JOURNAL OF VIROLOGY, Sept. 1974, p. 552-559

Vol. 14, No. 3
Printed in U.S.A.

# Replication of Sindbis Virus

## V. Polyribosomes and mRNA in Infected Cells

DANIEL T. SIMMONS[1] AND JAMES H. STRAUSS

*Division of Biology, California Institute of Technology, Pasadena, California 91109*

Received for publication 17 April 1974

Cells infected with wild-type Sindbis virus contain at least two forms of mRNA, 26*S* and 49*S* RNA. Sindbis 26*S* RNA (molecular weight $1.6 \times 10^6$) constitutes 90% by weight of the mRNA in infected cells, and is thought to specify the structural proteins of the virus. Sindbis 49*S* RNA, the viral genome (molecular weight $4.3 \times 10^6$), constitutes approximately 10% of the mRNA in infected cells and is thought to supply the remaining viral functions. In cells infected with *ts2*, a temperature-sensitive mutant of Sindbis virus, the messenger forms also include a third species of RNA with a sedimentation coefficient of 33*S* and an apparent molecular weight of $2.3 \times 10^6$. Hybridization-competition experiments showed that 90% of the base sequences in 33*S* RNA from these cells are also present in 26*S* RNA. Sindbis 33*S* RNA was also isolated from cells infected with wild-type virus. After reaction with formaldehyde, this species of 33*S* RNA appeared to be completely converted to 26*S* RNA. These results indicate that 33*S* RNA isolated from cells infected with either wild-type Sindbis or *ts2* is not a unique and separate form of Sindbis RNA.

In cells infected with group A arboviruses (alphaviruses), at least two species of virus-specific single-stranded RNA are manufactured (4, 15, 16). In the case of Sindbis virus, these two species of RNA have sedimentation coefficients of 49*S* and 26*S*. Sindbis 49*S* RNA is the viral genome and has a molecular weight of $4.3 \pm 0.3 \times 10^6$ (3, 13). Sindbis 26*S* RNA has a molecular weight of $1.6 \times 10^6$ and consists of one-third of the viral genome (13).

It has been determined that 26*S* RNA functions as a messenger in the infected cell (5, 8, 12). Since 26*S* RNA has only one-third of the coding capacity of the genome, it is expected that the other two-thirds of the viral functions are specified by other species of Sindbis-specific mRNA. Kennedy (5) and Mowshowitz (8) described a second species of Sindbis mRNA having a sedimentation coefficient of 33*S* and a molecular weight of 2.2 to $2.4 \times 10^6$. Rosemond and Sreevalson (12) described several species of mRNA smaller than 26*S* RNA in infected cells. In an earlier publication (14), we obtained indirect evidence for the existence of a species of single-stranded RNA with a molecular weight of $2.8 \times 10^6$ which contains two-thirds of the base sequence information present in 49*S* RNA but none of the information present in 26*S* RNA. We hypothesized that this species of RNA functions as a messenger in infected cells.

In this paper, we support the evidence that 26*S* RNA is the predominant messenger in cells infected with Sindbis virus, and we show that 49*S* RNA, the viral genome, serves as the second form of messenger in infected cells. We also present evidence that Sindbis 33*S* RNA is a structural variant of 26*S* RNA and not a separate species of Sindbis-specific RNA. No other unique species of RNA was detected in preparations of mRNA.

## MATERIALS AND METHODS

Except as described below, the materials and methods used in these experiments have been previously described (13). These include the methods for sucrose gradient and formaldehyde sucrose gradient velocity sedimentation, analytical and preparative acrylamide gel electrophoresis, and hybridization-competition.

**Isolation of Sindbis-specific polysomes.** Chicken embryo fibroblasts were infected with Sindbis virus as previously described (13). The cells were labeled with [5-$^3$H]uridine (specific activity 22.4 Ci/mmol) for various periods of time and/or with [$^{14}$C]amino acids (Schwarz hydrolysate, specific activity 50 mCi/mmol) for 2 min. In some cases, the cells were treated with 50 μg of cycloheximide per ml (Nutritional Biochemicals Corp.) for 7 min before harvesting the infected cells.

The flask of cells was rapidly cooled to 0 C in an ice-water slurry, and the cells were washed three times

[1] Present address: National Institutes of Health, National Institute of Allergy and Infectious Diseases, Bethesda, Md. 20014.

in ice-cold 0.01 M Tris, 0.01 M KCl, and 0.0015 M $MgCl_2$ (pH 7.4). The cell monolayer was scraped off the surface of the flask with a rubber policeman into 0.5 ml of the same buffer. After 10 min at 0 C to allow the cells to swell, the cells were homogenized with 10 to 15 strokes of a glass Dounce homogenizer. Nuclei and unbroken cells were removed by centrifugation at 1,000 × *g* for 8 min at 0 C. The supernatant was made 0.5% with respect to deoxycholate (Matheson, Coleman & Bell), and particulate material was removed by sedimentation at 10,000 × *g* for 10 min. The polysomes and other cytoplasmic material present in the clarified supernatant were sedimented in a linear 15 to 40% sucrose gradient in the above buffer for 2.25 h (unless otherwise stated) at 40,000 rpm and 1.5 C in the Spinco SW40 or SW41 rotor.

