Semliki Forest Virus Intracellular RNA: Properties of the Multi-stranded RNA Species and Kinetics of Positive and Negative Strand Synthesis

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SUMMARY

Three replicative forms of RNA (RF I, RF II, and RF III) have been isolated from BHK cells infected with Semliki Forest virus. Using analytical and rate-zonal sedimentation the mol. wt. of these replicative forms were estimated to be $8.5 \times 10^6$, $5.5 \times 10^6$ and $3.1 \times 10^6$ respectively. After continuous labelling from 1 to 6 h post-infection, RF I constituted more than 80% of the total replicative forms. Competition hybridization experiments showed that one strand of RF I was 42S RNA which had opposite (negative) polarity to that found in the virus particle. The positive strand of RF I was 42S RNA. The negative strand of replicative intermediate (RI) was also found to be 42S RNA. No evidence was found for an RI with a 26S negative strand. RF I was shown to contain non-hydrogen bounded poly A at or near the 3' end of the component 42S positive strand. Isolation and analysis of the poly A tract from RF I on an acrylamide gel showed it to be of essentially the same average size as the poly A tract from virus particle RNA. About 30% of the RI molecules contained non-hydrogen bonded poly A. No poly U was detected in either RF I or RI. The kinetics of positive and negative strand synthesis were investigated during virus multiplication. These experiments showed that the rate of negative strand synthesis reaches a maximum at 2.5 h post-infection and thereafter rapidly falls. The rate of positive strand synthesis increases rapidly up to 3 h post-infection and then remains constant for a further 3 to 4 h.

INTRODUCTION

The genome of alphaviruses such as Semliki Forest virus (SFV) and Sindbis virus is single-stranded RNA which is infectious (Friedman, Levy & Carter, 1966) and which has a mol. wt. of $4.2 \pm 0.3 \times 10^6$ (Levin & Friedman, 1971; Simmons & Strauss, 1972a; Martin & Burke, 1974) and a sedimentation coefficient of about 42S (Sonnabend, Martin & Mécès, 1967; Cartwright & Burke, 1970). In addition to this RNA, infected cells contain three other single-stranded RNAs. These are the 26S RNA (mol. wt. $1.8 \pm 0.2 \times 10^6$), a major species, and two minor species the 38S RNA (mol. wt. $3.1 \pm 0.2 \times 10^6$) and 33S RNA (mol. wt. $2.3 \pm 0.2 \times 10^6$) (Levin & Friedman, 1971; Kennedy, 1972; Martin & Burke, 1974). The 33S RNA is closely related to the 26S species (Simmons & Strauss, 1974b). All the single-stranded species have the same polarity, i.e. positive polarity (Martin & Burke, 1974), and there is direct evidence that both the 42S and 26S RNAs have messenger
function (Cancedda & Schlesinger, 1974; Cancedda, Swanson & Schlesinger, 1974; Clegg & Kennedy, 1974b; Simmons & Strauss, 1974a; Smith et al. 1974; Wengler, Beato & Hackemack, 1974; Clegg & Kennedy, 1975).

As with many other viruses containing RNA, the multiplication of alphaviruses involves the intermediate synthesis of structures containing RNA of both positive and negative polarity. On extraction from the infected cell these structures give rise to two hydrogen-bonded forms of RNA. These are the replicative intermediate (RI) which contains single-stranded non-hydrogen-bonded regions and the replicative form (RF) which is much more extensively hydrogen-bonded. These RNAs sediment in sucrose gradients between 16S and 28S (Pfefferkorn, Burge & Coady, 1967; Friedman, 1968; Yin & Lockart, 1968). Free single-stranded RNA of negative polarity has not been isolated from infected cells (Simmons & Strauss, 1972b; Martin & Burke, 1974) and it is presumed that RNA of this polarity is extracted as a component of the RF and RI structures. Recently, Simmons & Strauss (1972b), Segal & Sreevalsan (1974) and Martin & Burke (1974), identified three forms of RF in infected cell extracts and showed that a study of RNA hydrogen-bonded structures can provide information on the rate of virus-specified RNA synthesis, the number of polymerase molecules per RNA replication complex and the possible existence of different replication complexes for the synthesis of 42S and 26S single-stranded RNA.

In the present paper we describe the characterization of the RFs isolated from SFV-infected BHK cells by salt precipitation, cellulose column chromatography and preparative acrylamide gel electrophoresis. We report on the mol. wt. of the RFs and on the strand composition and non-hydrogen-bonded homopolyribonucleotide content of the major RF and of RI. We also describe the kinetics of positive and negative strand synthesis during virus multiplication.

