Sindbis Virus RNA Replication. I. Properties of the 38S RNA Species

By CHRISTINE W. CZARNIECKI* AND T. SREEVALSAN

Department of Microbiology, Georgetown University Medical and Dental Schools, Washington, D.C. 20007, U.S.A.

(Accepted 16 March 1979)

SUMMARY

Four species of single-stranded virus RNA (49S, 38S, 33S and 26S) were detected in chick embryo fibroblasts infected with Sindbis virus. The relative amounts of these RNAs were unaffected by the m.o.i. There was also no significant difference in the molar proportions of the four RNA species when purified virion RNA was used as the inoculum. These findings suggest that the 38S and 33S species represent products of the transcription of non-defective virion RNAs. Kinetic analyses of RNA synthesis indicated that during a 1 min pulse more radioactivity was associated with the 38S than with the 49S RNA and as the length of the pulse increased, the ratio of 38S/49S decreased, with the 49S appearing as the predominant species. Furthermore, addition of cycloheximide within the first 3 h p.i. resulted in detection of only the 49S species. Synthesis of all four species was unaffected when the drug was added after this time period. These data suggest that the 38S species may represent newly synthesized 49S molecules and some protein(s) synthesized within the first 3 h p.i. is necessary for maintaining the 38S conformational form.

INTRODUCTION

In addition to the 49S virion RNA, three additional species of single-stranded RNAs are found in cells infected with Sindbis virus (SBV; Levin & Friedman, 1971; Rosemond & Sreevalsan, 1973). The two major classes, 49S and 26S (interjacent RNA), have estimated mol. wt. of $4.9 \pm 0.3 \times 10^6$ and $1.84 \pm 0.2 \times 10^6$ respectively (Simmons & Strauss, 1972). The two minor species with sedimentation coefficients of 38S and 33S observed in infected cells (Levin & Friedman, 1971; Rosemond & Sreevalsan, 1973) have mol. wt. of $3.1 \times 10^6$ and $2.3 \times 10^6$ respectively, as determined by polyacrylamide gel electrophoresis under non-denaturing conditions (Levin & Friedman, 1971). All four RNA species are polyadenylated and possess the same polarity as the virion RNA (Clegg & Kennedy, 1974).

The SBV genome has a high degree of secondary structure (Sprecher-Goldberger, 1964) and the presence of 'panhandles', as seen by the electron microscope suggests that there are regions of complementary sequences near the termini of the molecules (Hsu et al. 1974). The virion RNA is infectious, i.e. it has a positive polarity (Friedman et al. 1966; Scheele & Pfefferkorn, 1969) and is translated in the infected cell to yield the non-structural (NS) polypeptides that are components of the virus-specified RNA-dependent RNA polymerase (Clegg et al. 1976; Clewley & Kennedy, 1976). The 26S RNA has been shown by competition hybridization to comprise only one third of the nucleotide sequence of the 49S species (Simmons & Strauss, 1972), is located at the 3' end of the genome (Simmons &
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Strauss, 1974b; Kennedy, 1976; Wengler & Wengler, 1976) and acts as the predominant mRNA specifying structural proteins in infected cells (Kennedy, 1972; Mowshowitz, 1973; Rosemond & Sreevalsan, 1973; Simmons & Strauss, 1974b). Little is known about the 38S and 33S RNA species. The 33S species has been reported to be a structural variant of the 26S species (Simmons & Strauss, 1974a). Additionally, the 38S and 33S RNAs behave as conformational forms of the 49S and 26S RNA species, respectively, on polyacrylamide gels run under denaturing conditions (Kennedy, 1976).

The present investigation was carried out to further characterize the 38S RNA and to determine what role(s), if any, it plays in the replication cycle of the virus. Our results suggest that the newly synthesized 49S RNA synthesized in infected cells may exist in the 38S conformational form. Experiments are also reported which indicate that the addition of cycloheximide to cells early during the infectious cycle results in the appearance of only the 49S species of RNA.