If the gradient was to be analyzed for radioactivity, it was first pumped through a flow cell in a Gilford recording spectrophotometer to determine the position of the monosome band. The material in each fraction was precipitated with trichloroacetic acid and counted in toluene-fluor scintillation fluid.

If the labeled RNA in the gradient containing Sindbis-specific polysomes was to be analyzed by velocity sedimentation, the gradient was first fractionated directly into 2-ml beakers containing 20 μliters of 10% sodium dodecyl sulfate (SDS). The RNA from selected fractions was subjected to sucrose gradient velocity sedimentation (14) for 4.5 h at 40,000 rpm in the Spinco SW41 rotor.

**Preparation of mRNA from infected cells.** To prepare mRNA, polysomes were sedimented through a sucrose gradient such that those consisting of more than 10 ribosomes were pelleted in the centrifuge tube. The gradient above the pellet was carefully removed by aspiration, and the pellet was resuspended in 0.2 ml of 0.01 M Tris, 0.01 M KCl, and 0.0015 M $MgCl_2$ (pH 7.4). The solution was made 0.01 M in EDTA to release the mRNA from ribosomes and 100 μg/ml in dextran sulfate (Sigma Chemical Co.) to inhibit RNase. To separate the released mRNA from nucleocapsids present in the polysomal pellet, the material was sedimented in a sucrose gradient, as described above, for the sedimentation of polysomes with the exception that the time of centrifugation was increased to 3 h. The gradient was collected in beakers containing 20 μliters of 10% SDS, and a volume of each fraction was counted for radioactivity. The RNA in the pooled fractions was made 0.2 M in potassium acetate (pH 6.0) and precipitated with 2.5 volumes of ethanol overnight at −20 C. The RNA was resuspended in 0.01 M Tris, 0.06 M NaCl, 0.001 M EDTA, and 1% SDS (pH 7.2) and extracted twice with phenol-chloroform (9) at 0 C and twice more at room temperature. The RNA was then subjected to electrophoresis in an acrylamide gel as previously described (13).

**Preparation of Sindbis 33S RNA.** Sindbis 33*S* RNA was prepared from chick cells infected with either wild-type Sindbis virus of *ts2*.

When the source of the 33*S* RNA was cells infected with wild-type Sindbis (HR), total cellular RNA was prepared as previously described (13, method A, but without diethyl oxydiformate). The RNA was subjected to sedimentation in a 15 to 30% sucrose gradient in 0.01 M Tris, 0.06 M NaCl, 0.001 M EDTA, 0.2% SDS (pH 7.2) for 4.5 h at 40,000 rpm and 23 C in the Spinco SW41 rotor, and the fractions corresponding to Sindbis 26*S* RNA and 33*S* RNA were pooled together. The RNA was precipitated with ethanol, and the resuspended RNA was subjected to preparative gel electrophoresis (13). The fractions containing 33*S* RNA were combined and the RNA was concentrated by ethanol precipitation.

When the source of the 33*S* RNA was cells infected with *ts2* at 39 C, polysomes were prepared and subjected to sedimentation, as described above. The fractions containing polysomes with 4 to 10 ribosomes were made 1% with respect to SDS, and the samples were extracted with phenol-chloroform (9). The species of 33*S* RNA was purified by electrophoresis in a preparative acrylamide gel (13).

**Preparation of viral nucleocapsids.** Nucleocapsids were isolated from detergent-treated Sindbis virus which had been purified as previously described (10). The virus solution was diluted threefold in 0.01 M Tris, 0.01 M KCl, and 0.005 M EDTA (pH 7.4), and Triton X-100 (Sigma Chemical Co.) was added to a final concentration of 0.5%. The nucleocapsids were sedimented in a 15 to 40% sucrose gradient in the same buffer for 3 h at 40,000 rpm and 1.5 C in the Spinco SW40 rotor. The fractions containing the nucleocapsids were pooled and stored frozen.

## RESULTS

**Polysomes of infected cells.** Polysomes prepared from cells infected with Sindbis virus served as the source of the mRNA in these experiments. It was therefore important to analyze the size of these polysomes and to determine if they were involved in protein synthesis.

When cells infected with wild-type (HR strain) Sindbis virus were briefly labeled for 2 min with $^{14}C$-labeled amino acids, the sedimentable radioactivity was associated primarily with monosomes, disomes, and trisomes (Fig. 1), indicating that these structures were involved in protein synthesis. Smaller amounts of amino acid label were incorporated into larger polysomes. The polysomes were also labeled with [$^3H$]uridine which was added at 30 min after infection in the presence of actinomycin D (in order to label only virus-specific RNA). In addition, the uridine label was incorporated into viral nucleocapsids which sediment at 140*S*. In these gradients, most of the nucleocapsids sedimented slightly faster than trisomes and at the position shown by bar b in Fig. 1. Relatively little amino acid label was associated with nucleocapsids after only 2 min of labeling.