METHODS

Materials. Actinomycin D was a generous gift from Merck, Sharpe and Dohme Research Laboratories, N.J., U.S.A. Sodium dodecyl sulphate (SDS; especially pure grade), formamide and dimethyl sulphoxide (both AnalAr) were obtained from British Drug Houses Ltd, Poole, Dorset. Deoxyribonuclease (ribonuclease-free) and venom phosphodiesterase (exonuclease) were purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Pancreatic ribonuclease A (A grade) and ribonuclease T1 and T2 (both Sankyo products) were obtained from Calbiochem Ltd, London. Agarose was supplied by L'Industrie Biologique Française S.A., Gennevilliers, France, and oligo (dT)-cellulose by G. D. Searle and Co. Ltd, High Wycombe, Bucks. Minimal essential medium (suspension culture grade; MEMS) was obtained from Flow Laboratories Ltd, Irvine, Scotland. Griffin and George Ltd, London, supplied the Whatman CF11 cellulose. Sephadex G-200, Sepharose 2B, poly U-Sepharose 4B and CNBr-Sepharose 4B were obtained from Pharmacia, London. [5-3H]-uridine-5'-diphosphate (4-7 Ci/mmol), [5-3H]-uridine 24 Ci/mmol), [2-3H]-adenosine (22 Ci/mmol), [32P]-orthophosphate (88 to 100 Ci/mg phosphorus) and [2-3H]-adenosine-5'-diphosphate (3-1 Ci/mmol) were all obtained from The Radiochemical Centre, Amersham, Bucks. Acrylamide and N,N'-methylenebisacrylamide (both from Kodak Ltd., Kirkby, Liverpool) were recrystallized from chloroform and acetone respectively (Loening, 1967). Phenol, ethanol and ether were all re-distilled and formamide recrystallized at -8 °C before use. All other chemicals were the best grade available commercially.

Virus. Three-times plaque-purified wild-type ts+ SFV was used throughout and plaque
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assayed as previously described (Walters, Burke & Skehel, 1967; Kennedy & Burke, 1972). Virus inocula were prepared by a single further passage in suspensions of chick embryo fibroblasts (Kennedy & Burke, 1972).

**Cells and media.** Monolayer cultures of BHK cells, clone 13, were grown in 14 cm plastic Petri dishes or in 2.5 l smooth-walled roller bottles as described by Morser, Kennedy & Burke (1973). BHK cells adapted to grow in suspension were cultivated in MEMS supplemented with 0.3 % tryptose phosphate broth and 10 % calf serum. Cultures, contained in water-jacketed vessels, were maintained at 37 °C, and the pH automatically adjusted to 7.4 by gassing with 95 % air/5 % CO₂.

**Infection and labelling of suspension culture BHK cells.** Immediately before infection 2 l of BHK cells at a density of approx. 7.5 × 10⁵ cells/ml were collected by centrifuging, washed 3 times with 250 ml of warmed 20 mM-HEPES-buffered phosphate-free MEMS containing 2 % dialysed calf serum and 1 μg/ml actinomycin D and suspended in 50 ml of the same medium. The culture was cooled to 4 °C and virus added to an input multiplicity of 20. After 1 h, 2 l of warmed medium was added and the infected culture replaced in the growth vessel. At the times indicated in the text (specified relative to the end of the adsorption period) a 100 ml portion of the infected culture was withdrawn into a 500 ml conical flask and 1 mCi [³²P]-orthophosphate added. After gently shaking at 37 °C for 5 min the cells were collected by centrifuging, washed twice with ice-cold PBS, once with 50 mM-tris containing 1 mM-EDTA and 0.1 M-NaCl (pH 7.4) and total nucleic acids extracted as previously described (Clegg & Kennedy, 1974a).

**Preparation of [³H]-labelled virus particle 42S RNA.** SFV was grown in suspensions of chick embryo fibroblasts containing 10 μCi/ml of either [³H]-uridine or [³H]-adenosine and purified as described previously (Kennedy, 1974). Virus particle 42S RNA was extracted using the phenol/SDS/2-mercaptoethanol procedure of Martin & Burke (1974), purified by sucrose gradient sedimentation (Martin & Burke, 1974) and precipitated with 2.5 vol. of ethanol at −20 °C.

**Fragmentation of 42S virus particle RNA.** Purified 42S RNA was recovered from alcohol, washed twice with 70 % ethanol containing 50 mM-NaCl and dissolved in 20 mM-tris containing 1 mM-EDTA (pH 7.2) to a concentration of 400 μg/ml. This solution, was sonicated in a 3 ml polypropylene tube for 5 min at 1A using a 3 mm diam. probe (Soniprobe type 7530A; Dawe Instruments Ltd, London). To aid cooling, the tube was immersed in crushed ice and the current was switched off for 60 sec every alternate min. The fragmented RNA was stored at −70 °C. Analysis on a sucrose velocity gradient showed that it had an average S value of 4.7.

