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Replication of Semliki Forest Virus: Polyadenylate in Plus-Strand RNA and Polyuridylate in Minus-Strand RNA

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The 42S RNA from Semliki Forest virus contains a polyadenylate [poly(A)] sequence that is 80 to 90 residues long and is the 3'-terminus of the virion RNA. A poly(A) sequence of the same length was found in the plus strand of the replicative forms (RFs) and replicative intermediates (RIs) isolated 2 h after infection. In addition, both RFs and RIs contained a polyuridylate [poly(U)] sequence. No poly(U) was found in virion RNA, and thus the poly(U) sequence is in minus-strand RNA. The poly(U) from RFs was on the average 60 residues long, whereas that isolated from the RIs was 80 residues long. Poly(U) sequences isolated from RFs and RIs by digestion with RNase T1 contained 5'-phosphorylated pUp and ppUp residues, indicating that the poly(U) sequence was the 5'-terminus of the minus-strand RNA. The poly(U) sequence in RFs or RIs was free to bind to poly(A)-Sepharose only after denaturation of the RNAs, indicating that the poly(U) was hydrogen bonded to the poly(A) at the 3'-terminus of the plus-strand RNA in these molecules. When treated with 0.02 μg of RNase A per ml, both RFs and RIs yielded the same distribution of the three cores, RFI, RFII, and RFIII. The minus-strand RNA of both RFI and RFIII contained a poly(U) sequence. That from RFII did not. It is known that RFI is the double-stranded form of the 42S plus-strand RNA and that RFIII is the experimentally derived double-stranded form of 26S mRNA. The poly(A) sequences in each are most likely transcribed directly from the poly(U) at the 5'-end of the 42S minus-strand RNA. The 26S mRNA thus represents the nucleotide sequence in that one-third of the 42S plus-strand RNA that includes its 3'-terminus.

The genome RNA of the alphaviruses Semliki Forest virus (SFV) and Sindbis virus sediments at 42S and 49S, respectively (9, 17, 37, 41). The RNA from both viruses is infectious and functions as RNA early during infection (17). The virion plus-strand RNA of both viruses contains a polyadenylate [poly(A)] sequence reported to range in length from 50 to 100 nucleotides (6, 14, 15, 20), but some sequences were found as long as 150 to 250 nucleotides (15). The length of the poly(A) in the individual RNAs was not critical to infection since those RNAs that contained a poly(A) sequence of 50 or 150 nucleotides were equally infectious (15). A poly(A) sequence of some length, however, was necessary for infectivity. About 10% of the Sindbis virion plus-strand RNAs had no detectable poly(A) sequence, and these RNAs were 10% as infectious as the RNAs that contained a poly(A) segment (T. K. Frey and J. H. Strauss, Abstr. ICN-UCLA Winter Conf. Mol. Cell. Biol. 1976, p. 47). Previously, it was reported that the poly(A) sequence in Sindbis virion RNA was near the 3'-terminus of the virion RNA (3, 18), but it was not the 3'-terminal sequence (10). The recent experiments of Frey and Strauss cited above have, however, established that the poly(A) is at the 3'-terminus of the Sindbis virion RNA.

Two major species of single-stranded RNAs are found in alphavirus-infected cells, the genome RNA and an RNA sedimenting at 26S (17, 21, 22, 25, 29, 32, 34, 37, 39, 41). The 26S RNA contains approximately one-third of the total nucleotide sequence of the genome RNA (37), and it is the mRNA for all the viral structural proteins (4, 7, 23, 40, 46). A poly(A) sequence of 60 to 70 nucleotides was found in the 26S mRNA (6, 14). In addition, the SFV replicative forms (RFs) and replicative intermediates (RIs) also contained a poly(A) sequence in their plus-strand RNA (3, 6).

The synthesis of poly(A) in viral RNAs can occur by one of two mechanisms: by the addition of adenylate residues without any requirement for template, or by the direct transcription of a complementary poly(U) sequence in the template strand. The poly(A) in mRNA's of negative-strand viruses is added post-transcriptionally, since no poly(U) sequence has been

detected in the genome RNAs (28). The poly(A) sequence in poliovirus RNA appears to be directly transcribed from a poly(U) sequence located at the 5'-terminus of the minus-strand RNA (44, 47, 48). Support for the direct transcription of the poly(U) in minus-strand RNA into the poly(A) of the plus-strand poliovirus RNA came from in vitro experiments that utilized poliovirus replication complexes shown to be free of detectable terminal adenylate transferase activity (11).

The experiments described in the present paper were designed to determine whether the poly(A) sequence in the plus-strand alphavirus RNAs was added after transcription in the absence of template or whether, as in the case for the poliovirus poly(A), the poly(A) in SFV plus-strand RNAs was also directly transcribed from a poly(U) in the minus-strand RNA. We report that the latter mechanism is the likely mode of synthesis of the poly(A) in SFV plus-strand RNAs. We have examined the Semliki Forest virion RNA and the RFs and RIs for size of their poly(A) and have verified that the poly(A) sequence is the 3'-terminal sequence of the virion RNA. We show that a poly(U) sequence at the 5'-end of the minus strand of the RFs and RIs is long enough to code for most or all of the poly(A) sequence in the plus-strand RNAs.

MATERIALS AND METHODS

Cell culture. BHK-21 cells, clone 13, a continuous cell line derived from baby hamster kidney cells, were obtained from Michael Stoker and were grown in plastic roller bottles (Corning Glass Works, Corning, N.Y.) at 37°C in Eagle minimal essential medium (MEM) (13) supplemented with 5% fetal bovine serum.

Virus. SFV, prototype strain obtained from J. R. Henderson, Yale University School of Medicine, was inoculated into the brains of newborn Swiss mice obtained from The Rockefeller University. When tremors developed, 12 to 24 h later, the animals were sacrificed and the brains were harvested. A 20% suspension in phosphate-buffered saline (PBS) (12) containing 0.5% bovine plasma albumin (BPA) was prepared by grinding the brains with carborundum in a sterile mortar. The suspension was cleared of carborundum by centrifugation at 250 × *g*. The supernatant fluid, which was used as starting material for preparation of virus stock, was stored in small aliquots at −90°C. For stock virus, BHK cells were infected at a multiplicity of infection (MOI) of less than 0.1 PFU/cell. Growth of virus and determination of its infectivity by plaque assay were as described (21). The harvest from the second passage of virus in BHK cells was the stock virus used in all the experiments described in this paper.

Preparation of SFV 42S virion RNA. Monolayers of BHK cells were washed thoroughly with MEM, and 25 ml of fresh MEM containing 0.2% BPA, 0.15 μg of actinomycin D (AMD) per ml, and SFV at 0.01 to 0.1 PFU/cell was added to each bottle. After 1 h at 37°C, the inoculum was removed and replaced with 25 ml of fresh MEM containing 0.2% BPA and AMD at 0.15 μg/ml. For the preparation of ^{3}H-labeled virion RNA, [^{3}H]adenosine was added to cultures after the adsorption period to a final concentration of 40 μCi/ml. For the preparation of virion RNA labeled with [^{32}P]orthophosphate, the cultures were washed thoroughly with phosphate-free MEM containing 0.2% BPA and AMD at 0.15 μg/ml. After the 1-h adsorption period, the inoculum was removed and replaced with 25 ml of phosphate-free MEM containing 0.2% BPA, AMD at 0.15 μg/ml, and [^{32}P]orthophosphate at 100 to 400 μCi/ml.

All infected cultures were then incubated for 16 h at 37°C or until a complete cytopathic effect was observed. The medium was removed and centrifuged at 250 × *g* for 15 min to remove large particulate matter. The supernatant fluid was then centrifuged at 100,000 × *g* for 1 h at 4°C in the SW27 rotor. The pelleted material containing virus was resuspended in PBS containing 0.5% BPA; it was layered on a potassium tartrate gradient, 5 to 50% in PBS; and it was centrifuged in the SW27 rotor at 103,000 × *g* for 3 h at 4°C. The visible SFV band at a density of 1.19 g/ml was collected. After dilution with PBS containing 0.5% BPA, the SFV was collected into the pellet by centrifugation in the SW27 rotor at 100,000 × *g* for 1 h at 4°C. The virus was resuspended in PBS, to which was added sodium dodecyl sulfate (SDS) at a final concentration of 1% for disruption of virus. The suspension was layered onto a sucrose gradient, 15 to 30% in 0.2% SDS buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride, pH 7.5, 0.001 M EDTA, and 0.2% SDS), for centrifugation in the SW27 rotor at 87,000 × *g* for 14.5 h at 20°C. Fractions of 1 ml were collected from below, and each was assayed for absorbancy at 260 nm in the Zeiss spectrophotometer or for radioactivity. The fractions containing the SFV 42S RNA were pooled, 1/10 the volume of a solution containing 3 M sodium acetate and 20 mM EDTA, pH 5.5, was added, and the RNA was precipitated overnight at −20°C after the addition of 2 volumes of ethanol.

Preparation of cellular extracts. After washing with MEM, BHK monolayers were overlaid with 25 ml of MEM containing 0.2% BPA, 0.15 μg of AMD, and SFV at a concentration necessary to obtain an MOI of 100. If the time of labeling with radioactive precursors began at 0 h, the adsorption medium was not removed until harvest. If the time of labeling started later than the 1 h for adsorption, the adsorption medium was removed and replaced with MEM containing 0.2% BPA and 0.15 μg of AMD per ml. For the preparation of SFV RNAs labeled with [5,6-^{3}H]uridine or [2,8-^{3}H]adenosine during the first 2 h of infection, the monolayers were exposed to 0.15 μg of AMD per ml for 30 min before addition of SFV and radioactive precursor at a final concentration in the medium of 40 μCi/ml. For the preparation of ^{32}P-labeled SFV RNAs, BHK cells were prelabeled before infection. Where indicated, AMD at 0.15 μg/ml was added at 210 min before infection. At 180 min before infection, the medium was replaced with 25

ml of phosphate-free MEM containing 0.2% BPA, AMD at 0.15 μg/ml, and [^{32}P]orthophosphate at 250 to 500 μCi/ml. At 0 h, SFV, at a concentration to yield an MOI of 100, was added in a small volume directly to this labeled medium.

At the times indicated, the medium was removed, and the cells were washed with ice-cold PBS-PVS (PBS containing polyvinyl sulfate at 20 μg/ml) and with RSB-PM (0.01 M Tris-hydrochloride, pH 7.4, 0.01 M KCl, 0.0015 M $MgCl_2$, 0.001 M 2-mercaptoethanol, and PVS at 20 μg/ml). The cells were scraped into RSB-PM and, after swelling, they were disrupted with 25 strokes of a tight-fitting Dounce homogenizer. The homogenate was centrifuged at 250 × *g* for 10 min. The pelleted material was resuspended in 0.8% Tween 40 and 0.4% sodium deoxycholate in RSB-PM, and the suspension was recentrifuged. Both supernatant fluids were combined and adjusted to 5 mM EDTA and 0.5% SDS. The cytoplasmic fraction was extracted with phenol-chloroform-isoamyl alcohol (50:48:2), followed by two extractions with chloroform-isoamyl alcohol (96:4). The aqueous phase was adjusted to 0.3 M sodium acetate by the addition of 1/10 the volume of a solution containing 3 M sodium acetate and 20 mM EDTA, pH 5.5, and the total RNA in the cytoplasmic extract was precipitated by the addition of 2 volumes of ethanol. The precipitated RNAs were dissolved in 0.1 M sodium acetate, pH 5.5, containing 0.02 M EDTA for LiCl treatment.

