JOURNAL OF VIROLOGY, Nov. 1974, p. 1189-1198

Vol. 14, No. 5
Printed in U.S.A.

Defective Interfering Passages of Sindbis Virus: Nature of the Intracellular Defective Viral RNA

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Received for publication 17 June 1974

BHK cells infected with defective-interfering passages of Sindbis virus accumulate a species of RNA (20S) that is about half the molecular weight of the major viral mRNA (26S). We have performed competitive hybridization experiments with these species of RNA and have established that 20S RNA contains approximately 50% of the nucleotide sequences present in 26S RNA. Our further studies, however, demonstrate that 20S RNA is unable to carry out the messenger function of 26S RNA. We found very little of the defective RNA associated with polysomes in vivo. In addition, it was unable to stimulate protein synthesis in vitro under conditions in which 26S RNA was translated. We have also examined viral RNA synthesis in BHK cells infected with standard or defective-interfering passages of Sindbis virus. This comparison suggests that defective particles do not synthesize a functional replicase.

Undiluted serial passaging of Sindbis virus in BHK-21 cells leads to the generation and accumulation of defective-interfering (DI) particles (6, 14, 15). Although these noninfectious particles have not been isolated free of standard infectious virions, their presence has been clearly established by a number of criteria (7, 18). (i) Late-passage virus populations inhibit the formation of standard virus. (ii) This interfering activity migrates with infectivity in a sucrose gradient and bands at the same density as infectious virus. (iii) Cells infected with such interfering passages of virus contain a new species of RNA (20S RNA, mol wt of approximately 0.86×10^6), considerably smaller than the predominant RNA species (26S RNA, mol wt of about 1.6×10^6) found in cells infected with standard virus. (iv) The appearance of this DI specific RNA is accompanied by a marked drop in 26S RNA synthesis as well as a large reduction in intracellular, assembled 140S nucleocapsid structures.

The present study was designed to examine the chemical and functional relatedness of 20S and 26S RNA. We have carried out competition hybridization experiments to establish possible sequence homology between these two species of RNA and have examined the ability of 20S RNA to function as a mRNA, a role established for 26S RNA in viral protein synthesis both in vivo and in vitro (2, 10).

Our results enable us to conclude that 20S RNA contains approximately 50% of the nucleotide sequences present in 26S RNA. In addition, based on our studies of the association of 20S RNA with polysomes, both in vivo and in vitro, and on the synthesis of virus-specific proteins in vitro, this deleted form of the major mRNA species (26S) in infected cells appears unable (or very weakly able) to serve as a mRNA. These results lead us to propose that the defective virions of Sindbis virus cannot independently direct the production of viral RNA and proteins.

MATERIALS AND METHODS

Cell cultivation, virus growth, and interference assay. Sindbis virus was grown on monolayers of BHK-21 cells and passaged according to published procedures (14). Plaque assays were carried out using chicken embryo fibroblast cells as described previously (14). Late passages of Sindbis virus were tested for interference activity by the method detailed by Weiss and Schlesinger (18). These passages were tested at several multiplicities of infection, with standard virus kept constant at a multiplicity of either 10 or 30. Only those late passages that, at a multiplicity of infection one-tenth that of the standard virus, caused an inhibition of virus yield greater than 50% were used in the experiments. It was necessary to test each passage, as the degree of defectiveness and interfering potential was highly variable. For

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example, two different preparations of passage 9 were used in these experiments. The passage 9 used to obtain the data in Fig. 3 contained less defective particles than the passage 9 described in Table 2.

Preparation of cell extracts. For the analysis of polysomes and the isolation of RNA, infected cells were washed twice with sterile buffer containing 10 mM Tris-hydrochloride (pH 7.4), 10 mM NaCl, and 1.5 mM $MgCl_2$ and were lysed with this buffer containing 1% Triton X-100 and polyvinyl sulfate (100 μg/ml). Unbroken nuclei and cell debris were removed by low-speed centrifugation. For the direct isolation of RNA, sodium dodecyl sulfate was added to the supernatant fraction to a final concentration of 1%. In experiments designed to isolate the nucleocapsid, an EDTA-containing buffer (10 mM EDTA, 20 mM NaCl, and 10 mM Tris, pH 7.2) was substituted for the $MgCl_2$ buffer used above.

Polysome analysis. Cell extracts prepared as described above were layered over 12-ml, 10 to 40% sucrose gradients prepared in a buffer containing 0.5 M NaCl, 5 mM $MgCl_2$ and 10 mM Tris, pH 7.4. All solutions were sterilized before use. Centrifugation was carried out in an International SB283 rotor at 40,000 rpm for 200 min.

Nucleocapsid analysis. Identical gradient conditions were used as for the polysome analysis, except that the gradients were prepared with the EDTA buffer described above.