**Preparation of RFs.** The RFs were isolated from SFV-infected BHK cells labelled from ½ to 6 h post-infection with 1.5 mCi [³²P]-orthophosphate/culture as described by Clegg & Kennedy (1974a). After the second CF11 cellulose chromatography step the RFs were separated by preparative acrylamide gel electrophoresis using apparatus and buffers essentially identical to those described by Hagen & Young (1974). The 7 cm gel contained 1.7 % (w/v) acrylamide, 0.085 % bisacrylamide and 0.5 % agarose as described previously (Clegg & Kennedy, 1974a). The gel was run at 80 V fixed potential at room temperature with buffer recirculation and radioactivity in the eluate counted by Čerenkov radiation. Appropriate fractions were pooled and RNA precipitated by adding 2.5 vol. ethanol at −20 °C.

**Preparation of RI.** A deoxyribonuclease-treated total nucleic acid extract from SFV-infected BHK cells labelled from ½ to 6 h post-infection with 1.5 mCi [³²P]-orthophosphate/culture was prepared as described before (Clegg & Kennedy, 1974a). This material was
dissolved in 50 mM-tris containing 0·1 M-LiCl, 1 mM-EDTA and 0·1 % lithium dodecyl sulphate (pH 7·4; TLEL) and chromatographed through a 70 × 1·5 cm column of Sepharose 2B at 4 °C using TLEL as eluant. Carrier tRNA was added to the material eluting in the void volume and the solution made 2 M in LiCl and kept at 4 °C for 48 h. Precipitated material was collected by centrifuging at 20000 g for 40 min at 4 °C and the RI further purified by two successive cycles of CF11 cellulose chromatography (Martin & Burke, 1974).

Sucrose gradient sedimentation analysis. Samples dissolved in 10 mM-tris containing 0·06 M-NaCl, 0·001 M-EDTA and 0·2 % SDS (pH 7·2) were layered over 12 ml 15 to 30 % (w/v) linear sucrose gradients in the same buffer and centrifuged at 195000 g for 10 h at 20 °C. Gradients were unloaded by upward displacement and 7 drop fractions collected. A sample of each fraction was counted in 3 ml of Triton X-100:toluene scintillator.

Analytical ultracentrifugation. Sedimentation coefficients were determined using a 30 mm Kel F band forming centrepiece. Each analysis was done on 10 µg of sample dissolved in 40 µl of 50 mM-tris (pH 7·2). The sedimentation solvent was 50 mM-tris containing 1·0 M-NaCl (pH 7·2). Sedimentation was performed at 20 °C at 40000 rev/min using the AN-E rotor of the Beckman Model E ultracentrifuge. Photographs were taken automatically at 4 min intervals and the $s_{obs}$ corrected to $s_{20,w}$ as described by Bauer & Vinograd (1971).

Analytical RNA polyacrylamide gel electrophoresis. Single-stranded or total virus-specified RNA was analysed on 20 cm gels as described by Clegg & Kennedy (1974a). For the determination of the mol. wt. of SFV RFI, gels containing 2·5, 2·9 and 3·3 % acrylamide were used. The double-stranded RNAs from reovirus (kindly supplied by Dr Luis Garcia of this department) were used as markers. Polyacrylamide gel electrophoresis of poly A derived from RFI and 42S virus particle RNA was performed as described by Yogo & Wimmer (1973). Formaldehyde denatured tRNA was used as marker. After electrophoresis gels were sliced into 1 mm segments, solubilized and counted as previously described (Kennedy & Burke, 1972).

