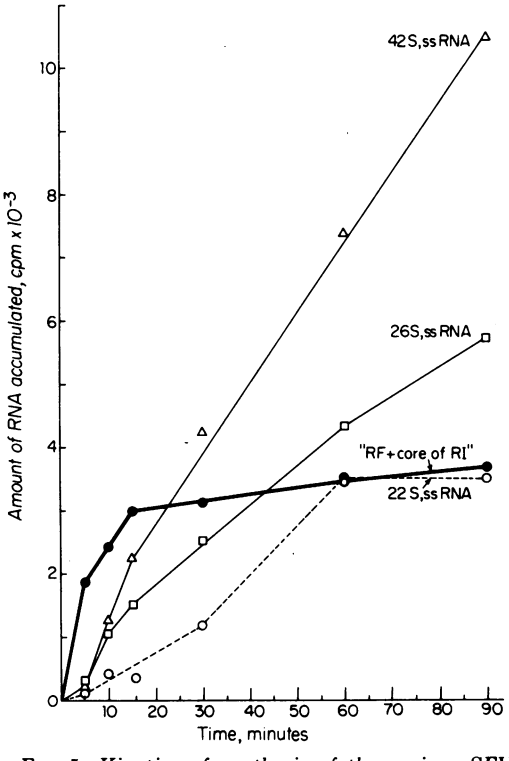


FIG. 5. *Kinetics of synthesis of the various SFV RNAs synthesized in vitro. The supernatant fluid no. 1 was obtained from BHK cells harvested at 4 h. Equal samples of supernatant fluid no. 1 were incubated at 28 C in the standard assay mixture containing ^{32}P-GTP. RNA synthesis was terminated at the indicated time by the addition of the SDS-BRIJ-EDTA detergent mixture. The detergent-treated samples were layered over 15 to 30% sucrose density gradients in TNE, centrifuged, and analyzed for radioactivity in RNAs and optical density. The individual RNA species were identified as described in the text.*

The kinetics of synthesis of the double-stranded RNAs, including the RF and RNase-resistant core of RI, was identical and is presented for clarity as the composite of the two. They appeared first and increased in amount until the maximal level of accumulation was reached at about 15 min. Thereafter, the double-stranded RNAs did not accumulate significantly, and their amount remained constant. After a 5-min delay in the onset of their appearance, the 42 or 26*S* single-stranded RNAs were synthesized at a linear rate from 5 to 15 min, and at a slower linear rate from 15 to 90 min, the last time point analyzed. The synthesis of the 22*S* single-stranded RNA was linear from 5 to 60 min, after which time the 22*S* RNA no longer accumulated. The initial synthesis of all the SFV RNA species by the enzyme in the pelleted fraction followed kinetics similar to that shown for the supernatant fluid no. 1. Thus the onset of the appearance and maximal rate of synthesis in vitro of all species of SFV single-stranded RNAs occurred later than those of the SFV double-stranded RNAs.

If the double-stranded RNAs are the precursors to the single-stranded RNAs, their synthesis would have to continue after 15 min, the time point at which no further accumulation of double-stranded RNAs occurred. This must be so, as the synthesis of single-stranded RNAs continues with linear kinetics beyond the first 15 min. To demonstrate that the synthesis of double-stranded RNAs does continue after the first 15 min, we analyzed the RNA species synthesized in vitro during successive 30-min intervals. After incubation in the presence of the four unlabeled nucleoside triphosphates, ^{32}P-GTP was added at the beginning of each interval at a known specific activity, and incubation was continued. The virus-specific RNAs synthesized during the next 30 min were analyzed by subsequent sucrose density gradient centrifugation. The rate of synthesis was fastest during the first 30 min and subsequently decreased to a constant level (Fig. 6). The time when this constant rate of synthesis was reached was 60 min later for the 42 and 26*S* single-stranded RNAs than for the double-stranded RNAs and for the 22*S* single-stranded RNA. Thus, despite the fact that, after the first 15 min, the amount of newly synthesized double-stranded RNAs did not increase, their synthesis did continue and was matched by their conversion into other species or by their hydrolysis.