Isolation of RNA. The procedure for the preparation of RNA from BHK cells infected with Sindbis virus was essentially that described by Cancedda and Schlesinger (2). RNA was extracted from cell extracts with a 1:1 mixture of buffer-saturated phenol and chloroform-isoamyl alcohol (24:1) in the presence of 1% sodium dodecyl sulfate and 100 μg of polyvinyl sulfate per ml. Fresh redistilled phenol was used, and all glassware and solutions were sterilized before extraction. Two extractions with the phenol-chloroform mixture and one extraction with chloroform-isoamyl alcohol alone were done. The aqueous phase was precipitated overnight at −20 C with 2.5 volumes of 95% alcohol in the presence of 0.1 M NaCl. For the in vitro protein synthesizing reaction, the RNA was precipitated with 2 M LiCl and eluted from an oligo (dT) cellulose column by the procedure of Cancedda and Schlesinger (2). For hybridization the RNA was not precipitated with LiCl but was applied directly to the column.

Labeling procedures. For the polysome experiments, monolayers of BHK cells were incubated with actinomycin D (1 μg/ml) at the time of infection and labeled with [^{3}H]uridine (20 μC/ml), 24.3 Ci/mmol) from 3 to 8 h postinfection.

For the large scale isolation of [^{3}H]uridine-labeled 20*S* and 26*S* RNA, 1 mCi of [^{3}H]uridine in 50 ml of medium was added to each of two roller bottles (containing approximately 5×10^8 cells) at 1 h postinfection. Actinomycin D was added at the time of infection. For the late-passage virus infections, three additional unlabeled, infected roller bottle cultures were used, and the cells from five bottles were combined 12 h after infection for the isolation of RNA to give a low-specific-activity [^{3}H]uridine-labeled RNA preparation for both hybridization and in vitro protein synthesis experiments. In the early-passage infections only one roller bottle of cells was labeled and combined with two unlabeled, infected cultures.

For the preparation of ^{32}P-labeled viral-specific RNA, cells were incubated in PO_4-free medium for 2 h prior to infection. Actinomycin D was added at the time of infection, and 5 mCi of $^{32}P_i$ was added 2 h later. The cells were harvested 12 h after infection.

The [^{14}C]uridine-labeled BHK ribosomal RNA used as markers was obtained according to published procedures (18).

Polyacrylamide gel electrophoresis. The electrophoretic procedure for the analysis of RNA was identical to that previously described (18). For the isolation of specific RNA species, gel slices were eluted overnight at 4 C in sterile water or buffer. Over 50% of the radioactivity present in each slice was recovered. A small portion from each eluted gel was counted to locate the position of each radioactive species. The eluted RNA was either used directly for hybridization or passed through an oligo (dT) cellulose column. For the in vitro translation of 20*S* RNA, the eluted RNA was always subjected to the latter procedure.

The slab gel electrophoresis procedure used to analyze ^{35}S-methionine-labeled proteins synthesized in the in vitro translation systems has been described in detail (2).

Preparation of cell-free extracts for in vitro protein synthesis. The preparation of the rabbit reticulocyte and Krebs ascites cell-free extracts was identical to that described at length by Cancedda and Schlesinger (2). In addition, the various components of the reaction mixture and their preparation are given by these authors.

Preparation of double-stranded RNA. The procedure adopted was essentially that devised by Simmons and Strauss (16). Purified, unlabeled intracellular viral RNA was prepared in large quantity with roller bottle cultures of BHK cells infected with standard virus at a multiplicity of approximately 50. Cells were harvested at 12 to 17 h after infection, and RNA was extracted as described above. The total, purified RNA, after ethanol precipitation, was dissolved in 2 × SSC (0.15 M NaCl plus 0.015 M sodium citrate) and digested with 10 μg of pancreatic ribonuclease per ml for 30 min at 37 C. The enzyme was removed by phenol extraction, and the resulting RNase-resistant RNA was alcohol precipitated, suspended in a solution containing 1.0 mM EDTA and 50 mM NaCl (pH 7.0), and applied in 0.5 ml portions to a Sephadex G-150 column. The RNA present in the void volume was used in hybridization experiments.

Competition hybridization. Hybridization was carried out in 4 × SSC in the presence of saturating amounts of diethylpyrocarbonate (17). Each reaction mixture contained unlabeled double-stranded RNA (2.5 μg), about 500 counts/min of ^{32}P-labeled 26*S* RNA, and increasing amounts of [^{3}H]uridine-labeled 26*S* or 20*S* RNA. Samples were dried under vacuum and suspended in 50 μliters of 4 × SSC previously equilibrated with diethylpyrocarbonate. (Approximately 45 μliters of diethylpyrocarbonate per 3-ml

volume of solution was vortexed and heated at 37 C for 10 min before use.) Each reaction mixture was sealed in sterile capillary tubes, heated for 3 min in an ethylene glycol bath held between 110 and 120 C, and immediately transferred to an oven at 66 C for 17 h. At this time each tube was opened, and the samples were quantitatively transferred to 1 ml of 2 × SSC. Control samples in duplicate were precipitated directly with 10% trichloroacetic acid. The experimental samples were incubated with 40 μg of pancreatic ribonuclease per ml for 30 min at 37 C, precipitated with trichloroacetic acid and carrier tRNA (50 μg/ml), filtered, and counted.