Isolation of the component strands of RFI and RI. The component positive and negative strands of SFV RFI and the negative strand of RI were isolated by hybridization techniques using fragmented virus particle RNA to displace the hydrogen-bonded positive strands (Roy & Bishop, 1970). Specifically, 0·1 to 5 µg of the purified [32P]-labelled RFI or RI was mixed with a 20- to 50-fold weight excess of fragmented [3H]-labelled 42S virus particle RNA to give a final volume of 150 µl in 20 mM-tris containing 1 mM-EDTA (pH 7·2). Dimethyl sulphoxide was added to a concentration of 95 % and the sample incubated at 50 °C for 5 min. The sample was then mixed with 20 mM-tris containing 1 mM-EDTA and 2·4 M-NaCl to reduce the dimethyl sulphoxide concentration to 30 % and increase the NaCl concentration to 0·4 M. Incubation was then continued for 24 h at 37 °C and the RNA precipitated with 3 vol. of ethanol at −20 °C. The remainder of the procedure was determined by whether positive or negative strands were to be isolated. Negative strands, present at this stage as negative strand-fragmented 42S virus particle RNA hydrogen-bonded complexes, were isolated by LiCl precipitation and cellulose chromatography. The hydrogen-bonded complexes were then denatured by sedimentation on a 5 to 20 % (w/v) linear sucrose gradient in 99 % dimethyl sulphoxide (Kelly & Sinsheimer, 1967; Bishop et al. 1969) and [32P]-labelled RNA was recovered by alcohol precipitation and analysed on polyacrylamide gels. Positive strands were isolated directly by cellulose chromatography and then analysed on polyacrylamide gels. To determine the distribution
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of radioactivity in the positive and negative strands of RI and RF, these RNAs were denatured and reannealed using a 30-fold excess of unlabelled, intact virus particle RNA and then digested with pancreatic and T₁ ribonucleases (Clegg & Kennedy, 1974a). Radioactivity resistant to digestion was taken as a measure of negative strands and this value subtracted from the total TCA-precipitable count as a measure of positive strands in multi-stranded RNA.

Preparation of poly A-Sepharose. CNBr-Sepharose 4B was washed with 1 mM-HCl and distilled water, and 2 g mixed with 60 mg of poly A in 12 ml of 0-1 M-NaHCO₃ and 0-5 M-NaCl, pH 8-0 (CN buffer). Coupling was performed at 21 °C for 2 h on an end-over-end roller and the gel washed extensively with CN buffer. Remaining active groups were blocked with 0-5 M-glycine in CN buffer at 4 °C for 16 h and the gel washed using the acetate/borate procedure recommended by the manufacturers. Finally the gel was washed with binding buffer (see below) and stored at —20 °C in 50 % glycerol.

Chromatography through poly A and poly U-Sepharose. A jacketed 60 × 5 mm column of either poly A- or poly U-Sepharose was washed with 85 % dimethyl sulphoxide containing 0-15 M-EDTA, pH 7-4 (elution buffer; Yogo & Wimmer, 1973) and equilibrated with 50 mM-tris containing 0-7 M-NaCl, 10 mM-EDTA, 0-1 % SDS and 25 % formamide, pH 7-5 (binding buffer) at 21 °C. The sample was dissolved in binding buffer and applied to the column in a volume of 1 to 1-5 ml at 10 °C. Unbound material was eluted with 20 ml of binding buffer at 21 °C. The temperature was raised to 80 °C and bound material eluted with 15 ml of elution buffer. Using [³H]-poly U the binding capacity of the poly A-Sepharose was > 100 μg of poly U per ml of gel.

Hybridization of poly A and poly U to RF I. Hybridization of [³H]-poly A or [³H]-poly U (prepared as described by Clegg & Kennedy (1974a) to asp. act. of 162 to 170 × 10⁶ cl/min/μg) to [³²P]-labelled RFs was performed as described by Gillespie, Marshall & Gallo (1972). Hybrid formation was detected by sucrose gradient sedimentation (see above).

Detection of poly U in denatured RF I and RI. This was performed as described by Marshall & Gillespie (1972) and Yogo & Wimmer (1973).

Treatment of RF I with venom exonuclease. Digestion was with 50 μg/ml of Dowex 50 purified enzyme (Laskowski, 1966) at 21 °C for 20 min in 0-01 M-tris containing 0-01 M-MgCl₂ and 0-1 M-NaCl, pH 8-5. After treatment, SDS was added to 1 % and the RNA phenol extracted and alcohol precipitated with 100 μg carrier tRNA (Clegg & Kennedy, 1974a).

Isolation of the poly A tracts from RF I and virus particle RNA. Purified RF I was denatured by incubating at 130 °C for 3 min in 200 μl of 0-4 M-NaCl containing 1 mM-EDTA, pH 7-4. This solution was rapidly cooled to 37 °C and then immediately mixed first with 200 μl of [³H]-adenosine labelled virus particle RNA in 0-1 M-tris containing 0-4 M-NaCl and 1 mM-EDTA, pH 7-4, and then with 20 μl of a mixture of pancreatic and T₁ ribonucleases (final concentrations of 10 μg/ml and 50 U/ml respectively). After incubation at 37 °C for 30 min the poly A tracts were isolated by oligo(dT)-cellulose chromatography as previously described (Clegg & Kennedy, 1974a).

