Failure of Defective Interfering Particles of Sindbis Virus Produced in BHK or Chicken Cells to Affect Viral Replication in *Aedes albopictus* Cells

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Whereas defective interfering particles of Sindbis virus are readily produced in BHK-21 cells or chicken embryo fibroblasts by the techniques of serial undiluted passage, similar methods failed to generate such particles in *Aedes albopictus* cell cultures. In addition, Sindbis virus stocks produced in BHK-21 cells or chicken embryo fibroblasts and which contained defective interfering particles, when tested in *A. albopictus* cells, failed (i) to interfere with the replication of standard Sindbis virus and (ii) to change the pattern of intracellular viral RNA synthesis from that produced by infection with standard Sindbis virus alone. We conclude that defective interfering particles of Sindbis virus generated in chicken or hamster cells are silent or inert in mosquito cells.

When Sindbis virus (SV) is serially passaged without dilution in chick embryo fibroblasts (CEF) or in BHK-21 cells, viral stocks are produced which contain, in addition to normal infectious virions, defective interfering (DI) particles (7, 9, 13, 16). Although the generation of DI particles in viral stocks is sometimes suggested by noting cyclic variations in the titer of infectious virus during the passage series, their presence is demonstrated more conclusively by showing interference with the replication of standard SV (SV_{STD}) in mixing experiments.

Another hallmark of infection with virus populations containing DI particles is the finding of an altered pattern of viral RNA species in the infected cells (6, 16). Whereas in CEF or BHK-21 cells infected SV_{STD} the major viral RNA species found are 22S double-stranded (ds)RNA and 42S and 26S single-stranded (ss)RNA, after infection with stocks containing DI particles, the synthesis of these species is depressed and prominent new species appear. In addition, an analysis of the intracellular viral RNA species produced during passage in CEF has shown a progressive decrease in the size of the new or defective intracellular viral (DICV) RNAs (7). Thus, the most prominent new ssRNA and dsRNA species evolved during passage decreased in size from 33S to 24S, 22S, and 20S, and from 20S to 18S, 15S, and 12S, respectively. At any given passage level, the size of the new dsRNA correlated well with that of the new ssRNA.

Although particles smaller than normal viri-

ons have been observed in stocks containing DI particles (8), it has not proved possible to separate DI particles from standard Sindbis virions in a routine or reproducible fashion (7, 15). It is therefore necessary to study SV DI particles as part of a mixed population.

Johnston et al. (8) reported also, however, that extraction of RNA from SV stocks containing DI particles yielded, in addition to the 42S found in SV_{STD}, discrete new smaller species of RNA. As with the intracellular viral RNAs, the RNAs associated with extracellular particles also diminished progressively in size as the passage series was continued. Similar findings have been obtained by G. Guild in this department (in preparation).

Because of their mode of transmission in nature, alphaviruses such as SV are also included in the large complex of arthropod-borne viruses (arboviruses). These viruses, by definition, have an obligatory alternate transmission cycle between vertebrate and arthropod hosts; in the case of most alphaviruses, the latter is a mosquito. The questions thus arise of (i) what the capabilities and properties of DI particles generated in a vertebrate host might be in the alternate host and (ii) whether DI particles of SV are also produced in mosquito cells.

The results presented in this report show that (i) after serial undiluted passage of SV in mosquito cells we were unable to find any evidence pointing to the presence of DI particles in the passaged viral stocks; (ii) DI particles of SV produced in CEF or BHK-21 cells appeared in-

FIG. 1. Serial undiluted passage of Sindbis virus in *A. albopictus* cells. Monolayer cultures of *A. albopictus* cells (2×10^6 to 4×10^6 cells/60-mm petri dish) were inoculated with an undiluted stock of SV_{STD} (MOI = 100 PFU/cell). Cultures were maintained at 28°C for 16 h, after which the progeny virus was harvested. This virus-containing medium was then used undiluted to infect fresh *A. albopictus* cells following the procedure just described. In this way virus was passed up to 48 times. Virus infectivity was titrated by plaque assay on CEF at 34°C (○—○) and 39.5°C (●---●).

ert in, or were not recognized as such by, mosquito cells.