RESULTS

Sequence homology between 26S and 20S RNA. BHK cells infected with DI passages of Sindbis virus accumulate a species of RNA (20S) that is about half the molecular weight of the virus-specific 26S RNA (18). To determine if this new RNA is a truncated form of 26S RNA, we performed competition hybridization experiments. For these experiments we purified 20S RNA by elution of the species from polyacrylamide gels after electrophoresis of [^{3}H]uridine-labeled RNA isolated from BHK cells infected with passage 12 Sindbis virus (Fig. 1). 26S RNA labeled with either $^{32}P_i$ or [^{3}H]uridine was obtained in a similar manner from cells infected with early-passage virus. Unlabeled viral-specific double-stranded RNA was prepared from cells infected with early-passage virus by a modification of the method described by Simmons and Strauss (16). Figure 2 shows the hybridization of ^{32}P-labeled 26S RNA to double-stranded RNA in the presence of increasing amounts of [^{3}H]uridine-labeled 20S or 26S RNA. The competition observed between the two different species reached a plateau at about 50%. This result demonstrates that 20S RNA is a unique species containing approximately 50% of the sequences present in 26S RNA.

The role of 20S RNA in protein synthesis in vivo. Since 20S RNA is a deleted form of the major mRNA in Sindbis virus-infected cells and appears to contain poly A sequences as judged by its ability to bind to oligo (dT) cellulose columns (a step used in its purification), and by the preliminary finding that approximately 30% of [^{3}H]adenosine-labeled 20S RNA is RNase A and T_1 nuclease resistant as compared to 5% for [^{3}H]adenosine-labeled 49S RNA (unpublished observations), it appeared reasonable to assume that this RNA could serve as a functional mRNA for the synthesis of certain viral structural proteins. To test this proposal, we deter-

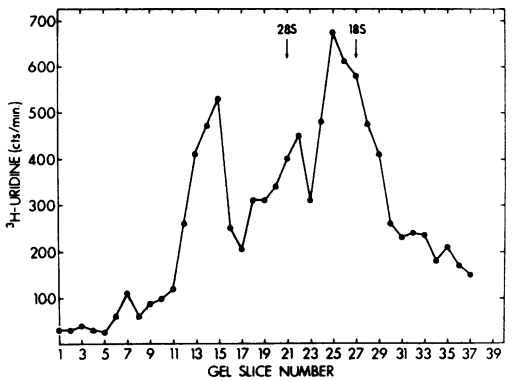

FIG. 1. *Polyacrylamide gel electrophoresis of [^{3}H]uridine-labeled intracellular viral-specific RNA from BHK cells infected with passage 12 virus. The isolated RNA was eluted from an oligo (dT) cellulose column and alcohol precipitated before electrophoresis. The RNA, in 50 μliters of buffer, was layered on a 0.5% agarose-1.8% polyacrylamide gel, and electrophoresis was carried out for 2.5 h at 5 mA/gel. The gel was sliced, and each slice was eluted overnight at 4 C in 0.2 ml of sterile water. Portions (20 μliter) from each eluted slice were counted. The origin is at the left. The viral RNA species were identified by the use of a companion gel containing ribosomal RNA markers. The 20S RNA eluted from the peak gel slices (25 through 28 in the gel shown) was either pooled or used separately in the hybridization experiments (see Fig. 2), depending on the activity of the sample.*

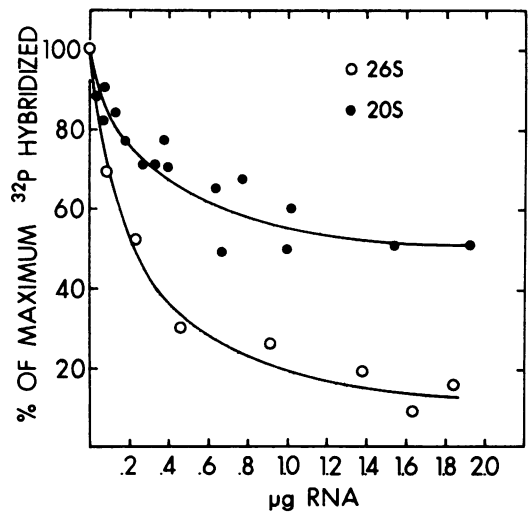

FIG. 2. *Determination of the degree of sequence homology between 26S and 20S RNA by competition hybridization. Each sample contained unlabeled viral-specific double-stranded RNA (2.5 μg), 500 count/min of ^{32}P-labeled 26S RNA, and increasing amount of [^{3}H]uridine-labeled 26S or 20S RNA. The conditions of hybridization and assay of ribonuclease resistance are given in Materials and Methods. The data for two different experiments with two independent samples of 20S RNA are shown.*