In cells infected with certain mutants of Sindbis virus (*ts2*, *ts5*, *ts13*, and *ts106*), the synthesis of viral nucleocapsids is temperature

sensitive (1, 2, E. Strauss, personal communication). When polysomes were prepared from cells infected with *ts2* at the restrictive temperature, uridine label was found in polysomes as expected, but relatively little uridine had been incorporated into nucleocapsids (Fig. 2) (nucleocapsids, if present, would be found near fraction 27 in this gradient). These polysomes were also labeled with amino acids in a 2-min pulse showing that all classes of *ts2* polysomes were involved in protein synthesis.

Because polysome preparations from cells infected with *ts2* were free from large amounts of nucleocapsids, we were able to calculate the relative amounts of [$^{14}$C]amino acids to [$^{3}$H]uridine incorporated into each class of polysomes. Thus, in Fig. 2, the ratio of [$^{14}$C]amino acid to [$^{3}$H]uridine label increased as the polysomes got larger. This was expected on the basis that larger polysomes contain a greater total length of polypeptide chains per unit length of mRNA than do smaller polysomes. The $^{14}$C-to-$^{3}$H ratio in the polysomes of Fig. 1 could not be calculated due to the presence of large amounts of nucleocapsids in the gradient (see below). However, these results suggest that polysomes from cells infected with wild-type Sindbis virus are smaller than polysomes from cells infected with *ts2*.

Furthermore, to show that polysomes were not degraded during the isolation procedure, we prepared polysomes from uninfected cells after a brief labeling period with amino acids. Most of the sedimentable radioactivity was associated with large polysomes consisting of five or more ribosomes, whereas relatively little label was in monosomes or disomes (Fig. 3). Moreover, as shown below, the RNA isolated from polysomes of infected cells was usually undegraded. These results indicate that, under the conditions of infection, polysomes from cells infected with either wild-type Sindbis or *ts2* were smaller than those from uninfected cells.

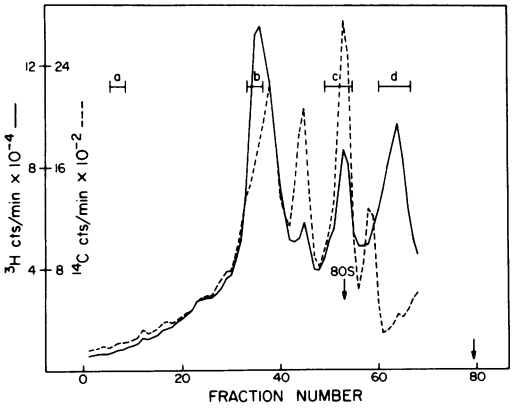


FIG. 1. *Polysomes from cells infected with wild-type Sindbis virus. 1.5 × 10$^{7}$ chicken embryo cells were infected with wild-type (HR) Sindbis virus and labeled with 20 μCi of [5-$^{3}$H]uridine per ml from 0.5 to 4.5 h after infection and with 100 μCi of [$^{14}$C]amino acids per ml (Schwarz hydrolysate) for 2 min before cell harvesting. The polysomes were prepared and sedimented. The bars labeled a, b, c, and d refer to certain fractions which were pooled for analysis of the labeled RNA (see Fig. 4). The arrow on the right in this and all other figures represents the top of the gradient. Symbols: –, [$^{3}$H]uridine label; -----, [$^{14}$C]amino acid label.*

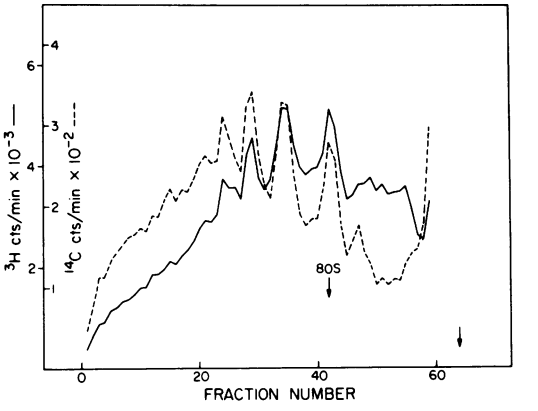


FIG. 2. *Polysomes from cells infected with ts2. The experimental procedures were as described in the legend to Fig. 1 with the exception that the cells were infected with ts2 at 39 C. Symbols: –, [$^{3}$H]uridine label; -----, [$^{14}$C]amino acid label.*