Precursor-product relationship between the various species of virus-specific RNAs. Regardless of the duration of incubation at 28 C,

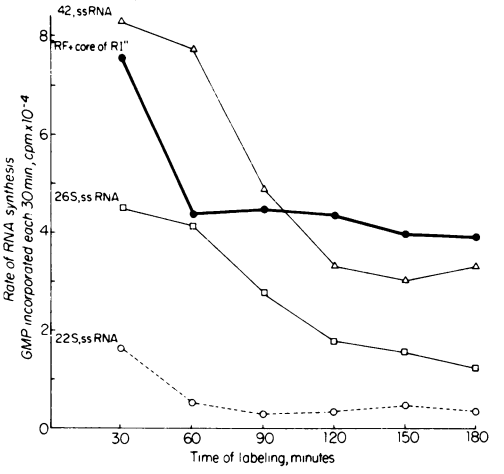


FIG. 6. *Rate of synthesis in vitro of the various SFV RNAs during successive 30-min intervals of incubation at 28 C. The pelleted, large particle fraction was obtained from BHK cells harvested at 4 h after infection. Equal samples of the large particle fraction as enzyme were incubated at 28 C in the standard assay containing unlabeled GTP. At the beginning of each 30-min interval, including that from 0 to 30 min, ^{32}P-GTP was added at a known specific activity to one, of the samples. After the 30-min labeling period, the sample was separated into its three subfractions as described in Fig. 1. Each was treated with the SDS-BRIJ-EDTA detergent mixture, and layered over 15 to 30% sucrose density gradients in TNE. Centrifugation and analysis for RNAs labeled with radioactivity and for optical density were as described in Materials and Methods. The individual RNA species were identified as described in the text.*

all the SFV-specific RNA-synthesizing enzyme with its template could be pelleted and was available in the P-250 $\times$ *g* for continued synthesis. The pelleted enzyme-template complex, when resuspended in new assay mixture, synthesized during any given time the same RNAs, both in amount and distribution, as did the enzyme which was not pelleted, but to which labeled precursor was added for the same period of time. After pelleting the enzyme-template complex, the pulse-label could be removed by aspiration and the pelleted material resuspended for further incubation in fresh assay mixture. Pulse-chase experiments were thus possible.

A. Pulse in standard assay, chase in standard assay. Figure 7A shows the pulse-labeled RNAs remaining with the pellet after the 30-min incubation period in standard assay in the presence of ^{32}P-GTP. The RNAs were 75% of the RF's and RI's and 20% of the single-stranded RNAs (similar to Fig. 1B and 1C). Figure 7B shows the fate of these molecules after a 30-min chase under standard conditions. There was no net incorporation of ^{32}P-GTP (the pulse-label) during the chase, and an approximately equal amount of the pulse-labeled RNAs present at the end of the pulse was recovered in RNAs at the end of the chase. The results show that, during the chase, about 50% of the labeled RNA in double-stranded RNAs disappeared coincident with an appearance of an approximately equal amount of pulse-labeled RNA as 42*S* single-stranded RNA (Fig. 7B). The chase of the pulse-labeled RNAs was essentially complete after 10 min. No further label could be chased even when the chase period was prolonged to 60 min. These results provide strong evidence that double-stranded RNA is the precursor of at least the 42*S* single-stranded RNA.

B. Pulse in standard assay containing 0.15 M NaCl, chase in standard assay. The double-stranded RNAs were also precursors to the 26 and 22*S* single-stranded RNAs. In the presence of salt, the enzymatic synthesis of the double-stranded RNAs was separated from the synthesis of single-stranded RNAs. When NaCl was added to the standard assay mixture to 0.15 M, the amount of newly synthesized double-stranded RNAs in the 30-min product of the large particle fraction was 50 to 60% of that synthesized by the same fraction in the absence of salt, whereas the amount of the single-stranded RNAs was less than 5% (Fig. 8). This small amount of single-stranded RNAs was synthesized during the first 10 min, and no increase occurred thereafter.

The marked inhibition of synthesis of single-stranded RNAs was not due to irreversible inactivation by 0.15 M NaCl of the SFV enzyme or separation of the enzyme from its template. When the large particle fraction was removed by centrifugation from the salt-containing mixture, all the virus-specific single- and double-stranded RNAs were synthesized in amounts similar to those synthesized by the enzyme which had never been exposed to 0.15 M NaCl. A pulse-chase experiment was thus possible and the results are shown in Fig. 9A and B. During the chase under standard conditions, 44% of the pulse-label in the double-stranded RNAs synthesized in the presence of 0.15 M NaCl disappeared coincident with the appearance of pulse label in RNAs, most of which were RNase sensitive (Fig. 9B). The RNase-sensitive RNAs in the product after the chase (Fig. 9B) were precipitated with 2 M LiCl, and the LiCl-insoluble RNAs were analyzed by PAGE. The pulse label in these RNAs had mobilities during PAGE which were coincident with those of 42, 26, and 22*S* virus-specific single-stranded RNAs. During the chase shown in Fig. 9B, there was a 30%