Purification of the RFs and RIs. Treatment of the total cytoplasmic RNAs with 2 M LiCl for 16 to 18 h at 0°C was used to separate the SFV RFs from the RIs (1). After centrifugation in the SW41 rotor at 2°C for 1 h at 39,000 × *g*, the pelleted LiCl-insoluble RNA containing the RIs was processed directly, whereas the LiCl-soluble RNA containing the RFs was precipitated first from the supernatant fraction with 2 volumes of ethanol. The LiCl-insoluble and -soluble RNAs were dissolved in 1 ml of digestion buffer (0.06 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4, and 7 mM $MgCl_2$). DNase, RNase-free, was added at a final concentration of 20 μg/ml, and the mixture was incubated at 37°C for 5 min. The incubation was interrupted by the addition of EDTA and SDS to 2 mM and 0.5%, respectively. The RNA was then chromatographed at room temperature through a column (70 by 1.5 cm) of Sepharose 2B in 0.5% SDS buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride, pH 7.4, 0.02 M EDTA, 0.5% SDS) as previously described (1, 47). Fractions of 0.5 ml were collected, and the distribution of RNA in each was determined by counting an aliquot in a toluene-based scintillation fluid containing Triton X-100. The LiCl-soluble RFs and LiCl-insoluble RIs eluted in the void volume and were collected by precipitation with 2 volumes of ethanol at −20°C for 18 h.

Poliovirus RF was purified from HeLa cells at 7 h after infection exactly as described for SFV RFs. The infected cells were a generous gift of Raphael Fernandez-Munoz.

Isolation of poly(A) sequences. Affinity chromatography on poly(U)-Sepharose was used to isolate poly(A) sequences free of other RNA fragments. The poly(U)-Sepharose was prepared by coupling poly(U) to cyanogen bromide-activated Sepharose 4B (27). After the coupling reaction, the Sepharose was treated for 12 h at 4°C with 0.1 M glycine in 0.05 M potassium phosphate, pH 8.0, to block unreacted groups. It was then washed extensively with 0.05 M potassium phosphate, pH 8.0, and then with sterile water. The poly(U)-Sepharose was suspended in 0.1 M NaCl, 0.01 M EDTA, 0.01 M Tris-hydrochloride, pH 7.4. It can be stored for several months at 4°C. Columns (4 cm) of poly(U)-Sepharose were used for chromatography at room temperature. Before use, the columns were treated in the following way: (i) they were extensively washed with 0.2 NETS (0.2 M NaCl, 0.01 M EDTA, 0.01 M Tris-hydrochloride, pH 7.4, and 0.2% SDS); (ii) 1 mg of yeast RNA in 1 ml of 0.2 NETS was applied, followed by 5 to 10 ml of 90% formamide in ETS (NETS with no NaCl); and (iii) they were thoroughly washed with 0.2 NETS. Essentially all of an applied sample of commercially obtained, radiolabeled poly(A) bound to the poly(U)-Sepharose. Thus, the selection of poly(A) sequences on poly(U)-Sepharose was 95 to 100% efficient.

Virion RNA, RFs, or RIs in 2 ml of 0.001 M EDTA were denatured by heating at 100°C for 2 min; the solutions were quickly cooled and adjusted to final concentrations of 0.2 M NaCl, 0.01 M EDTA, and 0.01 M Tris-hydrochloride, pH 7.4. The RNAs were digested for 30 min at 37°C with RNase T1 at 50 U/ml and RNase A at 2.5 μg/ml. After digestion, the RNA was extracted three times with an equal volume of phenol-chloroform-isoamyl alcohol (50:48:2) before chromatography. Alternatively, digestion was at 37°C for 30 min with RNase T1 alone, at 50 U/ml, in 0.02 M Tris-hydrochloride, pH 7.4, 0.001 M EDTA, and yeast RNA at 50 μg/ml. This yielded poly(A) sequences of the same purity as digestion by both of the above enzymes, and phenol extraction before chromatography was not necessary.

After digestion, the sample was heated at 60°C for a few seconds; the necessary components were added to achieve the respective concentrations in 0.2 NETS; and the sample was applied to the poly(U)-Sepharose. The poly(U)-Sepharose then was washed extensively with 0.2 NETS, followed by a wash with 5 to 10 ml of ETS. The bound RNAs either were (i) step-eluted by first washing the column with 5 to 10 ml of 10% formamide in ETS and then eluting the remaining bound RNA with 90% formamide in ETS or, alternatively, with 0.5 ml of 90% formamide in ETS followed by 50% formamide in ETS; or (ii) after the ETS wash, eluted with an exponential gradient of formamide, from 0 to 90% in ETS (31).

The ^{32}P-labeled RNAs in each fraction were determined directly by monitoring radioactivity by Cerenkov radiation, and ^{3}H-labeled RNAs were identified by determining the radioactivity in an aliquot of each fraction dissolved in a toluene-based scintillation fluid containing Triton X-100. The RNAs eluting in 90% formamide or eluting as a peak of RNA in the 0 to 90% formamide gradient were diluted twofold with ETS, LiCl was added to a final concentration of 0.2 M, and the RNAs were precipitated in the presence of 100 μg of yeast RNA as carrier by the addition of 2 volumes of ethanol.

Isolation of poly(U) sequences. The column material was poly(A)-Sepharose 4B, which was the generous gift of S. L. Marcus (27). The washing

procedure and conditions used during chromatography, including washing, elution, and analysis, were identical to those used above for chromatography through poly(U)-Sepharose. Before application of the sample to the poly(A)-Sepharose column, the SFV RFs and RIs (0.1 to 1 μg of RNA), dissolved in 2 ml of 0.001 M EDTA containing 1 to 2 μg of poly(U), were denatured by heating for 2 min at 100°C. After quickly cooling, Tris-hydrochloride, pH 7.4, and yeast RNA were added to final concentrations of 0.02 M and 50 μg/ml, respectively. Digestion with RNase T1 at 50 U/ml was at 37°C for 30 min. The sample was adjusted to 0.2 NETS before application to the poly(A)-Sepharose column.

Determination of base composition and end-group analysis. The RNAs were dissolved in 0.3 M KOH and hydrolyzed for 18 h at 37°C. Potassium ions were removed by chromatography on CM-82 paper (19), and the eluate, to which carrier mononucleotides were added, was spotted on Whatman 3MM paper and subjected to electrophoresis for 4.5 h at 68 V/cm. The buffers used during electrophoresis were 20% acetic acid adjusted to pH 3.5 with concentrated ammonium hydroxide, or 5% acetic acid, 0.5% pyridine, 5 mM EDTA, pH 3.5. Digestion with RNase T2 at 10 U/ml was at 37°C for 18 h in 0.05 M ammonium acetate, pH 4.5, and 0.005 M EDTA.

For the identification of 5'-phosphorylated residues in poly(U) segments from purified RFs or RIs, digestion with RNase T2 at 10 U/ml and RNase A at 20 μg/ml was for 1 h at 37°C in 0.05 M ammonium acetate, pH 4.5, and 0.005 M EDTA. The ammonium acetate in the digest was removed by repeated suspension in water followed by lyophilization. The digest was applied to DEAE-81 paper, and electrophoresis was for 4 h at 3,000 V in 5% acetic acid and 0.005 M EDTA adjusted to pH 3.5 with pyridine. Purified ^{32}P-labeled pUp residues (the generous gift of Raphael Fernandez-Munoz; 16) and [^{3}H]UTP were used as markers. The paper was cut into 1-cm strips, 0.5 ml of NEN solubilizer, Protosol, was added, and the radioactivity in each was determined in a toluene-based scintillation fluid. Where indicated, the material migrating to the positions of pUp and ppUp residues and remaining at the origin was eluted with 30% triethylaminebicarbonate, pH 8.5. The eluted material was digested for 1 h at 37°C with alkaline phosphatase at 20 U/ml in 0.02 M Tris-hydrochloride, pH 8.0. After extraction with phenol-chloroform-isoamyl alcohol (50:48:2), the digest was lyophilized, resuspended in a small volume of water, and electrophoresed on Whatman 3MM paper for 3 h at 5,000 V in the same buffer system used for the electrophoresis on DEAE-81 paper. The paper was cut into 1-cm strips, and the radioactivity in each was determined as described above. Under these conditions of hydrolysis with alkaline phosphatase, [^{3}H]UTP was completely converted to uridine.

Polyacrylamide gel electrophoresis. Gels (0.7 by 10 cm) containing 15% acrylamide, 8 M urea, 10% glycerol, and 0.075% bisacrylamide in Peacock-Dingman gel buffer (0.09 M Tris-hydrochloride, pH 8.3, 0.025 M EDTA, 0.09 M boric acid; 33) were prepared and were pre-run in gel buffer containing 0.2% SDS and 10% glycerol for 30 to 60 min at 150 V. The RNA samples were dissolved in 50 to 100 μl of gel buffer containing 8 M urea, 20% glycerol, 0.4% bromophenol blue, and 0.2% SDS. Electrophoresis was at 3 to 4 mA/gel for 4 h or until the blue dye was at the bottom of the gel. The gels were cut into 2-mm slices; the slices were dissolved at 60°C for 2 h in 0.5 ml of Protosol; and the radioactivity in each was determined in a Packard scintillation spectrometer after addition of a toluene-based scintillation fluid.

Determination of the distribution of homopolymer lengths. The amount of radioactivity in homogeneously labeled homopolymers of different size varies directly with the length of the polymer. If a distribution of molecules of different sizes is to be obtained, a correction must be applied for their length. In acrylamide gels containing 8 M urea, the mobility of poly(A) and heteropolymeric 4*S* and 5*S* cellular RNAs is linear and varies inversely with the log of the molecular weight (S. Sawicki, personal communication). This result was obtained by elution from various regions of the gel of poly(A) sequences, randomly labeled with [^{3}H]adenosine, followed by determination of the length of these poly(A) sequences from the ratio of radioactivity in AMP versus adenosine. Poly(U) sequences of different lengths fall on the same straight line. To obtain the relative amounts of homopolymers of a particular size, the radioactivity (counts per minute) in each 2-mm gel slice was divided first by the average length of the polymers in that slice. Because the mobility during electrophoresis of segments of different sizes varies exponentially with changes in molecular weight, it is necessary to correct also for the range of lengths in each gel slice. The number so obtained for each gel slice is expressed in the text as a percentage of these numbers in the whole gel.

