VIRAL RNA SPECIES IN BHK-21 CELLS INFECTED WITH SINDBIS VIRUS SERIALLY PASSAGED AT HIGH MULTIPLICITY OF INFECTION

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SUMMARY. Only one species of double-stranded RNA (20S) is found in BHK-21 cells after infection with plaque-purified Sindbis virus. A smaller species of double-stranded RNA (12S), which is shown to contain viral sequences by hybridization experiments, is present when BHK-21 cells are infected at high multiplicities with a Sindbis virus stock derived by repeated passage at high multiplicity of infection. Several small single-stranded RNA species (0.9 to 2.2 x 10^5 MW) not found in cells infected with plaque-purified virus are present in infected cells which contain 12S DS RNA.

INTRODUCTION. Multiple double-stranded (DS) RNA species have been described in Sindbis virus (SV)-infected cells[1,2,3] and in cells infected with Semliki Forest virus, a closely related group A arbovirus.[4] For example, SV-infected BHK-21 cells contained 12S and 20S DS RNAs.[3] We now show that the presence of the smaller species of DS RNA (12S) in infected BHK-21 cells depends on the infection of cells at high multiplicity with a virus stock derived by repeated passage at high multiplicity of infection (MOI). These observations may be related to the recent reports by S. Schlesinger et al.[5] and Inglot and Chudzio[6] describing incomplete SV subsequent to serial passage of the virus at high MOI.

MATERIALS AND METHODS.

Cells, Media and Virus. The BHK-21 cells and the media in which they were propagated have been described.[3] The stock of plaque-purified SV was prepared from virus which was plaque purified three times in chick cells and then grown in primary chick embryo cultures which were infected at a multiplicity of 10^{-3} plaque-forming units/cell.

Analysis of Viral RNAs. Intracellular viral RNA species were isolated and purified by phenol extraction of whole cells.[3] After precipitation with ethanol at -20°C, the RNA was redissolved in either 1 mM tris, pH 7.4 containing 1 mM $MgCl_2$ for sucrose gradient analysis or in electrophoresis buffer containing 1% SDS. Sucrose gradient analysis was carried out in 5-20% linear sucrose gradients which contained 10 mM sodium acetate, pH 5.1, 50 mM NaCl, and 1 mM $MgCl_2$. Electrophoresis was in gels 90 mm long and 6 mm in diameter containing 2.4% acrylamide, 0.125% N, N'-methylenebisacrylamide and 0.5% agarose. The electrophoresis buffer contained 40 mM tris, 60 mM sodium acetate, 3 mM EDTA, 0.5% SDS, and 10% glycerol, adjusted to pH 7.2 with glacial acetic acid. Gels were cut into 1.4 mm slices on a Mickle gel slicer. Each slice was incubated overnight at room temperature in 5 ml of Packard Permafluor scintillation liquid containing 8% NCS and then counted in a liquid scintillation counter. The ribonuclease and immunochemical binding assays for the detection of DS RNA have been described.[3]

Preparation of ^{32}P-labeled Sindbis RNA. Primary chick embryo cells were infected with plaque-purified SV at a multiplicity of 0.01 plaque-forming unit/cell, and maintained in phosphate-free Eagle's medium[7] containing 2% calf serum, 0.04 µg/ml Actinomycin D and 35 µC/ml carrier-free [^{32}P]phorphoric acid. At 20 hours after infection, the virus was precipitated from the medium with ammonium sulfate,[8] resuspended in TNE buffer (50 mM tris, pH 7.5, 100 mM NaCl, 1mM EDTA) and pelleted through 10 ml of 15% sucrose prepared in TNE. This pellet was resuspended in 1 ml of TNE, and then the virus was centrifuged in a 15-30% sucrose gradient prepared in TNE. ^{32}P-labeled RNA was obtained from the virus-containing region of the gradient by phenol extraction.