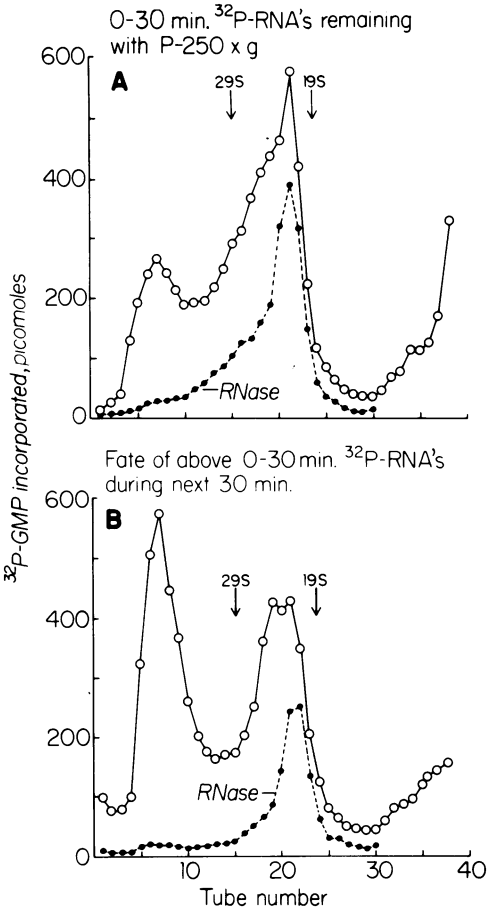


FIG. 7. *Conversion of double-stranded RNAs synthesized in vitro under standard conditions during a pulse into single-stranded RNAs during a chase. The pelleted, large particle fraction was obtained from BHK cells harvested at 4 h after infection. Two equal samples of the large particle fraction as enzyme were incubated at 28 C for 30 min in the standard assay mixture containing ^{32}P-GTP. After incubation, both samples were centrifuged for 10 min at 250 × g, and the supernatant fluid no. 2 containing the pulse label, ^{32}P-GTP, was removed by aspiration. The resulting pellet no. 2 of one sample was directly used to determine the RNAs newly synthesized during the pulse and remaining with pellet no. 2. For this, pellet no. 2 was resuspended in the Tween 40-DOC detergent mixture. Supernatant fluid no. 3 and pellet no. 3 were obtained as described in Fig. 1. Both were analyzed separately for virus-specific RNAs and are presented in the upper panel as the composite of the subfractions as described in Fig. 1. The pellet no. 2 of the second sample was used to determine the fate of the pulse-labeled RNAs during a subsequent chase. For this, pellet no. 2 was resuspended in fresh standard assay mixture containing unlabeled GTP and was incubated at 28 C for an additional 30 min. After the chase, the mixture was also separated into supernatant fluid no. 3 and pellet no. 3 by the Tween 40-DOC treatment. The subfractions were analyzed for virus-specific RNAs in an identical manner to that described above and are presented as the composite of the subfractions (lower panel).*

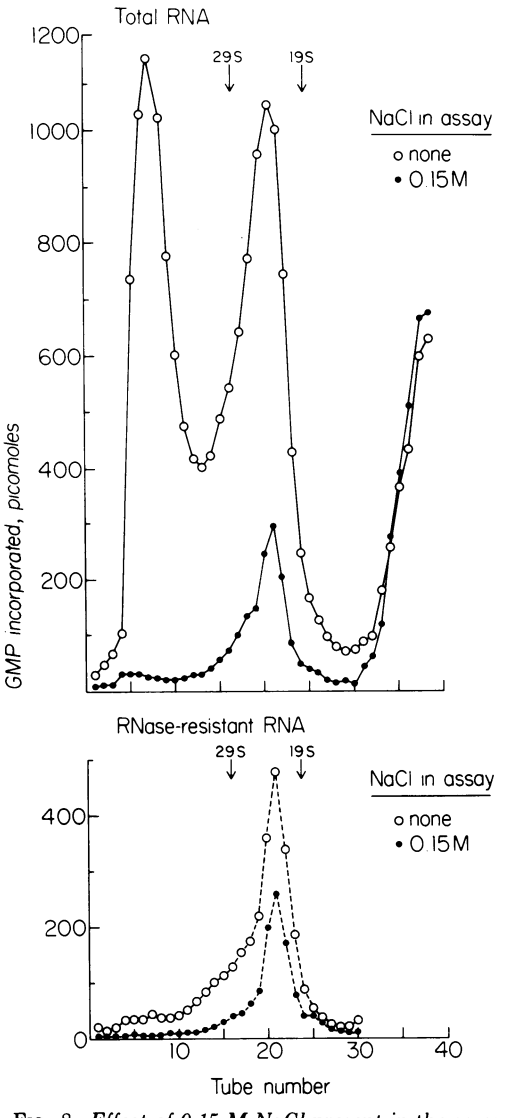


FIG. 8. *Effect of 0.15 M NaCl present in the assay mixture on the synthesis in vitro of SFV RNAs by the large particle fraction. The pelleted, large particle fraction was obtained from BHK cells harvested at 4 h after infection. Two equal samples were incubated in vitro at 28 C for 30 min, one in the standard assay mixture and the other in the standard assay mixture to which NaCl was added to 0.15 M final concentration. ^{32}P-GTP was the labeled precursor. The virus specific RNAs were analyzed by sucrose density gradient centrifugation and are presented as the composite of the subfractions as described in Fig. 1. RNase treatment was as described in Fig. 1.*