RESULTS

Sedimentation coefficient and mol. wt. of SFV RFs

SFV RFs were isolated from the total nucleic acid extract of infected cells by LiCl precipitation, chromatography through CF₁₁ cellulose using the ethanol elution procedure of Franklin (1966) and preparative polyacrylamide gel electrophoresis. The gel elution
profile of the RFs isolated from cells labelled with $^{32}$P-orthophosphate from $\frac{1}{2}$ to 6 h post-infection (Fig. 1), shows three species of RF. In increasing order of electrophoretic mobility, they are RF I, the major species, and two minor species, RF II and RF III. The distribution of radioactivity (expressed as a percentage of the total RF radioactivity) was RF I, 82%; RF II, 11%; and RF III, 7%. Although these percentages varied, both with pulse length and as a function of time of pulse post-infection, RF I was always the major species, accounting for over 70% of the total radioactivity (Martin & Burke, 1974).

Recently Michel & Gomatos (1973) reported a value of $4.4 \times 10^6$ for the mol. wt. of the major RF extracted from SFV-infected BHK cells. This value was obtained using polyacrylamide gel electrophoresis and we therefore attempted to determine the mol. wt. of purified RF I using this technique. The value of $4.3 \times 10^6$ obtained (Fig. 2) was in good agreement with that of Michel & Gomatos (1973). In contrast to this value however, Simmons & Strauss (1972b) reported that the largest RF from Sindbis virus-infected BHK cells has a mol. wt. of $8.8 \times 10^6$ as determined by sucrose gradient sedimentation. It appeared therefore that the largest RF from SFV-infected cells was only half the mol. wt. of the largest RF from Sindbis virus-infected cells. This apparent difference in mol. wt. is hard to reconcile with the close similarities observed between other SFV-specified RNAs and their counterparts in Sindbis virus-infected cells. In an attempt to clarify this point we determined the $s$ value of RF I using analytical ultracentrifugation. The $s_{20,w}$ value obtained
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Fig. 2. Determination of the mol. wt. of RFI by polyacrylamide gel electrophoresis. $[^{32}P]$-labelled RFI purified as described in the legend to Fig. 1 was mixed with $[^{3}H]$-labelled double-stranded RNA from reovirus and analysed on 20 cm gels: △△△, 3.3%; ○○○, 2.9%; ●●●, 2.5% polyacrylamide. After electrophoresis the gels were fractionated and radioactivity determined in 1 mm segments. The mol. wt. of reovirus double-stranded RNA are taken from Shatkin, Sipe & Loh (1968). The mol. wt. was 22.9. Using the Studier (1965) equation $s = M^{0.346}$ this corresponds to a mol. wt. of $8.5 \times 10^6$. This value is almost twice that obtained by gel electrophoresis and close to that obtained by Simmons & Strauss (1972b). As will be apparent when we report on the strand composition of RFI, it is the higher of these two mol. wt. that is correct and we conclude that gel electrophoresis as employed both in the present study and by Michel & Gomatos (1973) gives a grossly inaccurate value for the mol. wt. of SFV RFI RNA. The $s_{20,w}$ of RFI was 19.8S and RFI was determined on sucrose density gradients using RFI as internal marker. The values obtained were 19.8S and 16.7S, which correspond to mol. wt. of $5.6 \times 10^6$ and $3.1 \times 10^6$ respectively.

Strand composition of RFI

The strand composition of RFI was investigated using displacement hybridization techniques (Roy & Bishop, 1970). The negative strand was isolated by denaturing $[^{32}P]$-labelled RFI using dimethyl sulphoxide in the presence of excess $[^{3}H]$-labelled fragmented 42S virus particle RNA. (The use of dimethyl sulphoxide as chaotropic agent greatly improved the yield of intact single-stranded RNA from RFI.) After reannealing the $[^{32}P]/[^{3}H]$-hybrid was isolated as described in Methods, denatured again and analysed on sucrose gradients containing dimethyl sulphoxide. The result of one such analysis is shown in Fig. 3(a). All the $[^{3}H]$-radioactivity was present at the top of the gradient, confirming the complete denaturation of the $[^{32}P]/[^{3}H]$-hybrid. A single peak of $[^{32}P]$-radioactivity
was observed at a position corresponding to a sedimentation coefficient of approx. 40 to 44S. This RNA, which contained 38% of the input [32P]-RF radioactivity, was precipitated with ethanol in the presence of carrier tRNA and analysed by polyacrylamide gel electrophoresis (Fig. 3b). Again a single peak of radioactivity was observed which had an electrophoretic mobility identical to that of virus particle 42S RNA and is thus, presumably, of identical size. In order to confirm the polarity of this RNA as negative, a portion was mixed with virus particle RNA under annealing conditions and then digested with pancreatic and T1 ribonucleases. Over 97% of the [32P]-radioactivity was made RNAse resistant by this procedure.