Materials. AMD was purchased from Merck, Sharp and Dohme (West Point, Pa.). [5,6-^{3}H]uridine (28 Ci/mmol), [2,8-^{3}H]adenosine (32.6 Ci/mmol), and [^{32}P]orthophosphate were obtained from New England Nuclear Corp. (Boston, Mass.), and [^{3}H]poly(A) was purchased from Miles Laboratories (Elkhart, Ind.). SDS (Matheson, Coleman and Bell) was recrystallized according to Mandel (26). Sodium deoxycholate, enzyme grade, was obtained from Schwarz/Mann (Orangeburg, N.J.); polyvinyl sulfate, potassium salt, was from General Biochemicals (Chagrin Falls, Ohio). *N,N,N',N'*-tetramethylethylenediamine (TEMED), acrylamide, and *N,N'*-methylene bisacrylamide were purchased from Eastman Organic Chemicals (Rochester, N.Y.). Cyanogen bromide was a product of J. T. Baker (Linden, N.J.). RNase A, crystallized once; DNase, electrophoretically purified; and unlabeled poly(U) and poly(A) were purchased from P-L Biochemicals (Milwaukee, Wisc.). Alkaline phosphatase (*Escherichia coli*) was purchased from Worthington Biochemicals Corp. (Freehold, N.J.). RNase T1 and RNase T2 were obtained from Calbiochem (La Jolla, Calif.), and Sepharose 2B and 4B were products of Pharmacia (Uppsala, Sweden).

RESULTS

Poly(A) sequence in virion RNA. The poly(A) in Semliki Forest virion RNA has been reported to be heterogeneous in length, varying

from 50 to 250 nucleotides (15, 20), and its exact location in the genome RNA was as yet not known. As a basis for our study of the size of the poly(A) segments in the SFV RFs and RIs and for the finding of a complementary poly(U) sequence in minus-strand RNA, we designed our experiments to determine whether there was a particular length of poly(A) present in most of the genome RNA molecules and whether the poly(A) sequence was at the 3'-terminus. If the length of the poly(A) determined from the percentage of total radioactivity in 42S RNA that was present in poly(A) was close or equal to the number of nucleotides in poly(A) determined directly, it would indicate that a poly(A) sequence was present in all or almost all of the 42S RNA molecules. The genome RNA has a molecular weight of 4×10^6 (9, 17, 29, 37) and contains approximately 11,000 nucleotides.

SFV 42S RNA, radiolabeled with [^{32}P]orthophosphate, was isolated from purified virions and clarified of fragmented RNA by sucrose density gradient centrifugation. After digestion of the 42S RNA with RNase A and RNase T1, the virion poly(A) sequence was purified by affinity chromatography on poly(U)-Sepharose. From 0.73 to 0.82% of digested ^{32}P-labeled 42S RNA bound to poly(U)-Sepharose and was eluted with 90% formamide. This percentage range indicates that the poly(A) segment in each 42S RNA was 80 to 90 nucleotides long, assuming that each virion RNA had a poly(A) sequence. Figure 1A shows the mobility of the poly(A) during polyacrylamide gel electrophoresis. The data in Fig. 1B are derived from those in Fig. 1A and show the distribution of poly(A) of different sizes in the gel. More than 60% of the poly(A) segments from purified 42S RNA were 70 to 90 nucleotides long, a range close to that determined from the percentage of total RNA that was in poly(A). Together, the results of these two analyses indicate that there is only one poly(A) sequence per 42S virion RNA, and they suggest that there is a poly(A) segment in most, if not all, genome RNA molecules. The same range in sizes for the poly(A) sequence was obtained when the 42S RNA was digested with RNase and directly electrophoresed on polyacrylamide gels (data not shown).

If the isolated poly(A) sequence was located at the 3'-end of the 42S RNA, adenosine would be its 3'-terminal residue. To identify whether adenosine residues were present in the purified poly(A), virion 42S RNA labeled with [^{3}H]adenosine was digested with RNase A and RNase T1, and the poly(A) was isolated by electrophoresis of the digest on polyacrylamide gels. The poly(A) segments were electrophoresed from the gel, and the eluted material was

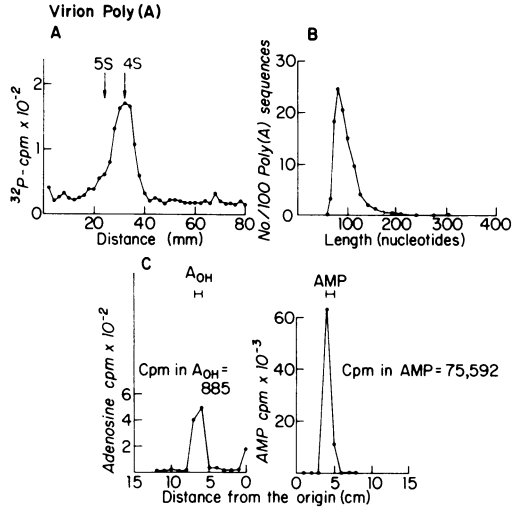

FIG. 1. *Analysis of the length of the Semliki Forest virion poly(A). The 42S SFV RNA, labeled with [^{32}P]orthophosphate or [^{3}H]adenosine, was obtained after sucrose density gradient centrifugation of RNA released from purified virus by exposure to 1% SDS. The virion RNA was digested with RNase A and RNase T1. The poly(A) segments were isolated on poly(U)-Sepharose columns and were analyzed as follows. (A) For mobility by electrophoresis in 15% polyacrylamide gels containing 8 M urea. HeLa cellular 4S and 5S RNAs, labeled with [^{3}H]uridine, were run in the same gel as markers. (B) For length distribution of virion poly(A) segments. The number of poly(A) segments having a given length was determined as described in the text from the mobility of the poly(A) shown in panel (A). (C) For the presence of a 3'-terminal adenosine residue. The poly(A), labeled with [^{3}H]adenosine, was obtained by electrophoresis of RNase A- and RNase T1-digested 42S RNA in 15% polyacrylamide gels containing 8 M urea and elution of poly(A) from the respective area of the gel. The eluted RNA was purified by chromatography on poly(U)-Sepharose, the bound RNA was hydrolyzed with RNase T2, and the digest was analyzed by electrophoresis at pH 3.5 on Whatman 3MM paper. All procedures were as described in the text.*

extracted with a mixture of phenol and chloroform before chromatography on poly(U)-Sepharose. The bound poly(A) was eluted from the column with 90% formamide and, after ethanol precipitation, the purified poly(A) was hydrolyzed with RNase T2 as described in Materials and Methods. The T2 RNase used in these experiments did not possess any phosphatase activity. Figure 1C shows the electrophoretic pattern of the digest. Radioactive label was detected in two areas, one corresponding to that of marker AMP and the other to marker adenosine. In the analysis shown, a value of 86 was obtained for the average length of the Semliki Forest virion poly(A) from the ratio of total

counts in adenosine plus AMP to the counts in adenosine. A second determination gave a value of 82 for the average length. These values are both well within the range indicated above, which were obtained by two different procedures. The finding of adenosine in amounts consistent with there being one adenosine for each poly(A) of the size obtained independently established that the poly(A) sequence is the 3'-terminus of the 42S virion RNA.

Characterization of the SFV RFs and RIs. Is the intracellular poly(A) sequence in virus-specific RNAs longer than virion poly(A)? We chose to study the poly(A) in newly synthesized plus-strand RNAs that were in purified RFs and RIs isolated 2 h after infection. Harvest at this early time during infection, 1.5 h before onset of exponential growth of virus, was selected because it was a time when both plus and minus strands were known to be newly synthesized (3, 21). The SFV RFs and RIs were separated from each other by their differential solubility in 2 M LiCl, and any single-stranded RNAs present in either were removed by chromatography on Sepharose 2B (1). At 2 h after infection, the amount of radioactivity in purified RFs was 1.4 times that in purified RIs, indicating that at this time of infection the RFs were the predominant of these two species of RNA.

The RF RNA, labeled with [^{32}P]orthophosphate, was greater than 95% resistant to hydrolysis by RNase A at 3 μg/ml, but was completely hydrolyzed to mononucleotides by alkali. As has been reported before for RFs isolated from extracts obtained at 6 h after infection (3), more than 80% of the RFs isolated at 2 h sedimented at 22S in sucrose gradients (Fig. 2A). There were two additional species comprising the remaining 20%, and these sedimented at approximately 18S and 15S. Twenty to 30% of the ^{32}P-labeled RIs was rendered acid soluble after digestion with RNase A at 3 μg/ml. The SFV RIs sedimented heterogeneously from 35S to 20S (Fig. 2C).

After treatment of either SFV RFs or RIs with 0.02 μg of RNase A per ml, we found three cores of double-stranded RNA in the same relative distribution (Fig. 2B,D). The three cores from RFs or RIs have been designated as RFI, RFII, and RFIII (3, 29, 38), even though it has not been directly determined that the information that each contains when derived from RFs is identical to that when they are derived from

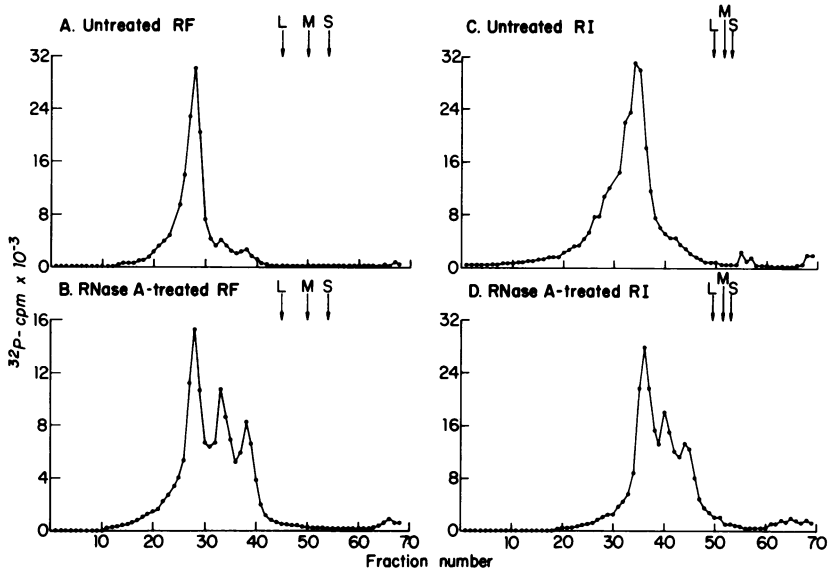

FIG. 2. *Sedimentation on sucrose gradients of SFV RFs and RIs isolated 2 h after infection. The RFs and RIs, labeled with [^{32}P]orthophosphate, were purified by LiCl precipitation followed by chromatography on Sepharose 2B in 0.5% SDS buffer. Equal amounts of LiCl-soluble RFs were analyzed in (A) and (B). (A) Untreated RFs. (B) RFs treated for 15 min at 25°C with RNase A at 0.02 μg/ml before centrifugation. Equal amounts of LiCl-precipitable RIs were used for (C) and (D). (C) Untreated RIs. (D) RIs treated for 15 min at 25°C with RNase A at 0.02 μg/ml before centrifugation. Centrifugation was on linear 15 to 30% sucrose gradients in 0.2% SDS buffer at 20°C for 16 h at 154,000 × g for (A) and (B) and for 16 h at 98,000 × g for (C) and (D) in the SW41 rotor. Fractions were collected from below and analyzed for labeled, acid-insoluble RNA in a toluene-based scintillation fluid. ^{3}H-labeled reovirus RNAs were included in each gradient as markers: L at 14.5S = 2.5 × 10^{6} daltons; M at 12S = 1.4 × 10^{6} daltons; and S at 10.5S = 0.8 × 10^{6} daltons (2, 35).*

RIs. After treatment of RFs with RNase, it is clear that the nuclease treatment generated the two smaller cores, RFII and RFIII. The RFI may be present as such in the total RF population and not modified in any way by treatment with RNase. After RNase treatment, 50 to 60% of the radioactive label was in RFI, and the remaining 40 to 50% was in RFII and RFIII (Table 1). A value for k of 0.089 was obtained in the Studier equation ($S = kM^{.346}$; 45) using the known molecular weights and sedimentation values for reovirus double-stranded RNAs (2, 35). The molecular weights that we obtained for the three SFV cores confirm those already in the literature (3). Relative to reovirus RNAs, RFI sedimented at 22 to 23S and had a molecular weight of 8.1×10^6 to 8.6×10^6; RFII sedimented at 18 to 19S and had a molecular weight of 4.8×10^6 to 5.3×10^6; and RFIII sedimented at 15 to 15.5S and had a molecular weight of 2.7×10^6 to 2.9×10^6.