mined if 20S RNA was associated with polysomes. Monolayers of BHK cells were infected with early or late passages of virus with multiplicities of infection between 50 and 60 and were labeled with [^{3}H]uridine between 3 and 8 h postinfection. The distribution of labeled RNA in cell extracts was analyzed by sucrose gradient sedimentation under conditions that displayed the polysomes. Two independent experiments performed with two different defective and standard passages of virus are illustrated in Fig. 3. Almost all of the viral RNA from cells infected with passage 3 virus was found in the heavy region of the gradient (region II). In contrast, when cells were infected with passage 9 or passage 11 virus, the labeled RNA was shifted toward the top of the gradient (region I). In the experiment comparing the polysomes from cells infected with passage 3 or passage 11 virus, the location of the nucleocapsid was established in a separate gradient with cell extracts prepared in the presence of 10 mM EDTA. Its position is indicated by the arrow at 140S (Fig. 3). Very little nucleocapsid was present in the late-passage infection, a finding previously reported (6, 18).

RNA was extracted from the two regions of the gradients and was analyzed by polyacrylamide gel electrophoresis (Fig. 4 and 5). The RNA pattern for region I from passage 3 is not shown, as it represents a negligible fraction of the total virus-specific cellular RNA. The distribution of each species of viral RNA between the polysome and nonpolysome fraction of the cell extract was calculated from Fig. 4 and 5 and is presented in Table 1. In addition, the distribution of each species of RNA as the percentage of total intracellular viral-specific RNA was determined. These latter data were obtained for passage 3 and 11 in two ways: (i) RNA was extracted from a sample of the original extract and analyzed by polyacrylamide gel electrophoresis (Fig. 6), and (ii) the values for total RNA were calculated by adding the amounts in region I and II of the sucrose gradient and the amount of each species in these two regions

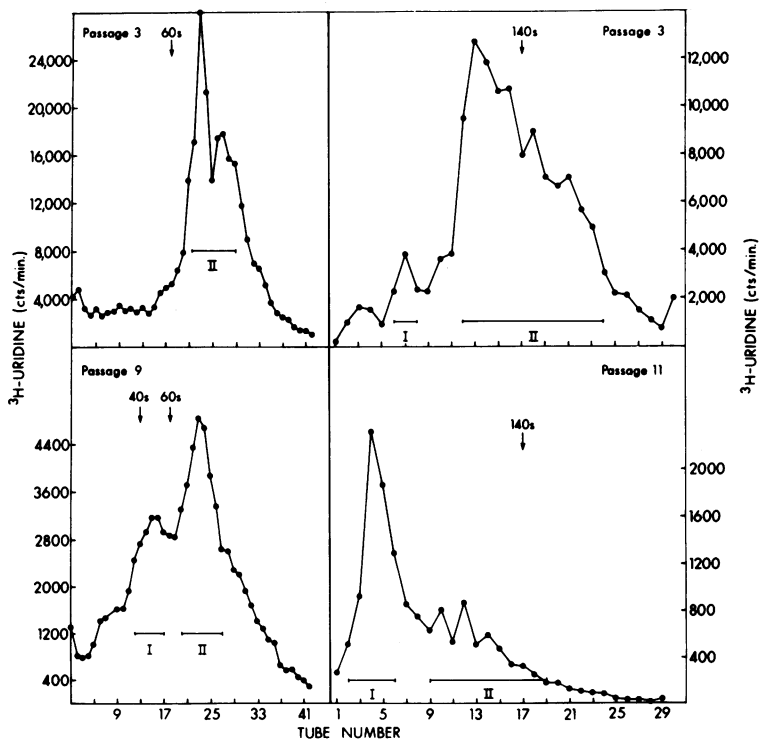

FIG. 3. *A comparison of polysome profiles obtained from cells infected with early- or late-passage virus. The results obtained for passages 3 and 9 (shown on the left) and 3 and 11 (on the right) represent two independent experiments. The arrow at 140S refers to the position of nucleocapsid, and those at 40S and 60S refer to ribosomal markers run concurrently. The RNA from Regions I and II of the gradients were pooled for further analysis.*

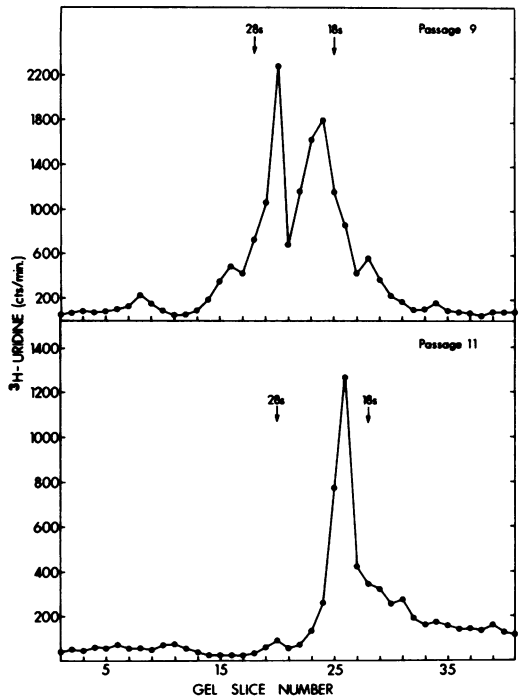

FIG. 4. *A comparison of polyacrylamide gel electrophoresis patterns of RNA isolated from region I, the nonpolysome region of the sucrose gradients, for passages 9 and 11 depicted in Fig. 3. Internal [^{14}C]uridine-labeled BHK ribosomal RNA markers were included in each gel, and migrated as indicated by the arrows.*

determined from the gels shown in Fig. 4 and 5. Both methods gave identical results, and only method (ii) was used for passage 9.