To identify the positive strand of RF I, an analogous approach was used. After the formation of the [32P]/[3H]-hybrid, the released [32P]-labelled positive strand RNA was precipitated with LiC1 and purified by CF1 I chromatography. This material, when analysed on a polyacrylamide gel (Fig. 3c), consisted almost entirely of 42S RNA. From these experiments we conclude that RF I consists of a duplex of 42S positive and negative strands. Such a structure would have a mol. wt. of approx. 8.4 x 10^6 (see previous section).
Fig. 4. Identity of the negative strand of RI. [\(^{32}\)P]-labelled RI was purified from a total nucleic acid extract of SFV-infected BHK cells as described in Methods. The negative strand of the RI was isolated by displacement hybridization as described in the legend to Fig. 3. This RNA (○---○) and [\(^{3}H\)]-labelled total virus-specified RNA (●---●) were analysed on two separate 1.7 (w/v) acrylamide + 0.5 agarose gels. Migration is towards the right.

**Nature of the negative strand of RI**

The negative strand of purified replicative intermediate was isolated exactly as described above for RF I. On a polyacrylamide gel (Fig. 4) this material consisted predominantly of 42S RNA together with some smaller fragments probably derived from partially degraded RI molecules. No evidence was obtained for a negative strand of the 26S size class.

**Presence of non-hydrogen-bonded homopolynucleotide tracts in RF I and RI**

We have previously shown that SFV RFs bind to oligo (dT)-cellulose; a property indicative of the presence of non-hydrogen-bonded poly A (Clegg & Kennedy, 1974a). In the present study we examined purified RF I and RI for the presence of both non-hydrogen-bonded poly A and poly U. Two approaches were used in these studies. First, synthetic [\(^{3}H\)]-poly A or poly U (both with essentially the same sp. act.) was incubated with RF I under annealing conditions and any hybrid formed between the synthetic homopolymer and its complement in RF I was detected by gradient sedimentation. Secondly, RF I and RI, either alone or after annealing with unlabelled poly A or poly U, were chromatographed through poly A-Sepharose and poly U-Sepharose. Using these techniques to analyse RF I for poly A we obtained the results shown in Fig. 5. Panel (a) shows that [\(^{3}H\)]-poly U annealed to RF I and (b) that about 75% of the RF I molecules bound to poly U-Sepharose. This binding can be prevented by prior incubation of RF I with poly U. These results suggest that most of the RF I molecules contain non-hydrogen bonded poly A. In contrast no non-hydrogen bonded poly U could be detected in RF I using analogous techniques (Fig. 6).

When purified RI was chromatographed through poly U-Sepharose, 27% of the radioactivity bound to the column. This binding could be reduced to 2% by prior incubation with poly U. No binding of RI to poly A-Sepharose could be detected. From these results we conclude that a small proportion of the RI molecules contain non-hydrogen bonded poly A but that non-hydrogen bonded poly U is not present.
Fig. 5. Analysis of RF I for non-hydrogen bonded poly A. (a) [3H]-poly U both alone and after mixing with purified RF I were incubated under annealing conditions (Gillespie et al. 1972) and fractionated on 15 to 30 % (w/v) sucrose gradients. Sedimentation is from right to left. Radioactivity in fractions from the gradients containing [3H]-poly U alone (●—●) and RF I+[3H]-poly U (○—○) was determined. The arrow indicates the position of purified [32P]-labelled RF I run on a parallel gradient. (b) [32P]-labelled purified RF I was chromatographed through poly U-Sepharose as described in Methods. X indicates the change from 10 °C to 21 °C and Y the change to elution buffer and 80 °C. Whole fractions (1 ml) were counted by Čerenkov radiation (○—○). In a separate experiment unlabelled poly U was incubated with RF I in binding buffer before chromatography (●—●).
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Fig. 6. Analysis of RF I for non-hydrogen bonded poly U. (a) [3H]-poly A both alone and after mixing with purified RF I were incubated under annealing conditions and fractionated as described in the legend to Fig. 5. Sedimentation is from right to left. Radioactivity in fractions containing [3H]-poly A alone (●—●) and RF I+[3H]-poly A (○—○) was determined. The arrow indicates the position of purified [32P]-labelled RF I run on a parallel gradient. (b) [32P]-labelled purified RF I was chromatographed through poly A-Sepharose as described in Methods. The significance of X and Y are as in Fig. 5. Whole fractions (1 ml) were counted by Čerenkov radiation (○—○). In a separate experiment unlabelled poly A was incubated with RF I in binding buffer before chromatography (●—●).
Fig. 7. Formaldehyde-polyacrylamide gel electrophoresis of the poly A tracts from RF I and virus particle RNA. Purified [\(^{32}P\)]-labelled RF I and [\(^{3}H\)]-adenosine-labelled 42S virus particle RNA were mixed, denatured and then digested with pancreatic and T\(_{1}\) ribonucleases as described in Methods. The poly A tracts from RF I (○○) and from virus particle RNA (●●) were recovered by oligo(dT)-cellulose chromatography and analysed on a 10 % (w/v) polyacrylamide gel containing formaldehyde. The arrow indicates the position of denatured tRNA run on a separate gel.