METHODS

Materials. Actinomycin D was a gift from Merck, Sharp and Dohme, Rahway, N.J., U.S.A., and was used at a concentration of 1 μg/ml to inhibit the synthesis of cellular RNA. The following materials were purchased from Sigma Chemical Co., St Louis, Miss.: D(+)glucosamine hydrochloride, cycloheximide, the sodium salts of p-aminosalicylic acid and N-lauroyl sarcosine (Sarkosyl). SDS was purchased from British Drug Houses, Poole, Dorset, U.K. Polyvinyl sulphate (PVS) was obtained from K & K Laboratories, Plainview, N.Y. 3H-uridine (sp. act. > 50 mCi/mmol), 14C-uridine (sp. act. > 50 mCi/mmol) and Biofluor, were purchased from New England Nuclear Corp., Boston, Mass. Tris-EDTA-saline (TES) consisting of 0.01 M-tris-(hydroxymethyl)-aminomethane, pH 7.4, 1.0 mM-EDTA and 0.1 M-NaCl was used for suspending nucleic acids.

Viruses and cells. The HR strain and ts24 mutant of Sindbis virus were used. Virus stocks, free of defective particles were prepared by methods similar to those used by Stampfer et al. (1971). Primary cultures of chick embryo (CE) cells were prepared and grown in Eagle’s minimal essential medium (MEM; Sreevalsan & Lockart, 1966). Monolayers of CE cells (1 × 10⁶ cells) were infected with virus that had been plaque-purified through three successive cycles (100 p.f.u./culture). After 30 min of incubation at room temperature, an agar overlay consisting of MEM, 1% calf serum, 1% Bacto-agar and 0.08% protamine sulphate was added and the cultures were incubated at 37 °C for 15 h. At this time, the agar overlay and monolayer of cells were harvested into a large centrifuge tube, MEM containing 3% calf serum was added and the suspension was incubated with shaking, at 4 °C. After 24 h the suspension was centrifuged at 12,500 g for 10 min. The supernatant was removed and the precipitate was washed once with MEM containing 3% calf serum. The supernatants were pooled, assayed for infectious virus and stored at −80 °C to serve as seed virus. The seed virus was used to prepare stock virus by infecting CE monolayers at a m.o.i. of 0.1 p.f.u./cell. After 30 min of adsorption at 27 °C, MEM containing 3% calf serum was added and the cultures were incubated at 27 °C for 10 h at which time the supernatant (stock virus) was harvested, assayed for infectious virus and stored at −80 °C.

The above method was also used for the preparation of ts24 seed and stock virus, with the following modifications: the infected cultures were incubated at 27 °C and infectivity was assayed at both 27 °C and 42 °C to detect the amount of revertants present. All of the ts24 stock preparations used in this study contained less than 0.001% revertants.

Infection and labelling of cells. The methods used were similar to those described previously (Sreevalsan & Yin, 1969). However, the following procedure was adopted for short-term labelling of infected cells. Growth medium containing actinomycin D was added to monolayers of CE cells infected with stock virus and the cultures were incubated at 27 °C.
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Approximately 1 h before the cells were to be pulse-labelled, the cells were removed from the plates by scraping with a rubber policeman and centrifuged at 500 g for 10 min at 37 °C. The cell pellet was carefully resuspended in pre-warmed growth medium lacking calcium and magnesium and incubated, slowly stirring in a 37 °C warm room. The cells were allowed to equilibrate to 37 °C for 15 min, at which time 3H-uridine diluted in growth medium (1 mCi/ml) was added. At the end of the desired pulse period (either 1 or 2 min) the suspension was poured into ice-cold PBS, and centrifuged at 500 g for 10 min at 4 °C. This halted further incorporation of the radioactive nucleoside. Unincorporated radioactivity was then removed by further washes with cold PBS.