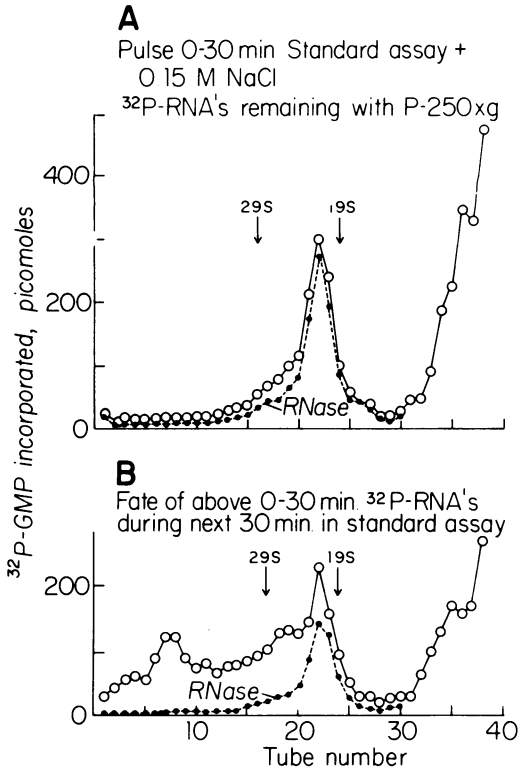


FIG. 9. *Conversion of SFV double-stranded RNAs synthesized in vitro in the presence of 0.15 M NaCl into single-stranded RNAs during a chase in the absence of 0.15 M NaCl. The pelleted, large particle fraction was obtained from BHK cells harvested at 4 h after infection. The experimental conditions and procedures were identical to those described in Fig. 7, save that, during the 30-min pulse, NaCl was present in the standard assay mixture at 0.15 M final concentration. As for Fig. 7, the chase was done in the presence of the standard assay mixture, containing unlabeled GTP and no added NaCl.*

increase of total radioactivity in RNAs due to continued incorporation of the labeled precursor which had been present during the pulse. This was due to an inability to separate well by centrifugation the large particles from the pulse label, since salt caused the large particle fraction to be fluffy and fragile. This increase in pulse-label in RNAs during the chase cannot account for the preponderance of pulse label in single-stranded RNAs after the chase.

The kinetic and the pulse-chase experiments indicate that there are at least two steps involved in the synthesis of SFV single-stranded RNAs: namely, (i) that the synthesis of double-stranded RNAs occurs first; and (ii) that the double-stranded RNAs are the precursors of all the single-stranded RNAs. The results of the pulse-chase experiment shown in Fig. 9A and B can be interpreted in two ways: (i) that the synthesis of single- and double-stranded SFV RNAs is catalyzed by different enzymes; or (ii) that only one round of "initiation" occurs, and termination and/or release of the newly synthesized RNAs is inhibited by 0.15 M NaCl.

The number of replicative forms. As the 42, 26, and 22*S* single-stranded RNAs represent discrete RNA species with possibly different functions, we undertook to define whether or not there was more than one replicative form involved in their synthesis. We analyzed the RNase-resistant RNAs synthesized in vivo during infection and in vitro by the enzyme-template complex. The results were identical. We present here only those obtained with the in vitro-synthesized RNAs.

The in vitro-synthesized double-stranded RNAs to be ultimately analyzed by PAGE, polyacrylamide gel electrophoresis (Fig. 10 and 12), were initially divided into three fractions by sucrose density gradient centrifugation (inset of Fig. 10): (i) RNAs sedimenting faster than 22*S* relative to ribosomal RNAs (fraction A); (ii) RNAs sedimenting at 22*S* (fraction B); and (iii) RNAs sedimenting from 14 to 22*S* (fraction C). The RNAs in the three fractions were further separated by treatment with 2 M LiCl, yielding the LiCl-soluble RF and the LiCl-insoluble RI precipitated along with single-stranded RNAs.