TABLE 1. *Distribution of SFV RFs and RIs after sucrose gradient centrifugation with and without prior treatment with RNase A*[a]

S value of RNAs	Untreated		RNase-treated	
	Percent of total RNAs in RFs or RIs	Relative no. of moles[b]	Percent of total RNAs in RFs or RIs	Relative no. of moles[c]
RFs				
22	84	100.0	54	61.0
18	11	22.0	28	31.2
15	5	20.0	18	42.9
RIs				
35 to 20	92	60.8		
22			60	42.1
18	5	7.6	24	21.4
15	3	6.7	16	29.0

[a] BHK cells were labeled with [^{32}P]orthophosphate in the presence of AMD from 3 h before infection, and the RFs and RIs present at 2 h after infection were purified and analyzed on sucrose gradients as described in Fig. 2.

[b] Obtained by dividing the counts per minute in 22S, 18S, and 15S RNA by the average molecular weights for these three RNAs: 8.4×10^6, 5.1×10^6, and 2.8×10^6, respectively. The RIs isolated at 2 h were estimated to have a molecular weight of 10.5×10^6, which is equal to one equivalent of double-stranded RNA and one-half equivalent of single-stranded RNA, as $N = 1$ (1). The calculation assumes that the specific activity of all species of RNAs is the same. The value obtained in the calculation for 22S RNA in the untreated RFs was made equal to 100.0, and the other values were adjusted to this figure. The total counts per minute analyzed on sucrose gradients were 139,000 for the untreated RFs and 99,100 for the untreated RIs. After RNase treatment, 100% of counts per minute in RFs were recovered as acid insoluble, and approximately 80% of those in RIs were recovered as acid insoluble.

[c] Before calculating the relative number of moles of RNA released from the 22S RFs or the 35 to 20S RIs by RNase A treatment, the amount of labeled RNA sedimenting at 18S and 15S in the gradients of untreated RFs and RIs was subtracted from the total labeled RNA present in these areas of the gradients after RNase A treatment.

The small amounts of RFII (18S RNA) and RFIII (15S RNA) present in RF and RI preparations that were not treated with RNase were present in equimolar amounts (Table 1). When treated with RNase, the RFs and RIs yielded RFII and RFIII in unequal molar amounts, with as much as 1.4 times more RFIII than RFII. This inequality in molar amounts assumes that RFII and RFIII were labeled with [^{32}P]orthophosphate to the same specific activity. A correction would have to be applied if RFII and RFIII were not labeled equally, a situation that would result if proportionally less product was synthesized from RFII than from RFIII at 2 h after infection.

SFV RF and RI poly(A) sequence. The poly(A) sequence was purified from the digested RFs and RIs by chromatography on poly(U)-Sepharose. Before chromatography, the RFs and RIs, labeled with [^{32}P]orthophosphate, were denatured by heating at 100°C in 1 mM EDTA and were then digested with RNase A and RNase T1. Less than 3% of the total digested RNA was acid precipitable after the nuclease treatment. Digestion with RNase T1 alone also released poly(A) relatively free of other sequences, but the denaturation, digestion, and chromatography had to be repeated a second time. As a monitor for the nuclease treatment and the chromatography, we analyzed similarly the RF obtained from HeLa cells infected with poliovirus for 7 h and labeled from 1 to 7 h after infection. Between 0.8 and 1.1% of the digested ^{32}P-labeled SFV RFs or RIs isolated 2 h after infection bound to poly(U)-Sepharose (Table 2). The percentage obtained was two to three times the expected value for one poly(A) sequence per randomly labeled SFV RF or RI of the same length as in virion RNA. This higher value did not result from incomplete digestion with nucleases, since after T1 RNase and chromatography the base composition of the bound SFV RNA indicated that it was poly(A): Cp = 0.6%; Ap = 94.9%; Gp = 0.0%; and Up = 4.5%. In addition, the value obtained of 1.17% of the total radioactivity in poliovirus RF that was in poly(A) was well within the range reported in the litera-

TABLE 2. *Percentage of total ^{32}P-labeled RNA in poly(A) sequences*[a]

Sample	Time of labeling relative to 0 time of infection (h)	Percent expected[b]	Percent obtained
SFV 42S RNA	1 to 18	0.73	0.73; 0.82
SFV RF	1 to 2	0.36	1.14
	−3 to 2		0.82
SFV RI	1 to 2		1.05
Poliovirus RF	1 to 7	0.90–1.33	1.17

[a] The poly(A) sequences were purified from heat-denatured and RNase A- and RNase T1-digested SFV and poliovirus RNAs by chromatography on poly(U)-Sepharose columns as described in the text.

[b] Calculated for one poly(A) sequence 80 residues long for each SFV RF (22,000 nucleotides) or each 42S RNA (11,000 nucleotides) and for one poly(A) sequence 140 to 200 residues long for each poliovirus RF (15,000 nucleotides; 42, 47).

ture, namely from 0.90 to 1.33% (42, 47). The absence of labeled GMP residues in the base composition analysis indicated that the SFV poly(A) was located at the 3'-terminus of one of the strands in the RFs, most likely the plus strand.

When analyzed by polyacrylamide gel electrophoresis, the poly(A) segments isolated from the total population of SFV RFs or RIs migrated similarly to each other and to virion poly(A) (Fig. 3). That they were similar in size was confirmed by a comparison of the length distributions of the poly(A) (see lower panel, Fig. 7). In each, 60 to 70% of the poly(A) segments were 60 to 90 nucleotides long. Only 4 to 6% of the poly(A) in the total population of RFs and RIs was as long as 140 to 200 residues, and less than 3% was longer than 200 nucleotides. If the individual strands in SFV RFs and RIs were labeled equally, the percentage of total radioactivity that was in poly(A) (Table 2) indicates that, if each randomly labeled RF or RI had one poly(A) sequence, the poly(A) would contain between 190 and 250 nucleotides. The size of the average poly(A) in SFV RFs and RIs directly determined was 60 to 90 nucleotides, and thus our data suggest that there is at least one poly(A) sequence per RF or RI of approximately the size of that in virion RNA, namely 80 to 90 nucleotides.

We analyzed whether any of the poly(A) segments in SFV RFs or RIs that had not been denatured or digested with nucleases were free to bind to poly(U)-Sepharose. Only 40 to 50% of either the undenatured RFs or the RIs bound to poly(U)-Sepharose (Table 3). The RFs that did not bind during the first chromatography on poly(U)-Sepharose failed to bind when rechromatographed, indicating either that approximately half of the RF molecules, and presumably also half of the RI molecules, did not contain a poly(A) sequence or that the poly(A) sequence in each was masked in a way that prevented its binding to poly(U)-Sepharose. To determine whether a poly(A) sequence was present in the RFs in both populations, those that bound to poly(U)-Sepharose and those that did not bind, the two populations of RFs obtained after the first chromatography on poly(U)-Sepharose were individually denatured. They were digested with RNase A and RNase T1 and then rechromatographed on poly(U)-Sepharose. Poly(A) segments were found in both populations of RFs. The size of the poly(A) sequences in each was analyzed by electrophoresis on polyacrylamide gels (Fig. 4). The poly(A) sequence was 75 to 85 nucleotides

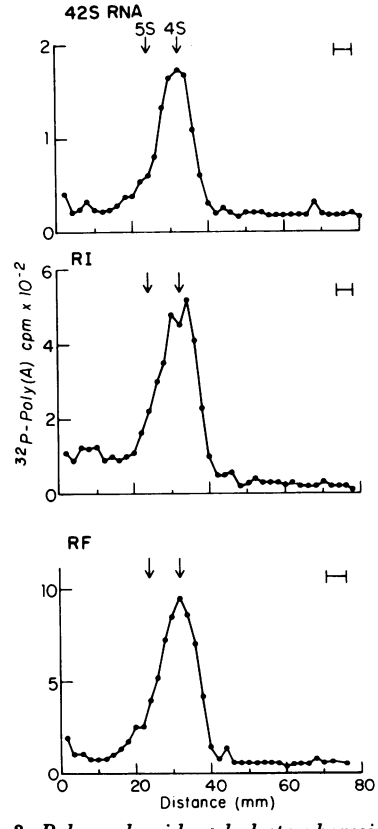

FIG. 3. *Polyacrylamide gel electrophoresis of the poly(A) sequence in ^{32}P-labeled 42S virion RNA and in ^{32}P-labeled RIs and RFs isolated 2 h after infection. The poly(A) was obtained from each and was analyzed in 15% polyacrylamide gels as described in the text. The horizontal bar indicates the position of the bromophenol blue used as marker.*

TABLE 3. *Distribution during sucrose density gradient centrifugation of SFV RFs and RIs in fractions obtained during chromatography on poly(U)-Sepharose columns*[a]

Fraction	Percent of total RF or RI RNA applied to column	Relative no. of moles[b]						
		Untreated before centrifugation				RNase A-treated before centrifugation		
		35-20S	22S	18S	15S	22S	18S	15S
RFs								
Flow-through	50		100	47	15	63	43	33
10% Formamide	20		49	4	12	35	13	19
90% Formamide	26		62	8	13	45	16	20
RIs								
Flow-through	54	73		19	6	43	24	27
10% Formamide	17	25		2	2	18	8	8
90% Formamide	27	38		4	6	21	7	7

[a] SFV RFs or RIs, labeled with [^{32}P]orthophosphate, were chromatographed on poly(U)-Sepharose in 0.2 NETS buffer without prior denaturation or digestion with RNase, and each fraction was then analyzed on 15 to 30% sucrose gradients as described in Fig. 2. The RNAs in the flow-through fraction are those which failed to bind to poly(U)-Sepharose. In addition to the three fractions shown, RNA was also present in an ETS wash and represents the difference between the amounts shown and 100% of the RFs or RIs labeled with radioactivity that were applied to the column.