Preparation of [5-^{3}H]Uridine-labeled DS RNA. Labeled 12S and 20S DS RNAs were prepared from BHK-21 cells infected with an uncloned stock of SV at a multiplicity of 10 plaque-forming units/cell. The whole cells

were phenol extracted, and single-stranded RNA was precipitated by adding LiCl to a final concentration of 2 M.[9] The DS RNAs were then precipitated from the resulting supernatant with ethanol, resuspended in buffer and sedimented through sucrose gradients as described above.

RESULTS AND DISCUSSION. BHK-21 cells infected with a stock of SV prepared from plaque-purified virus contained only one species of DS RNA which sedimented at 20S (Fig. 1A). This plaque-purified virus was then passaged serially through BHK-21 cells as described in Table 1. The titer dropped approximately 80-fold in passages 9 through 11 and then rose to greater than 10^8 plaque-forming units/ml in all subsequent passages (Table 1). After 11 passages (Fig. 1C) infected cells were found to contain a small peak of 12S DS RNA in addition to the 20S species. After 13 passages, the 12S species was the major DS RNA present (Fig. 1E) whether measured by RNase resistance or by an immunochemical binding assay.[3] Gel electrophoresis of the actinomycin resistant RNAs present in infected cells demonstrated that while mature viral (3.8×10^6 MW)* and interjacent (9×10^5 MW) RNAs constituted the major species of single-stranded RNA present in BHK-21 cells infected with unpassaged virus (Fig. 1B), passages 11 and 13 (Fig.1D, F) showed increasing amounts of smaller single-stranded RNA (0.9 to 2.2 x 10^5 MW) coincident with the appearance of 12S DS RNA.

When the passage 13 virus stock was diluted 10-fold and then used to infect BHK-21 cells (MOI=1.0), the amount of 12S DS RNA present in these infected cells was greatly reduced (Fig. 2A) relative to the amount present when an undiluted inoculum was used (Fig. 1E). Further, when the stock was diluted 1000-fold (MOI=0.01), the smaller RNA species was almost entirely missing (Fig. 2C). This suggests that the passage 13 stock contained incomplete particles which were responsible for the production

* Molecular weights were determined by the method of Peacock and Dingman[10] using BHK-21 ribosomal RNAs as marker.

TABLE 1. TITER OF SINDBIS VIRUS DURING SERIAL PASSAGE THROUGH BHK-21 CELLS.(1)