It is evident that most of the 20S RNA (70 to 80%) was not associated with polysomes, whereas from 57 to 86% of the 26*S* RNA was polysome associated. The small percentage of 20*S* RNA found in the polysome region may represent the association of this RNA with ribosomes or result from contamination of this region with some of the 20*S* RNA from the top of the gradient. Most of the 49*S* RNA in the polysome region was probably present in viral nucleocapsids.

The results obtained with passages 9 and 11 differ in two aspects. First, much more RNA was associated with polysomes in cells infected with passage 9 than in cells infected with passage 11 (Fig. 3). Second, the molar ratio of 20*S* to 26*S* RNA in passage 9 was approximately 2.4, whereas this ratio was about 7 for passage 11. Based on interfering ability, passage 11 was enriched in DI particles compared to passage 9. Thus, there are several effects that can be correlated with an enhancement of interfering potential. These include a decrease in virus titer, an increase in the ratio of 20*S* to 26*S* RNA, and a decrease in the content of messenger-bound ribosomes.

In vitro protein synthesis by late-passage viral-specific cellular RNAs. Although the in vivo data indicated that 20*S* RNA was a nonfunctional derivative of standard viral mRNA, we decided to further test this RNA for messenger activity in an in vitro protein-synthesizing

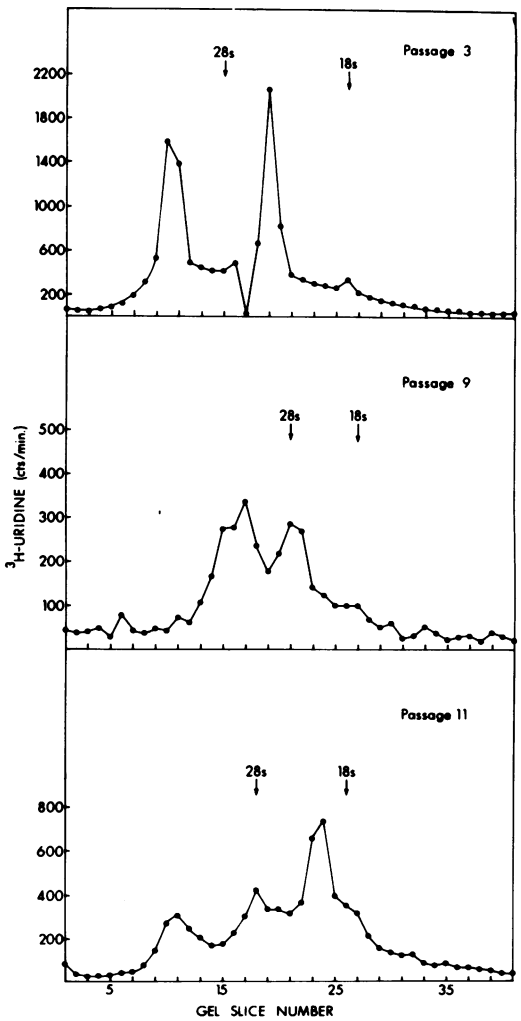

FIG. 5. *A comparison of polyacrylamide gel electrophoresis patterns of RNA isolated from region II, the polysome region, of the sucrose gradients illustrated in Fig. 3. The position of the internal [^{14}C]uridine-labeled ribosomal markers are indicated for each gel with arrows.*

TABLE 1. *Distribution of viral-specific RNAs in the polysome and nonpolysome fractions of infected cell extracts*

Passage	Fraction analyzed	Distribution of each RNA species[a]		
		20S	26S	49S
3	Total RNA		72	28
	Nonpolysome region I		14	0
	Polysome region II		86	100
9	Total RNA	42	34	23
	Nonpolysome region I	82	43	4
	Polysome region II	18	57	96
11	Total RNA	71	20	9
	Nonpolysome region I	72	18	18
	Polysome region II	28	82	82

[a] Expressed as the percentage of total concentration in the cell extract. The procedures for calculating the percentage of each species of RNA in the total intracellular viral-specific RNA are described in the text. The distribution of each species of RNA between the two regions of the sucrose gradient was calculated from Fig. 4 and 5.