[b] Obtained as described in Table 1, and the value obtained for the 22S RFs in the flow-through fraction was made equal to 100.0. All other values were adjusted to this figure. The total counts per minute analyzed on the poly(U)-Sepharose columns were 460,000 for the RFs and 330,000 for the RIs. Essentially 100% of the RF radioactivity and 80% of the RI radioactivity was recovered after RNase A treatment.

long in the RFs that did not bind to poly(U)-Sepharose when undenatured. The average size of the poly(A) sequence in the RFs that were eluted from poly(U)-Sepharose with 10% formamide was 100 residues, and it was 120 residues in the undenatured RFs eluted from poly(U)-Sepharose with 90% formamide.

Therefore, the inability of some RFs, when undenatured, to bind to poly(U)-Sepharose was not due to their lack of a poly(A) sequence or to the fact that the poly(A) sequence was unusually short in these molecules. Masking by hydrogen bonding to a complementary sequence in the RF molecules was more likely the reason why a double-stranded RNA with a poly(A) sequence of 75 to 85 nucleotides did not bind to poly(U)-Sepharose. If a complementary sequence was present in all RF molecules, the RFs that did bind to poly(U)-Sepharose had to contain a poly(A) sequence longer than the complementary sequence. We did find that the average length of the poly(A) in RFs that did bind was greater than in those that did not bind.

To determine whether any other difference could be detected, the two populations of RFs and RIs, those that bound to poly(U)-Sepharose and those that did not, were separately analyzed by sucrose density gradient centrifugation. The 22S RF was the major double-stranded RNA present in the two populations of RFs (Table 3). We were also concerned with the distribution between these two populations of the 15S and 18S double-stranded RNAs present in the original, unfractionated preparation of RFs. The small amounts of 15S double-stranded RNA originally present were distributed equally between the populations of RFs that did and those that did not bind to poly(U)-Sepharose (Table 3). In contrast, there was a decided enrichment of the 18S double-stranded RNA in the fraction of RFs that did not bind to the affinity column. Eighty percent of the total 18S double-stranded RNA originally present was in the population of RFs that did not bind to poly(U)-Sepharose. This suggests that 18S double-stranded SFV RNA (RFII) may not contain a poly(A) sequence.

Treatment of either the RFs that did or those that did not bind to poly(U)-Sepharose with RNase A at 0.02 μg/ml before sedimentation yielded (when corrected for the RFII and RFIII originally present) the three cores, RFI, RFII, and RFIII, in the same ratio found previously for the total RF population (Table 1). About 60 to 65% of the 22S RNA in each of the two populations of RFs retained a sedimentation value of 22S after RNase treatment (Table 3), and thus these RNAs were RFI. The 35 to 40% of labeled RNAs no longer sedimenting at 22S were found in RFII and RFIII. There is thus a 22S double-stranded RNA that binds to poly(U)-Sepharose through a poly(A) sequence at one of its ends and can be cleaved by RNase to yield RFIII. The absence of poly(A) in RFII taken together with the finding that RFII and

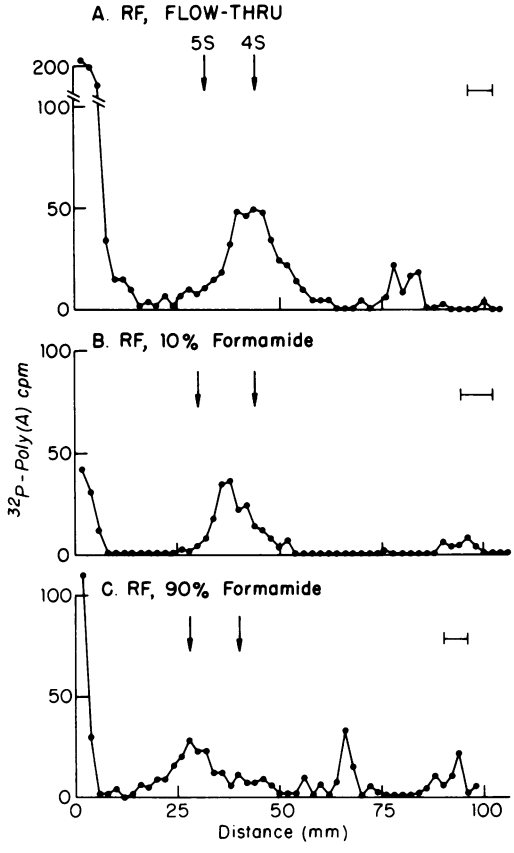

FIG. 4. *Polyacrylamide gel electrophoresis of the poly(A) sequence in the fractions of the total, undenatured RF population that were obtained from chromatography on poly(U)-Sepharose. The RFs, labeled with [^{32}P]orthophosphate, were isolated 2 h after infection, and the undenatured RFs were chromatographed on poly(U)-Sepharose as described in the text. The RFs in each of the three resultant fractions were then denatured by heating at 100°C for 2 min in 1 mM EDTA, and the poly(A) sequence in each was isolated and analyzed as described in Fig. 1. (A) Poly(A) sequences in RFs that failed to bind to poly(U)-Sepharose (flow-through fraction). (B) Poly(A) sequences in RFs that were eluted during chromatography with 10% formamide. (C) Poly(A) sequences in RFs that were eluted with 90% formamide. The horizontal bar indicates the position of the bromophenol blue used as marker.*

RFIII were produced in approximately equimolar amounts after RNase treatment of the RFs that bound to poly(U)-Sepharose strongly suggests that RFII and RFIII are originally part of the same 22S RF as proposed by Simmons and Strauss (38). In addition, our data extend their proposal by indicating that the poly(A) sequence is in the RFIII portion of that 22S RF.

The 50% of the RIs that did not bind to poly(U)-Sepharose sedimented heterogeneously from 35S to 20S. The RIs that were eluted in 10% formamide had a sedimentation pattern identical to that of those that did not bind. The RIs eluting in 90% formamide sedimented much more homogeneously, with the majority of molecules at 22S. As shown above for the RFs, there was no selection during the chromatography on poly(U)-Sepharose for RIs with a particular core structure. After RNase treatment, both the RIs that bound to poly(U)-Sepharose and those that did not yielded the three double-stranded cores in the expected distribution (Table 3). The molar ratio of RFI to RFIII indicated that about 50 to 60% of the RIs that did or did not bind to poly(U)-Sepharose had an RFI type of core structure.

Based on our results, poly(U)-Sepharose chromatography did not discriminate between RFs or RIs that contained an RFI type of core and those which after RNase digestion were cleaved to yield RFII and RFIII. Selection by poly(U)-Sepharose was therefore solely based on the presence of a poly(A) segment in the RFs or RIs that was free to bind to the column.

Determination of the presence of a poly(U) sequence in SFV RFs and RIs. A complementary poly(U) sequence in RFs and RIs would be expected to be in minus-strand RNA. To detect the poly(U) segment, enough radioactive precursor would have to be incorporated into the minus strands so that analysis of minus-strand RNA would be possible. We have found that, if the labeling period with [^{32}P]orthophosphate was from 0 to 2 h, there was preferential labeling with radioactivity of the plus strands in the RFs. This would yield an underestimation of the length of the poly(U) segment, if indeed it could be detected at all. To increase the radioactivity in the minus strand of the RFs, BHK cells were prelabeled in the presence of AMD with [^{32}P]orthophosphate for 3 h before infection. The radioactive precursor and AMD were also present throughout the adsorption period and infection. The RFs and RIs were purified from cellular extracts obtained at 2 h after infection. Even under these conditions of labeling, the minus-strand RNA of the RFs and RIs was labeled to a lower specific activity than the plus-strand RNAs. This would indicate that even though both minus and plus strands were synthesized during the 2 h of infection, new synthesis of plus-strand RNA in the double-stranded RNAs was predominant at 2 h. In any case, prelabeling the cells for 3 h before infection with [^{32}P]orthophosphate increased the amount of radioactivity recovered in the minus-strand RNA twofold over that found when the radioactive label was added at the same time as virus.

When the RFs and RIs were not denatured, they did not bind to poly(A)-Sepharose during chromatography. A different result was obtained when the RFs or RIs were denatured in the presence of unlabeled poly(U) and were digested with RNase T1 before chromatography on poly(A)-Sepharose. Approximately 0.2% of the digested SFV RF bound to the column (Fig. 5A). If the bound RNA was exposed to a second cycle of denaturation, digestion, and chromatography, essentially all of this RNA from the digested SFV RF bound to the poly(A)-Sepharose (Fig. 5B). The base composition of the

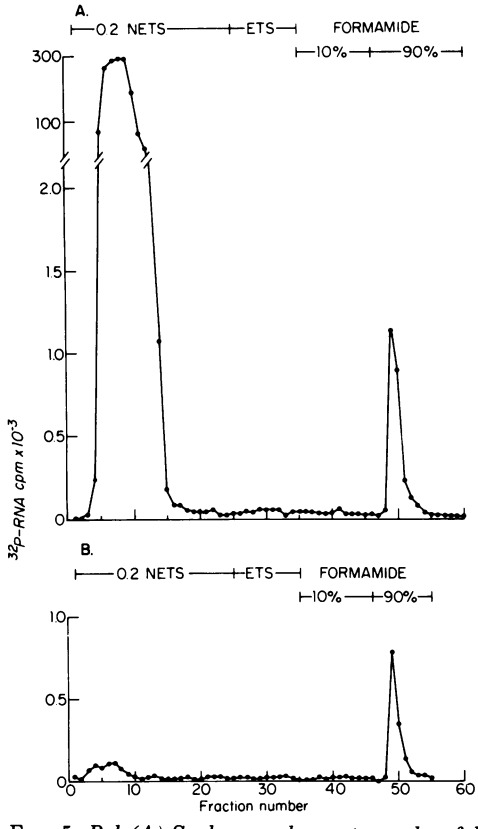

FIG. 5. *Poly(A)-Sepharose chromatography of denatured and digested ^{32}P-labeled RFs. The RFs, isolated 2 h after infection, were denatured by heating in the presence of unlabeled poly(U) and digested with RNase T1. The digest was analyzed by chromatography on poly(A)-Sepharose. All procedures are described in the text. (A) Chromatography of digested RF RNA. (B) Rechromatography on poly(A)-Sepharose of the RNA eluted with 90% formamide during the first chromatography shown in (A). Before the second chromatography, the RNA was again denatured by heating in the presence of excess unlabeled poly(U) and digested with RNase T1. Radioactivity in the fractions from the column were monitored for Cerenkov radiation.*

RNA binding during the chromatography identified it as poly(U). From 95 to 97% of its nucleotides were UMP (Table 4). In addition to UMP, a small amount of radioactive label was consistently found in GMP, indicating that the bound RNA was a homopolymer of uridylate terminated by a 3'-GMP residue. Table 4 also shows the base composition of the poly(U) segment obtained from the poliovirus RF. Approximately 90% of the nucleotides in the homopolymer obtained from the poliovirus RF were in UMP. This confirmed that there was little or no contamination of the poly(U) segment from either SFV RF or poliovirus RF with the poly(A) sequence at the 3'-terminus of the plus-strand RNA in either RF as a result of annealing to the excess poly(U) added during the denaturation.