Portions of this work have been presented earlier (12, 21; A. Igarashi, G. M. Guild, and V. Stollar, Int. Virol. Abstr., 3rd Int. Congr. Virol., Madrid, p. 214, 1975).

While this paper was in preparation, Eaton working with Semliki forest virus (SFV) also reported the failure of DI particles of SFV to interfere with or alter the pattern of viral RNA synthesis in mosquito cells (5).

MATERIALS AND METHODS

Cells and viruses. The primary CEF and the BHK-21 cells have been described previously (24). The *Aedes albopictus* cells (19) have recently been adapted to a medium consisting of 1 volume of the medium devised by Mitsuhashi and Maramorosch (10) and 9 volumes of Eagle medium (4), supplemented with nonessential amino acids (0.2 mM each) and 10% fetal calf serum. For experiments with *A. albopictus* cells, 60-mm petri dishes were seeded with 0.5×10^6 cells and grown in 5% CO_2 at 28°C for 3 days before being used for virus infection.

At the time of infection, plates contained 2×10^6 to 4×10^6 cells.

Stocks of SV_{STD} were prepared in CEF as described elsewhere (15). To prepare SV stocks containing DI particles, SV_{STD} was serially passaged without dilution in BHK-21 or CEF cells to produce SV_{BP} and SV_{CP} , respectively. Serial undiluted passage of SV in *A. albopictus* was performed in a similar fashion, but at 28°C instead of 37°C. In each case and at each passage level the progeny virus was harvested 16 h after infection. Titers of infectious virus were assayed by plaque titration on CEF or BHK-21 cells (14). Plaque assays were performed routinely at 34°C and in some experiments at 39.5°C.

Labeling and extraction of intracellular viral RNA. Monolayer cultures were inoculated with SV virus which was allowed to adsorb at 37°C in the case of BHK and CEF cells or at 28°C in the case of *A. albopictus* cells. After 1 h the inoculum was removed, and the cells were washed twice with PBS-2 (23). Cultures were then maintained in Eagle medium (at 37°C for BHK and CEF cells, at 28°C for *A. albopictus* cells) supplemented with non-

FIG. 2. Growth of SV_{STD} and SV_{BP-18} in *A. albopictus* and BHK-21 cells. Duplicate monolayer cultures of *A. albopictus* (B) and BHK-21 (A) cells were inoculated with stocks of SV_{STD} (○—○) or SV_{BP-18} (●---●) at an input MOI of 20 PFU/cell. After adsorption and removal of the unadsorbed virus, cells were incubated in Eagle medium containing nonessential amino acids and 2% fetal calf serum either at 28°C for *A. albopictus* cells or at 37°C for BHK-21 cells. Samples of medium were taken at the indicated times and infectivity was titrated on CEF at 34°C.

FIG. 3. Lack of interference with SV_{STD} by SV_{BP-18} in *A. albopictus* cells. Duplicate monolayer cultures of *A. albopictus* (B) and BHK-21 (A) cells were inoculated either with 0.2 ml of SV_{STD} alone (○—○) or with 0.1 ml of SV_{STD} plus 0.1 ml of SV_{BP-18} (●---●). The titer of SV_{STD} was 8.2×10^8 PFU/ml and that of SV_{BP-18} was 2.4×10^8 /ml. Procedures otherwise were similar to those described in the legend to Fig. 2.