In order to investigate the possibility that the secondary structure of RF I and/or RI masks any poly U tract, we denatured RF I and RI and searched for a poly U tract by the Marshall & Gillespie technique (1972). Again, no evidence for a poly U tract was found in either RF I or RI.

**Location and relative size of the poly A tract in RF I**

The location and structure of the poly A tract in RF I was investigated by treating RF I with venom exonuclease (a 3' exonuclease) and then examining the ability of the treated molecules to bind to poly U-Sepharose under conditions which minimized degradation of the RNA duplex. In addition, RNA recovered after treatment was centrifuged through a sucrose gradient (see Methods) and only those molecules with the same s value as native RF I were chromatographed through the affinity column. After treatment RF I completely failed to bind to the poly U-Sepharose column showing that the poly A tract is located at or near the 3' end of one or both of the component strands. Since the poly A tract in 42S virus particle RNA is located at the 3' end (Hsu, Kung & Davidson, 1974), it seems likely that the poly A tract in RF I is located at the 3' end of the component 42S positive strand. This was confirmed by the observation that isolated 42S negative strands from RF I did not bind to poly U-Sepharose.

To determine the relative size of the poly A tract in RF I the RNA duplex was denatured and the resultant single strands, together with added [\(^{3}H\)]-adenosine labelled virus particle 42S RNA, were digested with pancreatic and T\(_{1}\) ribonucleases and the poly A tracts recovered by oligo(dT)-cellulose chromatography and analysed on a polyacrylamide gel containing formaldehyde. The result (Fig. 7), shows not only heterogeneity in both
Kinetics of positive and negative strand synthesis during virus multiplication

The kinetics of synthesis of SFV intracellular positive and negative strand RNA were investigated in BHK cells as described in Methods. Since negative strand RNA is extracted from the cell as a component of RF and RI, these two classes of RNA were purified together using chromatography through Sepharose 2B and CF11 cellulose. Radioactivity in both the positive and negative strands of these RNA structures was then determined using displacement hybridization. Radioactivity in the free positive strands was measured using the same column fractionation techniques.

The radioactivity incorporated into positive and negative strands in 5 min periods at intervals throughout the virus growth cycle was determined and is shown in Fig. 8. The rate of negative strand synthesis reached a maximum 2½ h post-infection and then rapidly decreased. The rate of positive strand synthesis increased from 1½ to 3 h post-infection and thereafter continued at an almost constant rate till approx. 6 h post-infection.
the mol. wt. of each strand as approx. $4.2 \times 10^6$, this corresponds to a mol. wt. for RF I of $8.4 \times 10^6$ which is in good agreement with the value obtained from the analytical ultracentrifugation analysis. We do not know why our attempts and those of Michel & Gomatos (1973) and B. A. B. Martin (unpublished observation) to determine the mol. wt. of RF I by gel electrophoresis gave inaccurate values. Either the relationship between log (mol. wt.) and mobility of double-stranded RNA is not linear in the mol. wt. range of RF I, or RF I migrates anomalously through polyacrylamide gels.

Because of the relatively poor labelling of RF II and RF III (see Fig. 1) we were unable to determine the strand composition of these molecules. Therefore, even although the mol. wt. of RF II plus RF III is close to that of RF I it cannot be assumed that the minor species represent contiguous parts of RF I as has been suggested for the corresponding species isolated from Sindbis virus infected cells (Simmons & Strauss, 1972b).