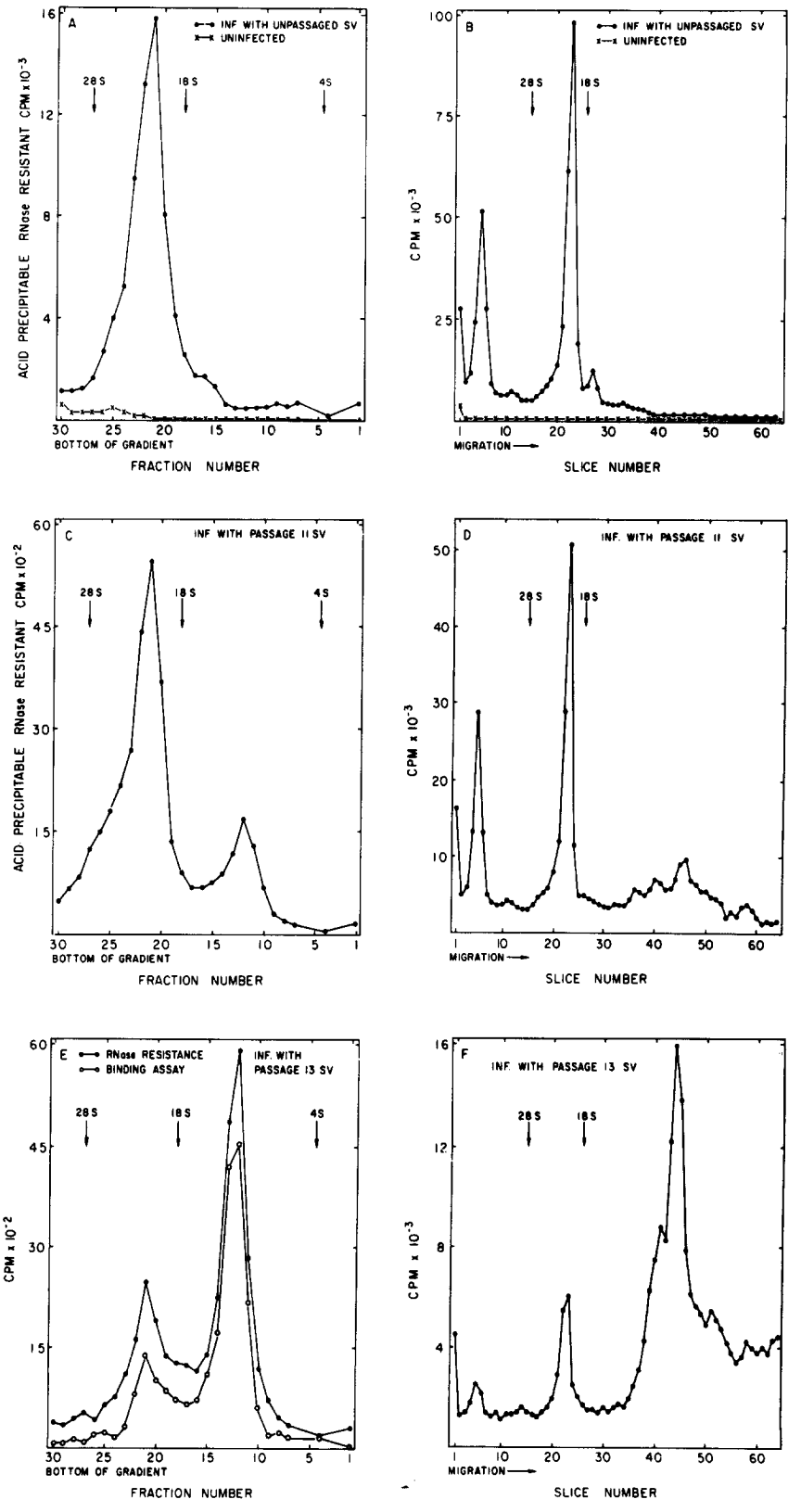
Passage	PFU/ml x 10^{-8}(2)	Passage	PFU/ml x 10^{-8}(2)
Cloned stock	2.0	9	0.12
1	8.8	11	0.10
3	8.5	13	1.0
5	8.9	15	1.5
7	7.1	27	1.7

(1) For each passage 1 ml of undiluted virus from the previous passage was used to infect 10^7 BHK-21 cells at 36°C. The medium from the infected cultures was harvested at 16 hr. after infection.
(2) Plaque-forming units/ml.

TABLE 2. ANNEALING OF ^{32}P-VIRAL RNA TO DS RNA SPECIES.(1)

Sample	RNase Resistant ^{32}P CPM per Reaction	% ^{32}P CPM RNase Resistant
^{32}P-viral RNA only	92.2 ± 6.3	4.2
^{32}P-RNA + 18S rRNA(2)	96.5 ± 5.1	4.4
^{32}P-RNA + 12S DS RNA(3)	279.0 ± 13.6	12.9
^{32}P-RNA + 20S DS RNA	442.1 ± 2.7	20.4

(1) DS RNA prepared as described in Materials and Methods was heated to 105°C for 7 minutes and then quenched in an ice-water bath. The denatured RNA (which was greater than 95% RNase sensitive) was then mixed with 2160 CPM of ^{32}P-labeled viral RNA in buffer containing 0.3 M sodium chloride, 0.03 M tri-sodium citrate, and 0.1% SDS in a final volume of 72 λ, sealed in an ampule, heated in a boiling water bath for 5 minutes, and then annealed at 70°C for 90 minutes. The mixture was digested with ribonucleases A and T1 (final concentrations 100 μg/ml and 50 units/ml respectively) for 30 minutes at room temperature, and the trichloroacetic acid precipitable ^{32}P CPM were determined. The data represents the mean ± standard deviation for three separate reaction mixtures.
(2) Reaction mixture received 10^{-2} μg of BHK-21 18S rRNA (determined from absorbance at 260 nm).
(3) Reaction mixture received 3.1 x 10^{-3} μg of denatured 12S or 20S DS RNA from SV-infected BHK-21 cells (determined by complement fixation assay using anti-DS RNA antibodies[3]).