Infection of cells using 49S virion RNA. Infectious 49S virion RNA was prepared according to the following procedure. Monolayers of CE cells were infected with SB virus at an m.o.i. of 5 to 10. The cultures were incubated with 3H-uridine and 15 h later, the supernatant fluid containing virus was harvested and centrifuged at 12500 g to remove cellular debris. The fluid was centrifuged at 54000 g for 2 h in a type 30 Spinco rotor to concentrate the virus. The pellet containing labelled virus was resuspended in TES containing 10% SDS, layered on to a gradient composed of 15 to 30% (w/w) sucrose in TES containing 0.25% SDS and centrifuged in a SW41 rotor at 195700 g for 165 min at 23 °C. The peak fractions, corresponding to 49S RNA were pooled and subjected to two more consecutive cycles of centrifugation using the above conditions. The majority of the radioactivity at the end of the third cycle of centrifugation was found in three fractions in the sucrose gradient and possessed a sedimentation rate of about 49S. This RNA was then used to infect cells according to the following method: 48 h-old CE cell monolayers were washed with 0.5 M-NaCl after which 0.5 ml of appropriate dilutions of the inoculum (diluted in 1.0 M-NaCl) was added. Adsorption was allowed to occur for 20 min at room temperature. The inoculum was drained from the cultures followed by successive washes with 0.5 M-NaCl and PBS (containing no calcium or magnesium). At this time either an agar overlay (for plaque assay) or growth medium (when newly synthesized RNA was to be labelled) was added to the cultures.

Isolation of RNA from infected cells. The method used was a modification of that described by Kirby (1968). CE monolayers were washed twice with ice-cold PBS containing PVS (20 μg/ml). The plates were drained to remove traces of PBS and the cultures received 1 ml/plate of a solution containing 4% Sarkosyl and 6% p-aminosalicylic acid. The lysed cells were transferred into a centrifuge tube and mixed with an equal vol. of a solution containing 1% NaCl and 1% SDS and an equal vol. of freshly distilled phenol:cresol:8-hydroxyquinoline (900:100:1). The mixture was shaken for 20 min at room temperature and centrifuged at 25000 g for 20 min at 4 °C. The aqueous phase and interphase were collected. NaCl, to make a final concentration of 3%, was added along with an equal vol. of phenol:cresol:8-hydroxyquinoline, while the phenol phase was re-extracted with a buffer containing 0.01 M-tris, pH 7.4, 0.01 M-KCl, 0.01 M-MgCl2. Both mixtures were shaken for 10 min at room temperature and centrifuged at 20000 g for 10 min at 4 °C. The resulting aqueous phases were pooled and precipitated with 2 vol. of ethanol:cresol (990:100) at -20 °C for at least 2 h. The precipitated nucleic acid was recovered by centrifugation at 20000 g for 30 min and subjected to successive washes with ethanol:NaCl:H2O (375 ml:5 g:125 ml); ethanol:H2O (3:1); and ethanol. The precipitate was then dried under vacuum and dissolved in TES buffer.

Differential precipitation of various virus RNAs. The method used was that previously described (Segal & Sreevalsan, 1974). Phenol-extracted RNA was mixed with an equal vol. of 4 M-LiCl and was allowed to precipitate for 48 h at -20 °C. Under these conditions we obtained complete precipitation of single-stranded RNA.

Preparation of 14C-uridine labelled SBV RNAs. Virus-specific RNA, uniformly labelled
with $^{14}$C-uridine, was prepared in the following manner: CE cells were infected with SBV as described earlier and incubated with actinomycin D. $^{14}$C-uridine (0.5 μCi/culture) was added to the cultures and at 6 h p.i. the cells were harvested and the nucleic acid was extracted. The phenol-extracted RNA was salt-precipitated using 4 M-LiCl. The resulting precipitate, containing the single-stranded RNAs was recovered and samples were used as markers for electrophoretic analyses.