TABLE 1. Tests for presence of DI particles in various Sindbis virus stocks by interference in BHK-21 cells^a

SV inoculated and MOI (PFU/cell)				24-h yield (PFU/ml)
SV_{STD}	SV_{BP-18}	SV_{BP-19}	$SV_{BP-18-AP-1}$	
0.2				3.5×10^9
0.2	1.1			1.3×10^8
0.2		4.2		4.3×10^7
0.2			2.0	1.9×10^9
	1.1			1.2×10^8
		4.2		7.8×10^7
			2.0	1.7×10^9

^a The virus stocks are described in the text. Virus was inoculated at the input multiplicities indicated for the various experiments.

essential amino acids (0.2 mM each), fetal calf serum (2%), and actinomycin D (1 μ g/ml). Labeling was with [5-³H]uridine (10 μ Ci/ml of medium, 27 Ci/mmol) from 3 to 8 h after infection. RNA was prepared by phenol extraction, ether extraction, and ethanol precipitation (7, 22). Sucrose gradient analysis and identification of dsRNA by RNase resistance were previously described (22).

Alternatively, after labeling with [3H]uridine, whole cells were dissolved in 1% sodium dodecyl

sulfate (SDS) prepared in 0.1 M NaCl, 0.01 M Tris, and 0.001 M EDTA (final pH = 7.4), and then applied directly to sucrose gradients (15 to 30%, wt/wt) containing 0.1 M NaCl, 0.05 M Tris, 0.001 M EDTA, and 0.1% SDS (final pH = 7.4). Centrifugation was in the SW41 rotor at 26,000 rpm for 16 h at 15°C. After fractionation, SDS was precipitated by addition of KCl (final concentration 0.4 M) overnight at 4°C. Total and RNase-resistant radioactivity were then measured in the supernatant (20).

Polyacrylamide-agarose gel electrophoresis was used to analyze phenol-extracted RNA (7, 17). After electrophoresis, gels were sliced into 1-mm fractions and solubilized in scintillation vials with 0.5 ml of dimethyl sulfoxide (room temperature, 15 min). Scintillation liquid (1 volume of Triton X-100 and 2 volumes of NEN formula 949) was then added and radioactivity was measured in a scintillation counter.

Purification of labeled virus. Monolayer cultures of BHK-21 cells were infected with SV as described above. [³H]uridine (10 μ Ci/ml of medium) was added 3 h after infection and the infected culture medium was collected 13 h later, i.e., 16 h after infection. Virus was purified as previously described (7, 15). Briefly, the procedure included ammonium sulfate precipitation and sucrose velocity gradient centrifugation, followed by equilibrium density gradient centrifugation in sucrose-D₂O.

Radioisotopes. [5-³H]uridine (27 Ci/mmol) and [2-¹⁴C]uridine (57 mCi/mmol) were obtained from New England Nuclear Corp., as was NEN formula 949.

RESULTS

Serial undiluted passage of SV in *A. albopictus* cells. To test for the generation of DI particles, cells were infected with SV_{STD} at a multiplicity of infection of 100 PFU/cell. The progeny virus was harvested 16 h postinfection and transferred without dilution to fresh cultures of *A. albopictus* cells. The same process was continued through a series of an additional 47 undiluted passages. Figure 1 shows the titers of viral infectivity during such a passage series.

Although there was moderate fluctuation in the levels of infective virus during these passages, the greatest difference between the highest and lowest titers (assayed at 34°C) was about 50-fold. Usually the variation was considerably less. This contrasts with the very marked fall (>10⁴-fold) in virus titer which was observed in the early passages of SV in CEF (7). In BHK-21 cells, serial undiluted passage of SV also produced fluctuation in the virus titers, less than that seen in the CEF passages but greater than that shown in Fig. 1. These observations suggested that, if serial undiluted passage leads to the production in *A. albopictus* cells of SV DI particles, they may be produced in lesser numbers than in vertebrate cells. Alternatively, if such particles are produced, the

response of mosquito cells to their presence may differ from that of CEF or BHK-21 cells.

Between passages 5 and 19 the viral titer was significantly lower when assayed at 39.5°C instead of at 34°C. A somewhat similar phenomenon was noted during certain passages of SV in CEF (7). It is noteworthy, however, that after passage 20 there was little, if any, difference between the titers as assayed at the two temperatures. These passaged virus stocks, therefore, also differed markedly from SV_{PI} (SV from persistently infected cultures of *A. albopictus*

cells) which has been shown to be characterized by marked temperature sensitivity (14).