**Characterization of the RNA from gradients of Sindbis-specific polysomes.** Polysomes labeled with [$^{3}$H]uridine were prepared from cells infected with wild-type (HR) Sindbis virus and subjected to velocity sedimentation in a sucrose gradient. In this gradient, the profile of $^{3}$H radioactivity was similar to the one shown in Fig. 1. Four different samples of this gradient were pooled as shown by the bars labeled a, b, c, or d in Fig. 1, and the structures in these samples were disrupted with SDS. The RNA in each sample was sedimented in a linear sucrose gradient in the presence of *E. coli* rRNA (Fig. 4). Sample (a), which apparently consisted of large polysomes, and sample (b), which consisted of trisomes, tetrasomes, and viral nucleocapsids, contained both Sindbis 26*S* RNA and 49*S* RNA (Fig. 4[a] and [b]). On the other hand, samples (c) and (d), which consisted of monosomes and slower sedimenting structures, respectively, contained primarily 26*S* RNA (Fig. 4[c] and [d]). The interpretation of these results is complicated by the presence of large amounts of nucleocapsids which sedimented

faster than 140S throughout the bottom half of the gradient containing the polysomes. For example, it was shown that after EDTA treatment and resedimentation of the material in Fig. 1a, 35% of the $^3H$ label was in viral nucleocapsids. This observation and the data shown in Fig. 4 probably indicate that the large majority of the 49S RNA in these gradients of Sindbis-specific polysomes was in the form of nucleocapsids, whereas most of the 26S RNA was associated with ribosomes.

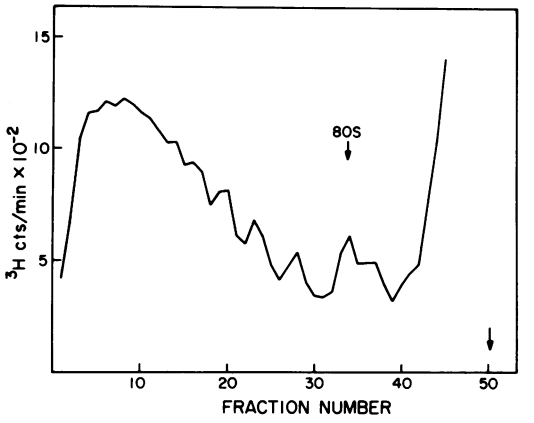


FIG. 3. *Polysomes from uninfected cells. A culture of uninfected chicken embryo cells was labeled with 100 μCi of [$^3H$]amino acids per ml (Schwarz hydrolysate) for 2 min before cell harvesting. The polysomes were subjected to velocity sedimentation, as described in the legend to Fig. 1 with the exception that the time of centrifugation was reduced to 2 h.*

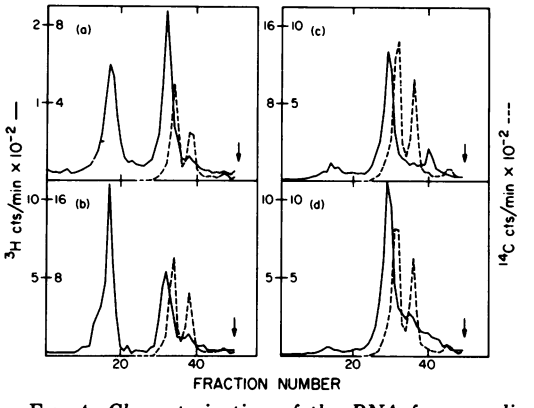


FIG. 4. *Characterization of the RNA from gradients of Sindbis-specific polysomes. Chick cells infected with wild-type Sindbis virus were labeled with 20 μCi of [5-$^3H$]uridine per ml from 0.5 to 4.5 h postinfection. The polysomes were prepared and sedimented, and the gradient was collected for analysis of the RNA. The RNA in each of the four samples, corresponding to the regions of the gradient shown by bars a, b, c and d in Fig. 1, was subjected to sedimentation in a 15% to 30% sucrose gradient at 40,000 rpm and 23 C for 4.5 h in the Spinco SW41 rotor using $^{14}C$-labeled E. coli rRNA as markers. Symbols: (a-d) –, $^3H$-labeled Sindbis-specific RNA in bars a-d of Fig. 1, respectively; -----, $^{14}C$-labeled E. coli rRNA.*

The RNA in each of the samples (a) through (d) was treated with RNase to detect the presence of labeled double-stranded RNA. Only sample (d) contained RNase-resistant RNA, and this was shown to consist of the three forms of Sindbis replicative forms (RF) (13) (data not shown). The conclusion was that only sample (d) contained the Sindbis replicative intermediates.

**mRNA in cells infected with wild-type Sindbis virus.** To isolate mRNA, polysomes from cells infected with wild-type Sindbis virus were sedimented such that those consisting of more than 10 ribosomes were pelleted in the centrifuge tube. For this experiment, the cells were treated with cycloheximide to increase the average size of the polysomes (7) and to reduce the relative amounts of nucleocapsids in the pelleted material. The polysomes in the pellet were dissociated with EDTA, and the components were sedimented in a sucrose gradient to separate released RNA (i.e., mRNA) from nucleocapsids (Fig. 5). The polysomal pellet contained relatively little uridine label in nucleocapsids as shown by the small amounts of $^3H$ radioactivity sedimenting with the nucleocapsid marker. Figure 5 shows as well that the RNA released by EDTA was associated with structures (presumably RNA-protein complexes) sedimenting at approximately 35S to 40S (sample I), and at 60S to 70S (sample II). After deproteinization, the RNA in each of samples I and II was subjected to acrylamide gel electrophoresis (Fig. 6). Sample I consisted primarily of 26S RNA (Fig. 6[a]), whereas 49S RNA was the predominant species of RNA in sample II (Fig. 6[b]). Other species of Sindbis mRNA could possibly be present in sample II since another band (or bands) could be hidden under the leading side of the 49S RNA peak in Fig. 6(b). Approximately 10% of the label in the mRNA fraction (samples I and II) was in 49S RNA. These results indicate that 26S RNA is the predominant species of mRNA in infected cells, but that 49S RNA has messenger function as well.