A
INF WITH UNPASSAGED SV
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28S
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ACID PRECIPITABLE RNase RESISTANT CPM x 10⁻³
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B
INF WITH UNPASSAGED SV
UNINFECTED
28S
18S
CPM x 10⁻³
MIGRATION
SLICE NUMBER
C
INF WITH PASSAGE 11 SV
28S
18S
4S
ACID PRECIPITABLE RNase RESISTANT CPM x 10⁻²
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MIGRATION
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RNase RESISTANCE
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INF. WITH PASSAGE 13 SV
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CPM x 10⁻³
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SLICE NUMBER

of the 12S DS RNA. As the amount of 12S DS RNA decreased, the smaller single-stranded viral RNAs also were diminished (Fig. 2B), further suggesting a relationship between these species, i.e., the 12S DS RNA could be derived from the replicative intermediate RNA from which the smaller single-stranded RNAs are produced.

In order to show that the 12S DS RNA species was indeed SV specific and not derived either from the host cell or another virus, hybridization experiments were performed. ^{3}H-labeled 12S and 20S DS RNAs were denatured and then annealed with ^{32}P-labeled SV RNA which was prepared from plaque purified virus. In each case a significant proportion of the ^{32}P-labeled SV RNA became ribonuclease resistant (Table 2) indicating that both 12S and 20S DS RNAs contain SV sequences.

The data presented in this report show that the presence of multiple DS RNA species in SV-infected BHK-21 cells depends on infection of cells with virus which has been serially passaged at a high MOI. In addition, the simultaneous appearance and disappearance of the 12S DS RNA and the small single-stranded RNA species points to a relationship between these forms.

The ability to dilute high passage virus with consequent loss of 12S DS RNA and the small single-stranded RNAs would be consistent with the

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Figure 1. Viral RNA species present in BHK-21 cells infected with plaque-purified or high passage SV. BHK-21 cells were infected at a MOI=10 or greater with either unpassaged SV(A,B), or SV passaged 11 (C,D) or 13 (E,F) times at high MOI. Viral RNA species were labeled with 30 μC/ml of [5-^{3}H] uridine (26.5 C/mM) in the presence of 5 μg/ml actinomycin D from 2 to 7 hours after infection. RNA was isolated and purified as described in Materials and Methods. 16.4 ml sucrose gradients each received 1.7 A_{260} units of RNA. Centrifugation was in a Spinco SW27 rotor (27,000 rpm, 18.5 hrs., 4°). 0.6 ml fractions were collected and treated with 10 μg/ml ribonuclease A in 0.2 M NaCl for 10 minutes at 37°C. The positions of 28, 18 and 4S RNAs were determined by their absorbance at 260 nm. Gel electrophoresis was for 4 hours at 6 mA/gel. Each gel received 1.0 A_{260} unit of RNA in a volume of 20-50 μl. The positions of 28 and 18S RNAs were determined by including ^{14}C-labeled BHK RNAs. The ^{3}H CPM of gel samples have been normalized to the same efficiency of counting as that obtained with sucrose gradient samples.
Fig. 1A and B: 0-0, RNA from BHK-21 cells infected with plaque-purified SV and labeled in the presence of actinomycin D; X-X, RNA from uninfected BHK-21 cells labeled in the presence of actinomycin D.
Fig. 1E: ●-●, DS RNA as determined by ribonuclease resistance; 0-0, DS RNA as determined by immunochemical binding assay.