Lack of interference by SV DI in *A. albopictus* cells. To examine the response of mosquito cells to SV DI particles, BHK-21 and *A. albopictus* cells were infected at similar multiplicities with SV_{STD} or SV_{BP-18}. The latter is a stock which was obtained after 18 serial undiluted passages in BHK cells and which contains DI particles. Figure 2 shows that, in agreement with previous results (13), the yield of infective virus was much less when BHK cells were infected with SV_{BP-18} instead of with SV_{STD}. In marked contrast, the yield from *A. albopictus* cells was similar in both infections.

A mixed stock of SV_{BP-18} and SV_{STD} was used to infect both BHK-21 and *A. albopictus* cells, and the yields were compared with those obtained after infection at a similar multiplicity with SV_{STD} alone (Fig. 3). As in the previous experiment, BHK cells were very sensitive to SV_{BP-18} and produced a significantly lower yield of virus in the mixed infection than after infection with SV_{STD} alone. Again, *A. albopictus* cells appeared indifferent to SV_{BP-18} and produced the same yield in its absence and in

TABLE 2. Test for interferences by SV_{CP-15} in CEF and *A. albopictus* cells^a

Inoculum (PFU/cell)		24-h yield from infected cultures (PFU/ml)	
SV _{STD}	SV _{CP-15}	<i>A. albopictus</i>	CEF
28		3.3×10^9	1.6×10^9
	32	1.8×10^9	1.5×10^8
0.3		2.0×10^8	2.2×10^9
0.3	32	2.8×10^9	1.5×10^8

^a The virus stocks are described in the text. Virus was inoculated at the input multiplicities indicated for the various experiments.

FIG. 4. Virus-specific double-stranded RNA synthesized in *A. albopictus* and BHK-21 cells infected with SV passaged in *A. albopictus* cells. Duplicate monolayer cultures (3×10^6 cells/60-mm dish) of *A. albopictus* (A-D) and BHK-21 (E-H) were inoculated with 0.2 ml of undiluted SV_{AP-10}, SV_{AP-20}, SV_{AP-30}, or SV_{AP-40} prepared by serial undiluted passage in *A. albopictus* cells. The number after AP indicates the passage level of the specific virus stock. RNA was prepared by phenol extraction of the infected cells which had been labeled with [³H]uridine (10 μ Ci/ml) 3 to 8 h after infection in the presence of actinomycin D (1 μ g/ml, 1 to 8 h after infection). dsRNA was identified by RNase treatment of sucrose gradient fractions as described in Materials and Methods.

its presence. These results suggested that the SV DI particles present in SV_{BP-18} are inert in mosquito cells and do not interfere with viral replication.

Failure of the DI particles to replicate in mosquito cells is confirmed by the experiment shown in Table 1. SV_{BP-18} was passaged once in BHK cells (to give SV_{BP-19}) or in mosquito cells (to give SV_{BP-18-AP-1}). As expected, both SV_{BP-18} and SV_{BP-19} interfered efficiently with viral replication. However, after one passage of SV_{BP-18} in mosquito cells (giving SV_{BP-18-AP-1}), the interfering capacity was almost completely lost. Thus, infection of BHK-21 cells with SV_{BP-18-AP-1} alone or together with SV_{STD} produced virtually the same viral yield as SV_{STD} alone. In contrast, single infection with SV_{BP-18} or SV_{BP-19} resulted in lower viral yields than did infection with either SV_{STD} or SV_{BP-18-AP-1} alone.

The lack of response by *A. albopictus* cells is not confined to SV DI particles produced in BHK-21 cells but applies as well to particles generated in CEF. Thus, infection with SV_{CP-15} produced the same viral yield in mosquito cells as did SV_{STD}, but a significantly lower yield in CEF (Table 2). Furthermore, addition of SV_{CP-15} to SV_{STD} actually increased the yield

from *A. albopictus* cells (presumably by increasing the effective MOI), but decreased that from CEF.