**Analysis of single-stranded virus RNAs by polyacrylamide gel electrophoresis.** Polyacrylamide gels containing 2–5% (w/w) acrylamide, 0.13% (w/w) bisacrylamide and 0.5% (w/v) agarose were prepared according to a modification of the method reported by Schincariol & Howatson (1972). Acrylamide-agarose solutions were prepared in batches of 25 ml as follows: 12.5 ml of 1% agarose was melted in a steam sterilizer for 30 min and cooled to 45 °C; 2.5 ml of the 10 × concentrated electrophoresis buffer consisting of 0.4 M-tris, 0.2 M-sodium acetate, 0.02 M-EDTA, pH 7.5, was mixed with 5.35 ml of water and 4.25 ml of stock solution containing 15% acrylamide and 0.75% bisacrylamide; 0.2 ml of a 10% (v/v) solution of TEMED in water and 0.2 ml of a 10% (w/v) solution of ammonium persulphate in water were added to the acrylamide; the two solutions were combined quickly and poured immediately into pre-warmed glass tubes sealed at one end with Parafilm. The gels were left at 37 °C for 1 h to allow the acrylamide to polymerize and were then transferred to room temperature and the agarose was permitted to solidify for at least 2 h before use. Both ends of the gel were sliced with a sharp scalpel to obtain flat surfaces before they were used. A piece of nylon cloth was used to seal one end of the glass tube and the gel was pre-electrophoresed at 100 V for 1 h using electrophoresis buffer containing 0.2% SDS. Sucrose, at a final concentration of 6 to 10% and trace amounts of bromophenol blue were added to the RNA samples which were then applied to the gels and electrophoresis was carried out at 100 V for 6 h at room temperature. The gels were fractionated using a Gilson Aliquogel fractionator and Biofluor was used for determination of radioactivity in the gel.

**RESULTS**

**Optimal conditions for detection of Sindbis virus RNAs**

The presence of four species of single-stranded RNAs has been reported in cells infected with alphaviruses (Levin & Friedman, 1971; Martin & Burke, 1974; Kennedy, 1976). The predominant species are 49S and 26S while the 38S and 33S represent minor species. Since one of the aims of the present study was to determine the nature of these minor species in SBV-infected cells, conditions for their detection had to be established.

Under conditions of a single-step growth cycle, Sindbis virus possesses a latent period of 2 to 2.5 h in CE cells, subsequent to which an exponential rise in progeny virus is observed (Rosemond & Sreevalsan, 1973; Segal & Sreevalsan, 1974). The maximum yield (2000 p.f.u./cell) is obtained by 12 h p.i. and the majority of infectious virus is produced between 6 and 12 h p.i. Preliminary experiments concerning the kinetics of the virus RNAs synthesized in infected cells showed that initiation of virus RNA synthesis occurred about 30 min after the addition of the virus to the cultures, reached a maximum value between 4 and 6 h and continued to decline thereafter. Therefore, cells infected at a m.o.i. of 10 were incubated with actinomycin D (1 μg/ml) at 1 h pre-infection and pulse-labelled for 30 min with $^3$H-uridine at 4.5 h p.i. The resultant labelled RNA was isolated and then precipitated in 2 M-LiCl to separate the single-stranded from the double-stranded forms of virus RNA; samples of the precipitate were mixed with $^{14}$C-uridine-labelled ribosomal RNAs (45S, 28S and 18S) and analysed by polyacrylamide gel electrophoresis. A typical profile of such an analysis is shown in Fig. 1. Four homogeneous peaks were observed with
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Fig. 1. Analysis of the single-stranded virus RNA species from SBV-infected cells by polyacrylamide gel electrophoresis. Salt-precipitable RNA was obtained from infected cells that were treated with actinomycin D and labelled with 3H-uridine (500 μCi/dish) for 30 min at 4.5 h p.i. A sample was mixed with 14C-uridine labelled ribosomal RNAs obtained from uninfected chick embryo cells, and co-electrophoresed as described in the Methods. Migration in this and all subsequent electrophoretograms is from left to right. • 3H-uridine labelled virus-specific RNA; ○ 14C-uridine labelled ribosomal RNAs. The apparent mol. wt. of the virus RNA species were estimated from the relative mobilities of the ribosomal RNAs (Δ —— Δ).
Fig. 2. Polyacrylamide gel electrophoresis of single-stranded RNA species present in cells under conditions of high and low m.o.i. Monolayers of CE cells were infected with SBV at (a) high (100) or (b) low (0·1) m.o.i. At 1 h p.i. growth medium containing actinomycin D (1 μg/ml) and glucosamine (20 mM) was added and the cultures were re-incubated for 1 h. 3H-uridine (250 μCi/dish) was then added and incorporation was allowed to occur for 30 min. Cultures were then washed with growth medium containing unlabelled glucosamine, cytidine and uridine and re-incubated for 30 min after which the nucleic acid was extracted and salt-precipitated. ••••••••••, 3H-uridine labelled virus-specific RNA; ○—○, 14C-uridine labelled virus-specific RNA, added as markers.