**mRNA in cells infected with ts2.** Since the virus-specific polypeptides synthesized in cells infected with *ts2* are different in size from the polypeptides synthesized in cells infected with wild-type Sindbis virus (17), we characterized the species of mRNA in *ts2*-infected cells. The mRNA forms were subjected to electrophoresis

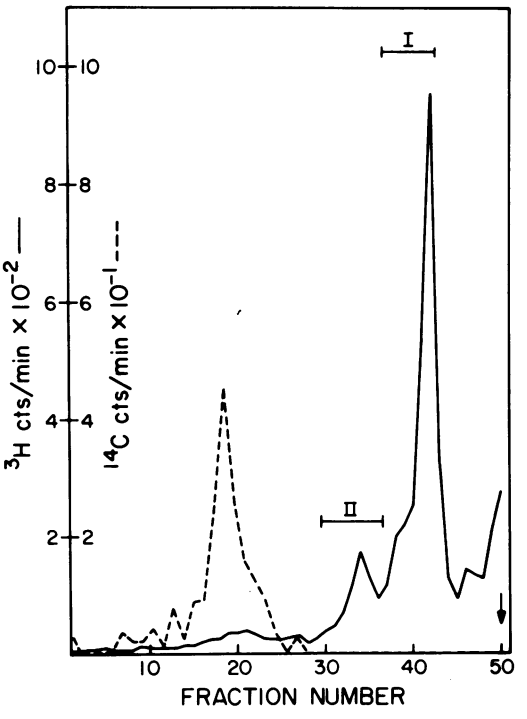


FIG. 5. *Disruption of polysomes with EDTA. 1.5 × 10⁷ chicken embryo cells were infected with wild-type (HR) Sindbis virus and labeled with 20 μCi of [5-³H]-uridine per ml from 3 to 5 h after infection. Seven minutes before cell harvesting, the medium was made 50 μg/ml with respect to cycloheximide to increase the average size of the polysomes. The polysomes were sedimented at 40,000 rpm for 2 h 15 min and 1.5 C in a Spinco SW40 rotor, and the polysomes in the pellet were treated with EDTA. The material was subjected to velocity sedimentation. ¹⁴C-labeled purified Sindbis nucleocapsids were added as a marker. Bars labeled I and II refer to certain fractions of the gradient which were pooled for analysis of the RNA by acrylamide gel electrophoresis (see Fig. 6). Symbols: —, [³H]uridine label; -----, ¹⁴C-labeled Sindbis nucleocapsids.*

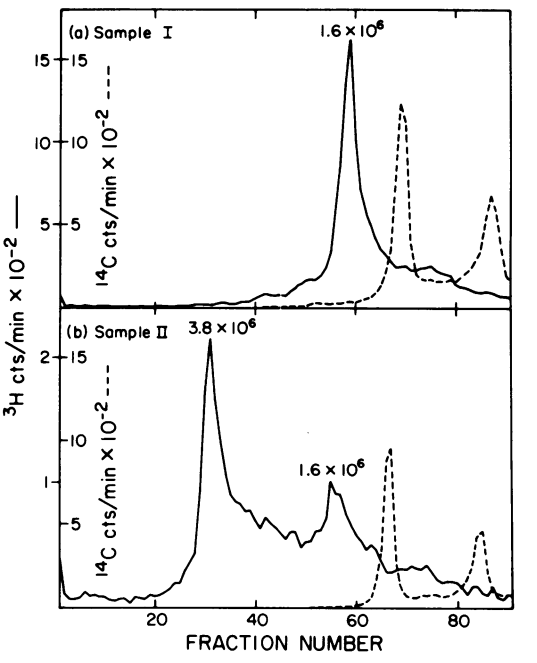


FIG. 6. *mRNA from cells infected with wild-type Sindbis virus. The RNA in each of samples I and II (Fig. 5) was subjected to acrylamide gel electrophoresis. ¹⁴C-labeled E. coli rRNA were added as markers and the direction of electrophoresis was from left to right. Symbols: (a) and (b) —, ³H-labeled Sindbis mRNA in sample I and in sample II, respectively; -----, ¹⁴C-labeled E. coli rRNA.*

in an acrylamide gel using purified Sindbis 26S RNA as marker (Fig. 7). They consisted primarily of 26S RNA and of small amounts of another species of RNA with an apparent molecular weight of 2.3 × 10⁶. This second species of RNA has been detected previously in cells infected with Semliki Forest virus or with Sindbis virus (6) and in mRNA preparations from these infected cells (4, 8). Although little 49S RNA was detected in this preparation of mRNA, we have found 49S mRNA in other preparations of polysomes from cells infected with *ts2* at either the restrictive or permissive temperature.