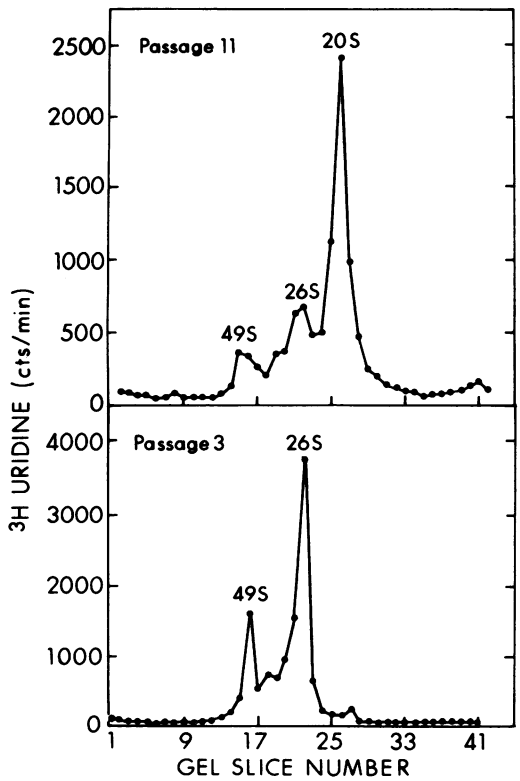

FIG. 6. *Polyacrylamide gel electrophoresis profiles of the total intracellular viral-specific RNA from infections with passages 3 and 11, at multiplicities of approximately 50. The identification of each RNA species was made with internal ribosomal RNA markers.*

system. The in vitro translation of both 49S and 26S RNA from Sindbis virus-infected BHK cells has been reported by Cancedda and Schlesinger (2). Using cell-free systems from rabbit reticulocytes or Krebs ascites cells these authors demonstrated a marked stimulation of the incorporation of [^{35}S]methionine into protein upon addition of viral RNA obtained from infected cells or purified virions. When similar experiments were performed with total viral-specific RNA prepared from cells infected with DI passages of Sindbis virus, no stimulation of incorporation was observed (Table 2). The predominant species of intracellular viral-specific RNA in this preparation was 20S RNA (the polyacrylamide gel pattern was essentially the same as that shown in Fig. 6). Before being tested in the in vitro system, the RNA had been partially purified by removal of double-stranded RNA with 2 M LiCl and by elution from an oligo (dT) cellulose column (2). To test if the lack of stimulation of protein synthesis could be due to the presence of an inhibitor in the preparation, the RNA was purified further by polyacrylamide gel electrophoresis. 26S RNA isolated by this procedure stimulated protein synthesis, but the 20S RNA showed no activity (Table 2). The labeled proteins synthesized in the in vitro reaction mixtures were also analyzed by electrophoresis in polyacrylamide

TABLE 2. *Stimulation of protein synthesis in cell-free extracts by Sindbis viral RNA*

Cell-free system	Source of RNA	RNA added (μg)	Counts/min incorporated into protein
Reticulocyte	None	0	12,910
	From cells infected with passage 2[a]	7.0	57,612
	From cells infected with passage 9[a]	8.0	8,974
Ascites	None	0	2,579
	From standard virions	2.5	8,281
	From cells infected with passage 9[a]	8.0	2,309
	20S RNA from passage 9 infection[b]	3.7	2,299

[a] After extraction from infected cells, RNA was precipitated with 2 M LiCl and eluted from an oligo (dT) cellulose column (2).

[b] The 20S RNA was isolated by polyacrylamide gel electrophoresis. 26S RNA isolated in this manner is active in in vitro translation.

slab gels. Only those protein bands formed in the endogenous reaction were detected with defective-passage RNA, whereas RNA from a standard infection produced a major new band identifiable as viral capsid protein, and some higher-molecular weight viral-specific proteins as described previously (Cancedda and Schlesinger, in press).

One additional test for the capacity of 20S RNA to act as a mRNA was to determine whether the RNA from defective-passage infections could associate with ribosomes to form polysome complexes in vitro. Figure 7 shows the results of an experiment comparing the interaction of early- or late-passage intracellular viral-specific RNA with ribosomes in vitro. The viral RNA obtained from cells infected with standard virus did form fast-sedimenting complexes, whereas no such complexes were detected with defective-passage viral-specific RNA. Thus the in vitro data support the in vivo observation that 20S RNA does not participate in protein synthesis.

The synthesis of RNA by DI particles. In a previous publication (18) we presented evidence that the incorporation of radioactive uridine into RNA was not disturbed by the presence of interfering levels of late-passage virus. However, in the experiments reported in Fig. 3, the incorporation of [^{3}H]uridine into viral-specific intracellular RNA was between 5- and 10-fold lower in passages 9 and 11 compared to passage 3. The reason for the variability observed in different experiments may be partially due to the fact that each late-passage virus population has a different ratio of standard and defective virions as judged by the RNA pattern they produced in infected cells and by the differences in their interfering ability. In an attempt to understand the role of defective particles in RNA replication itself, we examined the kinetics of RNA synthesis after infection with standard and defective passages of virus in more detail. Cells infected with passage 5 or 9 at the same multiplicity incorporated [^{3}H]uridine into RNA at the same rate during the exponential and early linear phase of RNA replication (Fig. 8). There is, however, a premature decrease and early termination of RNA synthesis in infections involving defective particles. The extent of this inhibition is highly variable.