input multiplicity of either 100 or 0·1 p.f.u./cell and incubated with MEM containing actinomycin D (1·0 μg/ml) and glucosamine (20 mM). The salt-precipitable virus RNA synthesized under the above conditions was analysed by polyacrylamide gel electrophoresis and the results are presented in Fig. 2. It can be seen from the results that all four species are represented. The amount of radioactivity associated with each species was determined. From cells infected at high multiplicity, the ratios of 38S/49S and 33S/26S were 0·3 and 0·2, respectively. From cells infected at low multiplicity, the ratio of 38S/49S was 0·4 and the ratio of 33S/26S was 0·3. Thus, there was no enhanced production of the 38S and 33S species in cells infected with a high m.o.i.

The other approach was to analyse the species of RNA found in cells infected with a purified preparation containing only the non-defective 49S virion RNA. The RNA used in the experiment was prepared as described in Methods. Purified virions were dissociated on SDS-sucrose density gradients and the peak fraction corresponding to 49S was recovered and further purified through two cycles of centrifugation on sucrose density gradients. The peak fraction, representing purified 49S was used to initiate infection in CE cells.
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Fig. 3. Electrophoretic analysis of the single-stranded virus-specific RNA species synthesized in cells infected with 49S virion RNA. CE cell cultures were infected with purified preparations of 49S virion RNA (input m.o.i. of 0·05) as described in the Methods. At 1 h p.i. MEM containing actinomycin D (1 μg/ml) and glucosamine (20 mM) was added and the cultures were re-incubated for 1 h. 3H-uridine (250 μCi/dish) was then added and incorporation was allowed to occur for 30 min. Cultures were then washed with MEM containing unlabelled glucosamine, cytidine and uridine and re-incubated for 90 min after which the nucleic acid was extracted and salt-precipitated.

- - - - ,3H-uridine labelled virus-specific RNA; O——O, 14C-uridine labelled virus-specific RNA, added as markers.

Actinomycin D and glucosamine were employed as described in the previous experiment and labelling conditions were identical to those described in the previous section. The salt-precipitable RNA was recovered, analysed by polyacrylamide gel electrophoresis and the results are shown in Fig. 3. The radioactivity ratios of 38S/49S and 33S/26S were, in both cases, 0·3. It has been reported that Sindbis virus defective interfering (DI) RNA can sediment at 49S (Shenk & Stollar, 1973) and purification of 49S RNA by non-denaturing techniques might not be adequate to remove DI RNA. However, the results indicating no enhancement of 38S or 33S species in this experiment suggest that these forms are not transcribed from defective RNAs.

For both experiments, glucosamine was used to ensure a single cycle of virus replication. This compound has been shown to inhibit maturation of several enveloped viruses (Kaluza et al. 1972; Hunter et al. 1974) and our preliminary experiments indicated that glucosamine had the same inhibitory effect whether cultures were infected with whole Sindbis virus particles or purified 49S virion RNA. Additionally, there was little or no inhibition in the amount of virus RNA synthesized in cells receiving glucosamine, as compared to RNA from untreated cells. Thus, glucosamine could be useful in determining the species of RNA synthesized in a single cycle of virus replication.