**Characterization of Sindbis 33S RNA.** The species of RNA with a molecular weight of 2.3 × 10⁶ (33S RNA) was isolated from polysomes of

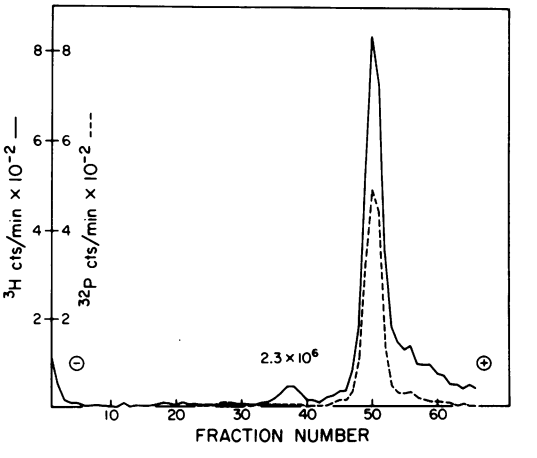


FIG. 7. *mRNA from cells infected with ts2. Chicken cells infected with ts2 at 39 C were labeled with 20 μCi of [5-³H]uridine per ml from 0.5 to 3.25 h postinfection. The mRNA fraction was isolated and the RNA was subjected to acrylamide gel electrophoresis using purified ³²P-labeled Sindbis 26S RNA as marker. Symbols: —, ³H-labeled ts2 mRNA; -----, ³²P-labeled purified Sindbis 26S RNA.*

cells infected with *ts2* at the restrictive temperature and purified by preparative gel electrophoresis. A hybridization-competition experiment using 33*S* RNA was performed to determine whether or not this form of Sindbis-specific RNA was separate and distinct from Sindbis 26*S* RNA. When 33*S* RNA was hybridized to Sindbis double-stranded RNA, unlabeled 26*S* RNA was able to effectively compete with it for the sites on the complementary strand of the double-stranded RNA (Fig. 8). Only about 10% of the base sequences in 33*S* RNA were not in competition with excess unlabeled 26*S* RNA. The curve for the control experiment where 26*S* RNA was hybridized to Sindbis double-stranded RNA in the presence of increasing amounts of unlabeled 26*S* RNA is also shown in Fig. 8. These results indicate that at least 90% of the base sequences in 33*S* RNA from cells infected with *ts2* are also present in 26*S* RNA. It is unclear whether the 10% nonhomology between 26*S* and 33*S* RNA represents a real difference between these molecules or is due to contamination of the 33*S* RNA preparation with fragments derived from 49*S* RNA.

If, as the hybridization-competition results indicate, 33*S* RNA is a structural variant of 26*S* RNA, denaturation of this form of RNA should convert it to 26*S* RNA. For the following experiment, 33*S* RNA was isolated from total cellular RNA after infection with wild-type Sindbis virus and was purified by electrophoresis in a preparative acrylamide gel. The native form of the RNA was subjected to sucrose gradient-velocity sedimentation with *E. coli* rRNA as markers (Fig. 9). In agreement with its size estimated by gel electrophoresis, the RNA had a sedimentation coefficient of 33.5*S*. However, when the purified RNA was denatured by heating in formaldehyde, it sedimented with an apparent molecular weight of only $1.4 \times 10^6$ (Fig. 10). After reaction with formaldehyde, Sindbis 26*S* RNA sedimented with this same apparent molecular weight (13). It appears, therefore, that when 33*S* RNA from cells infected with wild-type Sindbis virus was denatured with formaldehyde, it was converted to 26*S* RNA.

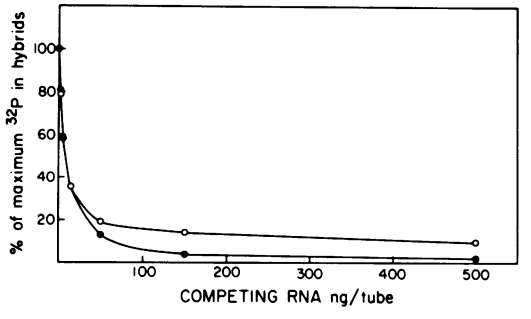


FIG. 8. *Hybridization-competition experiments with 33S RNA. $10^8$ chicken embryo cells were infected with ts2 at 39 C and labeled with 250 μCi of $H_3{}^{32}PO_4$ per ml from 0.5 to 4.5 h postinfection. The 33S RNA was isolated from polysomes. The annealing mixtures contained 20 ng of double-stranded RNA (13), 2 ng of $^{32}$P-labeled 26S or 33S RNA, and various amounts of unlabeled purified 26S RNA. The RNA was hybridized as previously described (13). Symbols: O, Hybridization of $^{32}$P-labeled 33S RNA; ●, hybridization of $^{32}$P-labeled 26S RNA.*

## DISCUSSION

Polysomes isolated from cells infected with either wild-type Sindbis virus or *ts2* appear to be smaller, under the conditions of infection, than polysomes of uninfected cells. This is in contrast with the results obtained on polysomes of cells infected with poliovirus (11).