The poly(U) sequence in both SFV RFs and RIs represented approximately 0.1 to 0.2% of the total radioactivity in RFs and RIs labeled with [^{32}P]orthophosphate (Table 5). There was no poly(U) sequence in SFV plus-strand RNA subjected to the same procedures, namely denaturation, digestion, and chromatography on poly(A)-Sepharose. An oligo(U) segment of only two to three nucleotides can be calculated from the 0.02% of the counts in SFV 42*S* plus-strand RNA that was retained during the chromatography. Thus, the poly(U) sequence in both SFV RFs and RIs was in their minus strands. The 0.51% of total radioactivity in poliovirus RF that was found in the poly(U) sequence (Table 5) is close to the expected value if both the minus and plus strands in the poliovirus RF are equally labeled with radioactivity. The lower-than-expected percentage of total found for the poly(U) sequence of the SFV RFs or RIs resulted from the difference in specific activity of the plus and minus strands of the SFV double-stranded RNAs.

When the RFs were isolated from cells infected for 2 h in the presence of [^{3}H]uridine, 0.8% of the total radioactivity in denatured, digested RFs bound to poly(A)-Sepharose and was, therefore, in poly(U). From the percentage of uridylate in the SFV plus-strand RNA, 19.5%, and minus-strand RNA, 27.3% (21), the value of 0.8% found corresponds to one poly(U) sequence 42 nucleotides in length in the minus strand of each 22*S* RF. The actual size of the poly(U) that bound to poly(A)-Sepharose from denatured, digested RFs and RIs was directly determined from its mobility during electrophoresis on polyacrylamide gels (Fig. 6). The majority of the poly(U) from RFs migrated slightly faster than the marker, 4*S* cellular RNA, whereas the majority of the poly(U) isolated

TABLE 4. *Base composition of RNA fragments from SFV and poliovirus RFs which after digestion with RNase T1 bound to poly(A)-Sepharose*

Fragment	Percent of total nucleotides		
	SFV		Poliovirus
	A	B	
Cp	1.2	0.3	6.2
Ap	0.0	1.0	2.0
Gp	3.5	2.2	2.4
Up	95.3	96.5	89.4

TABLE 5. *Percentage of total [^{3}H]uridine- or ^{32}P-labeled SFV RFs or RIs in poly(U) sequences*[a]

Sample	Time of labeling relative to 0 time of infection (h)	Percent obtained[b]	
		[^{3}H]uridine	^{32}P
SFV 42S RNA	1 to 16		0.02
SFV RFs	−3 to 2		0.17 (0.27)
	0 to 2	0.84; 0.79 (1.17)	0.10
	1 to 2		0.10
SFV RIs	0 to 2	0.64; 0.56 (1.10)	
	1 to 2		0.08 (0.29)
Poliovirus RF	1 to 7		0.51 (0.37; 0.43)

[a] The SFV RFs and RIs, isolated 2 h after infection, were denatured with heat in the presence of excess unlabeled poly(U). They were digested with RNase T1, after which they were chromatographed on poly(A)-Sepharose as described in the text. The bound RNA represents the poly(U) sequences and is expressed as a percentage of the total digested RNA applied to the columns.

[b] The value in parentheses is the expected value and is calculated as described in Table 2, using the data in Fig. 6, where the SFV RF poly(U) was found to be 60 nucleotides long and the RI poly(U) sequence was 80 nucleotides long. The poliovirus RF contains a poly(U) sequence 56 or 65 nucleotides long (44, 47). SFV plus-strand RNA contains 19.5% uridylate, and the minus-strand RNA contains 27.3% uridylate (21).

from RIs co-migrated with the 4S RNA. The poly(U) sequence in RIs was the same length as the majority of poly(A) sequences in the RIs and RFs (Fig. 7, lower panel). The poly(U) sequence in RFs was 20 nucleotides shorter. The difference in length of the poly(U) sequence in the minus strand of RFs and RIs was confirmed in an analysis of the length distribution of the poly(U) segment in each (Fig. 7, upper panel). Eighty percent of the poly(U) in RFs contained 50 to 70 residues, whereas 66% of the poly(U) in RIs was 70 to 100 nucleotides long. Since the directly determined length of the poly(U) sequence was in all cases larger than the value predicted from the percentage of ^{32}P- or [^{3}H]uridine-labeled poly(U) from denatured and digested RFs and RIs that bound to poly(A)-Sepharose, there can be only one poly(U) segment per 42S minus strand in either the RF or RI.

Once we identified a poly(U) segment in the minus strand of the total population of RFs, we wanted to determine whether the size of the poly(U) in SFV RFs was variable and in particular whether the poly(U) in the minus-strand RNA of RFs that bound to poly(U)-Sepharose was unusually short, thereby leaving the poly(A) sequence in the plus-strand RNA free to bind during the chromatography. The 50% of RF molecules that bound to poly(U)-Sepharose possessed a poly(A) segment about 50% longer than those that did not bind during chromatography (Table 3, Fig. 4). The RFs that did not bind and those that were eluted from poly(U)-

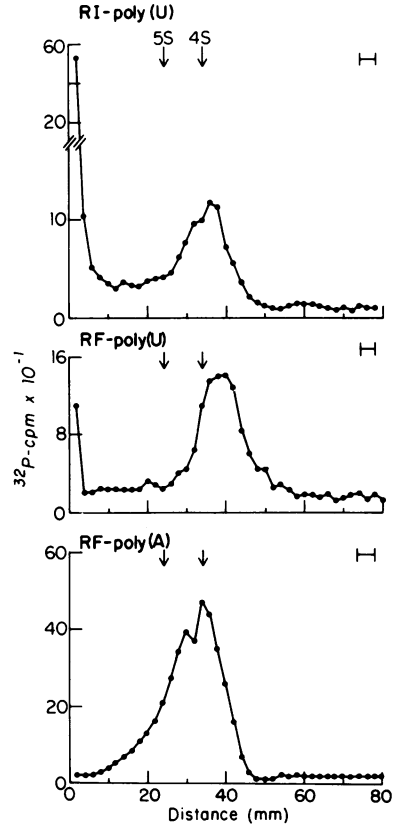

FIG. 6. *Polyacrylamide gel electrophoresis of poly(U) sequences in ^{32}P-labeled RFs and RIs isolated 2 h after infection. The material that bound to poly(A)-Sepharose and was eluted with 90% formamide was analyzed for size on 15% polyacrylamide gels. The conditions for electrophoresis are described in the text. (A) The electropherogram of the poly(U) sequences in RFs and RIs. (B) The electropherogram of the poly(A) sequences present in the same RF population analyzed in panel (A).*

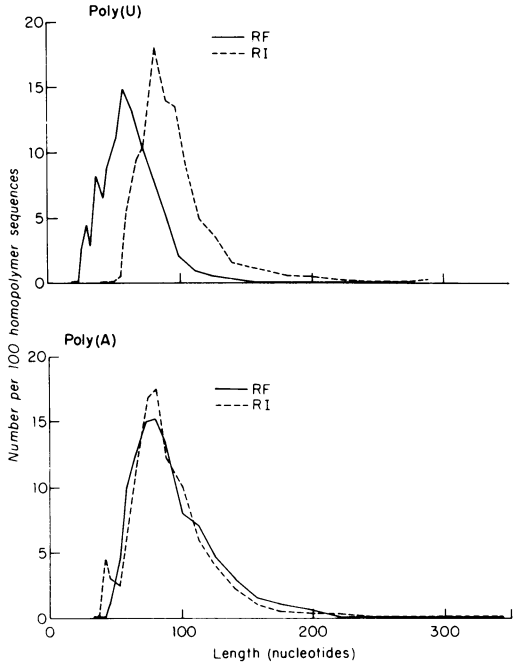

FIG. 7. *Length distribution of the poly(U) and poly(A) sequences in SFV RFs and RIs isolated 2 h after infection. The number of poly(U) or poly(A) sequences with a given length was determined as described in the text, from the mobilities shown in Fig. 6 and Fig. 3, respectively.*

Sepharose with 10% formamide or 90% formamide were analyzed separately for the size of their poly(U) segment. Each was denatured in the presence of excess unlabeled poly(U), after which the denatured RNAs were digested with RNase T1. The poly(U) sequences obtained by chromatography on poly(A)-Sepharose were analyzed for size by electrophoresis on polyacrylamide gels (Fig. 8). The majority of the poly(U) segments in RFs that bound to poly(U)-Sepharose and in those that did not bind had a length of 50 to 70 nucleotides, whereas the poly(A) sequence in all three fractions was longer (Fig. 9). The difference noted between the length of the poly(U) and poly(A) sequences in RFs was least in those RFs that did not bind to poly(U)-Sepharose. In the RFs eluted from the poly(U)-Sepharose with 10 or 90% formamide, the poly(U) segment in the RFs was 20 to 50% shorter than the corresponding poly(A) segment.

Terminal location of the poly(U) sequence in minus-strand RNA. Our data indicate that the poly(A) segment in SFV 42*S* RNA is the sequence at the 3'-terminus of the plus-strand RNA. If the poly(A) were transcribed directly from a complementary poly(U) sequence, the poly(U) would be at the 5'-end of the 42*S* minus-strand RNA, and it would have a 5'-phosphorylated residue of uridine at its 5'-terminus. We have already shown that GMP was present in the poly(U) segment obtained after T1 digestion of denatured RFs. This result established that the poly(U) segment was not the sequence at the 3'-terminus of the minus-strand RNA.