Kinetics of appearance of the minor RNA species during infection

Results from other laboratories (Bruton & Kennedy, 1975) as well as from ours, have indicated that the rates of synthesis of the positive-stranded species increase very rapidly from 1·5 h to slightly beyond 2·5 h p.i. Thereafter, synthesis continues at an essentially
Fig. 4. Species of single-stranded virus-specified RNA detected in infected cells during a 1 min pulse. CE cells were infected at an m.o.i. of 10 and pulse-labelled with $^3$H-uridine for 1 min as described in the Methods. Separate cultures of CE cells were labelled with $^{14}$C-uridine for 3 h. Samples of the $^3$H-uridine labelled salt-precipitable RNA (○—○) were mixed with $^{14}$C-uridine virus-specific RNA (●—●) serving as markers and analysed by polyacrylamide gel electrophoresis.

Constant rate until 6 h p.i. The synthesis of 49S RNA becomes maximal only during the phase of rapid release of virions (5 to 10 h p.i.) while the synthesis of 26S RNA occurs at a constant rate from 2.5 to 6 h p.i.

Since the 38S and 33S RNAs appear to be conformational forms of the 49S and 26S species (Simmons & Strauss, 1974b; Kennedy, 1976) it was of interest to determine the kinetics of their appearance during virus replication. Infected cells were pulse-labelled with $^3$H-uridine from 2 to 4, 4 to 6 and 6 to 8 h p.i. The salt-precipitable RNA was analysed by polyacrylamide gel electrophoresis and the amounts of radioactivity associated with each of the four species was determined for each time period. The ratios of 38S/49S and 33S/26S were constant, i.e. 0.2 to 0.3 and 0.1 to 0.2 respectively. Comparable values for the first 2 h p.i. could not be interpreted due to the predominance of negative-stranded 49S RNA synthesized during this time period.

The next step in determining the rates of synthesis of the 38S and 33S species was to analyse virus RNA labelled during a short pulse. Infected cells were exposed to $^3$H-uridine for different periods of time at 4.5 h p.i. a time at which the rate of intracellular virus RNA synthesis was maximal. The salt-precipitable RNA recovered from cells pulse-labelled for 1 min was analysed by polyacrylamide gel electrophoresis. The resulting profile, shown in Fig. 4, indicates that under these conditions the majority of the radioactivity was associated with the 26S species, followed by the 38S species. The amount of radioactivity present in 49S and 33S was comparatively small. In the above manner, the amount of radioactivity associated with each of the four single-stranded RNAs appearing during various lengths of pulse was determined and the corresponding ratios of 38S/49S and 33S/26S are shown in Table 1. It is apparent that regardless of the length of the pulse, the ratio of 33S/26S did not vary significantly. In contrast, the ratio of 38S/49S was high during a 1 min pulse but progressively decreased as the length of the pulse increased, reaching a constant value by 30 min.
Table 1. Distribution of radioactivity in single-stranded RNA species during various pulse periods

<table>
<thead>
<tr>
<th>Pulse period (min)</th>
<th>Ratio of radioactivity*</th>
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<tr>
<td></td>
<td>38S/49S</td>
</tr>
<tr>
<td>1</td>
<td>3.0 ± 0.40</td>
</tr>
<tr>
<td>2</td>
<td>2.8 ± 0.40</td>
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<tr>
<td>5</td>
<td>1.6 ± 0.40</td>
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<tr>
<td>30</td>
<td>0.2 ± 0.05</td>
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<tr>
<td>120</td>
<td>0.2 ± 0.05</td>
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* Values are expressed as the mean ± standard error of the mean of four determinations.

of labelling. Therefore, it appears that the 38S species may represent the newly synthesized form of virion RNA found in the infected cell.