The LiCl-soluble RF could be directly analyzed by PAGE, but little of the RI with its attached nascent chains entered the gels under our conditions, whereas the RNase-resistant core of the RI did enter. Treatment of the LiCl-insoluble RI from fractions A and B with 0.1 μg of pancreatic RNase per ml for 30 min at 37 C yielded only one RNase-resistant core of the RI which had the same sedimentation velocity as the bulk of the RF (18*S* relative to reovirus double-stranded RNAs), or a marginally greater one. There was no RI in the LiCl-insoluble pellet of fraction C. Treatment of the same LiCl-insoluble RI from fractions A and B with 3 μg of pancreatic RNase per ml for 30 min at 37 C resulted in fragmentation of the core, yielding double-stranded RNAs sedimenting heterogeneously from 8 to 18*S*. Before PAGE, the RNase-resistant cores of the RI were repurified through 15 to 30% sucrose density gradients. During PAGE, reovirus double-stranded RNAs served as markers, and as expected the logarithm of the molecular weights of the segments of reovirus RNA varied linearly with their respective relative mobilities (Fig. 11) (26, 31).

Figure 10 shows the relative mobilities of the LiCl-insoluble RNAs in fractions A, B, and C of the inset. The major form of the SFV RF had a

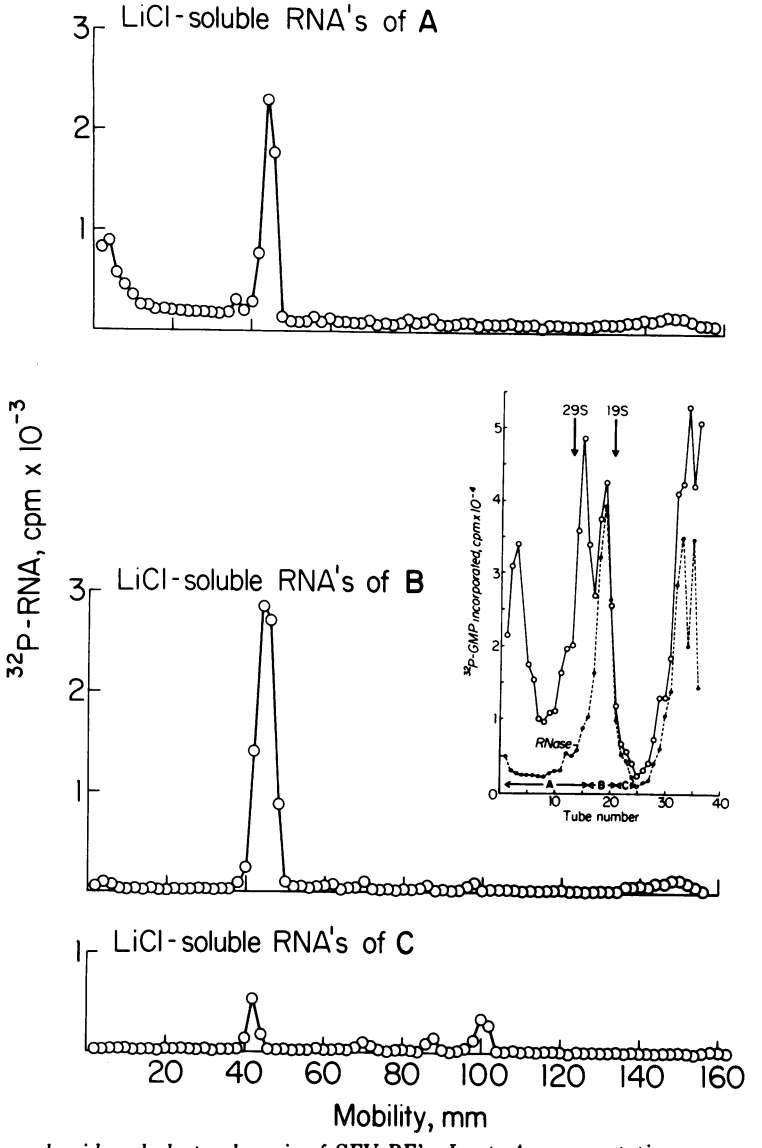


FIG. 10. *Polyacrylamide gel electrophoresis of SFV RF's. Inset: A representative sucrose density gradient centrifugation of SFV RNAs synthesized in vitro by the pelleted, large particle fraction at 28 C for 30 min in a standard assay mixture containing ^{32}P-GTP. After incubation, the mixture was separated into its three subfractions, treated with the SDS-BRIJ-EDTA detergent mixture, centrifuged, and analyzed for virus-specific RNAs as described in Fig. 1. The material in fractions A, B, and C of the inset was pooled separately and was divided into RNA soluble or insoluble in 2 M LiCl (for the latter, see Fig. 12). The procedures for obtaining the LiCl-soluble RNA, for suspending the RF's in electrophoresis buffer, and conditions of electrophoresis were as described in Materials and Methods. The time of electrophoresis was 44 h.*