Sindbis 26*S* RNA is the predominant messenger in cells infected with either wild-type Sindbis or *ts2*. Kennedy (5), Rosemond and Sreevalsan (12), and Mowshowitz (8) have reported similar conclusions. We found that Sindbis 49*S* RNA serves as messenger as well, but to a lesser extent than 26*S* RNA. The majority of the 49*S* RNA in our gradients containing Sindbis-specific polysomes was found in nucleocapsids, whereas the majority of the 26*S* RNA was probably associated with ribosomes.

Mowshowitz (8) also presented evidence that 49*S* RNA possesses messenger activity. In her experiments, treatment of the polysomes with EDTA or puromycin caused a decrease in the amounts of 49*S* RNA associated with fast-sedimenting structures and a corresponding increase of 49*S* RNA in structures sedimenting at 140*S* with viral nucleocapsids. Although it was assumed that the 49*S* RNA was released from polysomes, it is possible that some or all of the newly appearing 49*S* RNA in the 140*S* material was due to an altered sedimentation behavior of nucleocapsids in the presence of EDTA. We have found that fast-sedimenting structures do contain significant amounts of nucleocapsids which can be released by EDTA treatment. In our gradients, released 49*S* RNA sediments as a ribonucleoprotein complex at 60 to 70*S* versus 140*S* for nucleocapsids (or for released 49*S* RNA in the experiments of Mowshowitz [8]), and can be distinguished from nucleocapsids. It is also noteworthy that released 26*S* RNA sedimented as a ribonucleoprotein complex of 65*S* in the experiments of Mowshowitz (8), versus 35 to 40*S* in our experiments. The reason for these

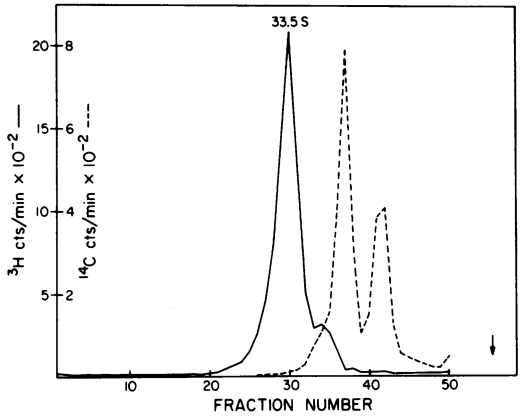


FIG. 9. *Sedimentation of 33S RNA in sucrose gradients. Chicken cells were infected with wild-type (HR) Sindbis virus and labeled with [5-$^3$H]uridine from 0.5 to 4.5 h postinfection. The 33S RNA was isolated from total cellular RNA and subjected to velocity sedimentation as described in the legend to Fig. 4. Symbols: –, $^3$H-labeled 33S RNA; -----, $^{14}$C-labeled E. coli rRNA.*

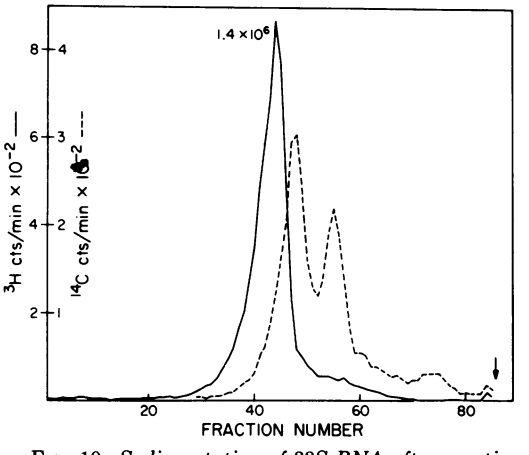


FIG. 10. *Sedimentation of 33S RNA after reaction with formaldehyde. 33S RNA was prepared, denatured by heating in the presence of formaldehyde (12), and sedimented in a formaldehyde-sucrose gradient for 10.5 h at 40,000 rpm and 23 C in the Spinco SW40 rotor. Formaldehyde-treated E. coli rRNA were used as markers. Symbols: –, $^3$H-labeled formaldehyde-treated 33S RNA; -----, $^{14}$C-labeled formaldehyde-treated E. coli rRNA.*

differences in sedimentation coefficients is not clear. It may be due to the different sedimentation conditions or to the use of different host cells.

In vitro and, by inference, in infected cells, 26S RNA codes for the structural proteins of the virus (D. T. Simmons and J. H. Strauss, J. Mol. Biol., in press; R. Cancedda and M. J. Schlesinger, Proc. Nat. Acad. Sci. U.S.A., in press). In infected cells, the remaining viral functions seem to be coded for by 49S RNA. The proteins specified by the 49S RNA genome are not known but they presumably include one or two RNA replicases and regulatory proteins needed in small quantities. In lysates of rabbit reticulocytes, 49S RNA specifies eight or nine polypeptides ranging in molecular weight from 60,000 to 180,000 (D. T. Simmons and J. S. Strauss, in press). It is not known if these polypeptides are synthesized in infected cells.