**Preferential inhibition of the synthesis of the single-stranded virus RNAs**

Scheele & Pfefferkorn (1969) observed that the addition of either puromycin or cycloheximide to cell cultures early during the course of virus infection (1.5 h p.i.) resulted in the preferential inhibition of 26S RNA synthesis. However, addition of the drugs at 5 h p.i. did not affect the synthesis of either 49S or 26S. A similar result, with respect to preferential inhibition of 26S RNA, was also obtained by the same authors when ts24, an RNA-mutant of SBV, was shifted from the permissive (29 °C) to the non-permissive (41.5 °C) temperature.

Our preliminary experiments confirmed these observations of Scheele & Pfefferkorn (1969). Therefore, it was of interest to determine the fate of the 38S and 33S species under the above conditions. The electrophoretic profiles of the salt-precipitable RNAs isolated from infected cells, incubated in the presence or absence of cycloheximide, added 30 min p.i., are shown in Fig. 5(a) and (b) respectively. As expected, all of the four species of RNAs are present in the control cultures, while the synthesis of the 26S RNA was inhibited in cultures incubated with cycloheximide. Although some radioactivity appears in the regions corresponding to the 38S, 33S and 26S RNAs, no well-defined species other than 49S were observed in the drug-treated cultures. Similar results were observed when cycloheximide was added at 60 or 90 min p.i. However, the profile of radioactivity obtained after analysis of RNA from cultures in which the drug was added at 240 min p.i. closely resembled that presented in Fig. 5(b) – control cultures – suggesting that there was no preferential inhibition of any of the four species of RNA by cycloheximide during the later stages of infection. Although not presented here, we have confirmed another observation originally made by the above authors. When cells infected with ts24 were shifted from 27 °C (permissive temperature) to 42 °C (non-permissive temperature) synthesis of the 26S RNA was inhibited. However, only the 49S species was present and the 33S and 38S forms could not be detected in infected cells under these conditions.

**DISCUSSION**

The data presented in this paper strongly suggest that 38S and 33S, the minor species of virus RNAs found in SBV-infected cells, do not represent defective forms. Although considerable effort was made to prepare virus stocks free of defective particles (see Methods), the possibility that defective particles might be present in these virus stocks can never be completely ruled out. However, no virions other than those containing the 49S RNA, were detected in culture supernatants derived from cells infected with our stock virus. Thus, it appears highly unlikely that defective virions or RNAs have any major influence on the
Fig. 5. Species of single-stranded virus RNAs detected in infected cells in the presence of cycloheximide. CE cell cultures were incubated with growth medium containing actinomycin D (1 μg/ml) and cycloheximide (100 μg/ml). After 30 min, stock SBV was added to the cultures at an input m.o.i. of 10 and the cultures were re-incubated at 37 °C for 1 h. The cultures were pre-treated with cycloheximide before and during adsorption in order to synchronize the infection. The cultures were then washed five times with PBS and incubated with growth medium. The time at which cycloheximide was removed from the cultures represented zero time p.i. At 30 min p.i. the growth medium was replaced with medium containing 3H-uridine (250 μCi/culture) (a) with or (b) without cycloheximide (100 μg/ml). The cultures were harvested 60 min later, the RNA was isolated, salt-precipitated and analysed on polyacrylamide gels. ••, 3H-uridine labelled virus-specified RNA; ©©, 14C-uridine virus-specific RNA, added as markers.