Only 42S RNA of negative polarity was isolated from RI. From studies on the nature and types of RF 'cores' derived by ribonuclease treatment of RI/RF, Simmons & Strauss (1972b) and Segal & Sreevalsan (1974) have proposed that the synthesis of 42S and 26S RNAs occurs on two distinct replication complexes. If this is the case, then the present study indicates that both complexes use 42S negative strand as template. The observation that RF I consists of an intact 42S positive and negative strand, eliminates the possibility that this form of RNA is derived from RI by ribonuclease digestion of nascent single strand tails. The status of such a duplex in RNA synthesis is however uncertain. On the one hand the kinetics of labelling of SFV, RI, RF and single-strands both in vivo (Martin & Burke, 1974) and using pulse-chase conditions in vitro (Michel & Gomatos, 1973) indicate that RF plays an intermediate role in the synthesis of single-stranded RNA. On the other hand experiments with poliovirus suggest that RF is a consequence rather than an intermediate of RNA replication (Noble & Levintow, 1970). It should however be borne in mind that RF I may arise during extraction by collapse of 42S positive and negative strands. If this were the case then the kinetic data for SFV might be explained by a collapse of RNA replicating structures which contain only a single replicate molecule located at the 5' end of the template strand. Experiments with cell-free RNA synthesising systems should clarify many of these issues.

Another aspect of this transcriptional problem is the mechanism of polyadenylation. The observation that RF I contains a non-hydrogen-bonded poly A tract at the 3' end of its component 42S positive strand suggests that addition of the polyadenylic acid tract occurs before nascent 42S RNA is released from the RNA-synthesizing complex. The limited binding of RI to poly U-Sepharose supports this idea if it is assumed that binding only occurs with those molecules containing a completed progeny 42S positive strand. It should be borne in mind, however, that we do not know to what extent this binding is by 'negative' RI molecules, i.e. molecules derived from complexes synthesizing negative strand RNA and using 42S positive strand as template. The absence of poly U in both RF I and RI indicates that the poly A tract is added by some mechanism other than transcription of a complementary poly U segment. This situation is different from that reported for poliovirus, where the RF contains both poly A and poly U. It would appear, therefore, that the alphaviruses, although belonging to the same group as the picornaviruses in the classification system of Baltimore (1971), acquire their polyadenylic acid tract by a post-transcriptional mechanism similar to that reported for reovirus (Stoltzfus et al. 1974) and several of the negative strand viruses such as Newcastle disease virus (Weiss & Bratt, 1974) and vesicular stomatitis virus (Ehrenfeld, 1974). Whether this polyadenylation is mediated by the replicate itself or by a separate enzyme or indeed whether the adenylc
acid residues are added singly or as a pre-formed segment remains to be determined. In this context it is interesting to note that we have found that cordycepin, an inhibitor of nuclear post-transcriptional polyadenylation (Adesnik et al. 1972), has no measurable effect either on SFV multiplication or on the average size of the 42S virus particle RNA poly A tract.

Our studies on the kinetics of positive and negative strand synthesis during virus multiplication reveal a number of interesting features. First, they show that the synthesis of negative strands is temporally regulated. From the earliest time of detection, about 1½ h post-infection, to 2½ post-infection the rate of negative strand synthesis increases rapidly. Thereafter the rate falls sharply and by 4 h post-infection is undetectable. Secondly, the rate of positive strand synthesis increases very rapidly during, and slightly beyond, the time when the rate of negative strand synthesis is also rising. Thereafter, positive strand synthesis continues at an essentially constant rate until 6 h post-infection. Taken together these observations strongly suggest a close correlation between the intracellular content of negative strands and the rate of positive strand synthesis. If the replicase responsible for positive strand synthesis does not turn over, then synthesis of this protein, and also of the negative strand replicase (if it is a different enzyme), could stop by 3 h post-infection. Support for this idea comes from the observations that (a) cycloheximide, if added after 4 h infection, has no effect on the rate of RNA synthesis (Friedman & Grimley, 1969) and (b) that almost all of the RNA negative mutants of the HR strain of Sindbis virus (Berge & Pfefferkorn, 1966) and 9 of the 12 RNA negative mutants of SFV (Tan, Sambrook & Bellett, 1969) produce essentially normal virus yields if left for 4 h at the permissive temperature before shifting to the restrictive temperature. This model would then divide the multiplication cycle of SFV in BHK cells into two stages. During the first stage, translation of 42S virus particle RNA gives the replicase(s) for negative and positive strand synthesis; this stage lasts until about 3 h post-infection. The first event of the second stage is the switch of virus-specified messenger RNA from 42S to 26S RNA. 26S RNA, the messenger RNA for the structural proteins of the virus particle (e.g. Clegg & Kennedy, 1974b; Simons & Strauss, 1974a), is the virus-specified RNA found on polysomes at 4 h post-infection (Kennedy, 1972). This switch frees 42S RNA from a messenger function, thereby allowing its direct encapsidation into progeny virus particles.

REFERENCES


SFV intracellular RNA


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