A 33S RNA species from arbovirus-infected cells was first detected by Levin and Friedman (6) and shown to have messenger activity by Kennedy (5) and by Mowshowitz (8). We have confirmed the existence of a 33S RNA species and its apparent messenger function. However, our results indicate that 33S RNA is not a unique species of Sindbis RNA. When 33S RNA isolated from cells infected with wild-type Sindbis virus was denatured with formaldehyde, it appeared to be completely converted to 26S RNA. Hybridization-competition experiments indicate that 33S RNA from cells infected with *ts2* shares at least 90% of its base sequences with 26S RNA.

In a previous publication (14), we presented a model for the replication of Sindbis-specific RNA which showed the synthesis of a hypothetical unique species of single-stranded RNA (molecular weight $2.8 \times 10^6$) equivalent to two-thirds of the genome. A rigorous search for this species of RNA has failed. We have abandoned the possibility that 33S RNA is the molecule in question. If this RNA molecule is ever released from the template, it must be rapidly degraded.

## ACKNOWLEDGMENTS

We wish to acknowledge the expert technical assistance of Edith M. Lenches and Sharman E. S. Christoph in preparing the cultures of chicken embryo fibroblasts. We also wish to thank Ellen G. Strauss for helpful suggestions in writing this manuscript.

This investigation was supported in part by the National Science Foundation grant no. GB 31763X and by the Public Health Service grant no. GM 06965 from the National Institute of General Medical Sciences. D.T.S. was supported by Public Health Service Training grant no. 5-T1-GM-86 from the National Institute of General Medical Sciences.

## LITERATURE CITED

1. Burge, B. W., and E. R. Pfefferkorn. 1966. Isolation and characterization of conditional-lethal mutants of Sindbis virus. Virology **30**:204–213.
2. Burge, B. W., and E. R. Pfefferkorn. 1968. Functional defects of temperature-sensitive mutants of Sindbis virus. J. Mol. Biol. **35**:193–205.
3. Dobos, P., and P. Faulkner. 1970. Molecular weight of Sindbis virus ribonucleic acid as measured by polyacrylamide gel electrophoresis. J. Virol. **6**:145–147.

4. Friedman, R. M., B. H. Levy, and W. B. Carter. 1966. Replication of Semliki Forest virus: three forms of viral RNA produced during infection. Proc. Nat. Acad. Sci. U.S.A. **56**:440-446.
5. Kennedy, S. I. T. 1972. Isolation and characterization of the virus-specified RNA species found on membrane-bound polyribosomes of chick embryo cells infected with Semliki Forest virus. Biochem. Biophys. Res. Commun. **48**:1254-1258.
6. Levin, J. G., and R. M. Friedman. 1971. Analysis of arbovirus ribonucleic acid forms by polyacrylamide gel electrophoresis. J. Virol. **7**:504-514.
7. Lodish, H. F., D. Housman, and M. Jacobsen. 1971. Initiation of hemoglobin synthesis. Specific inhibition by antibiotics and bacteriophage ribonucleic acid. Biochemistry **10**:2348-2356.
8. Mowshowitz, D. 1973. Identification of polysomal RNA in BHK cells infected by Sindbis virus. J. Virol. **11**:535-543.
9. Penman, S. 1966. RNA metabolism in the HeLa cell nucleus. J. Mol. Biol. **17**:117-130.
10. Pierce, J. P., E. G. Strauss, and J. H. Strauss. 1974. Effect of ionic strength on the binding of Sindbis virus to chick cells. J. Virol. **13**:1030-1036.
11. Rich, A., S. Penman, Y. Becker, J. E. Darnell, and C. Hall. 1963. Polyribosomes: size in normal and polio-infected HeLa cells. Science **142**:1658-1663.
12. Rosemond, H., and T. Sreevalsan. 1973. Viral RNA's associated with ribosomes in Sindbis virus-infected HeLa cells. J. Virol. **11**:399-415.
13. Simmons, D. T., and J. H. Strauss. 1972. Replication of Sindbis virus. I. Relative size and genetic content of 26S and 49S RNA. J. Mol. Biol. **71**:599-613.
14. Simmons, D. T., and J. H. Strauss. 1972. Replication of Sindbis virus. II. Multiple forms of double-stranded RNA isolated from infected cells. J. Mol. Biol. **71**:615-631.
15. Sonnabend, J., L. Dalgarno, R. M. Friedman, and E. M. Martin. 1964. A possible replicative form of Semliki Forest virus RNA. Biochem. Biophys. Res. Commun. **17**:455-460.
16. Sreevalsan, T., and R. Z. Lockart, Jr. 1966. Heterogeneous RNA's occurring during the replication of western equine encephalomeylitis virus. Proc. Nat. Acad. Sci. U.S.A. **55**:974-981.
17. Strauss, J. H., B. W. Burge, and J. E. Darnell. 1969. Sindbis virus infection of chick and hamster cells: synthesis of virus-specific proteins. Virology **37**:367-376.