Similar experiments with a number of SV_{AP} stocks (SV passaged in *A. albopictus* cells) also failed to demonstrate interference with SV_{STD}, whether tested in mosquito cells or in BHK-21 cells.

Viral RNA synthesis in cells infected with mosquito cell-passaged virus. The presence of DI particles in SV stocks can be readily recognized by the viral RNA patterns they produce in infected cells (6, 7, 9, 16). The precise pattern seen in a given infection depends to some extent on the cells used for the passage of the virus, but mainly on the passage level (i.e., number of passages) of a particular virus stock (7).

The passage series (Fig. 1) of SV in *A. albopictus* cells (producing SV_{AP}) suggested that, under the conditions used, VS DI particles may not be produced at all or not in great abundance in these cells. Failure of various SV_{AP} stocks to interfere with SV_{STD} further supported this contention. We therefore examined the viral RNA patterns in both BHK and mosquito cells infected with SV stocks obtained at 10, 20, 30, and 40 passages in mosquito cells. We chose in these

FIG. 5. Virus-specific double-stranded RNA synthesized in *A. albopictus* and BHK-21 cells infected with SV_{STD} or SV_{BP-18}. Duplicate monolayer cultures (3×10^6 cells/60-mm dish) of *A. albopictus* (A, B) or BHK-21 cells (C, D) were inoculated either with SV_{STD} (A, C) or SV_{BP-18} (B, D) at an input MOI of 25 PFU/cell. Labeling of the cells and analysis of dsRNA were performed as described in the legend to Fig. 4.

experiments to look only at ds viral RNA, but, as shown previously, the presence of a new DICV dsRNA species in vertebrate cells is always associated with the finding of a new DICV ssRNA of corresponding size.

In the experimental series depicted in Fig. 4, only one pair of panels (Fig. 4B, F; those representing infection with SV_{AP-20}) shows the appearance of small discrete peaks of dsRNA smaller than 22S (about 18S); there are small shoulders in the same region in the panels depicting infection with SV_{AP-40} (Fig. 4D, H). Even these small amounts disappeared after further passages. All other stocks in the passage series tested produced only 22S dsRNA, whether tested in BHK or *A. albopictus* cells. These findings fit well with the suggestion made earlier that those procedures which generate SV DI particles in CEF or BHK-21 cells fail to do so in *A. albopictus* cells.

SV DI particles do not alter the viral RNA patterns in *A. albopictus* cells. Having observed that DI particles of SV (present in SV_{BP} or SV_{CP}) did not interfere with SV_{STD} in mosquito cells, we next examined the pattern of viral RNA in mosquito cells after infection with SV_{CP} or SV_{BP} .

Infection of either BHK-21 or *A. albopictus* cells with SV_{STD} produced only 22S dsRNA (Fig. 5A, C). BHK cells infected with SV_{BP-18} produced, besides 22S dsRNA, an even larger amount of smaller (about 15S) dsRNA (Fig. 5D). In contrast, *A. albopictus* cells infected with SV_{BP-18} produced a pattern similar to that seen after infection with SV_{STD} , namely 22S dsRNA only (Fig. 5B).

With regard to total viral RNA patterns in cells infected with SV_{STD} and SV_{BP-18} (Fig. 6), the results agreed well with those presented in the preceding experiment. Thus, when BHK

FIG. 6. Actinomycin-resistant RNA synthesized in *A. albopictus* and BHK-21 cells infected with SV_{STD} or SV_{BP-18} . Duplicate cultures of *A. albopictus* (A, B, E) or BHK-21 (C, D) were infected with SV_{STD} or SV_{BP-18} and then labeled as described in the legend to Fig. 4. At the end of the labeling period (8 h after infection), the cells were dissolved in 1% SDS prepared in TNE buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride, 0.001 M EDTA, pH 7.4), and the RNA was fractionated by sucrose gradient centrifugation (15 to 30% sucrose [wt/wt] in TNE buffer containing 0.1% SDS; 26,000 rpm, 16 h, 15°C, SW41 rotor). Each fraction (0.4 ml) was mixed with an equal volume of 0.8 M KCl, and the SDS was precipitated overnight at 4°C. The total acid-insoluble (○—○) and the RNase-resistant, acid-insoluble (●---●) counts in the supernatant were measured.