mobility of 45 mm which, relative to the mobilities of reovirus RNAs, corresponded to a molecular weight of 4.4 $\times$ 10^6 (Fig. 11). In the electropherogram of fraction A (Fig. 10), there was a second peak of radioactivity at 36 mm. This peak is minimal in this experiment, but has been more prominent in others. As determined in several experiments, the average molecular weight of the RF at 36 mm was 5 $\times$ 10^6. No specific size class of RF's larger than 5 $\times$ 10^6 was found. Most of the RNAs in fraction A with mobilities less than 36 mm were RI's contaminating the LiCl-soluble RF's and could be made to enter the gels if the LiCl-soluble RNAs in fraction A were treated before electrophoresis with ribonuclease at 0.1 μg/ml. Fraction C contained, in addition to the major form of the SFV RF, minor species with mobilities corre-

sponding to molecular weights of 2.2×10^6, 2.5×10^6, and 3.3×10^6.

The mobility during PAGE of the core of the RI resistant to ribonuclease, 0.1 μg/ml, has been consistently greater than the major SFV RF (Fig. 12). As determined in several experiments, the average molecular weight of the core of the RI was 5.6×10^6. Its exact molecular weight, however, remains to be determined, as treatment of the RI with 3 μg of pancreatic RNase per ml to insure a complete hydrolysis of single-stranded portions of the RI resulted in its fragmentation (Fig. 12).

If the molecular weight of the 42S RNA is 4×10^6 (4, 5, 33), a double-stranded RNA derived from it would be expected to have double that molecular weight. It should be noted that significant amounts of RF's with molecular weights greater than 5×10^6 were not found in our SFV, in vitro-synthesized, 30-min product. As stated above, these results are identical to those obtained when extracts from infected BHK cells were analyzed for double-stranded SFV RNAs labeled with ^{3}H-uridine during infection.

DISCUSSION

In the study reported here, 75 to 90% of the SFV RNAs synthesizing enzyme with its associated template was found in the large particle fraction from infected BHK cells. The small amount of enzyme which did not sediment into the large particle fraction could be pelleted in the ultracentrifuge and had properties identical to the enzyme in the large particle fraction. The in vitro-synthesized products of the SFV enzyme were the species produced in vivo during infection of BHK cells, namely, the RF's and RI, and the single-stranded RNAs sedimenting at 42, 26, and 22S (18). None of the in vitro-synthesized RNAs was found free, but each was associated with large structures of different size. The 26S single-stranded RNA was in a structure which sedimented from 60 to 90S; the 42 and 22S single-stranded RNAs were in a structure(s) sedimenting at greater than 250S, but not large enough to sediment to the pellet at $250 \times g$ in 10 min; whereas most of the double-stranded RNAs sedimented in 10 min at $250 \times g$ to the pellet.

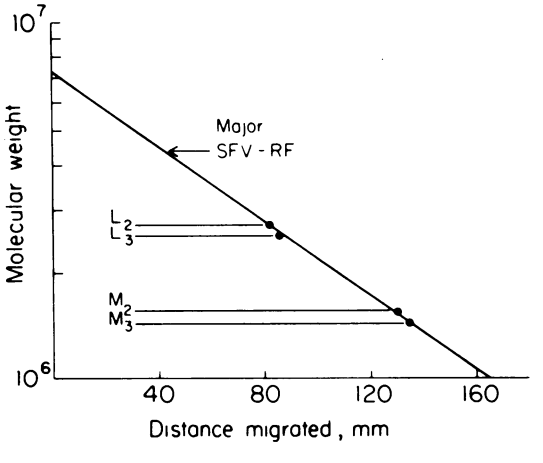


FIG. 11. *Relationship between electrophoretic mobility and molecular weight of L_2, L_3, M_2, and M_3 double-stranded RNA segments of reovirus and the major SFV RF. The electrophoretic run was that for Fig. 10 during which reovirus RNA labeled with ^{3}H-uridine was applied to two separate gels.*

The RF's and RI were the first species to be sythesized in vitro, and their synthesis preceded that of the single-stranded RNAs by about 5 min. Strong evidence that the RF's and RI were the precursors of the single-stranded RNAs was found in the pulse-chase experiments. Two features deserve comment. When the enzyme-template complex could be cleanly separated by centrifugation from the pulse-label, the amount of pulse-labeled RNAs in RF's and RI remaining with the enzyme-template complex decreased during the subsequent chase in an amount equivalent to that appearing in single-stranded RNAs. Secondly, regardless of whether the chase period was 10 min or 60 min, never more than 50% of the pulse-label in RF's could be chased into single-stranded RNAs. It is possible that the pulse label in the double-stranded RNAs which cannot be chased represents label in minus strand RNA (that strand complementary to viral RNA) which had been newly synthesized in vitro during the pulse. Whether both minus and plus strand RNAs of the duplex are synthesized in vitro can be determined experimentally.