The exponential phase of viral RNA synthesis in cells infected with early passages of Sindbis virus is strongly multiplicity dependent and thus is similar to data reported for poliovirus (1). Upon varying the multiplicity of infection with standard and defective passages of Sindbis

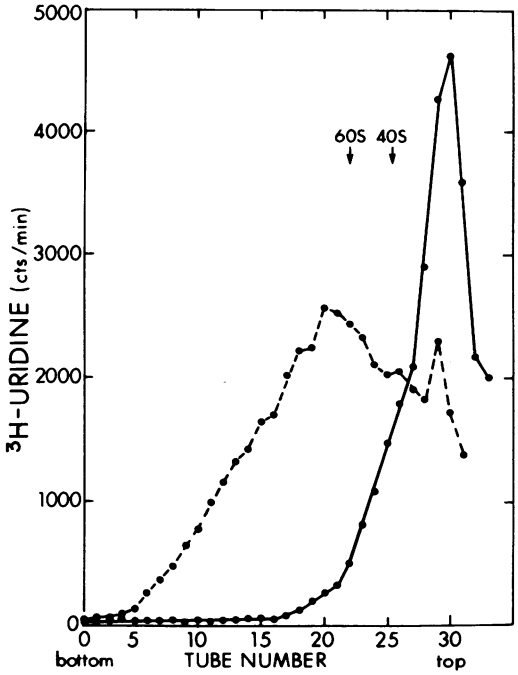

FIG. 7. *Polysome profiles generated in an in vitro system. Cell-free extracts prepared from lysed rabbit reticulocytes were incubated for 10 min at 29 C with the complete protein synthesizing system described previously (Cancedda and Schlesinger, 2) and RNA from BHK cells infected with early (●—●) or late (●——●) passage virus. The total incubation mixture was layered over a 12-ml 10 to 40% sucrose gradient and centrifuged in an International SB283 rotor for 200 min at 40,000 rpm.*

virus, we observed that the rate of RNA synthesis during the early period of replication is independent of the degree of defectiveness of the virus passage used (defectiveness being measured by the ability to interfere with standard virus replication) and reflects only the input multiplicity of infectious virus. The proportionality between the exponential rate of RNA synthesis and the multiplicity of infection, regardless of the passage of virus tested, is shown in Fig. 9. Such data suggest that defective particles do not independently contribute to their own RNA replication.

DISCUSSION

The competition hybridization experiments described here establish that 20S RNA produced in cells infected with DI passages of Sindbis virus is a deleted form of the viral 26S mRNA. This finding is in accord with our expectations based on the molecular weights of

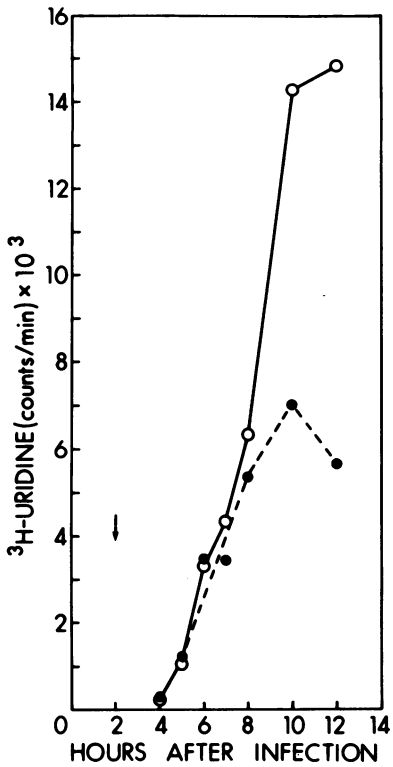

FIG. 8. *A comparison of the kinetics of incorporation of [^{3}H]uridine after infection with either passage 5 (○) or passage 9 (●) at a mulitplicity of 10. [^{3}H]uridine (1 μCi/ml, 2 × 10^{-6} M) was added 2 h after infection. Actinomycin D (1 μg/ml) was present from the time of infection.*

26S and 20S RNA and the increase in the ratio of 20S to 26S RNA as a function of the extent of viral interference. The apparent presence of Poly (A) and the marked reduction in the ability of 20S RNA to bind to ribosomes to form polysomes suggest that the deletion is in the 5′ terminal portion of the 26S RNA molecule.

The observation that, in vivo, the majority of 20S RNA molecules were not associated with polysomes, as well as the data showing no stimulation by 20S RNA of in vitro viral protein synthesis, imply that 20S RNA cannot be engaged as an active messenger molecule and thus cannot express even the limited viral structural protein information it contains. The appearance of a new protein (mol wt of 75,000) in electrophoretic patterns of ^{35}S-methionine-labeled cells infected with DI passages of virus originally suggested to us that 20S RNA was an active messenger coding for this protein (18). In light of the data presented here this seems unlikely. It is possible that this protein is a normal by-product that accumulates under interference conditions. We have detected a protein of about this size in low levels in several different cell lines infected with early-passage Sindbis virus. Furthermore, Morser and Burke have reported a similar protein in Semliki Forest virus-infected chicken embryo cells (9).