FIG. 7. SV-specific (actinomycin-resistant) RNA synthesized in BHK-21 cells infected with SV_{BP-19} or SV_{BP-18-AP-1}. Duplicate cultures of BHK-21 cells were inoculated with undiluted stocks of SV_{BP-19} (A) or SV_{BP-18-AP-1} (B) at the input MOIs indicated in Table 1. Whole cells labeled with [³H]uridine as described in the legend to Fig. 4 were treated with SDS and the RNA was fractionated and analyzed as described in the legend to Fig. 6. Total acid-insoluble (○—○) as well as RNase-resistant (●---●) acid-insoluble counts were measured.

cells were infected with SV_{BP-18} (Fig. 6D), the amount of both 42S and 26S ssRNA was markedly decreased compared with infection with SV_{STD} (Fig. 6C), and there was a large new peak of ss viral RNA at 18S. In contrast, *A. albopictus* cells contained the same viral RNA species whether infected with SV_{STD} or SV_{BP-18} (Fig. 6A, B). The corresponding dsRNA species

seen in this experiment were similar to those shown in Fig. 5. Uninfected *A. albopictus* cells (Fig. 6E) contained very little actinomycin-resistant dsRNA, but substantial amounts of actinomycin-resistant ssRNA smaller than 18S (see also Eaton [5]). The nature of this RNA is unknown.

In an earlier section it was shown that one passage of SV_{BP-18} in mosquito cells cleared this stock (SV_{BP-18-AP-1}) of its ability to interfere with SV_{STD}. Figure 7B shows that BHK cells infected with SV_{BP-18-AP-1} also showed a return to the normal pattern of viral RNA synthesis seen after infection with SV_{STD}. When SV_{BP-18} was passaged instead in BHK cells (producing SV_{BP-19}), the viral RNA pattern in the infected cells remained characteristic of infection with a stock containing DI particles (Fig. 7A).

Consistent with the lack of interference by SV_{CP-15} in mosquito cells (cf. Table 2), only 22S dsRNA was found in *A. albopictus* cells whether infection was with SV_{CP-15} or with SV_{STD} (Fig. 8A, B). In CEF, on the other hand, these two viral stocks produced markedly different patterns of dsRNA. (Fig. 8C, D).

Replicative forms (RF) of SV RNA from *A. albopictus* cells and from BHK cells. Simmons and Strauss (18) showed that, when RNA from BHK cells infected with SV was treated with RNase and then analyzed by sucrose gradient centrifugation, three species of RNase-resistant RNA were found. In order of decreasing size, these were called RF-1, RF-2, and RF-3, and had estimated molecular weights of 8.8×10^6 , 5.6×10^6 , and 2.9×10^6 , respectively. Because RF-2 and RF-3 are similar in size to various of the dsRNA molecules found in CEF or BHK-21 cells infected with DI particles of SV (7), it seemed possible that RFs 2 and 3 were in some way related to the generation of defective viral RNAs and thus to the production of DI particles. If this were so, the failure of mosquito cells to produce DI particles might be explained by finding very little or no RF-2 or RF-3 in RNA extracted from infected mosquito cells.

We therefore examined the RF molecules in *A. albopictus* cells to compare them with those found in BHK cells. RNA was extracted from BHK-21 and mosquito cells infected with SV_{BP-18} and was fractionated on sucrose gradients. In each instance the fractions containing the 22S dsRNA (identified by testing portions of each fraction for resistance to RNase) were pooled, treated with pancreatic RNase, and analyzed by polyacrylamide-agarose gel electrophoresis. In both cases, a similar pattern consisting of three peaks was found (Fig. 9A, B). These ranged in size between 16S and 22S and appear to correspond to RF-1, RF-2, and