To identify whether there was a 5'-phosphorylated residue at the 5'-end of the poly(U) sequence, the RFs and RIs were labeled during infection with [^{3}H]uridine and purified from extracts obtained 2 h after infection. The poly(U) was obtained from each by chromatography on poly(A)-Sepharose of RFs and RIs that were denatured in the presence of excess unla-

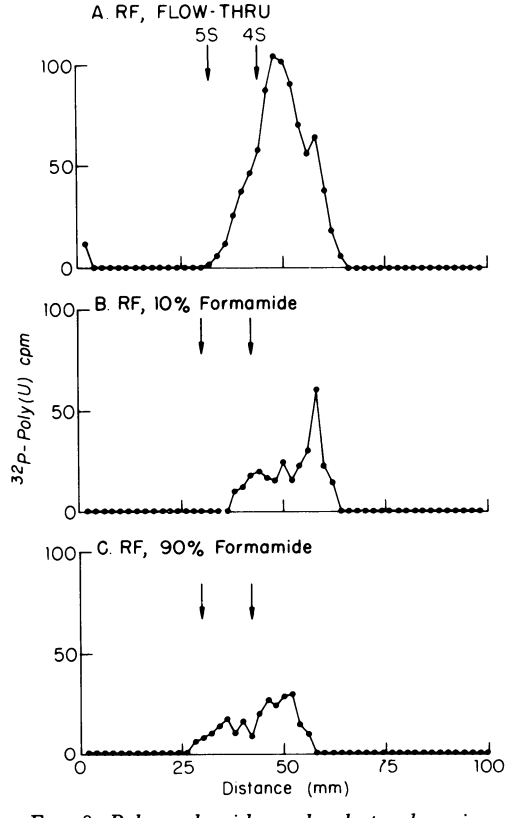

FIG. 8. *Polyacrylamide gel electrophoresis of poly(U) sequences in the RFs present in the three fractions obtained during chromatography on poly(U)-Sepharose of RFs that were not denatured. The material analyzed was that used in the experiments shown in Table 3 and Fig. 4. The poly(U) segment in RFs in the three fractions was obtained as described in Fig. 5. Electropherogram of poly(U) segments in RFs that: (A) did not bind to poly(U)-Sepharose (flow-through fraction); (B) eluted with 10% formamide during chromatography; and (C) eluted with 90% formamide.*

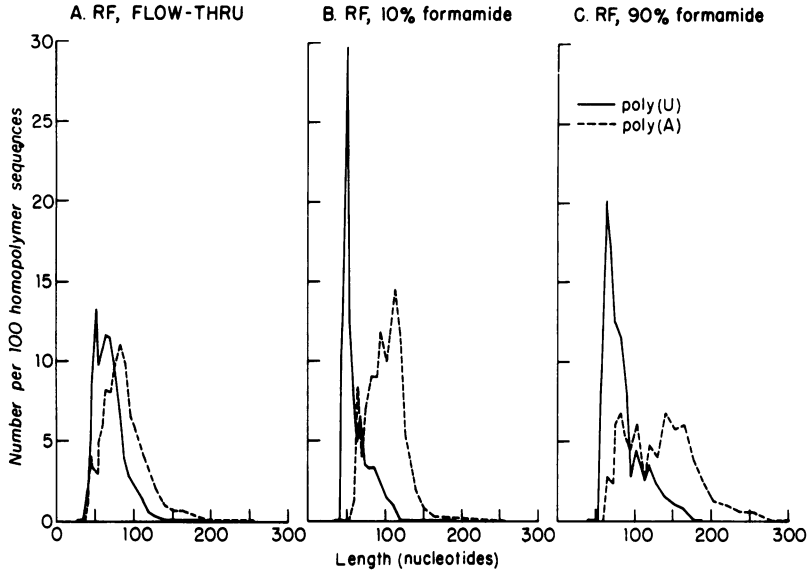

FIG. 9. *Comparison of the length distribution of poly(A) and poly(U) sequences in each of the three fractions obtained by chromatography on poly(U)-Sepharose of ^{32}P-labeled RFs that were isolated at 2 h and were not denatured. The data are derived from Fig. 4 and 8. The solid line represents the poly(U) sequences in RFs that (A) failed to bind to poly(U)-Sepharose (flow-through fraction); (B) eluted with 10% formamide; and (C) eluted with 90% formamide.*

beled poly(U) and digested with RNase T1. The purified poly(U) was hydrolyzed with RNase A and RNase T2, and the digest was subjected to electrophoresis on DEAE paper (Fig. 10). Most of the radioactively labeled residues from both RF and RI poly(U) migrated to the spot for authentic UMP marker. Three other peaks of labeled residues were found. One co-migrated with authentic pUp marker. The second migrated slightly slower than the UTP marker, a position expected for ppUp residues. The remaining radioactive material was at the origin.

To verify that the residues migrating with mobilities of pUp and ppUp were in fact those residues, the labeled residues from the corresponding areas in a duplicate electropherogram were eluted; they were digested with bacterial alkaline phosphatase; and electrophoresis on paper was repeated. All the labeled material from both now migrated as uridine (data not shown). This confirms that all the phosphates originally present in the residues were susceptible to removal by alkaline phosphatase and indicates that the residues found were those postulated, namely pUp and ppUp. About one-third of the radioactive material originally remaining at the origin during the first electrophoresis was recovered as uridine after alkaline phosphatase treatment, suggesting that pppUp residues may also be present at the 5'-terminus of the poly(U) segment. The other two-thirds migrated heterogeneously after the alkaline phosphatase treatment and paper electrophoresis, suggesting that this material may represent a small number of oligonucleotides that were incompletely hydrolyzed during the original digestion with RNase A and RNase T2.

The presence of ppUp and pUp residues in the poly(U) from RFs and RIs indicated that the poly(U) was the 5'-terminus of the minus-strand RNA. The length of the poly(U) sequence estimated from the ratio of counts in total poly(U) to the counts in pUp and ppUp gave values that were close to those determined directly from mobility of the poly(U) segments during polyacrylamide gel electrophoresis (Table 6) and confirmed that the poly(U) sequence in RIs was longer than that in RFs. We found that ppUp was the residue at the 5'-end of 60 to 80% of the poly(U) in RFs and RIs. Even though the residue pUp occurred less often, it was more likely to be found as the 5'-terminal residue of RFs than RIs.

Analysis of RFI, RFII, and RFIII for poly(U). RIs were isolated at 2 h after infection from cells exposed to [^{32}P]orthophosphate for 3 h before infection and throughout infection. The SFV RIs were treated with 0.02 μg of RNase A per ml, and the three resultant cores were separated from each other by sucrose density gradient centrifugation. The peak fractions of each were combined and purified by a second

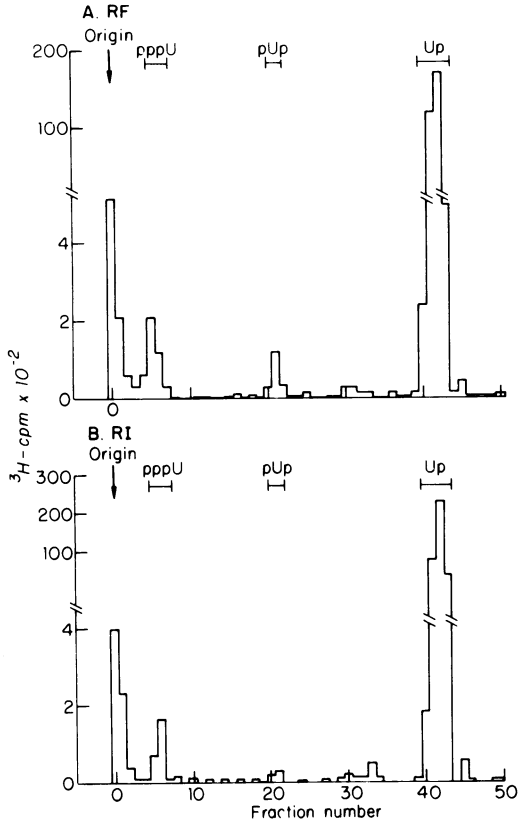

FIG. 10. *Electrophoresis on DEAE-81 paper at pH 3.5 of RNase A- and RNase T2-digested poly(U) segments from SFV RFs and RIs labeled with [^{3}H]uridine and isolated 2 h after infection. (A) Digest of the poly(U) isolated from RFs. (B) Digest of the poly(U) isolated from RIs. Procedures are described in the text. After electrophoresis, the paper was cut into 1-cm strips and the radioactivity in each was determined by counting in a toluene-based scintillation fluid in the presence of 0.5 ml of an NEN solubilizer, Protosol.*

TABLE 6. *Length of the SFV RF and RI poly(U) determined from end-group analyses*[a]

Residue	[^{3}H]uridine (cpm)		Length (nucleotides)	
	RF	RI	RF	RI
ppUp	390	237		
pUp	192	45		
ppUp + pUp	582	282	53	117
Up	30,063	32,632		

[a] The paper electrophoreses of digested, [^{3}H]uridine-labeled poly(U) sequences from RFs and RIs shown in Fig. 10 were used to determine the counts in 5'-terminal residues and in the internal uridylate residues. The length is obtained by dividing the total counts in poly(U) by the counts in the respective termini.

sucrose density gradient centrifugation. The cores were denatured by heating in the presence of an excess of unlabeled poly(U) and were separately chromatographed on poly(A)-Sepharose columns. To quantitate the recovery of poly(U) containing minus-strand RNAs, it was necessary to know how the radioactive label in the original RFs and RIs was distributed between the plus- and minus-strand RNAs.

From the directly determined value for the average length of the poly(A) segments in total ^{32}P-labeled RFs of 80 nucleotides (Table 2), 0.36% of the radioactive label should have been found in poly(A) if both strands of the RF, containing 22,000 nucleotides, were equally labeled. We actually found 0.82% of the total label in poly(A) (Table 2), suggesting that only plus-strand RNAs were labeled in these RFs even under our conditions of prelabeling. This conclusion was incorrect, since we found radioactive label in minus-strand RNAs. In preliminary annealing experiments utilizing virion RNA to displace the plus strand in denatured [^{32}P]orthophosphate-labeled SFV RFs, we found that we could replace at most 80% of the radioactivity in the denatured SFV RFs obtained from cells that had been prelabeled for 3 h before infection. Thus, in our preparation of SFV RFs, and also RIs analyzed similarly, approximately 80% of the radioactive label was in plus strands and 20% of the total label in RFs or RIs was in minus-strand RNA.

The minus strand in RFI, the double-stranded form of 42S RNA, contained a poly(U) sequence (Table 7). Greater than 75% of the total labeled minus-strand RNA present in RFI bound to poly(A)-Sepharose. When analyzed by sucrose density gradient centrifugation, 50% of the bound RNA sedimented at 42S (Fig. 11A). The remaining 50% sedimented slower and could have represented nascent minus-strand chains containing poly(U). The majority of the RNA that did not bind to poly(A)-Sepharose probably was comprised of nascent plus-strand chains. It sedimented at less than 40S (Fig. 11B), and only 5% contained poly(A) sequences,

TABLE 7. *Chromatography on poly(A)-Sepharose of denatured RFI, RFII, and RFIII obtained from RIs*[a]

Core	[^{32}P]RNA (cpm)	
	Not bound	Bound
RFI	114,404	19,362
RFII	17,928	909
RFIII	22,325	5,898

[a] Preparation and analysis of purified RFI, RFII, and RFIII from the RIs was as described in Fig. 11.