results or the conclusions presented here. The molar ratios of the four species of RNA were unaffected by the multiplicity of virus used for infection. There was no increase in the production of the 38S or 33S species in cells infected at high multiplicity, a condition which favours the production of defective virus RNAs in ribovirus-infected cells (Huang, 1973; Shenk & Stollar, 1973; Bruton & Kennedy, 1975). Moreover, the present results using the 49S RNA from SB virions to initiate infection, indicated no difference in the molar ratio of the four RNA species as compared to the ratio obtained using whole virions. Additionally, glucosamine was used to prevent multiple cycles of virus replication, thus inhibiting the amplification of defective genomes that might arise from re-infection. Thus, it appears that both the 38S and 33S species of RNAs, as studied here, do not represent defective forms.
While the present studies were in progress, Kennedy (1976) reported that the 38S and 33S species represented conformational forms of 49S and 26S respectively and the 38S species contained eight times as many panhandle forms as the 49S RNA by electron microscopic examination. Using the denaturing conditions described by Bailey & Davidson (1976), electrophoresis of a mixture of the four single-stranded RNA species from infected cells in agarose gels containing methylmercuric hydroxide revealed the presence of only two species with mol. wt. of $4.0 \times 10^6$ and $1.8 \times 10^6$ respectively (C. W. Czarniecki & T. Sreevalsan, unpublished data). This result is consistent with the notion that the 38S and 33S species may represent conformational forms of the 49S and 26S RNA. Experiments reported here indicate that the 38S species of RNA is labelled more rapidly than the 49S species (Fig. 4 and Table 1). The ratio of 38S/49S was high during a 1 min pulse but progressively decreased as the length of the pulse increased. These results suggest that the newly synthesized 49S molecules synthesized in infected cells may exist as the 38S form.

In the light of the above conclusions, the results presented here concerning the preferential inhibition of all single-stranded species except 49S in the presence of cycloheximide are puzzling. It could be argued that the results presented in Fig. 5 do not really show the alleged inhibition since a diffuse pattern of radioactivity, apparently due to some degradative steps, appeared in the 38S region of the gel. Although such an argument is valid it should be considered in the light of the observation that the preferential inhibition induced by the drug on SBV RNAs was time dependent, in the sense that addition of cycloheximide at 3 to 4 h p.i. did not result in inhibition of any of the RNA species and the profile of radioactivity was similar to that shown in Fig. 5(b). Thus, these results suggest that one or more proteins, synthesized early during infection, may be involved in not only initiating the synthesis of the 26S form of RNA, but also in maintaining the conformation of the 38S form. Apparently, the pool size of this protein(s) may be high enough later in infection so that cycloheximide has little discriminatory effect on the synthesis of SBV RNAs.

The non-structural protein NS p89 as well as the nucleocapsid or core protein have been implicated in the regulation of 26S RNA synthesis. Brzeski & Kennedy (1978) postulated that NS p89 was involved in the regulation of 26S RNA synthesis in cells infected with an RNA− mutant N2 of Semliki Forest virus. The core protein may also exert an effect on the synthesis of 26S messenger RNA in a fashion analogous to feedback inhibition (Brzeski et al. 1978). The four non-structural proteins, including NS p89, are derived from the cleavage of a polyprotein (Brzeski & Kennedy, 1977) apparently reducing the possibility of a differential pool size for any of the virus-specific non-structural proteins. The rate of synthesis of the structural proteins is low during the first 90 min p.i. (T. Sreevalsan, unpublished data). Several reports have indicated that cleavage of the core protein moiety from its precursor occurs during, or immediately following, translation (Wirth et al. 1977). Thus, at 3 to 4 h p.i. with increased amounts of 26S templates present, the pool size of the core protein would be greatly enhanced. Assuming that NS p89, along with the core protein, controls the synthesis of 26S RNA, the results obtained from the cycloheximide experiments can be explained. Besides playing roles in the formation of the nucleocapsid and the synthesis of 26S RNA, the core protein may also contribute to the stability of the 38S form of RNA. Direct proof for the above hypothesis might be obtained using a ts mutant which generates defective nucleocapsid protein at the non-permissive temperature and such a mutant has yet to be described.

The authors wish to thank Dr R. Friedman and Dr L. de la Maza for helpful discussions and criticism of the manuscript. We would also like to thank M. Milstein for expert preparation of the manuscript.

This investigation was supported by Public Health Service Grant No. AI-09355-06 and
Training Grant No. AI-00298, and was included in the dissertation submitted by C.W.C. in partial fulfilment of the requirements for the Ph.D. degree in Microbiology awarded by the Graduate School of Georgetown University.

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Properties of Sindbis 38S RNA


(Received 4 January 1979)