One would anticipate from the data presented that total viral protein synthesis would be reduced in proportion to the defectiveness of a given viral passage. Although we have observed a decrease in the synthesis of capsid protein relative to the envelope proteins, we have not noticed any marked effects on overall protein synthesis (18). However, it has been difficult to investigate viral-specific proteins in these late-passage infections because the greater the defectiveness of the passage studied, the poorer the inhibition of host protein synthesis. This results in a very high background of host proteins, effectively obscuring possible alterations in viral protein patterns.

We have previously reported that the RNA isolated from late-passage virions is heterogeneous (18), and we are still unable to identify the nature of the defect in the virion RNA. This leaves unanswered the question of how 20S RNA is produced during replication of viral-specific RNA. We have, however, directed our attention to the question of whether defective

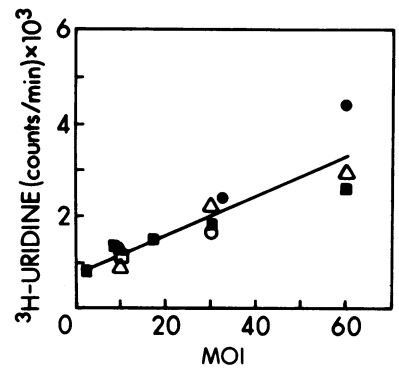

FIG. 9. *The relationship between the rate of incorporation of [^{3}H]uridine during the exponential phase of RNA replication and the multiplicity of infection. The open symbols represent standard virus infections (passages 3 through 5). The closed symbols represent defective-passage infection (passages 9 through 12). The different symbols indicate independent experiments. The conditions for each experiment were identical to those described in the legend to Fig. 8, except that the multiplicity of infection was varied. The ordinate represents the amount of [^{3}H]uridine incorporated into RNA between 2 and 5 h after infection.*

virion RNA can express any of the functions assumed to be coded for and expressed by the 49S RNA of the standard virus. Specifically, can the defective virion RNA direct the synthesis of functional replicase required for its own replication? The following indirect evidence suggests that it cannot. (i) Passages of virus-containing DI particles do not stimulate a higher rate of early RNA synthesis than standard passages at the same multiplicity of infection (Fig. 8 and 9). This suggests that DI particles do not contribute as independent entities to viral RNA synthesis. (ii) the reciprocal relationship between the amount of 20S and 26S RNA made suggests that 20S RNA is synthesized at the expense of 26S RNA. Thus, there appears to be a competition between these RNAs for a limiting amount of replicase made by the standard virion, and the replication of defective 20S RNA is favored.

Our proposal that DI particles of Sindbis virus are deficient in their ability to synthesize the viral replicase poses a dilemma in trying to understand the nature of the defect in the virion RNA. Our data have established that there is a deletion in the mRNA (26S) that codes for the virion structural proteins. This RNA almost certainly does not code for replicase, yet DI particles also appear to be defective in synthesizing viral-specific replicase. It is possible that the virion RNA contains a deletion covering the genes for some of the viral structural proteins as well as the replicase. Studies by Shenk and Stollar (15) have indicated that the RNA from DI particles of Sindbis virus can not be separated from standard virion RNA. Perhaps a relatively small deletion in virion RNA affects its conformation so that it cannot be translated. This abnormal conformation might also be invoked to explain how the defective virion RNA is processed to give rise to 20S RNA instead of the normal 26S RNA.

Our studies lead us to expect that the DI particles of Sindbis virus should behave in a manner more analogous to the T particles of vesicular stomatitis virus than the DI particles of poliovirus. In the poliovirus system (3, 4), defective virion RNA acts as an efficient mRNA for the synthesis of defective viral proteins and can also direct its own RNA synthesis in the absence of helper virus. Purified T particles, on the other hand, can neither induce RNA replication nor carry out viral protein synthesis (7, 8, 11, 12). In addition to these properties of T particles, Doyle and Holland (5) have shown that they can markedly alter the pathological course of infections with vesicular stomatitis virus both in vivo and in vitro. We have observed an analogous reduction in the development of cytopathological changes in BHK cells coinfected with standard and defective particles of Sindbis virus under conditions which normally lead to dramatic cell deterioration when standard virus alone is used. We are presently attempting to determine if Sindbis virus DI particles alone are able to perform any of the functions essential for virus replication.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation grants GB-37853 and GB-38657 (Cell Culture Facility Grant) and Public Health Service grant 1 ROI AI-11377 from the National Institute of Allergy and Infectious Diseases to S.S. B. W. was supported by Public Health Service training grant 5 ROI AI-00257, also from the National Institute of Allergy and Infectious Diseases.

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