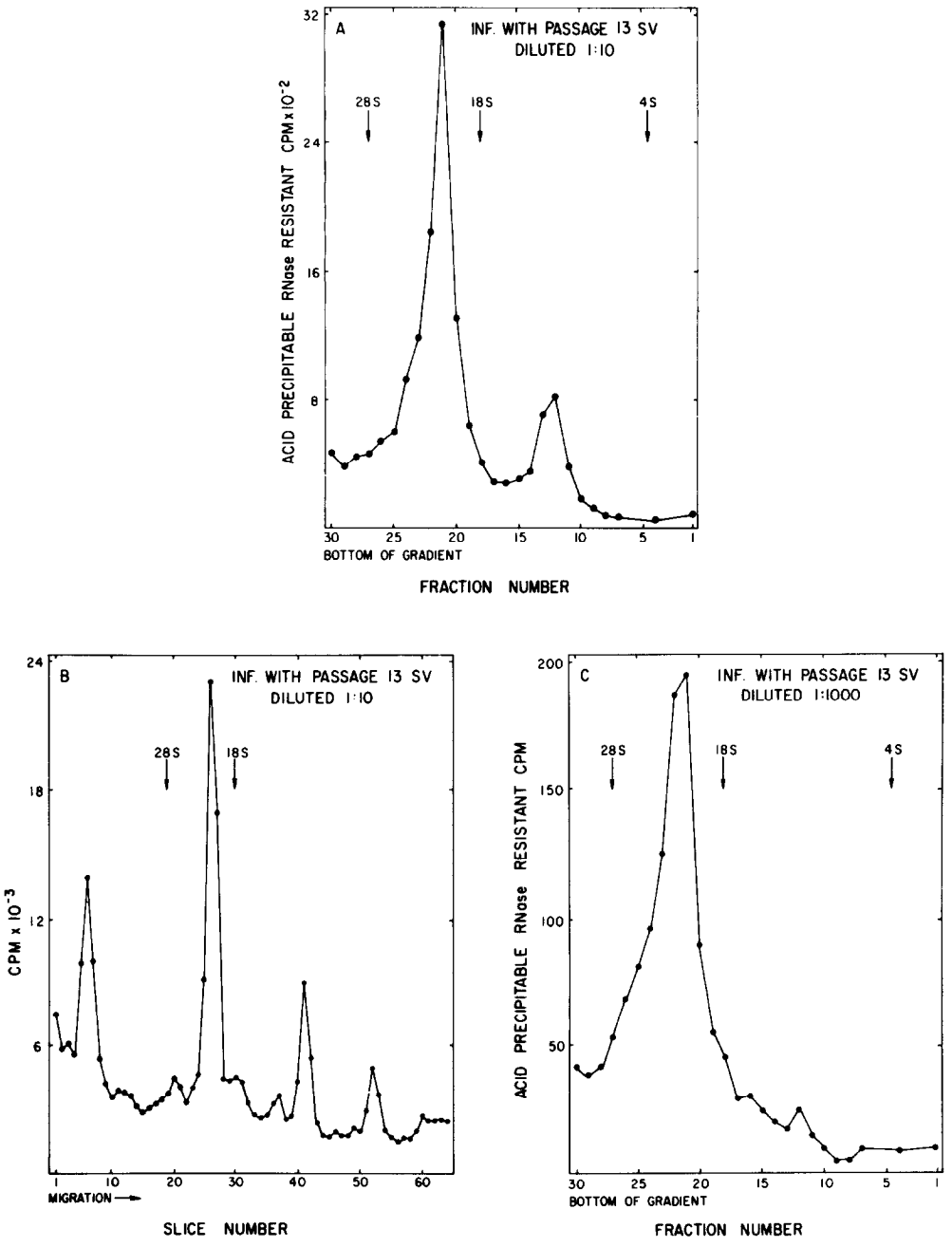

Figure 2. Viral RNA species present in BHK-21 cells infected with passage 13 SV stock at low MOI. BHK-21 cells were infected with either a 10-fold (A,B) or a 1000-fold (C) dilution of SV passaged 13 times at high MOI. Details are identical to Fig. 1, except that gel electrophoresis was for 4-1/2 hours at 6 mA/gel.

presence of incomplete Sindbis particles in high passage virus stock as described by S. Schlesinger et al.[5] However, the drop which we observed in the titer of stocks which were serially passaged at a high MOI was not as marked as that described by these authors.[5] Work is underway to isolate the presumptive incomplete particle and determine whether it can interfere with the replication of complete SV.

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