FIG. 8. Double-stranded RNA synthesized in *A. albopictus* and CEF cells infected with SV_{STD} or SV_{CP-15} . Duplicate cultures of *A. albopictus* (A, B) and CEF (C, D) were inoculated with SV_{STD} (A, C) or with SV_{CP-15} (B, D) at an input MOI of 30 PFU/cell. RNA was extracted with phenol from infected cells labeled with [3H]uridine (10 μ Ci/ml, 3 to 8 h after infection) in the presence of actinomycin D (1 μ g/ml, 1 to 8 h postinfection). After sucrose gradient centrifugation double-stranded RNA was localized by counting RNase-resistant, acid-insoluble radioactivity of the fractions.

RF-3 of Simmons and Strauss (18). Unpublished data by Guild and Stollar indicate that the 18S dsRNA, produced by infection of CEF cells with SV_{CP-6} , remains 18S after RNase treatment. These observations demonstrate that in *A. albopictus* cells, as in BHK-21 cells, the 22S dsRNA is composed of two kinds of molecules of which one (RF-1) is resistant to RNase and the other, upon RNase treatment, gives rise to RF-2 and RF-3. The inability of mosquito cells to generate DI particles therefore does not appear to be related to a fundamentally different spectrum of RF molecules.

Adsorption of SV_{STD} and SV_{BP-18} to BHK-21 and *A. albopictus* cells. To compare the adsorption to cells of SV_{STD} and SV_{BP-18} , [3H]uridine-labeled virus was prepared in BHK-21 cells. Virus purified by gradient centrifugation was diluted in Eagle medium containing 2% fetal calf serum to give the final infectivities and

counts indicated in Table 3. Duplicate cultures of *A. albopictus* or BHK-21 cells in 60-mm plates were infected with 0.2 ml of SV_{STD} or of SV_{BP-18} . After 1 h of adsorption (at 28°C for *A. albopictus*, at 37°C for BHK-21 cells), the unadsorbed virus was removed and its infectivity was measured. The cells were then washed twice with PBS-2 (23) and scraped into PBS-2 containing 1% SDS. The trichloroacetic acid-precipitable radioactivity was collected on GF/A filters (Whatman), which were then washed with 5% trichloroacetic acid, dried, and counted. Table 3 shows that, in terms of both infectivity and radioactivity, SV_{BP-18} adsorbed as efficiently as SV_{STD} to *A. albopictus* as well as to BHK-21 cells. These results do not support the hypothesis that the failure of *A. albopictus* cells to respond to DI particles produced in BHK-21 cells resulted from a failure of such particles to be adsorbed.

FIG. 9. Polyacrylamide-agarose gel electrophoresis of the RNase digests of 22S dsRNA extracted from *A. albopictus* and BHK-21 cells infected with SV_{BP-18}. RNA was extracted with phenol from *A. albopictus* and from BHK-21 cells (4×10^7 cells each) infected with SV_{BP-18} and labeled with [^3H]uridine (10 $\mu\text{Ci/ml}$, 3 to 8 h postinfection) in the presence of actinomycin D (1 $\mu\text{g/ml}$, 1 to 8 h postinfection). Each RNA was fractionated by sucrose gradient sedimentation and the dsRNA was localized by testing portions of each fraction for RNase-resistant, acid-insoluble radioactivity. For each gradient the fractions containing the 22S dsRNA were pooled and the RNA was digested with pancreatic RNase (10 $\mu\text{g/ml}$, 22°C, 10 min) in 0.3 M NaCl. RNA was then reextracted three times with phenol and three times with ether, and precipitated overnight at -20°C with 2.5 volumes of ethanol in the presence of carrier BHK cell RNA (100 μg). The precipitated RNA was collected by centrifugation (20,000 $\times g$, 30 min), dissolved in the electrophoresis buffer, and analyzed by polyacrylamide-agarose gel electrophoresis. Arrows indicate the position of 22S and 12S dsRNA internal marker prepared from BHK-21 cells infected with SV_{BP-18} and labeled with [^{14}C]uridine (1 $\mu\text{Ci/ml}$) in the presence of actinomycin D (1 $\mu\text{g/ml}$).