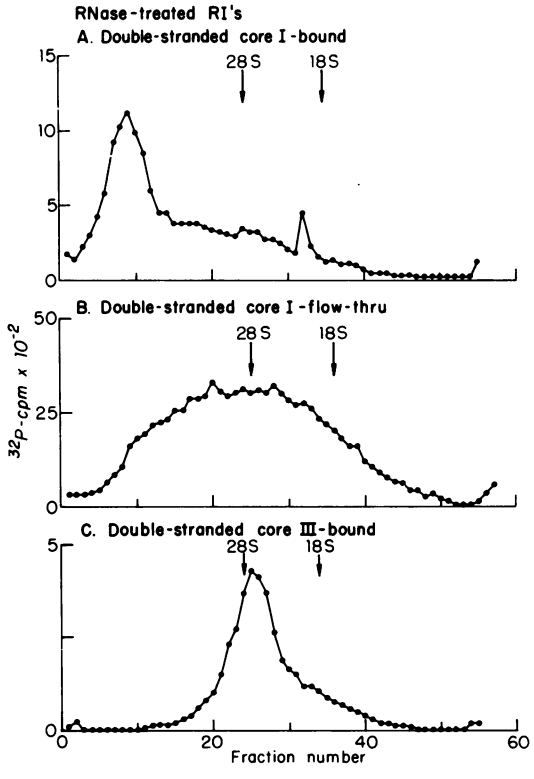

FIG. 11. *Sedimentation analysis on sucrose density gradients of the poly(U)-containing RNA in RFI and RFIII obtained from RIs. The RIs, labeled with [32P]orthophosphate, were obtained at 2 h after infection, and they were treated for 15 min at 25°C with RNase A at 0.02 μg/ml before centrifugation on sucrose density gradients as described in Fig. 2. The three cores obtained were purified by a second similar centrifugation of the peak fractions. After denaturation with heat in the presence of excess unlabeled poly(U), the RNA in each of the cores was chromatographed on poly(A)-Sepharose. Both the RNAs that did not bind and those that were eluted with 90% formamide were collected. Analysis for size was on sucrose density gradients in 0.2% SDS buffer. Centrifugation was in the SW41 rotor at 90,000 × g for 16 h at 20°C. Fractions were collected from below and analyzed for radioactive material by counting in a toluene-based scintillation fluid containing Triton X-100. (A) RNA containing a poly(U) sequence from core I or RFI which bound to poly(A)-Sepharose; (B) RNA from core I that did not bind to poly(A)-Sepharose; and (C) RNA containing a poly(U) sequence from core III or RFIII that bound to poly(A)-Sepharose.*

determined from the amount of this RNA that bound to poly(U)-Sepharose.

The minus-strand RNA in RFIII contained a poly(U) sequence (Table 7). Essentially all of the expected labeled minus-strand RNA of RFIII bound to poly(A)-Sepharose, and, when analyzed by sucrose density gradient centrifugation, most of the minus-strand RNA sedimented at 27S (Fig. 11C). The 900 counts in the RFII fractions that bound to poly(A)-Sepharose was less than 3% of the total radioactivity from the three RFs that did bind during chromatography. This amount of labeled RNA represented less than 20% of the total counts expected in the minus-strand RNA of RFII, indicating that if a poly(U) sequence were present in RFII minus-strand RNA, only one of every five RFII minus strands would contain this sequence with the expected length of 80 nucleotides. Because RFII has a sedimentation value intermediate between that of RFI and RFIII, but close to both of them, RFII would be the one most difficult to isolate free of the other two RFs. The most likely interpretation of our results is that RFII does not contain a poly(U) sequence, and that the small amount of RNA in RFII preparations that bound to poly(A)-Sepharose represented minus strands from RFI and RFIII that were present as contaminants in the preparation of RFII.

The finding of a poly(U) sequence in the minus strand of RFI and RFIII and its absence from the minus strand of RFII places the minus-strand RNA of RFIII at the 5'-end of the 42S minus-strand RNA. The minus-strand RNA of RFII would thus be that sequence represented in the remaining two-thirds of the 42S minus-strand RNA, which includes its 3'-end.

DISCUSSION

We have found that there is one poly(A) sequence in Semliki Forest virion RNA. Its length was relatively homogeneous, with about 60% of the poly(A) sequences having a length of 80 to 90 nucleotides. The poly(A) segment is the sequence at the 3'-terminus of virion RNA. The length of the poly(A) segment was derived from three independent findings: (i) from its mobility during gel electrophoresis; (ii) from the the percentage of total radioactivity in labeled 42S RNA that was found in poly(A); and (iii) from the ratio of the total ^3H label found in poly(A) to that in the 3'-terminal adenosine.

Our finding that the plus-strand RNA of both the SFV RFs and RIs contained a poly(A) sequence confirms previous reports (3, 6). The length of the majority of the poly(A) sequences found in SFV RFs and RIs obtained at 2 h after infection, and in preliminary experiments at 4 h after infection, was 80 to 90 nucleotides, the same length that was predominant in virion poly(A). The poly(A) segments in RFs and RIs apparently do not vary in length at different times during the growth cycle. When analyzed

at 6 h after infection, a time after the exponential growth of virus, the majority of the poly(A) segments in RFs were also 80 to 90 nucleotides long (3). This result differs from what occurs during poliovirus replication, where poly(A) sequences were found in some cytoplasmic poliovirus single-stranded RNAs that were twice as long as the poly(A) found in the same species of RNAs synthesized earlier during infection (42, 43, 47, 48).

The addition of poly(A) could result from post-transcriptional modification of the RNA or from transcription of a poly(U) sequence in the minus-strand RNA. No poly(U) sequence was found in virion RNAs from a number of negative-strand RNA viruses (28), indicating that most or all of the poly(A) sequence found in their plus-strand RNAs active as mRNA's during infection must result from addition of the sequence after transcription of the mRNA by the virion-associated transcriptase. The in vivo and in vitro experiments with poliovirus suggested strongly that the poly(A) sequence in poliovirus plus-strand RNA was directly transcribed from the poly(U) sequence in the minus-strand RNA (11, 44, 47, 48). Our results extend these observations and indicate that such transcription is not unique to poliovirus. The poly(A) sequence in Semliki Forest virion RNA and in the plus strand of both RFs and RIs can be, and most likely is, also transcribed directly from the poly(U) sequence that we identified in the SFV minus-strand RNA. Both poliovirus and the alphaviruses have in common three features: (i) a poly(A) sequence at the 3'-terminus of viral plus-strand RNA; (ii) a poly(U) sequence at the 5'-terminus of the viral minus-strand RNA; and (iii) no virion-associated transcriptase.

The poly(U) sequence in the RIs was the same length as its poly(A) sequence. In contrast, the poly(U) sequence in the minus strand of RFs was about 20 nucleotides shorter than the complementary poly(A) sequence present in plus-strand RNA. Even though care was taken in the isolation of RFs, the shorter poly(U) found in its minus-strand RNA could reflect some breakdown of the poly(U) during preparation of the RFs. This may not be the case, however, since the length of the RF poly(U) derived from the ratio of total radioactive label in poly(U) to that in its 5'-terminal residue gave an estimate for the length of the poly(U) close to that determined during electrophoresis on polyacrylamide gels. If in fact the poly(U) sequence in RFs is shorter than the poly(A) transcribed from it, the longer poly(A) could have been synthesized by slippage during transcription of the SFV polymerase on the minus-strand RNA used as template (5), or it could have resulted from the terminal addition (8, 43) of 15 to 20 adenylic acid residues to a poly(A) of 60 residues directly transcribed from the poly(U).

The rapid reannealing of the poly(U) sequence in minus-strand RNA with the poly(A) sequence in plus-strand RNA when the RFs and RIs were denatured in the absence of excess unlabeled poly(U) may explain the inability previously to detect the poly(U) sequence in SFV minus-strand RNA (3). In addition, it is known that synthesis of minus-strand SFV RNA decreases markedly at 3 h after infection and may cease altogether (3, 21). The RFs and RIs in which poly(U) was not detected were isolated from cells labeled continuously with radioactivity up to 7 h after infection. During the latter 4 h of the labeling period, only plus-strand RNAs were being newly synthesized, and thus it would be expected that a minimal percentage of the total radioactive label in the double-stranded RNAs would be in minus-strand RNA.

There was only one poly(U) sequence present per SFV RF or RI. This sequence was located at the 5'-terminus of the minus-strand RNA, since the poly(U) segment had a 5'-phosphorylated residue at its 5'-end. The finding of the 5'-phosphorylated residue of uridine at the 5'-terminus of minus-strand RNA does have important implications concerning the replication of SFV. It indicates that the SFV polymerase that catalyzes the synthesis of minus-strand RNA must bind to and copy the poly(A) sequence found at the 3'-end of 42*S* plus-strand RNA. The direct transcription into the poly(U) sequence at the 5'-end of the minus-strand RNA occurs before the enzyme continues to transcribe the remaining heteropolymeric sequence of the 42*S* virion RNA.

Our results support the model proposed by Simmons and Strauss (38): that RFII and RFIII are originally present as different parts of the same molecule, which is cleaved into two unequal parts by controlled treatment with RNase. Moreover, our data show that there is one poly(U) sequence per 42*S* minus-strand RNA and that this sequence is at its 5'-end. This same poly(U) is found in that part of the 42*S* minus-strand RNA that is the minus strand of RFIII. Therefore, the 26*S* minus strand of RFIII is the 5'-terminal third of the 42*S* minus-strand RNA. Thus, the 26*S* plus-strand mRNA is the sequence that is the 3'-terminal third of the genome 42*S* plus-strand RNA.

Our experiments also indicated that there

was no poly(U) sequence in either strand of RFII. This raised the possibility that there was no poly(A) sequence in the RFII portion of the SFV RFs. Over 80% of the 18S double-stranded RNA, RFII, which was present in the population of RFs not deliberately treated with RNase, did not bind to poly(U)-Sepharose. Equimolar amounts of RFII and RFIII were produced by RNase treatment from about 50% of the 22S RFs or the 20 to 35S RIs that bound to poly(U)-Sepharose. Thus, only when RFII was part of a larger molecule that contained a poly(A) segment could it bind to poly(U)-Sepharose. As RFII, it did not bind. An RNA of 2.8×10^6 daltons that would have the size expected for the plus-strand RNA of RFII was not detected in infected cells, indicating that it either did not accumulate during infection or was not produced at all (39). Since RFII and RFIII are present in the same molecule of RF or RI, the polymerase-replicating 26S mRNA appears able to recognize on the 42S minus-strand RNA an internal initiation site that is located at a distance two-thirds of genome size from the 3'-end of the template. No evidence has been obtained that the 26S plus-strand mRNA results from cleavage of a larger RNA.

During SFV infection, two mRNA's have been identified, the 26S mRNA and the genome 42S RNA. The 26S mRNA is the template for the synthesis of a polyprotein of 130,000 daltons that is the precursor to all structural viral proteins (7, 23, 40, 46). The 42S RNA is template for a different polyprotein of 300,000 daltons that is precursor to all nonstructural proteins, presumably including the SFV polymerase(s) (4, 24). The 42S RNA has the potential to code for both polyproteins, which together account for the total coding potential of the genome RNA. Two initiation sites for translation have been identified in the genome RNA (4, 40), but in experiments utilizing temperature-sensitive mutants only cleavage products from the protein of 300,000 daltons were found (24). This would indicate that the internal initiation site in the 42S genome RNA may not be recognized, a result similar to what has been found in studies of translation of some plant viral RNAs (36).

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