Simmons and Strauss have shown recently that the 26S RNA contains only one-third of the base sequences in viral RNA (33). The kinetics of appearance of the single-stranded RNAs shown in this paper also negates the suggestion that the 26S RNA is the precursor to all of the 42S RNA, and thus that both are one RNA which exists in two different configurations (37). In the in vitro reaction described here, the 42S RNA accumulated at a faster rate than did the 26S RNA. In addition, each of these RNAs was associated with a specific sized structure. After synthesis, the 26S single-stranded RNA was released in vitro from the enzyme-template complex in a form such that it sedimented between 60 to 90S. This structure closely resembles in size the 65S particle from the infected cell with which 26S RNA is found (8, 11, 19, 39). If the in vivo 65S particle and the in vitro-synthesized 60 to 90S particle are the same, the enzyme-template complex described here con-

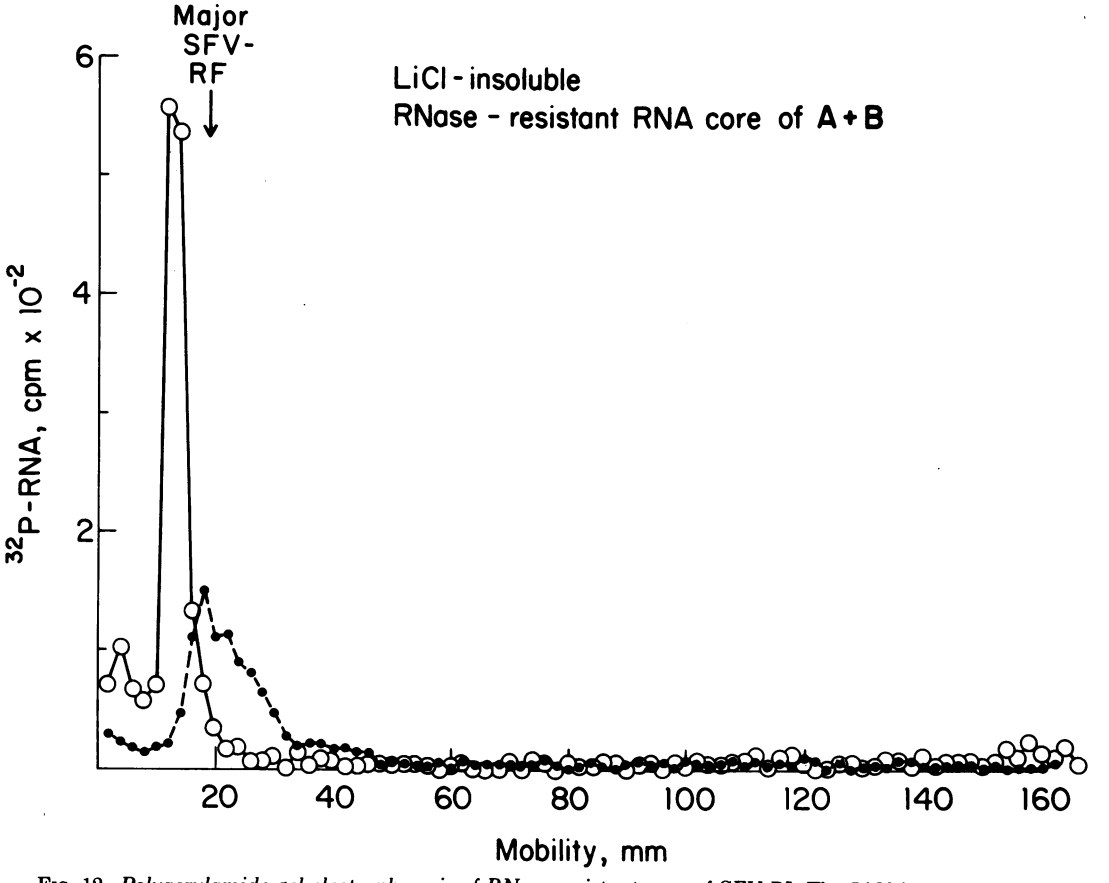


FIG. 12. *Polyacrylamide gel electrophoresis of RNase-resistant core of SFV RI. The LiCl-insoluble RNAs in fractions A, B, and C of the experiment shown in Fig. 10 were pooled, purified, and concentrated. After lyophilization, the LiCl-insoluble RNAs were resuspended in 1 × SSC and divided into two equal samples for treatment with pancreatic RNase for 30 min at 37 C at a concentration of 0.1 or 3 μg/ml. After enzyme treatment, both samples were analyzed by sucrose density gradient centrifugation. The RNase-resistant viral RNA of each was identified, pooled, precipitated with ethanol, resuspended in 1/10 × SSC, chromatographed through Sephadex G-50, lyophilized, and resuspended in electrophoresis buffer. Conditions for electrophoresis were as described in Materials and Methods. The time of electrophoresis was 24 h. The major forms of SFV RF and reovirus double-stranded RNAs were run in separate gels as markers. Symbols: (○), RI treated with 0.1 μg of RNase per ml; (●), RI treated with 3 μg of RNase per ml.*

tains the components necessary for coupling the in vitro-synthesized virus-specific 26S RNA with a preexisting viral protein(s) to form a defined structure.