DISCUSSION

The failure of mosquito cells to generate DI particles of SV during serial undiluted passage (SV_{AP}) contrasts sharply with the observations made when SV is similarly passaged in BHK-21 (SV_{BP}) or CEF (SV_{CP}) cells. The absence of DI particles is inferred from (i) failure to observe a marked decrease in viral titer during the undiluted passage in mosquito cells, (ii) lack of interfering ability in SV_{AP} stocks whether tested in vertebrate or mosquito cells, and (iii) the consistent absence of significant amounts of dsRNA smaller than 22S in cells infected with the mosquito cell-passaged virus.

It is worth noting that, when SFV was passaged serially in CEF, the presence of DI particles was not revealed until the passage stocks were used to infect mouse cells (9). It is unlikely that a similar phenomenon is operative in our system since, as just noted, stocks such as SV_{AP-20} failed to interfere with SV_{STD} whether tested in BHK or mosquito cells.

Our results have clearly demonstrated that, when stocks of SV_{BP} or SV_{CP} are used to infect *A. albopictus* cells, they neither interfere with SV_{STD} nor generate the aberrant smaller species of viral RNA characteristically found in BHK or chicken embryo cells infected with viral stocks containing DI particles. Thus, it appears that such particles are inert or silent in *A. albopictus* cells. This indifference or non-reaction to DI particles is such that one passage of SV_{BP-18} through mosquito cells is sufficient to clear the stock of DI particles.

At present we have no adequate explanation for this cell-specific difference. The inability to demonstrate any difference in adsorption to *A. albopictus* cells of viral particles from stocks of SV_{STD} or SV_{BP-18} seems especially significant, since DI particles may constitute between 60 and 80% of the population (8). It is unlikely that the temperature at which the cells are maintained after infection is critical, since similar results were obtained when *A. albopictus* cells were maintained after infection at 37°C and BHK-21 cells were maintained at 28°C (unpublished data).

At the present time we favor the idea that the primary block to DI particles in mosquito cells is at the level of RNA replication. Although we cannot offer a more precise mechanism, it is clear that interference by DI particles always goes hand in hand with the synthesis of the "defective" smaller viral RNA molecules and with inhibition of the synthesis of the standard viral RNA species.

Other examples in which expression of DI

TABLE 3. Adsorption of SV_{STD} and SV_{BP-18} to *A. albopictus* and BHK-21 cells^a

SV inoculated (0.2 ml)			Total PFU remaining in the medium after adsorption		Acid-insoluble cpm ^b bound to cells after adsorption (% of input)	
PFU	cpm		<i>A. albopictus</i>	BHK-21	<i>A. albopictus</i>	BHK-21
SV _{STD} 3.6 × 10 ⁷	1,115		1.5 × 10 ⁷	1.8 × 10 ⁷	520 (46.4)	569 (51.0)
SV _{BP-18} 2.8 × 10 ⁷	1,017		1.0 × 10 ⁷	1.4 × 10 ⁷	494 (48.5)	554 (54.4)

^a All the figures are the average of duplicate experiments. The methods used for measurement of adsorption are described in the text.

^b cpm, Counts per minute.

particles appears host controlled have been reported (2, 3, 11). In these cases also, the precise mechanism is unclear. Such observations do, however, point to a role of the host cell in the replication of viral RNA, be it normal or defective RNA.

In this report we have shown the inability of SV DI particles produced in vertebrate cells to be replicated as such in mosquito cells. Elsewhere we have reported that mosquito cells persistently infected with SV after a sufficient period of time generate virus populations which are predominantly temperature sensitive (14). Such virus would be strongly selected against in mammalian or avian cells maintained between 37°C and 41°C and even at 34°C in BHK cells (14).

The possible epidemiological implications of these findings and the role of host factors for the transmission and maintenance in nature of arthropod-borne togaviruses have been discussed elsewhere (12, 21).

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