Greater than 95% of the RNase-resistant RNAs, whether synthesized in vitro or obtained from infected BHK cells, were perfect duplexes as judged by their solubility in 2 M LiCl and by their unchanged *S* value (18S relative to reovirus RNAs) or mobility in PAGE when they were pretreated with RNase. The RF's were at least two, with molecular weights of 4.4×10^6 and 5×10^6. No RF's belonging to a larger size class were found, and thus these results are different from those described recently for Sindbis virus-infected cells (34). Preliminary results in our laboratory corroborate that there is no RF detectable in our in vitro product which consists of a duplex of 42S plus and minus viral RNA strands. Denaturation at 90 C, in the presence of 70% formamide of the LiCl-soluble RNAs in fractions similar to the A, B, and C of Fig. 10, yielded no 42S viral RNA, but only single-stranded RNAs sedimenting at about 26 and 22S.

Less than 5% of the RNase-resistant RNAs was present in an RI which was insoluble in 2 M LiCl and remained at the origin under our conditions of PAGE. The core of the RI resistant to 0.1 μg of pancreatic RNase per ml had a molecular weight of 5.6×10^6.

The RF's found in this study did not derive

from RI's which were originally multistranded and from which nascent strands had been stripped during their preparation. The extraction and analytical procedures used have been shown to preserve such multistranded RI's (2, 15). Since as much as 50% of the pulse-label in the RF's was chased into single-stranded RNAs, these RF's are not dead end products. It would appear that during synthesis of single-stranded RNAs, the RF's actually existed as RI's, but with only one nascent RNA chain held in place at either end of the parental duplex by a short region of hydrogen bonding with its complementary RNA. Our analysis of the double-stranded RNA and the product for which it was template was thus at a stage close to completion of the nascent plus RNA strand or shortly after initiation of its synthesis. If such were the case, the tenuous association of the RF's with the plus RNA strand being displaced, could have been disrupted during our analysis.

Since the label in the RF's and RI could be chased into the single-stranded RNA species, one or both of these RF's or the RI was the precursor to the 42, 26, or 22*S* single-stranded RNAs. Either of the two RF's or even the core of the RI is too small to be alone the total precursor to the 42*S* single-stranded RNA which is estimated to have a molecular weight of 4×10^6 (4, 5, 33). The 42*S* RNA must thus be derived from more than one RNA chain. Unless the genetic information in the 42*S* viral RNA is redundant, the RNA chains of the 42*S* RNA could be derived from different RF's or RI and the individual RNA segments joined together by a ligase to form one continuous polynucleotide chain (32). If such is the case, since 42*S* viral RNA was found in our in vitro product, it is implicit that such a ligase was present in our crude enzyme preparation.

If, as is reported, the 42*S* single-stranded RNA is a single continuous polynucleotide chain (1, 33), a basic question in the replication of SFV RNAs is: how does more than one RF appear during infection? Since 42*S* SFV RNA is infectious, the initial synthesis of viral proteins, specifically including the SFV RNA polymerase(s), must be directed by the parental inoculum RNA. One possibility is that, before translation, the 42*S* parental RNA dissociates into smaller segments. Shortly after infection, the inoculum RNA has been found to sediment at 20 to 26*S*, rather than at 42*S* (12, 13). These results were interpreted as conversion of parental RNA into RI or RF (27), but certainly could just as well be interpreted as separation of the parental RNA into subunits of different size, each of which would be the template for the synthesis of its complementary RNA in the formation of multiple RF's.

Alternatively, if the 42*S* RNA does remain intact after infection, the early events in RNA synthesis would be similar to those described for Sendai virus (28) or vesicular stomatitis virus (3, 17, 30). There would be more than one initiation site on the 42*S* RNA for the synthesis of more than one single-stranded RNA, each complementary to a different segment of the parental genome. The newly synthesized minus RNA strands would serve almost immediately as templates for plus RNA strand synthesis, as free minus RNA strands have never been found in arbovirus-infected cells (10, 18). Once the RF's were synthesized, they would serve as templates for subsequent synthesis of plus RNA strands.

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